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1 **Widespread vulnerability of Malagasy predators to the toxins of an introduced**
2 **toad**

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17 **Keywords:** invasive species, Madagascar, biodiversity, conservation, resistance,
18 poisoning, toxicity, bufonid,

19

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

25 **Highlights:**

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

30

31 **Summary**

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

46

47

48

49 **Main Text**

50 Invasive species are a key factor contributing to the global decline of biodiversity [1].
51 Therefore, understanding the mechanisms responsible is crucial if detrimental effects
52 are to be mitigated [1]. One such mechanism is the introduction of a novel defensive
53 strategy by which invasive species can disrupt native predator communities [2].
54 Significant disruption of such communities can produce trophic cascades and can
55 have an impact on a diverse array of taxa [2]. Madagascar, a globally significant
56 biodiversity hotspot, has recently experienced the introduction of a toxic bufonid
57 amphibian, the Common Asian Toad (*Duttaphrynus melanostictus*) [3]. Since its
58 invasion, the toad population has expanded rapidly, making control problematic and
59 eradication likely impossible [4]. Previous cases of bufonid introductions, such as the
60 infamous and ongoing spread of the cane toad (*Rhinella marina*) in Australia, have
61 resulted in the decimation of many indigenous species [2], prompting fears that
62 Madagascar may be similarly impacted [4]. Here we show that these fears are
63 warranted: we demonstrate that a wide diversity of Malagasy vertebrates are likely to
64 be susceptible to the toxic secretions of this invasive toad.

65 Bufonid toads secrete potent forms of cardiac glycosides known as bufadienolides to
66 defend themselves from predators [5]. These molecules exert toxic effects by binding
67 to the sodium-potassium pump ($\text{Na}^+/\text{K}^+\text{-ATPase}$) of cells, resulting in the inhibition of
68 ion transport, causing cardiotoxic effects and, ultimately, death [6]. Although
69 bufadienolides are highly toxic to naïve predators, many species from diverse animal
70 lineages (e.g., certain reptiles, amphibians and mammals) have evolved resistance
71 and readily consume toads without suffering ill effects [7]. Resistant species are
72 phylogenetically diverse, yet the adaptations that confer tolerance are remarkably
73 consistent, representing a fascinating example of convergent molecular evolution
74 (with only a few exceptions, see Supplemental Discussion 1). In each case, two
75 amino acid replacements, with at least one adding charge, in the first extracellular

76 domain (H1-H2) of the alpha 1 or alpha 3 Na⁺/K⁺-ATPase perturb binding
77 interactions with the bufadienolides, resulting in target site insensitivity [7]. The
78 universality of this resistance mechanism means that by sequencing a short portion
79 of the relevant gene, we can reliably predict a species' vulnerability to
80 bufadienolides.

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

91 We therefore sequenced the H1-H2 extracellular domain of the Na⁺/K⁺-ATPase from
92 77 Malagasy species, including 27 snakes, 2 lizards, 12 frogs, 8 mammals and 28
93 birds (GenBank accessions MH094669-MH094740), to examine the amino acid
94 composition in the bufadienolide binding site. In addition, we analysed data from the
95 genomes of 11 previously sequenced species found on Madagascar.

96 The Malagasy snakes sampled cover all three macrostomatan snake colonisations
97 of Madagascar [10]. All showed identical amino acid sequences in the H1-H2
98 extracellular domain of the Na⁺/K⁺-ATPase, matching other non-resistant snakes
99 [7,9] and providing strong evidence that the Malagasy species are likely to be highly
100 sensitive to the toxins of *D. melanostictus*. The two studied gerrhosaurid lizards

101 (*Zonosaurus* spp.) also exhibited the susceptible genotype, which matches the
102 demonstrably non-resistant Australian lizards [7,8]. Existing dietary studies lead us to
103 suggest that many of the sequenced reptile species will likely be directly impacted
104 via poisoning, as they are known to feed on amphibians [10]. However, the exact
105 nature of the effects on different species may be difficult to predict due to the
106 complexity of ecosystem-level trophic interactions (see Supplemental Discussion 2).

107 Of the 12 frog species sequenced, 11 showed genotypes with high degrees of
108 similarity to non-resistant frogs. We found a few species with amino acid
109 replacements in the middle of the H1-H2 extracellular domain, but the location and
110 physicochemical properties of these replacements seem unlikely to confer resistance
111 to bufadienolides, as none add charged amino acids, nor are any positioned at sites
112 previously associated with resistance [7]. Only the introduced Indian bullfrog
113 (*Hoplobatrachus tigerinus*) had amino acid replacements (including an insertion) that
114 might confer resistance; however, without further experimental evidence resistance
115 remains speculative.

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

122 We examined sequences of 34 bird taxa, 31 of which have a Na⁺/K⁺-ATPase H1-H2
123 domain that shows no evidence of amino acid replacements likely to confer
124 resistance to bufadienolides. While some of the endemic birds sampled are not at
125 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 manakin *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

151 relatedness could increase the chances of phylogenetically conserved vulnerability
152 [9,10]. Our findings stress the importance of the timely investment of resources to
153 monitor and control the spread of this alien species in order to prevent a worsening
154 biodiversity crisis in Madagascar.

155

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164 (Direction des Eaux et Forêts, DEF) for research permits (see supplemental
165 information).

166

167 **Author Contributions**

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

172

173 **Declaration of Interests**

174 The authors declare no competing interests.

175

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206

207 **Figure Legends**

208 Figure 1. Dated molecular phylogeny of the sampled diversity of taxa tested for
209 bufadienolide-resistant Na⁺/K⁺-ATPase genotypes, demonstrating a lack of
210 resistance across almost the entire breadth of the Malagasy vertebrate fauna.

211 Representative resistant non-Malagasy taxa have been included for phylogenetic
212 context.

213

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

Legend

- Species – non-resistant
- Species – likely sensitive
- Species – resistant

- – Madagascar endemic
- ◐ – Madagascar and islands endemic*
- – non-endemic
- ⊗ – not in Madagascar

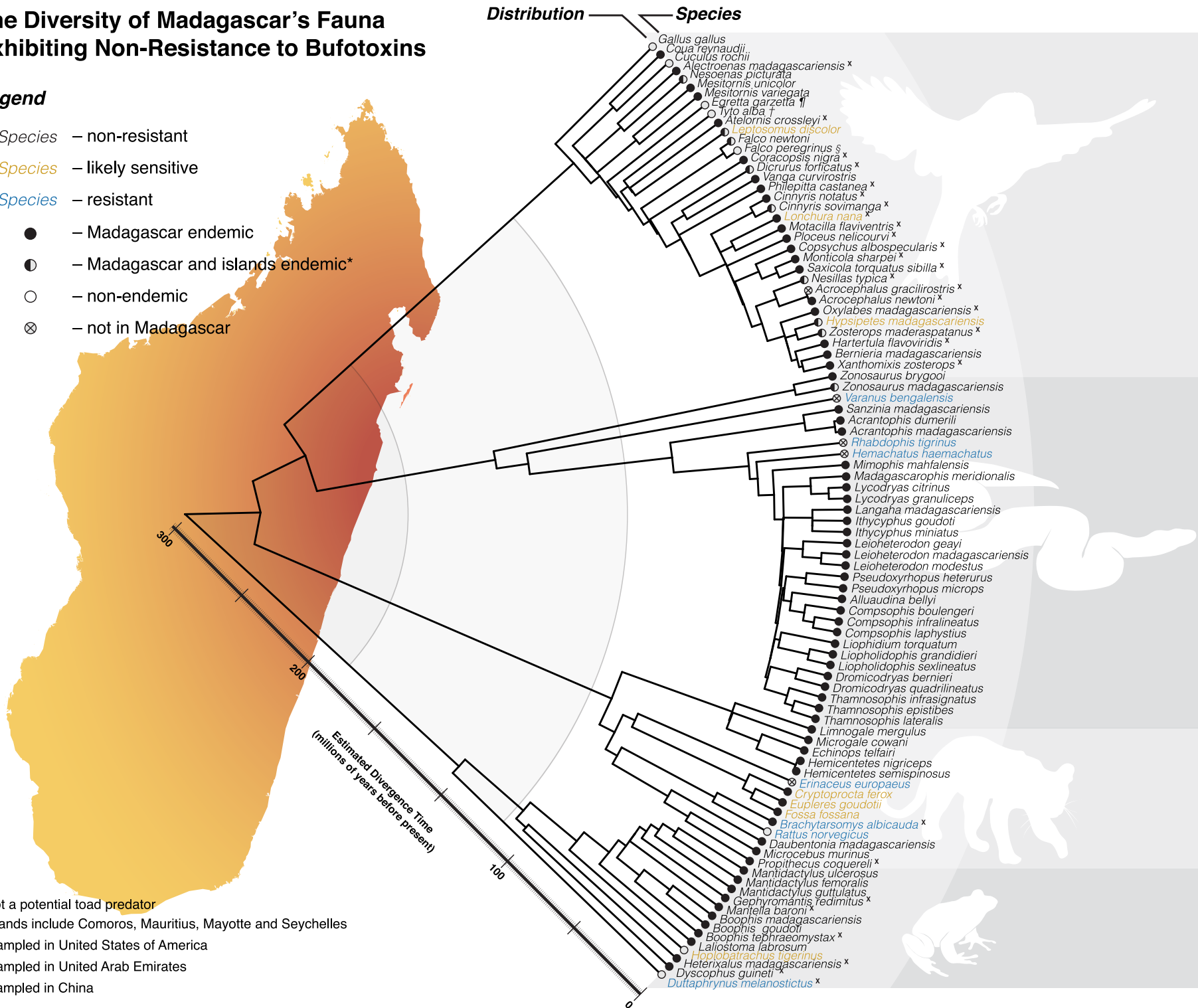
* Not a potential toad predator

* Islands include Comoros, Mauritius, Mayotte and Seychelles

† Sampled in United States of America

§ Sampled in United Arab Emirates

¶ Sampled in China



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

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5 Wolfgang Wüster

6

7 **Supplementary Methods**

8 **Experimental Model and Subject Details**

9 All field research, collecting of specimens, including in situ euthanasia of specimens
10 were approved by the Madagascan Ministère de l'Environnement, des Eaux et des
11 Forêts (Direction des Eaux et Forêts, DEF) under the following permits: 156-
12 MEF/SG/DGEF/DADF/SCB dated 12 December 2002; 238MINENVEF/SG/
13 DGEF/DPB/SCBLF dated 14 November 2003;
14 238MINENV.EF/SG/DGEF/DPB/SCBLF/ RECH dated 22 December 2004;
15 272MINENV.EF/SG/DGEF/DPB/SCBLF/RECH dated 8 November 2005;
16 298MINENV.EF/SG/DGEF/DPB/SCBLF/RECH dated 22 December 2006;
17 036/08MEEFT/SG/DGEF/DSAP/SSE dated 30 January 2008;
18 26/09/MEEFT/SG/DGEF/DSAP/ SLRSE dated 3 February 2009;
19 48/09/MEEFT/SG/DGEF/DSAP/SSE dated 9 March 2009;
20 188/09/MEEFT/SG/DGEF/DSAP/SSE dated 16 September 2009;
21 195/09/MEEFT/SG/DGEF/DSAP/SSE dated 28 September 2009;
22 314/10/MEF/SG/DGF/DCB.SAP/SCB dated 4 November 2010, and
23 232/12/MEF/SG/DGF/DCB.SAP/SCB dated 4 September 2012. Export of specimens
24 was approved by the DEF under permits: 063C-EA02/MG03, dated 26 February

25 2003; 094C-EA03/MG04, dated 1 March 2004; 103C-EA03/MG05, dated 15 March
26 2005; E1400/06, dated 1 June 2006; 055N-EA03/MG10, dated 25 March 2010.
27 Collection permit for bird samples (specimens released after sampling) were from 10
28 September 2003 (No. 0182 et 0184 /MINENVEF/SG/DGEF/ DPB/SCBLF), 19
29 October 2004 (No. 234 /MINENVEF/SG/DGEF/DPB/SCBLF/RECH), 4 November
30 2005 (No. 262 et 261/MINENVEF/SG/DGEF/DPB/SCBLF/RECH), 21 November
31 2006 (No. 275 et 276/MINENVEF/SG/DGEF/DPB/SCBLF/RECH), 4 December 2007
32 (No. 0296/07/MEEFT/SG/DGEF/ DPSAP/SSE), and renewals of No. 296/07 on 19
33 November 2010 (No. 335/10/MEF/SG/ DGF/DCB.SAP/SCB, No.
34 284/12/MEF/SG/DGF/DCB.SAP/SCB on 8 November 2012 and 7 October 2014 (No.
35 265/14/MEEF/SG/DGF/DCB.SAP/ScB).

36

37 **Method Details**

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

41 Amplification of the H1-H2 domain of the Na⁺/K⁺-ATPase was undertaken via
42 Polymerase Chain Reaction (PCR). PCR mixes were created using pure water, PCR
43 buffer Reddymix (Thermo Fisher) at 1X, forward and reverse primers at 0.3 μM, and
44 template DNA at around 20 ng/μl. For all reactions, a total volume of 15-16 μl was
45 used. The complete mixes were placed into a Bio-rad DNA Engine Tetrad 2 Peltier
46 Thermal Cycler. The PCR procedure entailed an initial denaturing at 94°C for 2
47 minutes, followed by 40 cycles of denaturing for 30 seconds at 94° C, annealing for
48 30 sec at primer-specific temperatures (51.5°C: ATP_178; 52°C: ATP1a1_PaPa1;

49 54°C: ATP1a1_GaGa2 and ATP1a1_FaPe2; 55°C: ATP1a1_EcTe1; 56°C: ATP1a3),
50 and an extension step at 72°C for 1 minute and a final extension step at 72°C for 5
51 minutes, followed by a cooling period at 4° C for 15 minutes.

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

57 Primers used to amplify the alpha 1 isoform of the H1-H2 domain of the Na⁺/K⁺-
58 ATPase for mammals and birds were designed using the National Center for
59 Biotechnology Information's (NCBI) Primer-Blast [S3]. Three pairs of primers (5'- >
60 3') were designed, based on the GenBank records for *Gallus gallus* (NM_205521.1),
61 *Falco peregrinus* (XM_005231095.2), *Panthera pardus* (XM_019457963.1) and
62 *Echinops telfairi* (XM_004714862.2): ATP1a1_GaGa2 (FWD =
63 ATGGGTMAAGTTCTGTCTCGGC, RVS = GCACCAWGTTCCTTGAASGACT),
64 ATP1a1_FaPe2 (FWD = CGGCAGCTCTTYGGAGGAT, RVS =
65 AACCAACAGCTGCCAACACRA), ATP1a1_PaPa1 (FWD =
66 ATGGGTCAAGTTCTGTCTCGGC, RVS = GAKAGKACCACRCCAAGATAS),
67 ATP1a1_EcTe1 (FWD = TSTTYGGGGGCTTCTCAATG, RVS =
68 GGAWAGCACCCACRCCRAGRT).

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

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

76

77 **Quantification and Statistical Analyses**

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

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

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

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

103 To visually represent phylogenetic relationships and divergence times among the
104 taxa, we first computed a phylogeny of all species included in our study plus a series
105 of additional, informative taxa on the basis of three mitochondrial genes (16S rRNA,
106 cytochrome oxidase subunit 1, cytochrome b) and one nuclear gene (recombination-
107 activating gene 1), under the maximum likelihood optimality criterion in MEGA7 with
108 a general-time reversible substitution model, and using a lungfish (*Protopterus*
109 *aethiopicus*) as outgroup. The resulting tree was manually adjusted to fit current
110 phylogenetic knowledge, as summarized in www.timetree.org and in recent
111 phylogenetic and phylogenomic studies [S6,S7]. We then used the adjusted tree
112 topology as user tree in a second maximum likelihood analysis in MEGA7, and the
113 resulting tree with branch lengths served in turn as input, along with our four-gene
114 matrix, for a RELTIME analysis in MEGA7 in order to obtain a timetree [S8]. For this,
115 nodes were time-constrained following settings in www.timetree.org [S9–S12]
116 The resulting tree was then edited using R [S13] and R studio [S14] with the “ggtree”
117 package [S15]. Final figure design was completed in Adobe Illustrator (CS5.1).

118

119 **Data and Software Availability**

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

123

124 **Supplementary Discussion 1.**

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

134 Within snakes there is evidence that some species have adaptations to help
135 counteract the impacts of bufadienolides instead of, or in addition to, the widespread
136 molecular changes detailed in Ujvari et al. [S5,S20,S21]. Some snakes have
137 different hormonal responses that can limit the impact of bufadienolides [S20,S21],
138 and there are species that use the resistant mutant Na⁺/K⁺-ATPase, described by
139 Ujvari et al. [S5] and utilised here for genotyping resistance in Malagasy vertebrates,
140 more effectively by concentrating it at critical organs [S22]. These additional
141 mechanisms are present in those snakes that specialise on bufonid prey, and are
142 even capable of sequestering bufadienolides it [S20]. Nonetheless, to date, every
143 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

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