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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 responsible is crucial if detrimental effects are to be mitigated [1]. One such mechanism is the introduction of a novel defensive strategy by which invasive species can disrupt native predator communities [2]. Significant 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 Common Asian Toad (*Duttaphrynus melanostictus*) [3]. Since its invasion, the toad population has expanded rapidly, making control problematic and eradication likely impossible [4]. Previous cases of bufonid introductions, such as the infamous and 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 wide diversity of Malagasy vertebrates are likely to be susceptible to the toxic secretions of this invasive toad.

Bufonid toads secrete potent forms of cardiac glycosides known as bufadienolides to defend themselves from predators [5]. These molecules exert toxic effects by binding 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). In 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 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.

The Malagasy snakes sampled cover 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 exhibited the susceptible genotype, which matches 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 with high degrees of similarity to non-resistant frogs. We found a few species with amino acid replacements in the middle of the H1-H2 extracellular domain, but the location and physicochemical properties 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]. Only the introduced Indian bullfrog (*Hoplobatrachus tigerinus*) had amino acid replacements (including an insertion) that might confer resistance; however, without further experimental evidence resistance remains speculative.

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 resistance to bufadienolides. While some of the endemic birds sampled are not at risk due to their diets, the 15 sampled species likely to consume amphibians are

126 probably vulnerable to toad poisoning since, in the absence of bufonids, they are
127 unlikely to have evolved behavioural mechanisms to avoid them as food.

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

137 The results reported here, demonstrating sensitivity to bufadienolides in virtually all
138 Malagasy predators with the potential to consume introduced toads, substantiate the
139 grave concerns surrounding the introduction of *D. melanostictus* to the biodiversity
140 hotspot of Madagascar [4] and strongly suggest that this invasive toad is likely to
141 have significant detrimental impacts on the native Malagasy predator fauna, in a
142 manner analogous to the introduced cane toad in Australia [2]. This makes trophic
143 cascades a distinct possibility by relieving pressure on non-susceptible rodents [2,4].
144 Given the taxonomic and ecological diversity of the apparently vulnerable species
145 sampled here, the impacts on each will be difficult to predict and, ultimately, will be
146 dependent on their natural histories, niche overlap with the toad and the adaptability
147 of the toads as they spread to different habitats, in particular undisturbed rainforests.
148 It is most likely that numerous species not sampled in this study will also be
149 vulnerable to bufadienolide poisoning, including many that are already critically
150 endangered. This may be especially true for Malagasy snakes, whose close

relatedness could increase the chances of phylogenetically conserved vulnerability [9,10]. Our findings stress the importance of the timely investment of resources to monitor and control the spread of this alien species in order to prevent a worsening biodiversity crisis in Madagascar.

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Author Contributions

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

Declaration of Interests

The authors declare no competing interests.

References

1. Kraus, F. (2015). Impacts from invasive reptiles and amphibians. Annu. Rev. Ecol. Evol. Syst. *46*, 75–97.

2. Shine, R. (2010). The ecological impact of invasive cane toads (*Bufo marinus*) in Australia. Q. Rev. Biol. *85*, 253–291.

3. Moore, M., Fidy, J.F.S.N., and Edmonds, D. (2015). The new toad in town: Distribution of the Asian toad, *Duttaphrynus melanostictus*, in the Toamasina area of eastern Madagascar. Trop. Conserv. Sci. *8*, 440–455.

4. McClelland, P., Reardon, J.T., Kraus, F., Raxworthy, C.J., and Randrianantoandro, C. (2015). Asian toad eradication feasibility report for Madagascar (Te Anau, New Zealand).

5. Flier, J., Edwards, M., Daly, J., and Myers, C. (1980). Widespread occurrence in frogs and toads of skin compounds interacting with the ouabain site of Na+, K+-ATPase. Science *208*, 503–505.

6. Lingrel, J.B. (2010). The physiological significance of the cardiotonic steroid/ ouabain-binding site of the Na,K-ATPase. Annu. Rev. Physiol. *72*, 395–412.

7. Ujvari, B., Casewell, N.R., Sunagar, K., Arbuckle, K., Wüster, W., Lo, N., O'Meally, D., Beckmann, C., King, G.F., Deplazes, E., *et al.* (2015). Widespread convergence in toxin resistance by predictable molecular evolution. Proc. Natl. Acad. Sci. *112*, 11911–11916.

8. Ujvari, B., Mun, H.C., Conigrave, A.D., Bray, A., Osterkamp, J., Halling, P., and Madsen, T. (2013). Isolation breeds naivety: Island living robs Australian varanid lizards of toad-toxin immunity via four-base-pair mutation. Evolution

67, 289–294.

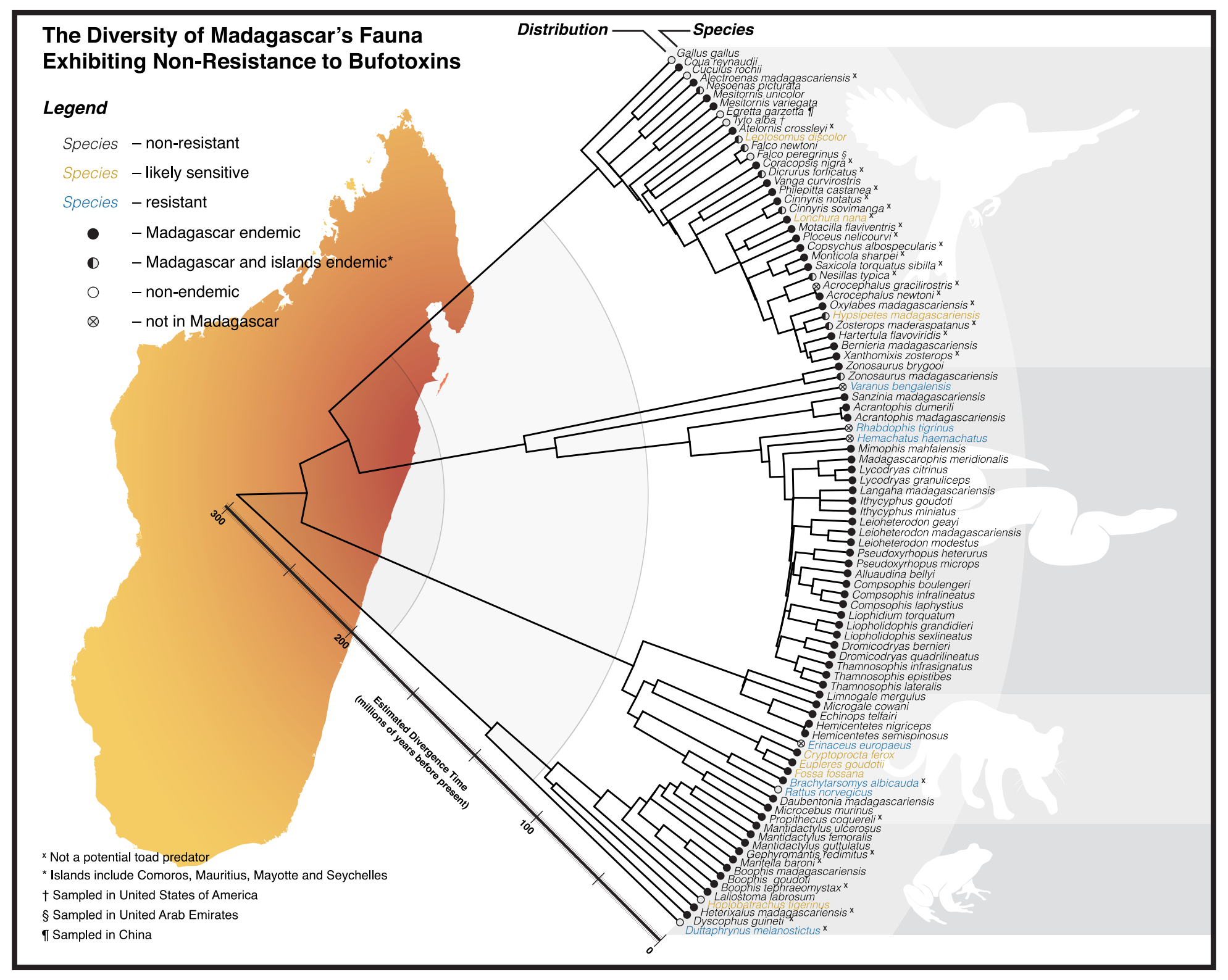
9. Mohammadi, S., Gompert, Z., Gonzalez, J., Takeuchi, H., Mori, A., and Savitzky, A.H. (2016). Toxin-resistant isoforms of Na+/K+-ATPase in snakes do not closely track dietary specialization on toads. Proc. R. Soc. B *283,* 20162111.

10. Glaw, F., and Vences, M. (2007). A Field Guide to the Amphibians and Reptiles of Madagascar 3rd ed. (Cologne: Vences & Glaw Verlag).

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.

The Diversity of Madagascar's Fauna Exhibiting Non-Resistance to Bufotoxins



Supplementary Material: Widespread vulnerability of Malagasy predators to the toxins of an introduced toad

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Supplementary Methods

Experimental Model and Subject Details

All field research, collecting of specimens, including in situ euthanasia of specimens were approved by the Madagascan Ministère de l'Environnement, des Eaux et des Forêts (Direction des Eaux et Forêts, DEF) under the following permits: 156-MEF/SG/DGEF/DADF/SCB dated 12 December 2002; 238MINENVEF/SG/ DGEF/DPB/SCBLF dated 14 November 2003; 238MINENV.EF/SG/DGEF/DPB/SCBLF/ RECH dated 22 December 2004; 272MINENV.EF/SG/DGEF/DPB/SCBLF/RECH dated 8 November 2005; 298MINENV.EF/SG/DGEF/DPB/SCBLF/RECH dated 22 December 2006; 036/08MEEFT/SG/DGEF/DSAP/SSE dated 30 January 2008; 26/09/MEEFT/SG/DGEF/DSAP/ SLRSE dated 3 February 2009; 48/09/MEEFT/SG/DGEF/DSAP/SSE dated 9 March 2009; 188/09/MEEFT/SG/DGEF/DSAP/SSE dated 16 September 2009; 195/09/MEEFT/SG/DGEF/DSAP/SSE dated 28 September 2009; 314/10/MEF/SG/DGF/DCB.SAP/SCB dated 4 November 2010, and 232/12/MEF/SG/DGF/DCB.SAP/SCB dated 4 September 2012. Export of specimens was approved by the DEF under permits: 063C-EA02/MG03, dated 26 February

2003; 094C-EA03/MG04, dated 1 March 2004; 103C-EA03/MG05, dated 15 March 2005; E1400/06, dated 1 June 2006; 055N-EA03/MG10, dated 25 March 2010.

Collection permit for bird samples (specimens released after sampling) were from 10 September 2003 (No. 0182 et 0184 /MINENVEF/SG/DGEF/ DPB/SCBLF), 19 October 2004 (No. 234 /MINENVEF/SG/DGEF/DPB/SCBLF/RECH), 4 November 2005 (No. 262 et 261/MINENVEF/SG/DGEF/DPB/SCBLF/RECH), 21 November 2006 (No. 275 et 276/MINENVEF/SG/DGEF/DPB/SCBLF/RECH), 4 December 2007 (No. 0296/07/MEEFT/SG/DGEF/ DPSAP/SSE), and renewals of No. 296/07 on 19 November 2010 (No. 335/10/MEF/SG/ DGF/DCB.SAP/SCB, No. 284/12/MEF/SG/DGF/DCB.SAP/SCB on 8 November 2012 and 7 October 2014 (No. 265/14/MEEF/SG/DGF/DCB.SAP/ScB).

Method Details

The DNA was extracted from tissue samples using Qiagen DNeasy Blood and Tissue Kits following standard Qiagen DNeasy protocol. The products were quantified using a Nanodrop Spectrophotometer ND1000.

Amplification of the H1-H2 domain of the Na^+/K^+-ATPase was undertaken via Polymerase Chain Reaction (PCR). PCR mixes were created using pure water, PCR buffer Reddymix (Thermo Fisher) at 1X, forward and reverse primers at 0.3 µM, and template DNA at around 20 ng/µl. For all reactions, a total volume of 15-16 µl was used. The complete mixes were placed into a Bio-rad DNA Engine Tetrad 2 Peltier Thermal Cycler. The PCR procedure entailed an initial denaturing at 94°C for 2 minutes, followed by 40 cycles of denaturing for 30 seconds at 94° C, annealing for 30 sec at primer-specific temperatures (51.5°C: ATP_178; 52°C: ATP1a1_PaPa1;

54°C: ATP1a1_GaGa2 and ATP1a1_FaPe2; 55°C: ATP1a1_EcTe1; 56°C: ATP1a3), and an extension step at 72°C for 1 minute and a final extension step at 72°C for 5 minutes, followed by a cooling period at 4º C for 15 minutes.

Snake and lizard alpha 3 isoforms were amplified using the primers ATP1a3Fwd (CGA GAT GGC CCC AAT GCT CTC A) and ATP1a3Rvs (TGG TAG TAG GAG AAG CAG CCG GT) [S1]; the amphibian 1 isoform amplicons were obtained using primers ATP1_178Fwd (CGA GAT GGC CCC AAT GCT CTC A) and ATP1_178Rvs (TGG TAG TAG GAG AAG CAG CCG GT) [S2].

Primers used to amplify the alpha 1 isoform of the H1-H2 domain of the Na^+/K^+-ATPase for mammals and birds were designed using the National Center for Biotechnology Information's (NCBI) Primer-Blast [S3]. Three pairs of primers (5′- > 3′) were designed, based on the GenBank records for *Gallus gallus* (NM_205521.1), *Falco peregrinus* (XM_005231095.2), *Panthera pardus* (XM_019457963.1) and *Echinops telfairi* (XM_004714862.2): ATP1a1_GaGa2 (FWD = ATGGGTMAAGTTCTGTCGGC, RVS = GCACCAWGTTCTTGAASGACT), ATP1a1_FaPe2 (FWD = CGGCAGCTCTTYGGAGGAT, RVS = AACCACAGCTGCCAACACRA), ATP1a1_PaPa1 (FWD = ATGGGTCAAGTTCTGTCGGC, RVS = GAKAGKACCACRCCAAGATAS), ATP1a1_EcTe1 (FWD = TSTTYGGGGGCTTCTCAATG, RVS = GGAWAGCACCACRCCRAGRT).

PCR products were cleaned using 2 µl of a mix comprising 1.6 µl of water, and 0.2 µl of both exonuclease and TSAP. Once the enzymes had been added the products were placed back into the thermal cycler running the following: incubation at 37º C, inactivation at 74º C, and stop at 4º C. All steps were run once for 15 minutes. The cleaned products for reptiles and amphibians were sent to Macrogen (Seoul, South

Korea) for sequencing. Products for mammals and birds were sequenced by LGC Genomics (Berlin, Germany).

Quantification and Statistical Analyses

Sequence data were examined and quality-checked using CodonCode Aligner (CodonCode Corporation – www.codoncode.com). Alignment of the consensus sequences was performed using MUSCLE in MEGA7 (V. 7.0.21) [S4]. In this analysis, sequences from GenBank were used as reference to identify the 11 codons of interest [S5]. Isoelectric points were identified using ProtParam tool (web.expasy.org/protparam/).

Additional sequences from the NCBI's GenBank were located via the NCBI's full genome annotation system (The NCBI Eukaryotic Genome Annotation Pipeline) and searching for genes annotated as ATP1a1. Additionally, previously confirmed sequences of the Na^+/K^+-ATPase were put into the NCBI's BLAST nucleotide search to find unannotated sequences. All identified sequences were aligned and reviewed in MEGA7 to confirm the presence and form of the H1-H2 domain of the Na^+/K^+-ATPase.

Four Malagasy mammals and six birds had large genome datasets available that covered the alpha 1 isoform of the Na^+/K^+-ATPase and so could be used to infer toxin resistance. These were *Daubentonia madagascariensis* (AGTM011609586.1), *Echinops telfairi* (XM_004714862.2), *Microcebus murinus* (XM_012761812.1), *Propithecus coquereli* (XM_012658471.1), *Gallus gallus* (NM_205521.1), *Egretta garzetta* (XM_009639091.1), *Falco peregrinus* (XM_005231095.2), *Tyto alba* (XM_009966040.1), *Leptosomus discolor* (XM_009949897.1) and *Mesitornis*

unicolor (XM_010192438.1). Also included are examples of known resistant species of various orders: *Varanus bengalensis* (KP238148.1), *Rhabdophis tigrinus* (KU738116.1), *Hemachatus haemachatus* (KU738087.1), *Erinaceus europaeus* (XM_007525504.1), *Rattus norvegicus* (NM_012504.1) and *Duttaphrynus melanostictus* (FJ976640.1).

To visually represent phylogenetic relationships and divergence times among the taxa, we first computed a phylogeny of all species included in our study plus a series of additional, informative taxa on the basis of three mitochondrial genes (16S rRNA, cytochrome oxidase subunit 1, cytochrome b) and one nuclear gene (recombination-activating gene 1), under the maximum likelihood optimality criterion in MEGA7 with a general-time reversible substitution model, and using a lungfish (*Protopterus aethiopicus*) as outgroup. The resulting tree was manually adjusted to fit current phylogenetic knowledge, as summarized in www.timetree.org and in recent phylogenetic and phylogenomic studies [S6,S7]. We then used the adjusted tree topology as user tree in a second maximum likelihood analysis in MEGA7, and the resulting tree with branch lengths served in turn as input, along with our four-gene matrix, for a RELTIME analysis in MEGA7 in order to obtain a timetree [S8]. For this, nodes were time-constrained following settings in www.timetree.org [S9–S12]

The resulting tree was then edited using R [S13] and R studio [S14] with the “ggtree” package [S15]. Final figure design was completed in Adobe Illustrator (CS5.1).

Data and Software Availability

The entire dataset is accessible at http://dx.doi.org/10.17632/rjzwxcpfrm.1. Sequences of sufficient length have also been deposited in the NCBI's GenBank under accession numbers MH094669-MH094740.

Supplementary Discussion 1.

Exceptions to the molecular resistance solution – To our knowledge the molecular changes to the Na^+/K^+-ATPase present the only solution vertebrates have evolved to allow consumption of bufadienolides. However, several species of invertebrates have impermeable membranes or midguts that prevent cardiac glycoside toxins from reaching sensitive areas [S16,S17]. Crayfish are able to feed on bufonid eggs, as well as tetrodotoxin producing amphibians, while at the same time having no apparent molecular-based resistance to tetrodotoxin [S18,S19]. It is possible that the same detoxification mechanism employed to deal with tetrodotoxin during feeding is also applied to prevent harm from bufadienolides.

Within snakes there is evidence that some species have adaptations to help counteract the impacts of bufadienolides instead of, or in addition to, the widespread molecular changes detailed in Ujvari et al. [S5,S20,S21]. Some snakes have different hormonal responses that can limit the impact of bufadienolides [S20,S21], and there are species that use the resistant mutant Na^+/K^+-ATPase, described by Ujvari et al. [S5] and utilised here for genotyping resistance in Malagasy vertebrates, more effectively by concentrating it at critical organs [S22]. These additional mechanisms are present in those snakes that specialise on bufonid prey, and are even capable of sequestering bufadienolides it [S20]. Nonetheless, to date, every snake species that is known to consume bufonids has been found to have "resistant"

144 molecular substitutions to their Na^+/K^+-ATPase [S1]. However, dietary records show
145 that many non-bufophagous snakes also harbour the same substitutions [S1]. This
146 mismatch between diet and resistance may suggest there are further modifications
147 required to gain the ability to consume toads without ill effects. It could also suggest
148 that the cost for maintaining these substitutions is low [S1]. Despite the imperfect
149 connection between the resistant Na^+/K^+-ATPase and bufophagy, it remains
150 apparent that the substitutions detailed in Ujvari et al. [S5] represent a prerequisite
151 for resistance to bufadienolides in snakes. Therefore, genotyping the Na^+/K^+-ATPase
152 remains a suitable way to detect vulnerability to bufadienolides.

153

154 **Supplementary Discussion 2.**

155 *The behavioural contingent and the adaptability of species* – The genetic evidence
156 we present demonstrates the potential for widespread poisoning of species. It does
157 not provide adequate information to predict the actual on-ground effects of the toad's
158 introduction to Madagascar.

159 Firstly, species will experience drastic differences in exposure to the toad. Some
160 species, like the almost exclusively arboreal snake *Langaha madagascariensis* may
161 rarely encounter the terrestrial *Duttaphrynus melanostictus* [S23], whereas other
162 species, such as Madagascar's large mammals, are predicted to experience
163 significant niche overlap with the toads [S24]. Differences in a species' natural
164 history, such as diet and circadian rhythm, need to be explicitly investigated to
165 predict the toad's impacts.

166 Secondly, there is evidence that species can rapidly adapt their behaviour and
167 physiology to new pressures [S25]. There are several examples in Australia,

168 covering three orders, where non-resistant native species have learnt to avoid
169 consuming toads. Bird species have been seen to selectively consume only the least
170 toxic parts of toads [S26], and there is evidence that reptile, amphibian and
171 mammalian species can learn to avoid toxic prey via taste aversion [S27–S31].

172 Thirdly, caution must be taken when extrapolating laboratory results into a whole
173 ecosystem context. Species do not exist in isolation and their trophic interactions
174 may dramatically alter how an invasive species affects them. In Australia, field
175 studies have not followed the predicted patterns of laboratory results [S32,S33] and
176 others see geographic variation [S34]. Furthermore, some species, despite being
177 sensitive to bufadienolides [S33], have actually benefited from the toads' presence,
178 as their main predators have been poisoned by the toads [S35]. Ultimately, species
179 interactions and the adaptability of species, both behaviourally and physiologically,
180 limit our ability to accurately predict the impacts of an invasive toxic toad. However,
181 the genetic insight presented here, where the vast majority of sampled species are
182 vulnerable to the toxic effects of the toad, strongly suggests that, while the precise
183 nature of the impact of the toads on individual species may be difficult to predict,
184 there is a high likelihood of significant perturbation of the dynamics of predator-prey
185 communities in Madagascar due to the selective rarefication or extinction of
186 particularly vulnerable predator species.

187

188 **Table S1. Related to Figure 1.** The amino acid sequence of each species' Na^+/K^+-
189 ATPase bufadienolide binding site and supplementary information relating to each
190 sample. Abbreviations correspond to: SMNS = Staatliches Museum für Naturkunde
191 Stuttgart, ZCMV = Zoological Collection Miguel Vences (field series of M. Vences),
192 FGZC = Frank Glaw Zoological Collection (Field series of Frank Glaw), ZSM =

193 Zoologische Staatssammlung München and MK = DNA extraction numbers (M.

194 Vences laboratory).

195

Supplementary References

S1. Mohammadi, S., Gompert, Z., Gonzalez, J., Takeuchi, H., Mori, A., and Savitzky, A.H. (2016). Toxin-resistant isoforms of Na+/K+-ATPase in snakes do not closely track dietary specialization on toads. Proc. R. Soc. B Biol. Sci. *283*, 20162111.

S2. Moore, D.J., Halliday, D.C.T., Rowell, D.M., Robinson, A.J., and Keogh, J.S. (2009). Positive Darwinian selection results in resistance to cardioactive toxins in true toads (Anura: Bufonidae). Biol. Lett. *5*, 513–6.

S3. Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., and Madden, T.L. (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics *13*, 1–11.

S4. Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol. Biol. Evol. *33*, 1870–1874.

S5. Ujvari, B., Casewell, N.R., Sunagar, K., Arbuckle, K., Wüster, W., Lo, N., O'Meally, D., Beckmann, C., King, G.F., Deplazes, E., *et al.* (2015). Widespread convergence in toxin resistance by predictable molecular evolution. Proc. Natl. Acad. Sci. *112*, 11911–11916.

S6. Crottini, A., Madsen, O., Poux, C., Strauss, A., Vieites, D.R., and Vences, M. (2012). Vertebrate time-tree elucidates the biogeographic pattern of a major biotic change around the K-T boundary in Madagascar. Proc. Natl. Acad. Sci. *109*, 5358–5363.

S7. Prum, R.O., Berv, J.S., Dornburg, A., Field, D.J., Townsend, J.P., Lemmon,

E.M., and Lemmon, A.R. (2015). A comprehensive phylogeny of birds (Aves) using targeted next-generation DNA sequencing. Nature *526*, 569–573.

S8. Tamura, K., Battistuzzi, F.U., Billing-Ross, P., Murillo, O., Filipski, A., and Kumar, S. (2012). Estimating divergence times in large molecular phylogenies. Proc. Natl. Acad. Sci. U. S. A. *109*, 19333–19338.

S9. Hedges, S.B., Dudley, J., and Kumar, S. (2006). TimeTree: A public knowledge-base of divergence times among organisms. Bioinformatics *22*, 2971–2972.

S10. Kumar, S., and Hedges, S.B. (2011). Timetree2: Species divergence times on the iPhone. Bioinformatics *27*, 2023–2024.

S11. Hedges, S.B., Marin, J., Suleski, M., Paymer, M., and Kumar, S. (2015). Tree of life reveals clock-like speciation and diversification. Mol. Biol. Evol. *32*, 835–845.

S12. Kumar, S., Stecher, G., Suleski, M., and Hedges, S.B. (2017). TimeTree: a resource for timelines, timetrees, and divergence times. Mol. Biol. Evol. *34*, 1812–1819.

S13. R Core Team (2017). R: A language and environment for statistical computing.

S14. R Studio Team (2016). RStudio: Integrated Development Environment for R.

S15. Yu, G., Smith, D.K., Zhu, H., Guan, Y., and Lam, T.T.Y. (2017). ggtree: an r package for visualization and annotation of phylogenetic trees with their covariates and other associated data. Methods Ecol. Evol. *8*, 28–36.

S16. Petschenka, G., Pick, C., Wagschal, V., and Dobler, S. (2013). Functional evidence for physiological mechanisms to circumvent neurotoxicity of

cardenolides in an adapted and a non-adapted hawk-moth species. Proc. R. Soc. B Biol. Sci. *280*, 20123089.

S17. Scudder, G.G.E., and Meredith, J. (1982). The permeability of the midgut of three insects to cardiac glycosides. J. Insect Physiol. *28*, 689–694.

S18. Wilson, N.J., and Williams, C.R. (2014). A critical review of freshwater crayfish as amphibian predators: capable consumers of toxic prey? Toxicon *82*, 9–17.

S19. Wilson, N.J., Stokes, A.N., Hopkins, G.R., Brodie, Jr., E.D., and Williams, C.R. (2014). Functional and physiological resistance of crayfish to amphibian toxins: tetrodotoxin resistance in the white river crayfish (*Procambarus acutus*). Can. J. Zool. *92*, 939–945.

S20. Mohammadi, S., French, S.S., Neuman-Lee, L.A., Durham, S.L., Kojima, Y., Mori, A., Brodie, E.D., and Savitzky, A.H. (2017). Corticosteroid responses of snakes to toxins from toads (bufadienolides) and plants (cardenolides) reflect differences in dietary specializations. Gen. Comp. Endocrinol. *247*, 16–25.

S21. Mohammadi, S., Mccoy, K.A., Hutchinson, D.A., Gauthier, D.T., and Savitzky, A.H. (2013). Independently evolved toad-eating snakes exhibit sexually dimorphic enlargement of adrenal glands. J. Zool. *290*, 237–245.

S22. Mohammadi, S., Savitzky, A.H., Lohr, J., and Dobler, S. (2017). Toad toxin-resistant snake (*Thamnophis elegans*) expresses high levels of mutant Na+/K+-ATPase mRNA in cardiac muscle. Gene *614*, 21–25.

S23. Glaw, F., and Vences, M. (2007). A Field Guide to the Amphibians and Reptiles of Madagascar 3rd ed. (Cologne: Vences & Glaw Verlag).

S24. Brown, K.A., Farris, Z.J., Yesuf, G., Gerber, B.D., Rasambainarivo, F., Karpanty, S., Kelly, M.J., Razafimahaimodison, J.C., Larney, E., Wright, P.C.,

et al. (2016). Modeling co-occurrence between toxic prey and naïve predators in an incipient invasion. Biodivers. Conserv. *25*, 1–19.

S25. Phillips, B.L., and Shine, R. (2006). An invasive species induces rapid adaptive change in a native predator: cane toads and black snakes in Australia. Proc. R. Soc. B Biol. Sci. *273*, 1545–1550.

S26. Beckmann, C., and Shine, R. (2011). Toad's tongue for breakfast: exploitation of a novel prey type, the invasive cane toad, by scavenging raptors in tropical Australia. Biol. Invasions *13*, 1447–1455.

S27. Brown, G., Shine, R., Somaweera, R., and Webb, J. (2011). Hatchling Australian freshwater crocodiles rapidly learn to avoid toxic invasive cane toads. Behaviour *148*, 501–517.

S28. Webb, J.K., Pearson, D., and Shine, R. (2011). A small dasyurid predator (*Sminthopsis virginiae*) rapidly learns to avoid a toxic invader. Wildl. Res. *38*, 726–731.

S29. Greenlees, M.J., Phillips, B.L., and Shine, R. (2010). Adjusting to a toxic invader: native Australian frogs learn not to prey on cane toads. Behav. Ecol. *21*, 966–971.

S30. Price-Rees, S.J., Webb, J.K., and Shine, R. (2011). School for skinks: can conditioned taste aversion enable bluetongue lizards (*Tiliqua scincoides*) to avoid toxic cane toads (*Rhinella marina*) as prey? Ethology *117*, 749–757.

S31. Jolly, C.J., Shine, R., and Greenlees, M.J. (2016). The impacts of a toxic invasive prey species (the cane toad, *Rhinella marina*) on a vulnerable predator (the lace monitor, *Varanus varius*). Biol. Invasions *18*, 1499–1509.

S32. Brown, G.P., Phillips, B.L., and Shine, R. (2011). The ecological impact of

invasive cane toads on tropical snakes: Field data do not support laboratory-based predictions. Ecology *92*, 422–431.

S33. Phillips, B.L., Brown, G.P., and Shine, R. (2003). Assessing the potential impact of cane toads on Austrialian snakes. Conserv. Biol. *17*, 1738–1747.

S34. Somaweera, R., Shine, R., Webb, J., Dempster, T., and Letnic, M. (2013). Why does vulnerability to toxic invasive cane toads vary among populations of Australian freshwater crocodiles? Anim. Conserv. *16*, 86–96.

S35. Doody, J.S., Castellano, C.M., Rhind, D., and Green, B. (2013). Indirect facilitation of a native mesopredator by an invasive species: Are cane toads re-shaping tropical riparian communities? Biol. Invasions *15*, 559–568.