Widespread vulnerability of Malagasy predators to the toxins of an introduced toad

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eTOC: The common Asian toad has recently been introduced to Madagascar, sparking fears that the toad's potent bufadienolide toxins will poison native species. Marshall et al. demonstrate that these fears are warranted, with toxin receptor genotyping revealing that the vast majority of Malagasy vertebrates are likely vulnerable to poisoning.

Highlights:

- There is widespread susceptibility to toad toxins in Malagasy fauna.
- Virtually all potential toad predators are toxin-sensitive.
- Widespread susceptibility suggests profound effects of toads on native wildlife.

Summary

Invasive and introduced species can pose major ecological challenges to vulnerable native wildlife. Toxic invaders can cause long-term disruptions of predator communities with consequent trophic cascade effects. Madagascar, a key global biodiversity hotspot, is experiencing an invasion by a toxic species, the toad *Duttaphrynus melanostictus*. Bufonid toads secrete bufadienolides that are fatal to many predator species by inhibiting the sodium-potassium-pump (Na^+/K^+-ATPase). However, multiple predator lineages have evolved resistance to these toxins through repeated, predictable and specific point mutations in the Na^+/K^+-ATPase gene. Here we analyse sequences of the Na^+/K^+-ATPase gene of a wide range of Malagasy species, including amphibians, birds, mammals and reptiles, and find that only one native species shows evidence of resistance to the novel toxin. The results strongly suggest that invasive toads are liable to have significant impacts on the native Malagasy fauna, and stress the importance of controlling the spread of this alien species to prevent a worsening biodiversity crisis.

Main Text

Invasive species are a key factor contributing to the global decline of biodiversity [1]. Therefore, understanding the mechanisms is crucial if detrimental effects are to be mitigated [1]. One such mechanism is the introduction of invasive species with defensive strategies, such as novel toxins, that can disrupt native predator communities. [2]. Disruption of such communities can produce trophic cascades and can have an impact on a diverse array of taxa [2]. Madagascar, a globally significant biodiversity hotspot, has recently experienced the introduction of a toxic bufonid amphibian, the Asian common toad (*Duttaphrynus melanostictus*) [3]. Since its invasion, the toad population has expanded rapidly, making control problematic and eradication extremely difficult [4]. Previous cases of bufonid introductions, such as the ongoing spread of the cane toad (*Rhinella marina*) in Australia, have resulted in the decimation of many indigenous species [2], prompting fears that Madagascar may be similarly impacted [4]. Here we show that these fears are warranted: we demonstrate that a many Malagasy vertebrates are likely to be susceptible to the toxins of this invasive toad.

Bufonid toads secrete potent forms of cardiac glycosides known as bufadienolides to defend themselves from predators [5]. These molecules bind to the sodium-potassium pump (Na^+/K^+-ATPase) of cells, resulting in the inhibition of ion transport, causing cardiotoxic effects and, ultimately, death [6]. Although bufadienolides are highly toxic to naïve predators, many species from diverse animal lineages (e.g., certain reptiles, amphibians and mammals) have evolved resistance and readily consume toads without suffering ill effects [7]. Resistant species are phylogenetically diverse, yet the adaptations that confer tolerance are remarkably consistent,

representing a fascinating example of convergent molecular evolution (with only a few exceptions, see Supplemental Discussion 1). n each case, two amino acid replacements, with at least one adding charge, in the first extracellular domain (H1-H2) of the alpha 1 or alpha 3 isoforms of the Na+/K+-ATPase perturb binding interactions with the bufadienolides, resulting in target site insensitivity [7]. The universality of this resistance mechanism means that by sequencing a short portion of the relevant gene, we can reliably predict a species' vulnerability to bufadienolides.

While most recent authors have assumed all potential Malagasy toad predators to be sensitive to bufadienolides [3,4], the distribution of resistance cannot be easily predicted from evolutionary origin or diet. For example, Australian monitor lizards appear to be descended from resistant Asian species but have lost that resistance after a prolonged period of allopatry with bufonids [8]. However, recent work on snakes has demonstrated that resistance to bufadienolides is far more widespread than bufophagy [9], suggesting phylogenetic conservatism. Since we cannot rely on dietary studies and/or evolutionary relatedness to predict resistance [9], the assumption that the Malagasy fauna will be vulnerable to bufadienolides due to lack of prior coexistence with toads needs to be explicitly tested.

We therefore sequenced the H1-H2 extracellular domain of the Na^+/K^+-ATPase from 77 Malagasy species, including 27 snakes, 2 lizards, 12 frogs, 8 mammals and 28 birds (GenBank accessions MH094669-MH094740), to examine the amino acid composition in the bufadienolide binding site. In addition, we analysed data from the genomes of 11 previously sequenced species found on Madagascar (Figure 1).

We sampled all three macrostomatan snake colonisations of Madagascar [10]. All showed identical amino acid sequences in the H1-H2 extracellular domain of the Na^+/K^+-ATPase, matching other non-resistant snakes [7,9] and providing strong evidence that the Malagasy species are likely to be highly sensitive to the toxins of *D. melanostictus*. The two studied gerrhosaurid lizards (*Zonosaurus* spp.) also possessed the susceptible genotype, which matches that of the demonstrably non-resistant Australian lizards [7,8]. Existing dietary studies lead us to suggest that many of the sequenced reptile species will likely be directly impacted via poisoning, as they are known to feed on amphibians [10]. However, the exact nature of the effects on different species may be difficult to predict due to the complexity of ecosystem-level trophic interactions (see Supplemental Discussion 2).

Of the 12 frog species sequenced, 11 showed genotypes highly similar to non-resistant frogs. We found a few species with amino acid replacements in the middle of the H1-H2 extracellular domain, but the nature of these replacements seem unlikely to confer resistance to bufadienolides, as none add charged amino acids, nor are any positioned at sites previously associated with resistance [7].

Among mammals we also identified likely vulnerability in lemurs and tenrecs. Only one native Malagasy species, the white-tailed antsangy (Rodentia: *Brachytarsomys albicauda*) shared the resistant Na^+/K^+-ATPase genotype of the brown rat (*Rattus norvegicus* [See Table S1]). These data suggest retention of ancestral rodent resistance, indicating either little cost of maintaining resistance or continued consumption of cardiac glycoside-producing plants.

We examined sequences of 34 bird taxa, 31 of which have a Na^+/K^+-ATPase H1-H2 domain that shows no evidence of amino acid replacements likely to confer

120 resistance to bufadienolides. While some of the endemic birds sampled are not at
121 risk due to their diets, the 15 sampled species likely to consume amphibians are
122 probably vulnerable to toad poisoning since, in the absence of bufonids, they are
123 unlikely to have evolved behavioural mechanisms to avoid them as food.

124 Our results for the remaining mammals and birds, specifically the endemic
125 mammalian carnivores (Eupleridae: Malagasy civet *Fossa fossana*, Eastern fanalouc
126 *Eupleres goudoti,* and fossa *Cryptoprocta ferox*) and three bird species (cuckoo
127 roller *Leptosomus discolor*, Madagascar bulbul *Hypsipetes madagascariensis* and
128 Madagascar mannakin *Lonchura nana*), are more equivocal: their sequences display
129 one of the two substitutions that could potentially perturb bufadienolide binding.
130 However, resistance has thus far only been identified in vertebrates that harbour two
131 substitutions, one towards each end of the H1-H2 extracellular domain [7],
132 suggesting that these Malagasy predators are likely to be sensitive to toad toxins.

133 The results reported here predict sensitivity to bufadienolides in virtually all Malagasy
134 vertebrate predators with the potential to consume introduced toads, substantiate the
135 grave concerns surrounding the introduction of *D. melanostictus* to the biodiversity
136 hotspot of Madagascar [4] and strongly suggest that this invasive toad is likely to
137 have significant detrimental impacts on the native Malagasy predator fauna, in a
138 manner analogous to the introduced cane toad in Australia [2]. This makes trophic
139 cascades a distinct possibility by relieving pressure on non-susceptible rodents [2,4].
140 Given the taxonomic and ecological diversity of the apparently vulnerable species
141 sampled here, the impacts on each will be difficult to predict and, ultimately, will be
142 dependent on their natural histories, niche overlap with the toad and the adaptability
143 of the toads as they spread to different habitats, in particular undisturbed rainforests.

144 It is most likely that numerous species not sampled in this study will also be
145 vulnerable to bufadienolide poisoning, including many that are already critically
146 endangered. This may be especially true for Malagasy snakes, whose close
147 relatedness could increase the chances of phylogenetically conserved vulnerability
148 [9,10]. Our findings stress the importance of the timely investment of resources to
149 monitor and control the spread of this alien species in order to prevent a worsening
150 biodiversity crisis in Madagascar.

151

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162

163 **Author Contributions**

164 N.R.C. and W.W. designed the research. M.V., F.G., F.A., A.R. and F.W. collected
165 the samples. B.M.M., G.Z. carried out the lab work. B.M.M. and N.R.C. analysed the
166 data. M.V. constructed the molecular dating tree. B.M.M. wrote the manuscript with
167 input from all other authors.

Declaration of Interests

The authors declare no competing interests.

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Figure Legends

Figure 1. Dated molecular phylogeny of the sampled diversity of taxa tested for bufadienolide-resistant Na^+/K^+-ATPase genotypes, demonstrating a lack of resistance across almost the entire breadth of the Malagasy vertebrate fauna. Representative resistant non-Malagasy taxa have been included for phylogenetic context.