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### Investigating the potential susceptibility of selected Malagasy species to the toxins produced by *Duttaphrynus melanostictus* (Asian Common Toad)

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**Investigating the potential susceptibility of selected  
Malagasy species to the toxins produced by *Duttaphrynus  
melanostictus* (Asian Common Toad)**

**Master of Science by Research in Biological Sciences  
Bangor University**

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2017

Supervised by Dr. Wolfgang Wüster

# **Investigating the potential susceptibility of selected Malagasy species to the toxins produced by *Duttaphrynus melanostictus* (Asian Common Toad)**

## **Abstract**

Invasive and introduced species can pose major ecological challenges to vulnerable native wildlife. Biodiversity hotspots, in particular, require protection from this significant cause of species loss. One hotspot, Madagascar, is experiencing the accidental introduction of a potentially ecologically damaging species – the toxin carrying bufonid toad, *Duttaphrynus melanostictus*. The presence of these toxic invaders drives fears that if such a species gains a foothold widespread poisoning of Malagasy predators could occur, mirroring the invasion of Australia by *Rhinella marina*. This includes numerous endemic and endangered species. The mechanism by which the toxin acts upon organisms has been previously identified via the study of toxin resistant versus toxin non-resistant taxa. Specific amino acid substitutions are required on the organism's  $\text{Na}^+/\text{K}^+$ -ATPase for them to be resistant to bufonid toxin. This solution to combat the toxin is widely consistent across taxa providing a method to discover and predict toxin resistance or vulnerability. Here I investigate the  $\text{Na}^+/\text{K}^+$ -ATPase gene to detect vulnerability of a selection of Malagasy fauna to the toxins of *Duttaphrynus melanostictus*. It is discovered that no tested species on Madagascar have the capacity to survive ingestion of the novel toxin. The vulnerability is found in all examined species, including snakes, frogs, lizards, lemurs and tenrecs. The results suggest that the invasive *Duttaphrynus melanostictus* is liable to have significant impact on Malagasy fauna.

# Contents

<b>Abstract</b> .....	<b>i</b>
<b>Contents</b> .....	<b>ii</b>
<b>List of Tables</b> .....	<b>iii</b>
<b>List of Figures</b> .....	<b>iii</b>
<b>List of Accompanying Material</b> .....	<b>iii</b>
<b>Acknowledgements</b> .....	<b>iv</b>
<b>Author's Declaration</b> .....	<b>v</b>
<b>Definitions</b> .....	<b>vi</b>
<b>Introduction and Background</b> .....	<b>1</b>
<b>The Cases of Immunity</b> .....	<b>6</b>
Snakes .....	6
Insects .....	7
Mammals.....	7
Varanids .....	8
Exceptions.....	8
<b>Past Invasions</b> .....	<b>8</b>
Island Examples.....	9
The Australia Situation.....	10
<b>Madagascar and Invasions</b> .....	<b>15</b>
Fears and Potential Impacts .....	19
<b>Methods</b> .....	<b>24</b>
<b>Results</b> .....	<b>28</b>
<b>Results of PCR</b> .....	<b>28</b>
<b>Sequence Data</b> .....	<b>28</b>
Snakes .....	29
Lizards.....	30
Amphibians .....	31
Mammals.....	32
<b>Isoelectric Point</b> .....	<b>32</b>
<b>Literature Search</b> .....	<b>33</b>
<b>Discussion</b> .....	<b>36</b>
<b>Herpetofauna</b> .....	<b>37</b>
Snakes .....	37
Lizards.....	42
Amphibians .....	45
Chelonians .....	48
Crocodiles .....	49
<b>Mammals</b> .....	<b>50</b>
Carnivora.....	50
Lemurs .....	52
Tenrecs .....	53
Implications and Behavioural Adaptations.....	54
<b>Conclusions</b> .....	<b>58</b>
<b>References</b> .....	<b>59</b>
<b>Appendix</b> .....	<b>97</b>

# List of Tables

Table 1 - Species count and endemism of Madagascar, excluding introductions. Figures from Table 1 in Goodman and Benstead (2005). (Does not include estimations of amphibians from Vieites et al. (2009) and Perl et al. (2014)).	2
Table 2 - H1-H2 extracellular domain of the alpha 3 subunit of the Na <sup>+</sup> /K <sup>+</sup> -ATPase of Malagasy snakes compared to the known resistant snake <i>Rhabdophis subminiatus</i> ...	29
Table 3 - The amino acids found in the H1-H2 extracellular domain of the alpha 3 and alpha 1 subunit of the Na <sup>+</sup> /K <sup>+</sup> -ATPase of Malagasy lizards. Displayed alongside the alpha 3 make-up of known resistant <i>Varanus bengalensis</i> .....	30
Table 4 - The amino acids found in the H1-H2 extracellular domain of the alpha 1 subunit of the Na <sup>+</sup> /K <sup>+</sup> -ATPase of Malagasy amphibians. Displayed alongside the alpha 1 make-up of known resistant toxic toad <i>Duttaphrynus melanostictus</i> .....	31
Table 5 - The amino acids found in the H1-H2 extracellular domain of the alpha 1 subunit of the Na <sup>+</sup> /K <sup>+</sup> -ATPase of Malagasy mammals. Displayed alongside the alpha 1 make-up of known resistant <i>Erinaceus europaeus</i> .....	32
Table 6 - A summary of some natural history traits for selected Malagasy species. Whether they have been reported eating frogs, forage terrestrial, are active during morning, evenings or night, occur near water bodies, and can live in anthropogenic areas. 1 denotes a trait they are confirmed to possess, -1 is a confirmation of the opposite, 0 is inconclusive. ....	34

# List of Figures

Figure 1 - <i>Duttaphrynus melanostictus</i> photographed in its native range in Thailand. ....	2
Figure 2 - Diagram of the H1-H2 extracellular domain of the Na <sup>+</sup> /K <sup>+</sup> -ATPase. Displayed across the top of the diagram are the discovered amino acid substitutions that can confer heightened resistance to bufotoxins and similar compounds. Redrawn from Ujvari et al. (2015).....	5

# List of Accompanying Material

Appendix 1 - Full list of samples and associated numbers. ....	97
Appendix 2 - Qiagen DNeasy Protocol Summary.....	98
Appendix 3 - Results of the DNA extractions.....	98
Appendix 4 - GenBank records used during the primer design process. ....	101
Appendix 5 - Example gel gradient results. ....	102
Appendix 6 - Genbank records accession numbers for squamate species as seen in Ujvari et al. 2013; 2015 and Mohammadi et al 2016b.....	103
Appendix 7 - Details on the attempts to amplify mammalian and African snake samples..	105
Appendix 8 - Genbank results for other mammals covering the H1-H2 domain of the alpha 1 subunit of the Na <sup>+</sup> /K <sup>+</sup> -ATPase. ....	107
Appendix 9 - Genbank results for primates covering the H1-H2 domain of the alpha 1 subunit of the Na <sup>+</sup> /K <sup>+</sup> -ATPase. ....	107

Appendix 10 - Genbank results for crocodilians covering the H1-H2 domain of the alpha 1 subunit of the Na <sup>+</sup> /K <sup>+</sup> -ATPase. Along with the alpha 3 isoform for <i>Crocodylus porosus</i> . .....	108
Appendix 11 - Genbank results for carnivores covering the H1-H2 domain of the alpha 1 subunit of the Na <sup>+</sup> /K <sup>+</sup> -ATPase. ....	108

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# Definitions

## Anurophagous

- A specialisation or tendency to consume anurans.

## Bufophagous

- A specialisation or tendency to consume toads belonging to Bufonidae.

## Bufotoxin

- The group of toxins produced by toads belonging to Bufonidae.

## Cardiac glycoside

- A group of compounds based on a core of four or five carbon rings with the ability to modify the function of muscle and cardiac systems. Specially the sodium/potassium-pump ( $\text{Na}^+, \text{K}^+$ -ATPase)

## Isoforms

- Variations of the same protein that share functionality. They will have the same purpose but can differ in the exact amino acid sequence.

## Ouabain

- A member of the cardiac glycoside group. It acts upon organisms in a mechanistically similar way to bufotoxins. It has often been the chemical used by studies investigating the function of the  $\text{Na}^+, \text{K}^+$ -ATPase.

# Introduction and Background

Invasive species are a widely recognised problem (Chornesky and Randall 2003; Vitousek et al. 1997). They play a role in the current trend of heightened species extinctions and significant global change (Ceballos et al. 2017; Clavero and García-Berthou 2005; Doherty et al. 2016; Vitousek et al. 1997; *but also* see Gurevitch and Padilla 2004). The invasions themselves are often very difficult to predict and study (Mack et al. 2000). Such understanding is critical to mitigating negative effects and prioritising conservation actions that are forced to make use of limited resources (Murdoch et al. 2007).

Perhaps the most infamous are those that have occurred on islands and contribute to the vulnerability of these isolated ecosystems (Pimm et al. 1994; Reaser et al. 2007; Simberloff 1995; Wiles et al. 2003). Madagascar is one of these isolated island ecosystems. Biodiversity hotspots, like Madagascar, overall account for 44% of all plant species and 35% of all vertebrate species; and have lost over 88% of their primary vegetation (Myers et al. 2000b).

Madagascar is one of the most diverse and valuable biodiversity hotspots on the planet (Ganzhorn et al. 2001; Myers et al. 2000b). Its separation from Africa, for ~88 Ma, is partly responsible for incredible endemism (Crottini et al. 2012; Samonds et al. 2012; Yoder and Nowak 2006); and shields these species from pressures found in Africa and Asia (Ujvari et al. 2013). Its more significant isolation has only occurred more recently when shifts in ocean currents precluded oceanic dispersal (Ali and Huber 2010; Samonds et al. 2013).

It is home to thousands of endemic species, 739 of which are endemic vertebrates, with many still to be and currently being described (Lambert et al. 2017; Perl et al. 2014; Scherz et al. 2017; Vieites et al. 2009) – see Table 1 for breakdown (Goodman and Benstead 2005). Many of these species are vulnerable to climate change (Bellard et al. 2014) and are under severe pressure from habitat loss and fragmentation (Ganzhorn et al. 2001), especially on the east coast (Geldmann et al. 2014). If we were to lose Malagasy species, we would lose over 2.8% of global vertebrate biodiversity (Myers et al. 2000b). Unfortunately, Madagascar has already



lost 90.1% of its primary vegetation (Irwin et al. 2010), and its megafauna (Burney et al. 2004; Crowley 2010). Losing even a portion of the remaining herpetofauna would be a huge blow for an already pressured group of taxa (Böhm et al. 2013; Jenkins et al. 2014). Mammals are also feeling the pressure of a rapidly changing world (Alford 2011; Hoffmann et al. 2010).



Figure 1 - *Duttaphrynus melanostictus* photographed in its native range in Thailand.

Current efforts in Madagascar are focused on the more charismatic primates and birds (Andreone et al. 2012). This is not to downplay their importance; all Malagasy fauna require a concerted effort if they are to be conserved. They face a multitude of threats, from habitat degradation to the pet trade, many of which have been exacerbated by recent political instability (Andreone et al. 2006; Andreone et al. 2012; Barrett and Ratsimbazafy 2009; Schuurman and Lowry II 2009). As of 2010-2014 Madagascar gained a new resident, the toxic producing bufonid *Duttaphrynus melanostictus* (common Asian toad) (Kolby 2014a).

*Duttaphrynus melanostictus*, previously known as *Bufo melanostictus*, is a true toad, a member of the Bufonidae family (see Figure 1) (van Dijk et al. 2016). It is

	# of Species	# of Endemic	% Endemism
<b>Birds</b>	209	109	52%
<b>Mammals (non-volant)</b>	101	101	100%
<b>Mammals (bats)</b>	30	18	60%
<b>Reptiles</b>	340	314	92%
<b>Amphibians</b>	199	197	99%
<b>Total</b>	879	739	84%

Table 1 - Species count and endemism of Madagascar, excluding introductions. Figures from Table 1 in Goodman and Benstead (2005). (Does not include estimations of amphibians from Vieites et al. (2009) and Perl et al. (2014)).

widespread across much of South East Asia. It is found as far west as Pakistan and has been introduced to islands throughout the East (Döring et al. 2017; van Dijk et al. 2016). Recent work has suggested that the toads across this range may constitute multiple species (Wogan et al. 2016).

Within that range the toad is only found below 1,800 m above sea level. It is considered a widely adaptable species making use of a variety of lowland habitats from human dominated to densely forested (Mathew 1999; van Dijk et al. 2016). It is capable of breeding in brackish waters or ephemeral ponds; and exceptionally quickly with females producing thousands of eggs per year (McClelland et al. 2015; Ngo and Ngo 2013). The eggs hatch within 48 hours, and their tadpoles' can react differently depending on predation risk (Csurhes 2010; Mogali et al. 2011). After 34-90 days they will metamorphose (Csurhes 2010). Their breeding is not physically limited to one season, although wild populations tend to breed in correspondence with monsoons (Huang et al. 1997; Jørgensen et al. 1986; Mathew 1999). This rate of reproduction is potentially greater than the famously problematic invader-of-Australia *Rhinella marina* (cane toad; Breder 1946 in Wingate 2011; ISSG 2016), and this is thought to be key to their success (Urban et al. 2007). All of these traits are known to make *D. melanostictus* an adaptable and successful generalist, capable of sustaining healthy populations in the face of anthropogenic environmental modifications. Many of these traits are critical to Bufonidae's success and cosmopolitan distribution (Van Bocxlaer et al. 2010).

Like other members of Bufonidae, *D. melanostictus* it secretes a potent poison comprising various bufadienolides (types of cardiac glycoside), such as bufalin (Bagrov et al. 1993; Krenn and Kopp 1998; Ujvari et al. 2013). These toxins are created from cholesterol (Santa Coloma et al. 1984) and the exact cocktail of toxins varies across Bufonidae, between species and location (Gao et al. 2010; Zhang et al. 2005). The toxin mixes also vary ontogenetically; with the egg stage being the most potent and varied, decreasing in both until they metamorphose to their terrestrial forms, when the potency will increase as they begin producing toxin of their own – presuming *R. marina* is typical of the family (Hayes et al. 2009). This pattern is also reflected in the ontogenetic selection of prey by *Tropidonophis mairii* (Llewelyn et al. 2012).

This toxic secretion is an effective defensive mechanism; targeting the predating organism's sodium/potassium-pump ( $\text{Na}^+, \text{K}^+$ -ATPase; Flier et al. 1980). The  $\text{Na}^+, \text{K}^+$ -ATPase is responsible for the gradient that allows the absorption of  $\text{Na}^+$  (Lingrel 2010). Specifically, the toxin targets the H1-H2 extracellular domain of the  $\alpha$  subunit (Price et al. 1990), which is responsible for creating the  $\text{Na}^+, \text{K}^+$ -ATPase's catalytic capabilities (Lingrel 2010). Therefore, preventing the correct balance of  $\text{Na}^+$  and  $\text{Ca}^{2+}$ , and leading to increased smooth muscle contraction and blood pressure (Bagrov et al. 1993; Lingrel 2010) leading to variable symptoms ranging from muscle paralysis to severe arrhythmia (Garg et al. 2007).

The pump is expressed in all animal cells in four isoforms (Köksoy 2002). The alpha 1 isoform is expressed universally in all tissues, whereas isoforms alpha 2, 3 and 4 have only been found in certain areas (Juhaszova and Blaustein 1997; Köksoy 2002). Of interest to this study are the alpha 1 and 3 isoforms. The alpha 3 has been the most widely investigated for squamates and is primarily found in nerve and brain tissue (Köksoy 2002; Mohammadi et al. 2016b; Ujvari et al. 2015). It appears to be a more refined version of other isoforms with a lower affinity for  $\text{Na}^+$ , and more sensitive to inhibition by ouabain (Jewell and Lingrel 1991; Jewell and Lingrel 1992; Juhaszova and Blaustein 1997).

Alpha 1 studies have covered a variety of species as earlier efforts had difficulties separating isoforms (Blanco and Mercer 1998; Jewell and Lingrel 1991). There is little concern about the variation between isoforms as this is a highly conserved area of the genome and it has been shown that over the entire  $\text{Na}^+, \text{K}^+$ -ATPase they only vary by a few percent (Blanco and Mercer 1998; Köksoy 2002). Additionally, variation in sensitivity to ouabain (another cardiac glycoside) between different isoforms with identical H1-H2 extracellular domains is several magnitudes lower than the resistance found in the wild required to consume a bufonid toad (Blanco and Mercer 1998; Price et al. 1990). Although, it must be noted that the H1-H2 extracellular domain varies more than central areas of  $\text{Na}^+, \text{K}^+$ -ATPase (Blanco and Mercer 1998).

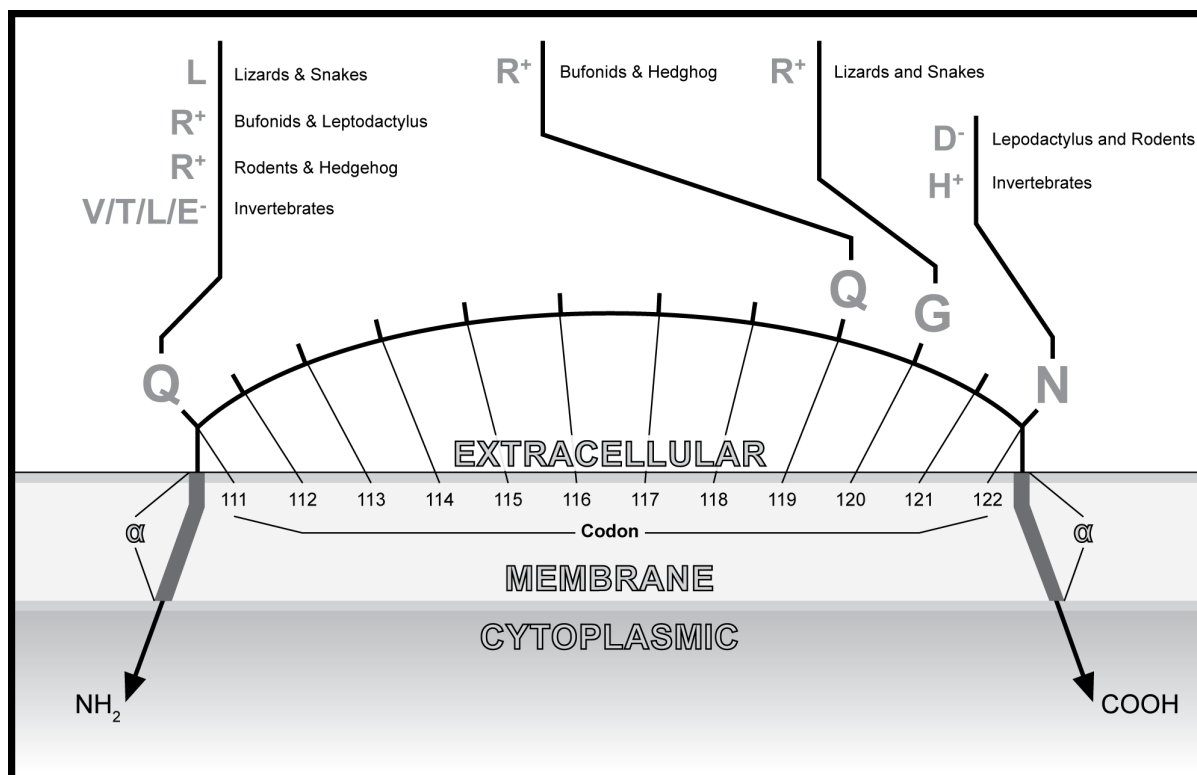


Figure 2 - Diagram of the H1-H2 extracellular domain of the Na<sup>+</sup>/K<sup>+</sup>-ATPase. Displayed across the top of the diagram are the discovered amino acid substitutions that can confer heightened resistance to bufotoxins and similar compounds. Redrawn from Ujvari et al. (2015).

It has been discovered that the primary amino acids mediating the cardiac glycoside interaction are located at each end of the H1-H2 extracellular domain – codon 111 and 119-122 (Price et al. 1990; Lingrel 2010). The primary means by which amino acids can confer resistance is by supplying a significant isoelectric charge (or residual charge), thereby inhibiting the binding of bufadienolides (Price and Lingrel 1988; Ujvari et al. 2015). Identification of select amino acids and the corresponding residual charge at either end of the H1-H2 domain allows detection of potential resistance or susceptibility to bufadienolides (Ujvari et al. 2015). Figure 2 illustrates a simplified structure of this critical binding site as well as the resistant conferring substitutions discovered so far. The previous identification of the important isoform for various taxa (isoform 1 for amphibians and mammals, isoform alpha 3 for squamates) means that detection of a known resistant pattern of amino acids or residual charge can provide a confident indicator of resistance (Ujvari et al. 2015).

However, some results indicate that hydrophobicity may also be a contributing factor, especially for the substitution occurring at codon 111 (Mohammadi et al. 2016b); and there is also evidence that substitutions other than those at H1-H2 extracellular

domain can confer a modicum of resistance (for summary see Croyle et al. 1997). These tend to be considerably weaker, and overall the aforementioned residual charges at either end of primary binding site, the H1-H2 domain, are the dominant determinant of toxin susceptibility (Croyle et al. 1997; Mohammadi et al. 2016b; Ujvari et al. 2013).

## The Cases of Immunity

There is a great diversity in the species that exhibit resistance to bufadienolides and similar compounds as the result of substitutions at codons 111 and 119, 120 or 122 (Ujvari et al. 2015). Bufonid toads themselves show amino acids 111R and 119D that confer resistance (Moore et al. 2009), and the greatest isoelectric charge (Ujvari et al. 2015).

### Snakes

Numerous snakes are bufophagous and have been found to be resistant, across a range of families (Mohammadi et al. 2013; 2016b; Ujvari et al. 2015). These snakes are all found to have the same mutation; Q111L and G120R (Mohammadi et al. 2016b; Ujvari et al. 2015), while those without do not display the same level of resistance (Mohammadi et al. 2016a). The most extreme example of resistance is perhaps *Rhabdophis tigrinus* (Asian tiger keelback). It is capable of not only consuming toads and their toxin but sequestering it for its own defence, a rare trait amongst vertebrates (Hutchinson et al. 2007).

Recent work by Mohammadi et al. (2016b) indicates that the resistance conferring point mutations are more widespread than bufophagy, suggesting there could be either no or little cost for the mutation in snakes. Comparisons between dog and rat kidneys have suggested that ouabain sensitivity does not hamper catalytic capabilities (Periyasamy et al. 1983). But this would be counter to findings in invertebrates, where the resistant forms of Na<sup>+</sup>,K<sup>+</sup>-ATPase are found to be less effective at ion transport (Dobler et al. 2012). Furthermore, snakes with resistance to other toxins do demonstrate trade-offs and selection (Brodie III and Brodie Jr 1999; Brodie Jr. et al. 2002). They additionally propose that these point mutations may be one fundamental part of a larger counter-toxin adaptation. Perhaps working in conjunction with adrenal glands (Mohammadi et al. 2013), or the mechanisms

present that allow sequestration (Hutchinson et al. 2007). Alternatively, gene duplication may have occurred providing the snake with a sufficient quantity of efficient non-resistant Na<sup>+</sup>,K<sup>+</sup>–ATPase to mitigate the costs of having resistant form Na<sup>+</sup>,K<sup>+</sup>–ATPase (Dalla and Dobler 2016; Mohammadi et al. 2016b). These duplications appear to be more important when considering evolutionary trade-offs than discerning resistance to bufadienolides. Regardless of additional mechanisms or duplications, discovering this resistance conferring mutation will serve as a predictor of susceptibility for bufonid-naïve populations, as for resistance to exist the resistant forms must be clearly present (Dalla and Dobler 2016; Mohammadi et al. 2016b; Ujvari et al. 2015).

### Insects

Insects similarly show resistance, allowing them to consume toxin generating plants and occasionally sequester the toxins (Agrawal et al. 2012; Duffey et al. 1978; Holzinger et al. 1992). Some only show one substitution, while the most resistant show two – 111V and 122H (Petschenka et al. 2013a). It is suggested that the double substitution is more connected to sequestration than just consumption (Petschenka et al. 2013a). The substitutions are found in butterflies, beetles, bugs and flies, all showing the 122H replacement that confers greater resistance (Dobler et al. 2012; Holzinger et al. 1992). The main difference between the insects and vertebrates is the precise amino acids involved, the mechanism and positions remain constant (Dobler et al. 2012).

### Mammals

Hedgehogs, a frequent toad predator and known to adorn themselves with the toxin to augment their own defence (Brodie Jr. 1977; Ewert and Traud 1978), can only do so because of charges at 111R and 119D (Ujvari et al. 2015). Studied rodents have the same replacements as *Erinaceus europaeus* (European hedgehog) (Price and Lingrel 1988; Ujvari et al. 2015). Even some that have had not been sympatric with toxic toads until recently can consume them, parotoid gland included (Cabrera-Guzmán et al. 2015). Once again possibly implying a limited cost to having a resistant Na<sup>+</sup>,K<sup>+</sup>–ATPase.

## Varanids

The patterns of immunity have also helped understand the evolutionary history of varanids (monitor lizards). They are found across South East Asia, Africa and down to Australia (Vidal et al. 2012). The species found in Africa and Asia are known toad predators and show resistance to the toxins they produce (Ujvari et al. 2013). By contrast their Australian cousins show no such resistance, having their populations decimated by the recently introduced *R. marina* (Ujvari and Madsen 2009; Ujvari et al. 2013). Ujvari et al. (2013) find the cause to be different amino acids on the codons 111, 119 and 120. It is then postulated that the Australian varanids lost their resistance in the millions of years since colonisation; potentially not as a consequence of negative selection but of relaxed selection (Ujvari et al. 2013). This naivety to toxins is expected on many islands whose species have limited shared evolutionary history with toads.

## Exceptions

While the substitutions at either end of the H1-H2 extracellular domain are the dominant solution to toxins, a few exceptions should be mentioned. Species of hawk moth have been shown to have impermeable membranes that prevent the toxins from reaching nerves (Petschenka et al. 2013b). Similarly, *Schistocerca gregaria* (locust) and *Periplaneta americana* (cockroaches) have impermeable midguts (Scudder and Meredith 1982). So far these adaptations are only apparent in invertebrates.

## Past Invasions

As alluded to, this toxin versus immunity arms race is not global, there are places where bufonids never dispersed naturally; Antarctica, Australasia, remote island chains, and Madagascar (Easteal 1981; Pramuk et al. 2008). In some of those places introduced toads are having significant effects. Toads like other invasive species can have a variety of impacts, understanding them is key to mitigation (Chornesky and Randall 2003; Mack et al. 2000). The impacts are apparent through a variety of channels: predation of native fauna by the invader, direct competition between native and invader species, introduction of novel pathogens, and via poisoning of native predators by the invaders novel defences among others (Kraus

2015). Here I present a summary of various toad/island invasions and the impacts that have arisen.

### Island Examples

Many island ecosystems are suffering from the damaging effects of introduced species (Pimm et al. 1994). Perhaps none so much as Guam. Other than the famously destructive introduction of *Boiga irregularis* (brown tree snake), there has been a series of botched and accidental introductions (McCoid 1993; Wiles et al. 2003). A mess of introduced rats, lizards (to control the rats), toads (to eat coconut plantation pests), and giant Africa snails (as a war time food source), has led to multiple cascading impacts ranging from poisoned lizards, increased domestic animal mortality and the release of pest species originally meant to be controlled (Lever 2001). Furthermore, Guam remains vulnerable to further introductions (Wiles 2000).

Jamaica is currently experiencing the introduction of a bufonid. Perhaps the most infamous invader, *R. marina*, was introduced in an attempt to control pests in the 19<sup>th</sup> century (Wilson et al. 2010). There the evolutionarily and behaviourally naïve *Epicrates subflavus* (Jamaican Boa) is being killed by *R. marina*' toxic secretions (Wilson et al. 2010). Lever (2001) gives an in depth review of all the areas *R. marina* has now become naturalised: from The Philippines and numerous Pacific islands; to Bermuda, the USA, and the Caribbean; and down to Papua New Guinea and Australia.

Timor-Leste is another in a string of islands that have had a some form of true toad introduced (McClelland et al. 2015; Trainor et al. 2009). Along with Bali where *D. melanostictus* is suspected to have out-competed native anurans (Church 1960); and American Samoa where *R. marina* is found to be consuming a wide variety of native prey (Grant 1996).

These introductions are further complicated as the ecological issues are paired with those of civil unrest and nation building meaning only recently have assessments started and much remains unknown (Kaiser et al. 2011; 2014).

Across these examples we find a range of outcomes, from the severe to the neutral. The introductions are especially worrying in biodiversity hotspots or when they threaten an especially unique and iconic species – e.g. *Varanus komodoensis*



(Komodo dragon) (Ujvari et al. 2014). Recent work with *V. komodoensis* highlights just how valuable these species could prove to be (Bishop et al. 2017).

### The Australia Situation

While many of these island introductions are not particularly well studied, one fortunately is – the introduction of *R. marina* to Australia. In 1935 *R. marina* was introduced to Queensland in an attempt to combat pests affecting sugar crops (Lever 2001). From then it has bred rapidly and expanded its range, with current estimates provided by Urban et al. (2007; 2008), building on those by Sutherst et al. (1996). The effects *R. marina* is having on native fauna are neither positive (with some exceptions), minor, nor limited to a few taxa (Shine 2010). Such effects have granted it a position amongst the 100 worst invasive species (ISSG 2016).

The most significant impact is on predators; naïve to the invaders' defences they consume poisonous prey items that results in widespread mortality (Shine 2010). As mentioned, Australian varanids no longer have the resistance that their forebears had, and Asian contemporaries have (Ujvari et al. 2013). This combined with behavioural naivety has led to reductions in varanid populations sympatric with *R. marina*; *Varanus mertensi* (Merten's water monitor) (Doody et al. 2009; 2014; Griffiths and McKay 2007), *Varanus mitchelli* (Mitchell's water monitor) (Doody et al. 2009), *Varanus panoptes* (Agus monitor) (Brown et al. 2013; Doody et al. 2009; Ujvari and Madsen 2009), *Varanus varius* (lace monitor) (Jolly et al. 2015; Jolly et al. 2016). And more are known to be vulnerable (Smith and Phillips 2006; Ujvari et al. 2013). Other large lizards such as *Bellatorias major* (land mullet), *Intellagama lesueurii* (Australian water dragon) and *Tiliqua scincoides intermedia* (northern blue-tongued skinks) are also vulnerable (Jolly et al. 2015; Pearson et al. 2014; Price-Rees et al. 2010). There may be evidence that these initial population crashes do not harm the long-term survival of species. Some areas and islands where *R. marina* have been introduced still have healthy populations suggesting the varanids there have learnt to avoid the toads (Easteal 1981; Jolly et al. 2016; Weijola et al. 2017). Although, learning may be minimal in some species (Llewelyn et al. 2014).

Many snakes are also poisoned by *R. marina*; *Antaresia children* (children's python), *Boiga irregularis* (brown tree snake), *Dendrelaphis punctulatus* (common tree snake), *Enhydris polylepsis* (Macleay's mud snake), *Stegonotus cucullatus* (slaty-

grey snake), *Acanthophis praelongus* (northern death adder), *Hemiaspis signata* (black-bellied swamp snake), *Pseudechis porphyriacus* (red-bellied black snake), *Pseudonaja textilis* (eastern brown snake) (Covacevich and Archer 1975; Phillips et al. 2003; Phillips and Fitzgerald 2004), and *Acanthophis praelongus* (Phillips et al. 2010; Webb et al. 2005). All these snakes have shown susceptibility to toad toxin and most are capable of consuming a toad large enough to deliver a fatal dose (Phillips et al. 2003). There are instances of population declines (Jolly et al. 2015; Phillips et al. 2010); and lower recruitment may lead to future prolonged declines (Phillips and Shine 2006a). Although these declines cannot be assumed to be spatially homogenous, nor guaranteed to follow patterns of resistance found in laboratory studies (Brown et al. 2011b), they remain a disturbing indication of *R. marina* impacts, whether sole instigator or part of a synergetic decline.

The impacts are not limited to populations. It has been shown that snakes' gape and head morphology will adapt depending on their prey (Queral-regil and King 1998; Bonnet et al. 2001). In areas of toad presence this has led to a reduction in head size among several Australian species (Phillips and Shine 2004). The introduction of toads has led to the negative selection of traits that make snakes more likely to be impacted by the toads (Phillips and Shine 2004; 2006b). Compounding these factors are likely to be sex and ontogenetic variation meaning impacts will vary within species as well as between species (Phillips et al. 2003; Webb et al. 2005).

Amongst the widespread vulnerability, there is one species that has been found to survive toad ingestion – *Tropidonophis mairii* (common keelback) (Covacevich and Archer 1975; Phillips et al. 2003). Unlike the Australian varanids, *T. mairii* has not lost this pre-adaptation developed during its sympatry with Bufonid toads (Phillips et al. 2003; Llewelyn et al. 2011). Why *T. mairii* maintains their resistance, while varanids lost theirs is still not fully understood. Llewelyn et al. (2011) suggest that perhaps the genes coding for resistance are also affecting other still-used traits, or that they are in the process of losing their resistance and their Asian ancestor was considerably more resistant than they are today. So, their present resistance may be due to arriving in Australia later than varanids.

Despite the inherited resistance, *T. mairii* appears to have not significantly incorporated *R. marina* into its diet (Llewelyn et al. 2010a; 2011); limiting its use in controlling *R. marina* populations. This may be due to several factors including

increased handling time, locomotion sacrifices and the lack of nutrition *R. marina* imparts to *T. mairii* (Llewelyn et al. 2009). This behaviour appears to be a product of evolutionary history as opposed to rapidly learnt (Llewelyn et al. 2011). However, they do appear to be benefiting from *R. marina*'s presence via the relief of predation pressure from varanids (Brown et al. 2013).

Evolutionary history may have a large role in defining the impacts of invasions (Llewelyn et al. 2011). This pattern may also be visible in crocodiles found in Australia. *Crocodylus porosus* (saltwater crocodile) is not particularly vulnerable to the toxins and is found to survive large dosages (Covacevich and Archer 1975; Smith and Phillips 2006). Their range reaches South East Asia as well as Northern Australia (Crocodile Specialist Group 1996); this sympatry and co-evolution has protected it from the bufotoxins (Letnic et al. 2008). *Crocodylus johnstoni* (freshwater crocodile), by contrast, is susceptible (Smith and Phillips 2006; Somaweera et al. 2013). Although the changes in populations convey a mixed story. Some populations remained steady when the toad arrived (Doody et al. 2009); but others have fallen by 73% in other areas (Doody et al. 2014; Letnic et al. 2008). These variations appear not to be caused by variation in toxic resistance (Somaweera et al. 2013); and are possibly linked to aridity and other factors affecting encounter rate (Letnic et al. 2008; Somaweera et al. 2013) or prey abundance (Britton et al. 2013; Doody et al. 2014). Crocodiles are additionally vulnerable due to their slow maturation, meaning fatal toad predation may undercut their recruitment more than for other species (Letnic et al. 2008). Alternatively, sub-adults may be capable of learning to avoid toads and *C. johnstoni* may ultimately benefit from reduced nest predation as varanids decline (Brown et al. 2011c).

There are other examples of toads affecting nesting. Doody et al. (2006) present an example where the nest predation on *Carettochelys insculpta* (pig-nosed turtles) has markedly decreased due to varanid declines. Conversely, Boland (2004) shows how the toads can directly harm nesting of native species, by usurping burrows and direct predation of young.

Mammals and birds are not exempt from the impacts of toads (Burnett 1997; Catling et al. 1999; Boland 2004). Species of quoll and dingoes are seeing declines (Burnett 1997; Catling et al. 1999), as well as various others in northern Australia (Woinarski et al. 2010). Whereas the relative paucity of research into birds leaves tentatively

positive impressions; suggesting their recent sympatry, and ability to selectively consume non-toxic prey components, are helping them weather the invasion (Beckmann and Shine 2009; Beckmann and Shine 2011; Covacevich and Archer 1975; Wilson et al. 2010). It should also be noted that several species of native rodent can consume toads without any apparent ill-effects (Shine 2010).

The impacts of a toad invasion have the potential to be mitigated by behavioural shifts. Quolls have been found to interrupt their predation of toads, and subsequently avoid them (Llewelyn et al. 2010b; Webb et al. 2008; 2011). Their ability to learn only requires one attempt at toad predation, immediately learning the scent cues to avoid (Webb et al. 2008; 2011). It is even suggested that a scheme to capture and condition Quolls against eating *R. marina* could help reduce their population declines (O'Donnell et al. 2010). Some varanid species have also demonstrated avoidance of toads; compared with other naïve populations it is clear that this new behaviour has only occurred within the last 70 years (Jolly et al. 2016; Llewelyn et al. 2014). These examples of adaptation present a less bleak future for some species, even if their body sizes are being impacted (Jolly et al. 2016).

Finally, amphibians are seeing a mixed response. Fears that the *R. marina* tadpole would consume native amphibian eggs and young are unfounded for some species (Crossland 1998), although these lab results may not hold entirely true in natural environments where *R. marina* exist in far denser numbers (Crossland 1998).

There is evidence that *R. marina* is having a very serious impact on some amphibian populations (Crossland et al. 2008); and is known to poison others (Crossland and Azevedo-Ramos 1999; Shine et al. 2009; Cabrera-Guzmán et al. 2011). Crossland et al.'s (2008) study suggests predation of *R. marina* eggs as a cause, but it is known that more complex interactions exist, for example the relative timing of egg laying could modify success and fitness of the invader versus native species (Crossland et al. 2009). Additionally, the specific characteristics of the species will alter the magnitude of effect (Crossland et al. 2008; Williamson 1999). Some can out-compete *R. marina* tadpoles (Cabrera-Guzmán et al. 2011; Cabrera-Guzmán et al. 2013a; Cabrera-Guzmán et al. 2013b). Such intense competition in the earlier stages of development can hamper the growth of *R. marina* potentially making them more vulnerable to predation and other mortality factors (Cabrera-Guzmán et al. 2011; Cabrera-Guzmán et al. 2013a; Pizzatto and Shine 2008; Pizzatto et al. 2008;

Ward-Fear et al. 2010a). There are suggestions that these species would make a useful component to an anti-toad campaign (Cabrera-Guzmán et al. 2011; Cabrera-Guzmán et al. 2013b).

Many frogs are likely to predate *R. marina* after metamorphosis also (Greenlees et al. 2010). Fortunately, similar to other taxa, Greenlees et al. (2010) have found that native frogs are capable of adapting behaviourally to avoid the toxic invader. This learning may not be restricted to full grown frogs (Crossland and Azevedo-Ramos 1999). After *R. marina*'s introduction other tadpole-eating species are less likely to attack tadpoles that superficially look like those of *R. marina* (Nelson et al. 2010). In this way, some native frog species may benefit from the learnt avoidance of tadpole predators; even if only for a short period (Nelson et al. 2011).

There is clearly a lot to learn from the Australian situation and the incursion of *R. marina*. Not only the mechanism for their success and potential impacts but avenues for their control (Shine 2012). Already mentioned is the use of resistant native species to out-compete and put pressure on *R. marina* during the developmental stages, but there are also efforts to investigate the possibility of using toad alarm pheromones to stress tadpoles and hamper their development (Hagman and Shine 2009). Alternatively, native invertebrates may hold the key to control. Several species of water beetles have been found to eat numerous *R. marina* tadpoles along with *Iridomyrmex reburrus* (Meat Ants) who can attack and consume metamorphosed toads (Cabrera-Guzmán et al. 2012; Ward-Fear et al. 2009; Ward-Fear et al. 2010a; Ward-Fear et al. 2010b)

All these impacts show just how dramatic and complex toad invasions can be, especially for reptiles that are not able to selectively eat non-toxic parts of toads nor survive predation to learn avoidance. Presently the toad is not causing outright extinctions but the genetic bottlenecks caused by significant declines could render species more vulnerable in the future (Madsen et al. 1996; 1999; 2004; Ujvari and Madsen 2009). It is expected over the long-term species will learn or adapt to the new addition (Webb et al. 2008; Shine et al. 2009), but this may be overly optimistic in situations encompassing multiple pressures.

## Madagascar and Invasions

It is also worth considering how Madagascar has coped with past invasions. Remarkably, Madagascar has had comparatively little attention directed towards invasive species (Kull et al. 2014); despite its island status and having already experienced numerous introductions since human colonisation 2,000 yrs BP (Bellard et al. 2014; Kull et al. 2014).

Introduced wild cats are found to be predating the vulnerable *Propithecus verreauxi verreauxi* (Verreaux's sifaka) (Andriaholinirina et al. 2014; Brockman et al. 2008), accompanied by Indian Civets and feral dogs harming other lemurs (Gould and Sauter 2007). Dogs are suspected to be partially responsible for the decline of *Cryptoprocta ferox* (fossa) (Barcala 2009), another vulnerable endemic (Hawkins 2016; Hawkins and Racey 2005). As introduced predators are excluding (Farris et al. 2016), and competing with endemic predators, there are fears of cascading effects as seen in other locations (Glen et al. 2007; Hawkins and Racey 2005; Johnson et al. 2007; Shine 2010). *Cryptoprocta ferox* with its diverse diet (Brown et al. 2016; Dollar et al. 2007), and possible keystone species status (Barcala 2009; Dollar 1999), means the effects of losing it could ripple through ecosystems.

Ultimately, it is difficult to discern whether these invasions are the primary factor in native fauna's declines (Gurevitch and Padilla 2004). What is certain is that they demand careful consideration if the native fauna are to be conserved.

Not that all introductions have been detrimental (Tassin and Kull 2015). *Eulemur cinereiceps* (white-collared brown lemur) has been studied eating introduced plants that have helped them overcome disturbance events (Ralainasolo et al. 2008). *Haplemur griseus* (Eastern lesser bamboo lemur) has incorporated a significant proportion of the introduced *Psidium cattleianum* (Strawberry Guava) into its diet as a more digestible alternative to bamboo (Grassi 2006). *Psidium cattleianum* is similarly consumed by *Pteropus rufus* (Madagascan Flying Fox), a more generalist herbivore that was found to consume a number of introduced species including the widely distributed and agriculturally utilised *Agave sisalana* (Sisal) (Long and Racey 2007). In fact, of the plant introductions, none had a markedly negative impact nor spread uncontrollably (Kull et al. 2014), and none have led to the extinction of a

native plant (Kull et al. 2012). Kull et al. (2012) only classify 8.7% of the non-native 1,379 plants catalogued as exhibiting behaviour consist with being invasive.

Despite some positive examples one introduction has particular potential to cause devastation to the rich and largely endemic Malagasy amphibian assemblages (Andreone et al. 2008; Goodman and Benstead 2005). Chytridiomycosis, a fungal disease caused by *Batrachochytrium dendrobatidis* (*Bd*), has the capacity to damage over a third of amphibian species and is spreading across the globe, isolated islands included (Lötters et al. 2011). Its presence was suggested in exported Malagasy amphibians, therefore implying its presence in populations (Kolby 2014b). Work with wild populations has prompted debate over the true extent of *Bd*. Some suggest a widespread prevalence (Bletz et al. 2015b; Bletz et al. 2015a), while others present a more sceptical interpretation of the evidence (Kolby et al. 2015; Kolby and Skerratt 2015). What remains clear, irrespective of *Bd*'s current status, is the threat that *Bd* and Chytridiomycosis pose to Malagasy amphibians (Bielby et al. 2008). Initial findings using Malagasy species *Dyscophus antongilii* (tomato frog) and *Plethodontohyla tuberata* (interior digging frog) indicate vulnerability to the disease (Lötters et al. 2011). But as of yet no study has satisfactorily assessed the susceptibility of Malagasy amphibians (Bletz et al. 2015b), although efforts are underway (Andreone et al. 2008). It has been postulated that *D. melanostictus* may amplify the spread of *Bd*, but current literature suggests it is not a vector (Bai et al. 2012; Kolby et al. 2015). The fears surrounding *Bd* and *D. melanostictus* rightly support increasingly stringent and effective biosecurity measures in Madagascar, especially when nearby countries are harbouring *Bd* (Kielgast et al. 2010). Plans are in place to monitor and protect Madagascar's amphibian assemblage and so far the undertaking is under-funded, hampered by political instability, but optimistically progressing (Andreone et al. 2012; Weldon et al. 2013).

Considering the information gleaned from other invasions and knowledge concerning Madagascar's isolation some predictions can be made concerning *D. melanostictus*' introduction. It has already been present for a number of years; likely to have arrived sometime before 2014 (Moore et al. 2015). Subsequently become essentially naturalised around the port of Toamasina (Kolby 2014a; Moore et al. 2015). It is likely that they arrived via shipping containers (Kolby 2014a); a well-documented vector for invasive species (Kraus 2009).

The current consensus is that there should be immediate action taken to limit and remove *D. melanostictus* to limit damage, costs, and the probability of wide-establishment (Andreone 2014; McClelland et al. 2015). Eradications have succeeded before in more limited contexts (McClelland et al. 2015; Myers et al. 2000a). Most notably on Nonsuch island in Bermuda where *R. marina* was successfully removed (Wingate 2011). Eradication efforts are more frequently effective on smaller islands (Zavaleta et al. 2001); Madagascar is considerably larger and poses a novel set of complications. So far the toad is restricted to the more degraded and urban areas (Andreone 2014); but this may be skewed by increased detectability in urban areas (McClelland et al. 2015). Earlier estimations suggest the climate of Madagascar is suitable for its proliferation; most likely initially avoiding the drier island interior (Pearson 2015). Although even drier areas may be colonised if *D. melanostictus* can adapt like *R. marina* or climates shift in its favour (Brown et al. 2011a; Cabrera-Guzmán et al. 2013c; Dukes and Mooney 1999; Sutherst et al. 1996). Since those initial estimations further work has been performed, narrowing down the origin and lineage of the introduced *D. melanostictus*, tentatively indicating that Pearson (2015) may have overestimated the climate suitability in Madagascar (Vences et al. 2017). Even this reduced range estimation, based on a narrower niche of the originating population, leaves *D. melanostictus* huge areas to colonise including the extremely biodiverse east coast (Brown et al. 2014b; Vences et al. 2017).

If expansion occurs at rates demonstrated by *R. marina* in Australia (Urban et al. 2008), then the nearby valuable protected areas (Gray et al. 2016; Jenkins et al. 2014; Kremen et al. 2008), such as the Betampona Nature Reserve, will shortly be feeling the impacts of *D. melanostictus* (Cressey 2014). Estimates currently suggest a range expansion rate of 2km per year (McClelland et al. 2015). The canal system connecting Toamasina to the South runs for 600km and may exacerbate the spread if the toad is capable of dispersing during its larval stage (Moore et al. 2015). Another dispersal aid could be roads that have been shown to aid *R. marina* dispersal in Australia (Brown et al. 2006), but not utilised by *R. marina* in dry areas of Hawai'i (Ward-Fear et al. 2016). There is no reason to think that the expansion will not occur, with no significant barriers surrounding the 98km<sup>2</sup> area presently occupied



(McClelland et al. 2015). They may also be spread further via anthropogenic actions, stowing away in goods vehicles (Vences et al. 2017).

Currently, reports are indicating a density of >500 toads per hectare and a population of four million adults (McClelland et al. 2015), at least two thirds of which are likely to be female (Lazell and Wei-Ping 1987; Mathew 1999). These densities are much higher than some reported on in their native range (Lazell and Wei-Ping 1987).

While the early measures of expansion potential are useful they must be taken as estimations with a great deal of uncertainty. Even in areas where there are decades of data on the invasive species, estimations have been wrong. For example, predictions made in the 1980s vastly underestimated *R. marina* colonising ability (Phillips et al. 2008). The controlling factors on invasive species expansion are still largely unknown and likely heterogeneous, both in terms of species and spatially (Lever 2001; Phillips et al. 2008). Phillips et al. (2008) review the progress made in Australia and promote using modelling efforts combining existing correlative data (based on current climate preference) and mechanistic data (based on the life history of the species (Kearney et al. 2008)). If this is to be implemented in Madagascar more research is required into the specific niche *D. melanostictus* is filling there, as well as what it can adapt to in the future, both behaviourally and physiologically (Phillips et al. 2008; Shine 2012; Urban et al. 2008). A greater understanding of *D. melanostictus* is especially important considering that it may be a species complex with subtle differences between the yet delineated species (Wogan et al. 2016). So greater attention should be paid to toads found in Cambodia and Vietnam were the invading population has originated (Vences et al. 2017).

Recognition of differences within the population is also required; the Australian example shows how toads on the invasion front differ from those naturalising behind (Gruber et al. 2017; Hudson et al. 2016c). In only 80 years a significant phenotypic change has occurred to allow toads to better colonise and utilise their new home (Hudson et al. 2016a; Hudson et al. 2016b; Ward-Fear et al. 2016). It is also important to consider the biogeographical aspects of the originating toad populations. Their native range, that informed range estimations, may be dictated by factors other than climatic suitability that may no longer constrain them in the foreign Malagasy environment (Vences et al. 2017).

In the wake of the alarmism there are researchers that caution the immediate and aggressive response to the introduction (Mecke 2014). They cite examples where the efforts to contain one species could damage the already vulnerable amphibian assemblages in the area. The east coast, where *D. melanostictus* is currently, contains the richest areas for reptile and amphibian diversity (Brown et al. 2014b; Jenkins et al. 2014). They warn against draining spawning pools and the destruction of spawn (Mecke 2014), currently advocated (Cressey 2014). Mentioning also an example where public engagement in toad eradication can have unacceptable error margins when identifying the correct targets (Somaweera et al. 2010). However, the appearance of *D. melanostictus* is sufficiently different from native amphibians that this should not be an issue in Madagascar (Moore et al. 2015). If these methods are indeed damaging they pose an additional threat to an already declining class of fauna (Andreone et al. 2008; Stuart et al. 2004; Stuart 2005; Pimenta et al. 2005), potentially proving terminal for vulnerable populations (Somaweera et al. 2010).

### Fears and Potential Impacts

Despite the warnings, the 2015 Asian Toad Eradication Feasibility Report for Madagascar by McClelland et al. strongly advocates eradication. Suggesting that if the toad is permitted to stay seriously detrimental cascades will occur. The addition of predator removal via poisoning will release another invasive species, *Rattus rattus* (black rat). *Rattus rattus* are already making excellent use of fragmented landscapes (Ganzhorn 2003), which are growing in abundance as forests are cleared (Irwin et al. 2010). Further growth of this invader is leading to human health ramifications, economic damage and increased competition with native species (Ganzhorn 2003; Hingston et al. 2005; McClelland et al. 2015). Although direct competition with some native species may be limited if rats remain restricted to disturbed forests (Ramanamanjato and Ganzhorn 2001). A similar rat boom was previously seen during the overexploitation of *Acrantophis spp.* (McClelland et al. 2015). In Australia, a cascade has been studied where a mesopredator, *Dendrelaphis punctulatus*, boomed due to relief from varanid predation, that are poisoned by *R. marina* (Doody et al. 2013). There is clear potential for similar poison mediated cascades in Madagascar.

Human health may also be affected by direct consumption. *Duttaphrynus melanostictus*, and other bufonids, have been documented causing illness and death

if consumed (Cheng et al. 2006; Chern et al. 1991; Trainor et al. 2009). Kuo et al. (2007) describe a case in Taiwan of an individual falling ill after the consumption of toad eggs. Keomany et al. (2007) report on several cases in Laos where the toad is commonly eaten in poorer rural areas; complimenting reports from Northern Thailand (van Dijk et al. 2016), but counter to those from India (Mathew 1999). They suggest that it is not lack of knowledge that causes these incidents and that there is likely a low report rate. Indicating that even if local Malagasy people are warned of *D. melanostictus*' effects there still may be substantial health implications. Currently the other introduced anuran species, *Hoplobatrachus tigerinus* (Indian bullfrog), constitutes a sizable portion of wild frog harvests (Jenkins et al. 2009a). Perhaps due to its freedom from tradition *fady* beliefs that can restrict harvest and exploitation of some native species such as *Crocodylus niloticus* (Nile crocodile) (Glaw and Vences 2007; Jones et al. 2008). *Duttaphrynus melanostictus*' size and readiness to live by human settlements may make it a prime target.

The impacts are not restricted to humans, the report warns of the vulnerability of many anurophagous snakes (Webb et al. 2005) – *Leioheterodon*, *Ithycyphus* and *Dromicodryas* spp. – and Moore confirms the demise of *Leioheterodon madagascariensis* (Madagascan giant hognose) (pers. comm. in McClelland et al. 2015). Others report *Acrantophis* spp. are already dying (Cressey 2014). Snake's restriction to consuming prey whole makes them vulnerable, unable to avoid the toxic skin or glands (Phillips et al. 2003). For those genetically non-resistant, survival chance may also depend on the fluency of ingestion (Kidera and Ota 2008), as well as the size of toad consumed (Phillips and Shine 2006a). With snakes being frequent predators of anurans (Toledo et al. 2007), they are unlikely to avoid serious impact.

The evolutionary history of Madagascar suggests that vulnerability will be widespread. Some Malagasy boid snakes, along with oplurid lizards, are thought to have occurred via vicariance around the time Madagascar fully separated – ~76Myr BP (Noonan and Chippindale 2006a; Noonan and Chippindale 2006b; Raxworthy 2004a; Yoder and Nowak 2006). This is approximately the same time as the origin of Bufonidae (Pramuk et al. 2008), therefore limiting or eliminating the two groups' interaction. There is evidence that terrestrial species spread via oceanic dispersal across from Africa until a change in currents ~20Myr BP (Samonds et al. 2012;

Vences et al. 2003; Yoder and Nowak 2006). Supported by the age of Malagasy Lamprophiids being younger than the possible vicariance origin (Nagy et al. 2003); along with evidence that some additional boids also underwent dispersal after the initial separation as well (Noonan and Chippindale 2006b; Vences et al. 2001). Amphibians present some of the strongest evidence of the existence of later oceanic dispersal, with their divergence dates being much closer to present (Vences et al. 2003), along with many other clades (Fritz et al. 2013; Raxworthy et al. 2002; Whiting et al. 2004; Wong et al. 2010). Carnivores and other mammals all have their own oceanic dispersal stories, all considerably after any possible vicariance event (Poux et al. 2005; Samonds et al. 2013; Yoder et al. 2003).

Even this later interaction date, determined by dispersal as oppose to vicariance, leaves a long time during which species could lose adaptations developed in what is now Africa or Asia. As seen in the Australian example, species lacking in shared evolutionary history are the most vulnerable to novel defensive adaptations (Llewelyn et al. 2011; Ujvari et al. 2013).

Impacts on snakes may be mitigated if the snake diversity is focused to non-anthropogenically altered areas (Irwin et al. 2010; Theisinger and Radianarivo 2015), unlike the toads (van Dijk et al. 2016), by virtue of a reduced encounter rate. This may factor in more if the toads' success is based heavily on capitalising on disturbed areas natives cannot utilise (Mack et al. 2000), as seen with *R. rattus* populations (Ramanamanjato and Ganzhorn 2001). A bias favouring disturbed areas may disproportionately impact invasive predators as opposed to natives by the same means, although there is still likely to be considerable niche overlap (Brown et al. 2016).

Other anurophagous predators they may harm range from lizards, such as *Zonosaurus* sp. (Glaw and Vences 2007; Heying 2001; Jovanovic et al. 2009), to mammals (Brown et al. 2016; Dollar et al. 2007; Dunham 1998; Goodman et al. 1997; Rasoloarison et al. 1995), and anurophagous or opportunistic amphibian species (Andreone and Nussbaum 2006; Vences et al. 1999b). Even if species learn to avoid *D. melanostictus* it could become a nutrient sink harming the entire community (Greenlees et al. 2006; McClelland et al. 2015).

Toads are also expected to damage endemic prey species (McClelland et al. 2015). This is likely to be skewed towards arthropods, irrespective of the preys defensive capabilities (Berry and Bullock 1962; Mathew 1999); there is no guarantee that invasive species will behave as their non-translocated cousins do (O'Shea et al. 2013; Pearson 2015), nor remain in their native niche (Shine 2012; Urban et al. 2007). There are reports of *D. melanostictus* consuming small reptiles (Hahn 1976; O'Shea et al. 2013); and *R. marina* consuming small vertebrates (Boland 2004; Covacevich and Archer 1975; Grant 1996; Lever 2001; Reed et al. 2007). Unsurprising given how large they can grow (Lu and Qing 2010). However, more recent and comprehensive studies have found *D. melanostictus* to focus on invertebrate prey (Döring et al. 2017).

In summary, the report firmly suggests a complete eradication of *D. melanostictus*, but acknowledges the difficulties of such an operation. If only 0.01% of the current population remain they would be able to rapidly recover, nullifying the eradication effort (McClelland et al. 2015). Even at current numbers, 2.5 million toads would be need to be removed every year for a successful eradication (McClelland et al. 2015). Eradications have worked on smaller islands and are currently under way on others attempting to remove a number of species (Mack et al. 2000; Morley et al. 2006; Morley 2006; Wingate 2011). Based on this report and the comparison of other toad invasions, namely Australia, there is a clear need to investigate the toxic susceptibility of Malagasy species. As toxicity and poisoning appears to be the mechanism that the most drastic population declines will be attributed (Shine 2010). Efforts must also be made to avoid acting upon unsupported anecdotal evidence (Shine et al. 2009).

The best estimations of invasive impacts will require background information on all species involved, behavioural studies clarifying predation and encounter rates, alongside long-term populations studies that can demonstrate trends despite obscuring stochastic variations (Brown et al. 2011b). In lieu of the extensive and long-term population studies required to reliably detect population falls (Woinarski et al. 2004), laboratory-based studies provide an excellent simpler alternative (Shine et al. 2016). This is especially true when the predator in question is a snake; snakes are notoriously difficult to study because of their cryptic nature (Durso and Seigel 2015), as demonstrated by the recent and repeated discovery of new species such

as *Madagascarophis lolo* (ghost snake) (Ruane et al. 2016). In theory, the genetic methodology investigating the mechanisms of the invasion should avoid any ambiguity concerning a species resistance or abstruse population changes (Shine 2010). For example *Dendrelaphis punctulatus* was indicated as resistant and non-resistant to bufotoxin by two different studies (Covacevich and Archer 1975; Phillips et al. 2003).

Information elucidating the impacts of *D. melanostictus*' toxin will help build an optimal conservation strategy and direct future research (Gurevitch and Padilla 2004; Shine et al. 2016). Especially if the eradication is abandoned in favour of containment/control or prioritised site/species. There is a clear indication from the Australian example and the long-term isolation of Malagasy anurophagous predators that poisoning by *D. melanostictus* is a distinct and significant possibility.

By investigating the structure of the bufotoxin binding site, the H1-H2 domain of the alpha subunit of the Na<sup>+</sup>,K<sup>+</sup>-ATPase, a definitive answer to whether poisoning is possible can be given.

This study aims to sequence the H1-H2 domain of the alpha subunit of the Na<sup>+</sup>,K<sup>+</sup>-ATPase of various anurophagous Malagasy predators. From these sequences, and the identification of amino acids present, I aim to detect whether the species will be vulnerable to poisoning via ingestion of *D. melanostictus* and elucidate the potential impacts of this introduced toxic toad.

# Methods

Tissue samples of all snakes, lizards, frogs and mammals were supplied by Prof. Miguel Vences and Dr. Frank Glaw, under previous ethical approval. Overall samples covering 69 species were obtained, alongside an additional five available from Dr. Wolfgang Wüster's existing library. All tissue samples were transported and stored in alcohol. Extracted *Crocodylus niloticus* DNA was obtained from Rob Gandola (full list see Appendix 1).

From the tissue samples, the DNA was extracted using Qiagen DNeasy Blood and Tissue Kits following standard Qiagen DNeasy protocol (Appendix 2).

The products were quantified using a Nanodrop Spectrophotometer ND1000. The ND1000 was first cleaned using purified water, then 'blanked' using 1 µl of buffer AE. This ensured that the equipment was free from contaminants and properly calibrated for reading DNA suspended in AE buffer. 1 µl of each sample was then placed into the ND1000 and all readings were recorded. Of note where the 260/280 measurements indicating the purity of the DNA. Values of approximately of 2 were accepted. Only one of the primary elution extractions failed. Second elution were only completed for early samples as well as *Cheirogaleus medius* (fat-tailed dwarf lemur) and *Cryptoprocta ferox* as these were to be used to test primer conditions. The other metric of note was the ng/µl values. For full details on sample extraction and the concentrations worked from see Appendix 3.

The primers used for snake and lizard species were the same as Mohammadi et al. (2016) – ATP1a3Fwd (CGA GAT GGC CCC AAT GCT CTC A) and ATP1a3Rvs (TGG TAG TAG GAG AAG CAG CCG GT). These primers are designed to amplify the H1-H2 extracellular domain of the Na<sup>+</sup>,K<sup>+</sup>–ATPase alpha subunit 3. For amphibians primers outlined by Moore et al. (2009) were used – ATP1\_178Fwd (WGA RAT CCT GGC ACG AGA TG) and ATP1\_178Rvs (GAG GMA CCA TGT TCT TGA AGG). These primers amplified the alpha 1 subunit that Moore et al. (2009) indicates as being primarily responsible for bufonid's resistance. These were the same primers also used by Shine et al. (2016). For some lizard species alternative primers detailed in Holzinger et al. (1992) were required, referred to in

this study by ATP1\_HoIAFwd (CTG TGG ATC GGT GCT ATT CT), ATP1\_HoIBFwd (CTG TGG ATC GGT GCG ATT CTT TGC TTT), and ATP1\_HoICRvs (ACC ATG TTC TTG AAC GAT TCC ATG ATC TT). These are the same primers as used by Ujvari et al. (2013).

New primers were designed for all the mammalian samples as well as for the *Crocodylus niloticus*. These primers were selected using the National Center for Biotechnology Information's (NCBI) Primer-Blast (Ye et al. 2012). This was accomplished by comparing sequences from the closest relatives to Malagasy mammals to identify conserved areas. Suitable sequences were found and obtained using GenBank (Appendix 4). This was achieved by using 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<sup>+</sup>,K<sup>+</sup>–ATPase were inputted into the NCBI's BLAST nucleotide search to find unannotated sequences. All identified sequences were aligned and reviewed in Mega (V. 7.0.21) to confirm the presence and form of the H1-H2 domain of the Na<sup>+</sup>,K<sup>+</sup>–ATPase.

From this selection of organisms five pairs of primers were designed. One pair (ATP1a1CFwd & Rvs) based on *Crocodylus porosus* with specificity checked on *Gavialis gangeticus* (gharial), *Alligator sinensis* (Chinese alligator) and *Alligator mississippiensis* (American alligator). The other pairs were based on *Panthera pardus* (leopard) (ATP1a1LFwd & Rvs as well as ATP1a1L2Fwd & Rvs), *Propithecus coquereli* (ATP1a1PFwd & Rvs), and *Echinops telfari* (lesser hedgehog tenrec) (ATP1a1TFwd & Rvs). All of them tested for specificity using available Afrosoricida, Carnivora and Primate sequences.

Polymerase chain reactions (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. One negative control was made for every PCR run containing all of the above bar the DNA template. The program used entailed an initial denaturing at 94°C for 2 minutes, a further denature at 94°C of 30 seconds, then an annealing step at 56°C for 30 seconds as suggested by (Moore et al. 2009). Following this was an extension step at 72°C for 1 minute. All, bar the initial denaturing, were repeated



another 39 times. To complete the program a final extension step at 72°C for 5 minutes was undertaken, followed by a cooling period at 4°C for 15 minutes. The annealing temperature was confirmed to be optimal by running a temperature gradient during the annealing stage using the sample for *Dromicodryas quadrilineatus* (four-striped snake). For ATP1a3 the optimal temperature was confirmed to be 56°C, whereas for ATP1\_178 discovered to work best at 51.5 °C for amphibians. An annealing temperature of 46°C amplified a single band for the mammals, however the amplicon was incorrect.

To confirm the success of the PCR and to gain rough estimates of product concentrations gel electrophoresis was performed (example in Appendix 5). Gels of 1 % were used in a ratio of 0.1 g of agarose to 10 ml of 1X TBE buffer. Safeview was added at a ratio of 1 µl to 10 ml of 1X TBE buffer. A 100 base pair DNA ladder was used to aid concentration and length estimations. Ladder, negative control and PCR products were run through the gel at 70 volts for usually 20 minutes (varied depending on gel tank). Resulting gel was placed in the Bio-rad Molecular Imager Gel Doc XR Imaging System – a UV trans-illuminator, where the brightness and position of bands relative to the ladder and contaminants in the negative control could be detected.

After this the PCR products required cleaning. 1µl of a mix comprising 0.8µl of water, and 0.1µl of both exonuclease and TSAP was added to samples 4936 through 4940. All other samples had 2 µl added to ensure complete cleaning. Once the enzymes had been added the products were placed back into the thermal cycler running the following: incubation at 37°C, inactivation at 74°C, and stop at 4°C. All steps were run once for 15 minutes.

The cleaned products were sent to Macrogen (Seoul, South Korea) for sequencing along with associated working aliquot primers. All samples had both forward and reverse sequencing to minimise erroneous or ambiguous base pair detection.

Sequence data was examined using CodonCode Aligner and contigs created using forward and reserve sequences where possible. Some samples did not produce sufficient PCR product for successful reverse reactions. Alignment of the consensus sequences was performed using MUSCLE in Mega (V. 7.0.21). In this analysis, reference sequences from GenBank were used to identify the 11 codons of interest

(see Appendix 6). Isoelectric points were identified using ProtParam tool ([web.expasy.org/protparam/](http://web.expasy.org/protparam/)).

Original sequence data was supplemented by data obtained from GenBank. Four Malagasy mammals had large genome datasets available that covered the H1-H2 alpha 1 sub-unit of the Na<sup>+</sup>,K<sup>+</sup>–ATPase and so could be used to infer toxin resistance. These were *Daubentonia madagascariensis* (Aye-aye), *Echinops telfari*, *Microcebus murinus* (grey mouse lemur) and *Propithecus coquereli* (AGTM011609586.1, XM\_004714862.2, XM\_012761812.1, XM\_012658471.1). The human Na<sup>+</sup>,K<sup>+</sup>–ATPase was also included for reference (NM\_000701.7).

I undertook a systematic review of the literature surrounding the Malagasy fauna used. I passed each species' binomial name plus the words 'diet', 'predation' or 'dietary' to the Google Scholar search engine. I scanned all available article abstracts and downloaded the article if I judged it likely to contain dietary information. Additionally, I ran searches only the species binomial name. I investigated these articles further if I deemed likely them to contain pertinent natural history information. I gained further details from Glaw and Vences (2007) on a species by species basis. Herpetological Review houses many one-off observations of predation attempts; however, their articles are not properly indexed for the Google Scholar search engine. To overcome this, I downloaded their catalogue of articles and searched it using the in-built text search function of a standard text reader. For these searches, I made sure to use old synonyms for species, as well as common alternative spellings.

# Results

## Results of PCR

The ATP1a3 primers were found to be the most consistent and most effective of the primer sets but failed to amplify for any species not a snake or *Zonosaurus sp.*. The products were approximately 300-400 base pairs in length.

For the ATP1\_178 primers the sequences were around the same length at around 300-400 base pairs. Unlike the ATP1a3 primers these yielded much less amplified product and in general their success varied more between species. They completely failed to amplify any DNA for samples *Ptychadena mascareniensis* (Mascarene grass frog), *Guibemantis timidus* and *Aglyptodactylus sp. ("inguinalis")* under a variety of conditions and concentrations. These species could not be sequenced so the make-up of their Na<sup>+</sup>,K<sup>+</sup>-ATPase remains unknown. There were attempts to use these primers to produce sequences for mammal samples. Single bands were produced but they were around 100 base pairs too short and upon sequencing it was made clear via a BLAST search that the incorrect area of the genome was amplified.

Designed primers and those from Holzinger et al. (1992) failed to produce any useable amplicons. All designed primers produced unspecific bands (full details in Appendix 7). Gel electrophoresis showed either many bands or smeared bands. Testing the primers under a variety of conditions and different PCR master mixes failed to produce any useable amplicons.

## Sequence Data

Sequencing produced clean and usable data for all 27 Malagasy snake samples, 12 Malagasy frogs, four lizards but no mammals. The snake samples covered 16 genera of both Alethinophidian families found in Madagascar, and 15 of the 23 total genera. Successful lizard sequences covered three families out of six present, and three of 30 are covered by this study. Frog sequences covered all four frog families found on Madagascar, of the 22 genera, nine are represented here. Lemurs included

half of the six lemur families, and three of the 15 genera. All nine tenrec genera belong to the same family, represented here by one a single species.

A few species appear to have heterozygous lengths. Fortunately, the differences did not overlap with the same set of base pairs required to discern toxin vulnerability. However, this did mean that the reverse sequencing reaction provided no usable data. Others had the reverse reaction produce poor quality results. The upshot being that data for *Amphiglossus astrolabi* (diving skink), *Boophis tephraeomystax* (Dumeril's bright-eyed frog), *Dyscophus guineti* (false tomato frog) and *Mimophis mahafalenis* were obtained by a single direction of sequencing. The instances of single direction sequencing used were fully clear of ambiguity in the area of interest.

## Snakes

All Malagasy snake species showed the exact same amino acid sequence on the Na<sup>+</sup>,K<sup>+</sup>-ATPase – QAGTEDDPAGDN (Table 2). They were no different from known non-resistant snake previously sequenced by Mohammadi et al (2016). A known resistant snake from the same study *Rhabdophis subminiatus* (red-necked keelback)

		Codon Position – α3											
		111	112	113	114	115	116	117	118	119	120	121	122
Colubridae	<i>Rhabdophis subminiatus</i>	L	A	G	T	E	D	D	P	S	R	D	N
	<i>Acrantophis dumerili</i>	Q	A	G	T	E	D	D	P	A	G	D	N
Sanziniidae	<i>Acrantophis madagascariensis</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Sanzinia madagascariensis madagascariensis</i>	Q	•	•	•	•	•	•	•	•	G	•	•
Lamprophiidae	<i>Alluaudina bellyi</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Compsophis boulengeri</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Compsophis infralineatus</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Compsophis laphystius</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Dromicodryas bernieri</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Dromicodryas quadrilineatus</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Ithyocyphus goudoti</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Ithyocyphus miniatus</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Langaha madagascariensis</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Leioheterodon geayi</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Leioheterodon madagascariensis</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Leioheterodon modestus</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Liophidium torquatum</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Liopholidophis grandidieri</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Liopholidophis sexlineatus</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Lycodryas citrinus</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Lycodryas granuliceps</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Madagascarophis meridionalis</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Mimophis mahfaleensis</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Pseudoxyrhopus heterurus</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Pseudoxyrhopus microps</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Thamnosophis epistibes</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Thamnosophis infrasignatus</i>	Q	•	•	•	•	•	•	•	•	G	•	•
	<i>Thamnosophis lateralis</i>	Q	•	•	•	•	•	•	•	•	G	•	•

Table 2 - H1-H2 extracellular domain of the alpha 3 subunit of the Na<sup>+</sup>/K<sup>+</sup>-ATPase of Malagasy snakes compared to the known resistant snake *Rhabdophis subminiatus* from Mohammadi et al. (2017).

is presented alongside for comparison (Genbank accession number: KP238137.1). Using these snakes as comparison it is clear to see that the H1-H2 domain of alpha 3 sub-unit of the Na<sup>+</sup>,K<sup>+</sup>–ATPase of all Malagasy snakes is the non-resistant form. This is a very clear indication that if a cardiac glycoside was introduced there would be nothing preventing it from inhibiting the Na<sup>+</sup>,K<sup>+</sup>–ATPase.

## Lizards

Both *Zonosaurus* spp. showed a similar pattern to the snakes. There is one difference at position 120, instead of Glycine (G) they had Asparagine (N) – QAGTEDDPANDN (Table 3). Instead of comparing the *Zonosaurus* spp. to snakes as before, sequence data from Ujvari et al. (2013) was used. It was found that the presence of Asparagine matched that of non-resistant *Varanus* spp. and *Tiliqua scincoides* from Australia as opposed to their resistant Asian cousins (Genbank accession numbers: KP238148.1-KP238176.1). We can conclude that the *Zonosaurus madagascariensis* (Madagascar plated lizard) and *Zonosaurus brygooi* (Brygoo's girdled lizard) do not have a resistant Na<sup>+</sup>,K<sup>+</sup>–ATPase alpha 3 sub-unit.

The above were sequenced using the primers outlined by Mohammadi et al. (2016). These primers failed to amplify DNA from other species. For *Geckolepis maculata* (Peter's spotted gecko) and *Ampiglossus astrolabi* primers ATP1\_178 from Moore et al. (2009) were used. These targeted the alpha 1 subunit as opposed to the less widely expressed but more sensitive alpha 3 sub-unit (Juhaszova and Blaustein 1997; Köksoy 2002). Due to the conserved nature of the Na<sup>+</sup>,K<sup>+</sup>–ATPase there is very little variation between alpha 1 and 3 (Blanco and Mercer 1998). Both these species had the same string of amino acids as the snakes (QAGTEDDPAGDN) indicating no resistance to bufotoxins (Table 3).

		Codon Position – α3											
		111	112	113	114	115	116	117	118	119	120	121	122
Varanidae Gerrhonosauridae	<i>Varanus bengalensis</i>	L	A	G	T	E	E	D	P	S	R	D	N
	<i>Zonosaurus brygooi</i>	Q	A	G	T	E	D	D	P	A	N	D	N
	<i>Zonosaurus madagascariensis</i>	Q	•	•	•	•	•	•	•	•	N	•	•
		Codon Position – α1											
Scincidae Gekkoniidae	<i>Ampiglossus astrolabi</i>	Q	A	G	T	E	D	D	P	A	G	D	N
	<i>Geckolepis maculata</i>	Q	•	•	•	•	•	•	•	•	G	•	•

Table 3 - The amino acids found in the H1-H2 extracellular domain of the alpha 3 and alpha 1 subunit of the Na<sup>+</sup>/K<sup>+</sup>–ATPase of Malagasy lizards. Displayed alongside the alpha 3 make-up of known resistant *Varanus bengalensis* from Ujvari et al. (2013).

## Amphibians

All amphibians sequenced had extremely similar amino acids on the H1-H2 domain – QAATEEEEPQNDN (Table 4). This series of amino acids matched the non-resistant form of Na<sup>+</sup>,K<sup>+</sup>–ATPase found in numerous species by Moore et al. (2009). This contrasted to a known resistant species, *D. melanostictus* itself, that has charged amino acids at the 119 position (Moore et al. 2009). Even species that are known to be sympatric with bufonids had non-resistant forms of the Na<sup>+</sup>,K<sup>+</sup>–ATPase such as *Hoplobatrachus tigerinus*. *Hoplobatrachus tigerinus* presented the most different sequence but all of the substitutions, compared to Malagasy natives, are neutrally charged. Unusually, for *H. tigerinus* it had an additional amino acid present in the sequence – TAATEEDTQNNNDN (Table 4). It is not clear why this would be the case but the fact that the amino acid in question (N) confers no charge it is unlikely to prevent binding of cardiac glycosides. Additionally, it harbours the substitution of Q111T, while at a known active position the neutral amino acid would not alter bufotoxin binding. Given *H. tigerinus*' long sympatry with toads and the novelty of its Na<sup>+</sup>,K<sup>+</sup>–ATPase I cannot conclusively state that it lacks resistance. This is the first instance of the addition of an amino acid in the H1-H2 domain of an amphibian judging by this study and Moore et al. (2009). However, it has been found in several rodent species (see Appendix 8).

		Codon Position – α1											
		111	112	113	114	115	116	117	118	119	120	121	122
Bufo	<i>Duttaphrynus melanostictus</i>	R	K	A	S	N	L	E	P	D	N	D	N
Hyperoliidae	<i>Heterixalus madagascariensis</i>	Q	A	A	T	E	E	E	P	Q	N	D	N
Mantellidae	<i>Boophis goudotii</i>	Q	•	•	•	•	•	•	•	Q	•	•	•
	<i>Boophis madagascariensis</i>	Q	•	•	•	•	•	•	•	Q	•	•	•
	<i>Boophis tephraeomystax</i>	Q	•	•	•	•	•	•	•	Q	•	•	•
	<i>Gephyromantis redimitus</i>	Q	•	•	•	•	•	•	•	Q	•	•	•
	<i>Laostoma labrosum</i>	Q	•	•	•	•	•	D	•	Q	•	•	•
	<i>Mantella baroni</i>	Q	•	G	•	•	•	E	•	Q	•	•	•
	<i>Mantidactylus femoralis</i>	Q	•	A	•	•	•	•	•	Q	•	•	•
	<i>Mantidactylus guttulatus</i>	Q	•	•	•	•	•	•	•	Q	•	•	•
Microhylidae	<i>Mantidactylus ulcerosus</i>	Q	•	•	•	•	•	•	•	Q	•	•	•
Dicroglossidae	<i>Dyscophus guineti</i>	Q	•	•	•	•	•	•	•	Q	•	•	•
	<i>Hoplobatrachus tigerinus</i>	T	•	•	•	•	•	D	T	Q	• (N)	•	•

Table 4 - The amino acids found in the H1-H2 extracellular domain of the alpha 1 subunit of the Na<sup>+</sup>/K<sup>+</sup>–ATPase of Malagasy amphibians. Displayed alongside the alpha 1 make-up of known resistant toxic toad *Duttaphrynus melanostictus* from Moore et al. (2009).

The only other differences in the amphibian samples were to *Mantella baroni* (Baron's mantella) and *Laliostoma labrosum* (Madagascar bullfrog), who had substitutions A113G and E117D respectively. The position and lack of charge of these substituted amino acids alter nothing concerning bufotoxin binding.

## Mammals

The GenBank acquired sequences show identical amino acids on H1-H2 of the Na<sup>+</sup>,K<sup>+</sup>-ATPase for two of the three primates – QAATEEEEPQNDN (Table 5). The same form as humans and all other examined primates take (Appendix 9). One primate *Daubentonia madagascariensis* (aye-aye) shows two substitutions compared to the others, E115D and P118S, neither of which would be capable of inhibiting the binding of bufotoxin to the Na<sup>+</sup>,K<sup>+</sup>-ATPase. The tenrec also showed markedly consistent Na<sup>+</sup>,K<sup>+</sup>-ATPase composition – QAGTEEDPQNDN – meaning it too harbours no resistance to bufotoxins (Table 5).

		Codon Position – α1											
		111	112	113	114	115	116	117	118	119	120	121	122
Hominidae	<i>Homo sapiens</i>	Q	A	A	T	E	E	E	P	Q	N	D	N
Cheirogaleidae	<i>Microcebus murinus</i>	Q	•	•	•	•	•	•	•	Q	•	•	•
Indridae	<i>Propithecus coquereli</i>	Q	•	•	•	•	•	•	•	Q	•	•	•
Daubentonidae	<i>Daubentonia madagascariensis</i>	Q	•	•	•	D	•	•	S	Q	•	•	•

		Codon Position – α1											
		111	112	113	114	115	116	117	118	119	120	121	122
Erinaceidae	<i>Erinaceus europaeus</i>	R	A	A	T	E	E	E	V	D	N	D	N
Tenrecidae	<i>Echinops telfairi</i>	Q	A	G	T	E	E	D	P	Q	N	D	N

Table 5 - The amino acids found in the H1-H2 extracellular domain of the alpha 1 subunit of the Na<sup>+</sup>/K<sup>+</sup>-ATPase of Malagasy mammals. Displayed alongside the alpha 1 make-up of known resistant *Erinaceus europaeus*. Genbank numbers in order of table: NM\_000701.7, XM\_012761812.1, XM\_012658471.1, AGTM011609586.1, XM\_007525504.1, XM\_004714862.2

## Isoelectric Point

Although the resistance of these species is clear by comparing them to known resistant or non-resistant counterparts, all sequences were confirmed not to have any residual charge capable of inhibiting binding of cardiac glycosides. All Malagasy species results showed four negatively charged amino acids and zero positively charged. The negatively charged amino acids occur in the centre of the H1-H2 domain conferring no advantage when combatting bufotoxins. For comparison

resistant squamates, would have one positively charged and four negatively charged, with the positive charge placed at a latter position (Ujvari et al. 2015). Resistant amphibians, however, would have a negatively charged amino acid at position 119 or 122 (Ujvari et al. 2015).

This rather straightforward comparison between native Malagasy species and those previously sequenced by other studies clearly shows a lack of resistance to bufotoxins in all sequenced Malagasy species.

## Literature Search

The systematic review produced 117 pieces of published literature that contained pertinent natural history information. Table 6 details the finding from those papers and summarised the most important traits. I prioritised the reporting of traits that would likely lead species to interact with *D. melanostictus* in some form by mainly via predation. The table also highlights how more work is required to fully understand the natural history of many Malagasy herpetofauna species.

<b>Snakes: Boidae, Lamprophiidae</b>						
<b>Species</b>	<b>Consumes Frogs</b>	<b>Forages Terrestrially</b>	<b>Nocturnal / crepuscular</b>	<b>Water Bodies</b>	<b>Anthropogenic Areas</b>	<b>Referecnes</b>
<i>Acrantophis dumerili</i>	1	1	1			5, 6, 22, 39,
<i>Acrantophis madagascariensis</i>	1	1	1	1	1	5, 15, 22, 34, 35, 39, 49,
<i>Sanzinia madagascariensis madagascariensis</i>	1	1	1		1	5, 39, 46, 78,
<i>Alluaudina bellyi</i>		1	1	1		5, 39,
<i>Compsophis boulengeri</i>	1	1	-1			5, 39,
<i>Dromicodryas bernieri</i>	1	1	-1		1	5, 31, 35, 39, 102,
<i>Dromicodryas quadrilineatus</i>		1	-1			5, 39,
<i>Geodipsas infralineata</i>	1	1	1	1		5, 12, 16, 39, 54,
<i>Geodipsas laphystia</i>	1	-1	1	1		5, 16, 39,
<i>Ithycyphus goudoti</i>		1	-1	1		5, 39, 54,
<i>Ithycyphus miniatus (cf.)</i>	1	1	-1			5, 23, 39,
<i>Langaha madagascariensis</i>		-1	-1			5, 39, 54, 58, 90, 104,
<i>Leioheterodon geayi</i>		1	-1			39,
<i>Leioheterodon madagascariensis</i>	1	1	-1		1	5, 39, 46, 69, 73, 74,
<i>Leioheterodon modestus</i>	1	1	-1			39, 72, 89,
<i>Liophidium torquatum</i>	1	1	-1			5, 39,
<i>Liopholidophis grandidieri</i>	1	1	-1			5, 21, 39,
<i>Liopholidophis sexlineatus</i>	1	1	-1	1	1	5, 39, 91,
<i>Lycodryas citrinus</i>	1	1	1			39,
<i>Lycodryas granuliceps</i>		-1	1		1	37, 39,
<i>Madagascarophis meridionalis</i>	1	1	1		1	5, 36, 39, 54, 93,
<i>Mimophis mahfalensis</i>	1	1	-1		1	5, 36, 39, 40, 70, 92,
<i>Pseudoxyrhopus heterurus</i>		1	1	1	1	5, 9, 17, 39, 54, 57, 85,
<i>Pseudoxyrhopus microps</i>	1	1	1	1	1	5, 39,
<i>Thamnosophis epistibes</i>	1	1	-1			5, 39, 53,
<i>Thamnosophis infrasinatus</i>	1	1	-1			5, 39,



### Plated Lizards, Skinks, Geckos, Chameleons, Amphibians, Chelodians, Crocodiles

Species	Consumes Frogs	Forages Terrestrially	Nocturnal / crepuscular	Water Bodies	Anthropogenic Areas	References
<i>Zonosaurus brygooi</i>		1	-1			39,
<i>Zonosaurus madagascariensis/haraldmeieri</i> (cf.)	1	1				39, 49, 53,
<i>Amphiglossus astrolabi</i>		1	1	1	-1	39, 71, 84,
<i>Geckolepis maculata</i>			1		1	39, 86,
<i>Oplurus cyclurus</i>						39, 88,
<i>Oplurus quadrimaculatus</i>						39, 88
<i>Calumma brevicorne</i>			-1			39,
<i>Calumma parsonii cristifer</i>		-1	-1			39, 52,
<i>Furcifer pardalis</i>		-1	-1		1	7, 32, 39, 52,
<i>Aglyptodactylus</i> sp. ("inguinalis")				1		3, 42, 106, 110,
<i>Boophis goudotii</i>	1	1	1	1	1	39, 75, 103, 108,
<i>Boophis madagascariensis</i>			1	1	1	3, 79,
<i>Boophis tephraeomystax</i>			1	1	1	39, 79, 111, 112,
<i>Dyscophus guineti</i>		1		1	-1	39, 96,
<i>Gephyromantis redimitus</i>		0	1	1	-1	39,
<i>Guibemantis timidus</i>		-1	1	1	1	39, 109, 115,
<i>Heterixalus madagascariensis</i>		-1	1	1	1	37, 39, 41, 107,
<i>Hoplobatrachus tigerinus</i>	1	1	1	1	1	33, 39, 50, 56, 76, 103,
<i>Laliostoma labrosum</i>		1		1	1	33, 39, 41, 94
<i>Mantella baroni</i>			-1	1	1	8, 39, 108, 110
<i>Mantidactylus femoralis</i>		1		1	-1	3, 39, 110,
<i>Mantidactylus guttulatus</i>			1	1	1	2, 39, 95, 112,
<i>Mantidactylus ulcerosus</i>	1		1	1		39, 110,
<i>Ptychadena mascareniensis</i>	1	1	1	1	1	3, 28, 33, 39, 41, 47, 76,
<i>Pelomedusa subrufa</i>	1			1	1	39, 66, 67, 68, 87,
<i>Pelusius castanoides</i>	1			1	1	39, 66, 67,
<i>Crocodylus niloticus</i>	1	1	1	1		39, 60, 116,

### Lemurs, Carnivores, Tenrecs, Rodents

Species	Consumes Frogs	Forages Terrestrially	Nocturnal / crepuscular	Water Bodies	Anthropogenic Areas	References
<i>Cheirogaleus medius</i>		-1	1			29, 61, 62,
<i>Daubentonia madagascariensis</i>		-1	1			100, 101
<i>Eulemur sanfordi</i>			-1			39,
<i>Propithecus coquereli</i>		-1	-1	-1		39,
<i>Cryptoprocta ferox</i>	1	1	1		-1	1, 14, 24, 38, 44, 82, 117,
<i>Eupleres goudoti</i>	1	1	1	1	-1	1, 14, 27, 38,
<i>Fossa fossana Fierenana</i>	1	1	1	1	-1	1, 14, 27, 38,
<i>Galidia elegans</i>	1	1	-1	1	1	1, 12, 14, 26, 27, 38,
<i>Echinops telfairii</i>		1	1			45,
<i>Hemicentetes nigriceps</i>		1			1	45, 99,
<i>Hemicentetes semispinosus</i>		1				45, 99,
<i>Limnogale mergulus</i>	1	1	1	1		11, 45, 98,
<i>Microcebus murinus</i>		-1	1		1	39,
<i>Microgale longicaudata</i>		1				45,
<i>Oryzoryctes hova</i>		1				65,
<i>Setifer setosus</i>		1	1		1	45, 64, 65,
<i>Brachytarsomys albicauda</i>		-1				39,
<i>Suncus murinus</i>		1			1	13,

Table 6 - A summary of some natural history traits for selected Malagasy species. Whether they have been reported eating frogs, forage terrestrial, are active during morning, evenings or night, occur near water bodies, and can live in anthropogenic areas. 1 denotes a trait they are confirmed to possess, -1 is a confirmation of the opposite, 0 is inconclusive.

1. (Albignac 1972)
2. (Altig and McDiarmid 2006)
3. (Andreone 1994)
4. (Andreone 2004)
5. (Andreone and Luiselli 2000)
6. (Andreone and Mercurio 2005)
7. (Andreone et al. 2005b)
8. (Andreone et al. 2006)
9. (Andreone et al. 2007)
10. (Baraud and Cadle 2016)
11. (Benstead et al. 2001)
12. (Britt and Virkaitis 2003)
13. (Brown et al. 2014a)
14. (Brown et al. 2016)
15. (Burney 2002)
16. (Cadle 1996)
17. (Cadle 1999)
18. (Cadle 2004a)
19. (Cadle 2004b)
20. (Cadle 2004c)
21. (Cadle 2014)
22. (Cressey 2014)
23. (Crottini and Rosa 2010)
24. (Dollar 1999)
25. (Dollar et al. 2007)
26. (Dunham 1998)
27. (Farris et al. 2015)
28. (Fatroandrianjafinonjasolomiovazo et al. 2011)
29. (Fietz and Ganzhorn 1999)
30. (Forsman 1993)
31. (Gandola et al. 2013)
32. (Gardner 2013)
33. (Gardner and Jasper 2009)
34. (Gardner et al. 2013)
35. (Gardner et al. 2015a)
36. (Gardner et al. 2015b)
37. (Gehring et al. 2010)
38. (Gerber et al. 2012)
39. (Glaw and Vences 2007)
40. (Glaw et al. 2003)
41. (Glos 2006)
42. (Glos and Linsenmair 2004)
43. (Goodman 2004)
44. (Goodman et al. 1997)
45. (Gould and Eisenberg 1966)
46. (Groves and Groves 1978)
47. (Hardy and Crnkovic 2006)
48. (Hawkins 2004)
49. (Heying 2001)
50. (Hirschfeld and Rödel 2011)
51. (Jenkins 2004)
52. (Jenkins et al. 2009b)
53. (Jovanovic et al. 2009)
54. (Kaloloha et al. 2011)
55. (Kerridge et al. 2004)
56. (Kosuch et al. 2001)
57. (Knoll et al. 2009)
58. (Krysko 2005)
59. (Kuchling and Garcia 2003)
60. (Kuchling et al. 2004)
61. (Lahann 2007)
62. (Lahann and Dausmann 2011)
63. (Lehtinen et al. 2003)
64. (Levesque et al. 2012)
65. (Levesque et al. 2013)
66. (Luiselli et al. 2000)
67. (Luiselli et al. 2004)
68. (Luiselli et al. 2011)
69. (McClelland et al. 2015)
70. (Mercurio et al. 2006)
71. (Miralles et al. 2011)
72. (Mori 2002)
73. (Mori and Randriamahazo 2002)
74. (Mori and Tanaka 2001)
75. (Ndriantsoa et al. 2014)
76. (Ndriantsoa et al. 2017)
77. (Nicol 2004)
78. (Rakotondravony et al. 1998)
79. (Raselimanana 2004)
80. (Raselimanana and Rakotomalala 2004a)
81. (Raselimanana and Rakotomalala 2004b)
82. (Rasoloarison et al. 1995)
83. (Raxworthy 2004b)
84. (Raxworthy and Nussbaum 1993)
85. (Raxworthy and Nussbaum 1994)
86. (Raxworthy et al. 2011)
87. (Rödel 1999)
88. (Rosa and Rakotozafy 2013)
89. (Rosa et al. 2010)
90. (Rosa et al. 2012a)
91. (Rosa et al. 2012b)
92. (Rosa et al. 2014)
93. (Ruane et al. 2016)
94. (Schmidt et al. 2009)
95. (Schulze et al. 2016)
96. (Segev et al. 2012)
97. (Somrimalala and Goodman 2004)
98. (Stephenson 1994)
99. (Stephenson and Racey 1994)
100. (Sterling 1994)
101. (Sterling et al. 1994)
102. (Struijk 2014)
103. (Surendran and Vasudevan 2013)
104. (Tingle 2012)
105. (Vallan 2000)
106. (Vences and Glaw 2004a)
107. (Vences and Glaw 2004b)
108. (Vences and Glaw 2004c)
109. (Vences and Glaw 2005)
110. (Vences et al. 1999b)
111. (Vences et al. 2002)
112. (Vences et al. 2003)
113. (Vences et al. 2004a)
114. (Vences et al. 2004b)
115. (Vences et al. 2015)
116. (Wallace and Leslie 2008)
117. (Wright et al. 1997)

# Discussion

The non-resistant form of the  $\text{Na}^+, \text{K}^+$ -ATPase is widespread across Malagasy fauna. No sampled species harbour the configuration of their  $\text{Na}^+, \text{K}^+$ -ATPase known to confer resistance to bufotoxins (Ujvari et al. 2015). The cross-section presented should prompt concern for other endemic species, and highlights how potentially widespread the impacts of a new toxic invader could be. Further, sampling effort would be required to confirm the breadth of vulnerability.

The chances that species have an alternative method of dealing with this form of toxin is extremely unlikely. Mohammadi et al. (2013) has suggested adrenal gland size may play a role in a several snake species' ability to consume highly toxic amphibians. More recent work has shown that bufophagous snakes may additionally use high levels of aldosterone to help combat the inhibition of the  $\text{Na}^+, \text{K}^+$ -ATPase (Mohammadi et al. 2017a). However, these additional anti-bufotoxin adaptations were found in the highly specialised *Rhabdophis tigrinus* and not other resistant but non-toxin sequestering snakes (Mohammadi et al. 2017a), leading to the conclusion that this is directly related to that sequestration ability and would not be found nor useful to snakes that have a non-resistant  $\text{Na}^+, \text{K}^+$ -ATPase. It has also been found that all studied bufophagous species have a resistant  $\text{Na}^+, \text{K}^+$ -ATPase, suggesting that it is at least a prerequisite (Mohammadi et al. 2016b). The breadth and diversity of taxa using this solution to combat cardiac glycosides, and bufotoxins, suggests not only that it is optimal but makes it an excellent predictor of resistance (Ujvari et al. 2015).

Madagascar does have snakes documented eating toxic frog species, but these prey species do not have comparable toxin to bufonids (Daly et al. 1984; Jovanovic et al. 2009). Unless they have a solution to deal with ingested toxins universally, out with of substitutions on the binding site of bufotoxins, these species will be equally as vulnerable as those who do not consume native toxic prey.

The discovery of widespread lack-of-resistance means that the relative impacts on species will be almost entirely dependent upon niche overlap and interspecific interactions, which can be extremely difficult to understand (Simberloff 1995). Life or

natural history traits will have a marked impact on how species weather the invasion (Feit and Letnic 2015). In fact the similarity between species' niches may be a better indicator of future impact by invasives than taxonomic relatedness (Saul et al. 2013). This is especially key when considering cases of convergent evolution (Saul et al. 2013).

Unfortunately, such data for Madagascar remains scarce and many species are in need of far more detailed studies. Dietary studies are particularly uncommon (Glaw and Vences 2007). This section aims to summarise natural history traits that would affect the chances of predation on *D. melanostictus* as well as adding additional detail on evolutionary history for selected species (Table 6).

## Herpetofauna

### Snakes

All of the 27 sequenced snake species were found to have a non-resistant form of the Na<sup>+</sup>,K<sup>+</sup>-ATPase. Three of them belong to Boidae – *Acrantophis dumerili* (Dumeril's ground boa), *Acrantophis madagascariensis* (Madagascar ground boa), *Sanzinia madagascariensis madagascariensis* (Madagascar tree boa). They represent all the boid species on Madagascar (Glaw and Vences 2007; Raxworthy 2004b; Vences et al. 2001). They are likely to have found their way to Madagascar around ~76 Myr BP and share characteristics from both Africa and South America (Noonan and Chippindale 2006b; Samonds et al. 2013; Vences et al. 2001). They are all known to forage terrestrially and to be primarily active at night (Andreone and Luiselli 2000; Glaw and Vences 2007; Raxworthy 2004b), and *S. m. madagascariensis* is also known to forage arboreally (Andreone and Luiselli 2000; Glaw and Vences 2007; Rakotondravony et al. 1998). While potentially beneficial, arboreal foraging does not preclude encountering *D. melanostictus* who have infrequently been reported to climb vegetation despite their apparent adaptation to terrestrial life (Norval et al. 2009).

Supporting these results is a captive dietary study indicating that *S. madagascariensis* does not survive ingestion of a bufonid (Groves and Groves 1978), although no details are provided concerning the size of this toad beyond "small" (Groves and Groves 1978:19). The reports of *A. madagascariensis* and *S.*

*madagascariensis* taking large lemurs indicate they are more than capable of consuming toads of sufficient size to deliver a lethal dose (Gardner et al. 2015a; Rakotondravony et al. 1998), and Phillips et al. (2003) indicates that even consuming smaller toads can prove fatal to numerous Australian snakes .

*Acrantophis madagascariensis* is the only one of the three who has no confirmed reports of amphibian consumption. However, an incident of failed predation of a toxic *Mantella laevis* (arboreal mantella) and the generalist diet of the other boids suggests anurans could be a prey item (Andreone and Mercurio 2005; Glaw and Vences 2007; Heying 2001). The reaction of the individual in Heying's (2001) observation indicate that palatability played a large role in why the *A. madagascariensis* did not complete its ingestion of *M. laevis*. The palatability of *D. melanostictus* may influence the mortality rate relative to predation attempts, presuming that the initial mouthing does not deliver a lethal dose. Returning to Groves and Groves' (1978) captive feeding trials a lethal dose was delivered from *Anaxyrus fowleri* (referred to as *Bufo woodhousei fowleri*; Woodhouse's toad) to *Leioheterodon madagascariensis* in only five seconds of mouthing. These larger snakes may be more vulnerable due to their increased inclination to take larger toad prey that contain more toxin (Pearson et al. 2014). The reports out of Madagascar currently indicate that their larger body sizes are not enough to prevent death (Cressey 2014; McClelland et al. 2015). In Australia there is a pattern of larger varanids being more likely to predate a *R. marina*, and still being significantly impacted by paratoid-gland-devoid specimens (Jolly et al. 2016). Further, Feit and Letnic (2015) show that the increased gape of large squamates has a greater negative impact on survival than any increase in body size.

Other than the Boidae, 24 members of Lamprophiidae formerly grouped in Colubridae were investigated (Vidal et al. 2007). The Lamprophiidae radiation occurred later than for Boidae and includes the majority of snake species on Madagascar. They are now diverse in their characteristics and niches. Around half of the sampled Lamprophiidae members demonstrate nocturnal behaviour, and the majority have been found to forage terrestrially (Andreone and Luiselli 2000; Cadle 2004b; Cadle 2014; Glaw and Vences 2007; Gehring et al. 2010; Kaloloha et al. 2011; Raxworthy and Nussbaum 1994; Rosa et al. 2012b; Struijk 2014), making them more likely to encounter the nocturnal *D. melanostictus* (Mathew 1999). Any of

these traits boosting encounter rate and inclination to take anurans make species more vulnerable (Feit and Letnic 2015). However, toad species are known to shift ontogenetically from diurnal to nocturnal activity (Pizzatto et al. 2008). The diurnal Malagasy snakes will still be threatened because even the smaller juvenile toads are likely to be fatally toxic (Phillips et al. 2003).

These are the broadest of predictors, within the scarcity of dietary records, 20 snakes are known to consume amphibians (Andreone and Luiselli 2000; Cadle 2004b; Cadle 1996; Cadle 2014; Glaw and Vences 2007; Gandola et al. 2013; Jovanovic et al. 2009; Mori and Tanaka 2001; Rosa et al. 2010). Some of those amphibians even produce toxins, although their toxins differ by being primarily alkaloid (Andriamaharavo et al. 2015; Daly et al. 1984; Vences and Glaw 2004c). These anurophagous species are expected to be especially vulnerable to an introduced amphibian with a novel toxin.

Anurophagous behaviour is expected to be more widespread than the current literature summary suggests. Species such as *Pseudoxyrhopus heterurus* (night brook snake) and *Ithycyphus goudoti* (forest night snake) have not been confirmed as eating anurans but are reportedly found near water bodies (Glaw and Vences 2007; Raxworthy and Nussbaum 1994). Given that snakes as a group are one of anurans biggest predators (Toledo et al. 2007), their diets will likely include anurans in some form. It must also be noted that predators can react in novel ways to new prey. In Australia there are a few reports of species killed by *R. marina* who have not previously been documented eating anurans (Pearson et al. 2014).

Another trait that *P. heterurus*, along with a few other species have, is an ability to survive in more disturbed anthropogenic areas (Andreone and Luiselli 2000; Gardner et al. 2015b; Glaw and Vences 2007; Kaloloha et al. 2011; Struijk 2014). Reports so far indicate *D. melanostictus* densities are greatest in those areas (McClelland et al. 2015; Moore et al. 2015). So, it would be reasonable to expect an increased encounter rate for those snakes making use of the more disturbed areas. By contrast *Liopholidophis grandidieri* (Grandidier's water snake) and *Liopholidophis sexlineatus* (six-lined water snake) are capable of living at very high altitudes (Cadle 2014; Glaw and Vences 2007). If the introduced *D. melanostictus* behaves similarly to those in their native range then these two species may be shielded from the invader at higher elevations.

One species that should be unlikely to encounter *D. melanostictus* is *Langaha madagascariensis* (Madagascar leaf-nosed snake). This species is very arboreal and hunts during the day (Andreone and Luiselli 2000; Glaw and Vences 2007; Krysko 2005; Tingle 2012). Studies of new hatchlings show that in captive environments they will only take arboreal prey (Glaw and Vences 2007). Unless reports of *D. melanostictus* moving arboreally become a common phenomenon in Madagascar *L. madagascariensis* should not be directly impacted by its presence. Although sounding initially unlikely, introduced *R. marina* has adapted rapidly to new environments to take advantage of arboreal prey in Hawaii and cliff-side habitats in Australia (Hudson et al. 2016b; Ward-Fear et al. 2016). There is even the possibility that arboreally foraging species that avoid bufonids will actually benefit from their arrival via reduced predation as seen with *Boiga irregularis* and *Dendrelaphis punctulatus* in Australia (Doody et al. 2013; Feit and Letnic 2015).

The snakes of Madagascar have been separated from bufonids and their toxins for over 20Myr (Nagy et al. 2003). This isolation may be partly to blame for the lack of resistance to the toxin, as demonstrated by the varanids of Australia (Ujvari et al. 2013). There it is suggested that there was a cost to maintain the resistant form of the  $\text{Na}^+, \text{K}^+$ -ATPase. Mori et al. (2012) go so far as to postulate that instances of “sudden death” seen in captive and wild *Rhabdophis tigrinus* may be a result of extreme bufotoxin resistance. Conversely, Mohammadi et al. (2016) suggest that in snakes the cost of maintenance is low. They discovered that many non-bufophagous snakes were still resistant, despite no continual selection pressure. It is suggested that bufophagous snakes may have non-resistance alpha1 and alpha2 isoforms of the  $\text{Na}^+, \text{K}^+$ -ATPase to counter any cost of a resistant alpha3 isoform (Mohammadi et al. 2016b). More recently they have found that *Thamnophis elegans* (western terrestrial garter snake) shows elevated expression of resistant  $\text{Na}^+, \text{K}^+$ -ATPase in the heart, kidney, liver and gut (Mohammadi et al. 2017b). This concentration to resistant  $\text{Na}^+, \text{K}^+$ -ATPase to key areas that are most affected by bufotoxins could go some way to reducing any cost of maintaining a less effective resistant  $\text{Na}^+, \text{K}^+$ -ATPase (Dalla and Dobler 2016; Mohammadi et al. 2017b). They do caveat their finding with the admission that investigations into the resistance and abundance of alpha1 and alpha2 isoforms in snakes are yet to be undertaken (Mohammadi et al. 2017b).

So while it may have been possible for Malagasy snakes to have maintained resistance during their long isolation and reduced selection pressure the alternative situation would be that they never had a resistant ancestor. Unfortunately, the mainland African relations of Malagasy lamprophiids were not fully explored by Mohammadi et al. (2016) who only sequenced *Boaedon fuliginosus* (African house snake). *Boaedon fuliginosus* was found not to be resistant and the ancestral reconstruction Mohammadi et al. (2016) performed indicated that there was a low chance that Lamprophiidae has a common resistant ancestor. The results of Malagasy snakes would further support this inference. While it does not preclude the existence of resistant snakes within Lamprophiidae, it would require an additional independent instance of toxic resistance evolution or multiple losses of that resistance if the assumption of a non-resistant ancestor is correct. As Mohammadi et al. (2016) suggests, more work is required on the African lamprophiids. This would also greatly help explain when or whether Malagasy species lost or ever had resistance.

Compared to the Malagasy snakes mentioned one species on Madagascar is even closer to its African ancestors – *Mimophis mahfalensis*. Unlike the Malagasy lamprophiids *M. mahfalensis* seems to have diverged much later, approximately 13 Myr BP (Cadle 2004b; Nagy et al. 2003). They likely rafted from Africa just before the ocean currents reached their present alignment that makes such events harder (Nagy et al. 2003; Samonds et al. 2012). They are more closely related to other African species than Malagasy (Kelly et al. 2008; Zheng and Wiens 2016). Several of those African species reportedly eat amphibians, and *Psammophylax variabilis* (Grey-bellied Grass Snake) will eat bufonids (Shine et al. 2006). There is also a report of *Psammophis longifrons* (stout sand snake), an Indian member of the subfamily Psammophiinae, accepting *D. melanostictus* (Vyas and Patel 2013). But this is anecdotal and occurred in captivity. Mohammadi et al. (2016) rightly calls for more work to be done in Africa and looking at the instances of bufophagy in Psammophiinae and among *Crotaphopeltis* spp. there may very well be a tenth instance of independently evolved bufotoxin resistance in snakes (Keogh et al. 2000).

The discovery of non-resistant Na<sup>+</sup>,K<sup>+</sup>-ATPase forms in *Mimophis mahfalensis* (this study) and *Boaedon fuliginosus* (Mohammadi et al. 2016b) begin to elucidate the



African situation but much more work is needed. Success would mean a more in depth understanding of African snake evolution and interaction with toxic prey. It is unfortunate that the primers, as well as trimmed primers, used to amplify the Malagasy snakes failed to do so for the available African snake species. More targeted work is required in the future.

Overall the many Malagasy snakes look extremely vulnerable to the introduction of *D. melanostictus*. There is widespread vulnerability to the toxins of *D. melanostictus* paired with widespread anurophagy. The combination of these will surely result in snake mortality. Predicting the extent of this mortality is far beyond the scope of this study but should be prioritised to inform conservation efforts if the initial efforts to eradicate the incursion fail. In the event that eradication efforts fail the snakes of Madagascar may mitigate the impacts by developing greater toxin resistance in the future (Phillips and Shine 2006b). This will not be achieved swiftly as the adaptations seen in Australia were achieved via selection rather than an individual's plasticity (Phillips and Shine 2006b).

### Lizards

All investigated lizards also have vulnerable  $\text{Na}^+, \text{K}^+$ -ATPase. Four lizards were successfully sequenced. Two belonging to Gerrhosauridae (Raselimanana et al. 2009). *Zonosaurus* spp. are likely to come across and attempt to consume *D. melanostictus* (Glaw and Vences 2007; Heying 2001; Jovanovic et al. 2009). *Zonosaurus madagascariensis* is documented as eating a toxic *Mantella* sp. (Heying 2001; Jovanovic et al. 2009). Unfortunately, the *Mantella*'s toxic are primarily alkaloid (Andriamaharavo et al. 2015; Daly et al. 1984)., therefore greatly reducing the chances that *Z. madagascariensis* adaptations will have any effect when facing bufotoxins. If anything, their ability to eat toxic and non-toxic frogs may put them at greater risk if they eat indiscriminately. This would equally be true for the snake *Thamnosophis lateralis* (lateral water snake), that has also been documented consuming a toxic *Mantella* sp. (Jovanovic et al. 2009). *Acanthophis praelongus* in Australia has demonstrated how native predators can react diversely to novel prey and how their usual methods of dealing with toxic frogs fail for toads (Hagman et al. 2009). One of the reasons they fail is that unlike some frogs, toad's toxin remains potent after its demise (Hagman et al. 2009; Phillips and Shine 2007). Hagman et al.'s (2009) study conclude that the foraging strategies make *Acanthophis*

*praelongus* more vulnerable. It is likely that non-resistant species in Madagascar will demonstrate similar differences in foraging strategy, and therefore differing levels of vulnerability.

Compared to *Z. madagascariensis*, *Z. brygooi* has even less literature concerning its diet. There is some information suggesting that it makes use of a different microhabitat, preferring more open habitats (Vences et al. 1999a). A preference for open habitats may lead to a greater encounter rate between *Z. brygooi* and *D. melanostictus*. Fortunately, these *Zonosaurus* spp. are largely diurnal (Vences et al. 1999a). An additional factor that should be considered outside of toxin resistance, is that they lay eggs below logs that could be used as day-time shelters for the toads (Raselimanana 2004). There is the possibility of nest site usurpation mirroring the Australian situation (Boland 2004; Raselimanana 2004).

One gekkonid and one scincid were sampled. The Gekkonid was *Geckolepis maculata*, a species known to frequent more disturbed anthropogenic areas (Raxworthy et al. 2011). *Geckolepis* spp. can get up to 70 mm snout to vent (Bauer 2004), therefore capable of taking small toads. The scincid was *Amphiglossus astrolabi*, one of the largest scincids on Madagascar (Miralles et al. 2011). *Amphiglossus astrolabi* is likely to encounter *D. melanostictus* if *D. melanostictus* ventures out from disturbed areas. *Amphiglossus astrolabi* avoid disturbed areas, are found near water bodies and will actively forage during night and day (Miralles et al. 2011; Raxworthy and Nussbaum 1993). Unlike the other squamates the only aspect of their Na<sup>+</sup>,K<sup>+</sup>-ATPase successfully sequenced was the alpha 1 isoform. This has not been investigated for resistant squamates (Mohammadi et al. 2017b). It is not known whether squamates require resistance in both alpha 1 and 3 isoforms to consume toads without ill effects. The alpha 1 isoform is found relatively ubiquitously in animal tissue (Juhaszova and Blaustein 1997; Köksoy 2002). This fact paired with the non-resistant form it takes suggests that they would feel some ill-effects or at least would require additional resources to overcome the toxin. I stress that without data on their alpha 3 isoforms no definitive answers can be given. A lone resistant alpha 3 isoform may be sufficient to protect an individual if it is paired with an ability to preferentially express that isoform at key organ that are targeted by the bufotoxin (Mohammadi et al. 2017b). Or when combined with other hormonal responses (Mohammadi et al. 2017a). The context of other squamate vulnerability makes this

seem like an unlikely prospect. Therefore like other species, the real impact of *D. melanostictus* will be dependent more on behaviour or niche overlap.

*Amphiglossus astrolabi* may have a greater chance of weathering the invasion. Gérard et al. (2016) shows that some skinks may be “predisposed” to avoid the toxins of bufonids. These skinks, *Caledoniscincus austrocaledonicus* (common litter skink), exist on isolated islands not until recently inhabited by *R. marina* but appear to use scent to avoid consuming the toxic prey (Gérard et al. 2016). This is in contrast to a tested gecko species, *Bavayia septuiclavis* (pale-stripe bavayia), that completely failed to avoid novel and native predator based on scent (Gérard et al. 2016). These differences in behaviour are crucial in deciphering the relative impacts on species in the face of the widespread vulnerability to bufotoxins. In addition to *A. astrolabi* two other closely-related and large skinks exist that share similar natural histories; *A. reticulatus*, and *A. waterloti* (Erens et al. 2017; Schmitz et al. 2005). There is every reason to think these skinks would see similar impacts to *A. astrolabi*.

Including these newly sequenced, very few lizard species outside of the varanids have had their  $\text{Na}^+, \text{K}^+$ -ATPase investigated. To my knowledge, outside of this study only four exist (Ujvari et al. 2015). All of which are non-resistant; bufotoxin resistance in squamates so far remains restricted to snakes and varanids.

Outside of these successfully sequenced species, lizards from Opluridae and Chameleonidae could not be amplified successfully. The two species of oplurid were *Oplurus cyclurus* (Merrem's Madagascar Swift) and *Oplurus quadrimaculatus* (Duméril's Madagascar Swift), which represent an arboreal and terrestrial species respectively (Chan et al. 2012). Like so many Malagasy herpetofauna, modern comprehensive dietary studies for these specific species are essentially non-existent, bar a suggestion of egg and lizard consumption by *O. quadrimaculatus* (Cadle 2004c). A sister species, *Oplurus cuvieri* (Madagascan collared iguana), has a modicum of data concerning its diet. They are found to be quite generalist, focusing on insects and vegetative matter, but will also consume small vertebrates (Rosa and Rakotozafy 2013). They are likely to be less impacted than the genuinely anurophagous or carnivorous species, perhaps doubly so if they consume toxic *Bryophyllum* spp. that contain compounds similar to bufotoxins (Price-Rees et al. 2012). Discovering the form of their  $\text{Na}^+, \text{K}^+$ -ATPase would provide insight into an endemic and potentially vulnerable family.

Chameleonidae are likely to be the least vulnerable to any invasion by a toxic toad, even if they are found to be non-resistant to the toxin. The three study species, *Furcifer pardalis* (panther chameleon), *Calumma parsonii cristifer* (Parson's chameleon) and *Calumma brevicorne* (short-horned chameleon), are extremely arboreal so are unlikely to ever encounter *D. melanostictus*. Especially for *C. parsonii* that is more associated with the primary forests of Madagascar (Jenkins et al. 2009b). *Furcifer pardalis* by contrast can live in more degraded areas and can often be found along road sides that mirror natural stream side microhabitats (Andreone et al. 2005b; Jenkins et al. 2009b). Others of the genus have been seen in more open areas (Gardner 2013). There are scattered reports of chameleons taking vertebrate prey but the validity of these remains questionable (Raselimanana and Rakotomalala 2004a). From the Chameleonidae family the most vulnerable to an introduced toad would likely be *Brookesia* spp. – Dwarf Chameleons. Their vulnerability has nothing to do with bufotoxins. Bufonid toads are voracious consumers and would have no issue eating a small terrestrial chameleon (Hahn 1976; O'Shea et al. 2013). Fortunately, the defence mechanism of *Brookesia* spp. may protect them from a visually stimulated predator (Hagman et al. 2009; Raxworthy 1991). Overall, while more knowledge surrounding their Na<sup>+</sup>,K<sup>+</sup>–ATPase would help elucidate their vulnerability, they are inherently at lower risk to bufonid poisoning than the other Malagasy reptiles.

### Amphibians

The widespread vulnerability to the toxins of *D. melanostictus* does not end with Malagasy squamates. All sequenced amphibians are vulnerable in much the same way. Even a species still known to be sympatric with bufonids – *Hoplobatrachus tigerinus*. These results raise the same fears as for the snakes as many sampled amphibians are known to eat other amphibians or vertebrates (Andreone 2004; Glos 2006; Hardy and Crnkovic 2006; Hirschfeld and Rödel 2011; Ndriantsoa et al. 2014; Raxworthy 2004a; Vences et al. 1999b; Vences et al. 2004b). Therefore, they are likely to consume the poisonous invader and suffer as a consequence, at least initially. In addition, due to the ecological similarity to toads native anurans will encounter a greater diversity of impacts other than prey toxicity (Shine 2014).

Of the 12 amphibians successfully sequenced, nine were from Mantellidae, one from Microhylidae, one from Hyperoliidae, and one from Dicroglossidae. Mantellidae is of

particular interest because they are endemic to Madagascar and the surrounding islands (Glaw and Vences 2007). Several of this family have been confirmed to eat other frogs or vertebrates – *Boophis goudotii* (Goudot's bright-eyed frog), *Mantidactylus femoralis* (fort Madagascar frog), *Mantidactylus ulcerosus* (warty Madagascar frog), and *Aglyptodactylus inguinalis* (Andreone and Nussbaum 2006; Vences et al. 1999b). Numerous mantellids are also found to occur outside of the pristine closed forests (Andreone 1994; Andreone 2004; Andreone et al. 2006; Cadle 2004a; Gardner and Jasper 2009; Glos and Linsenmair 2004; Glos 2006; Ndriantsoa et al. 2017; Schmidt et al. 2009; Segev et al. 2012; Vallan 2000; Vences and Glaw 2002; Vences and Glaw 2005; Vences et al. 2003; Vences et al. 2004a; Vences et al. 2004b). Therefore, they are likely to come into close contact with *D. melanostictus*.

*Ptychadena mascareniensis* and *Hoplobatrachus tigerinus* are two of the few amphibians not endemic to Madagascar. Both can be found in agricultural or urban areas (Ndriantsoa et al. 2017; Gardner and Jasper 2009; Vences et al. 2004a; Vences et al. 2004b) and are known to eat frogs or tadpoles (Fatroandrianjafinonjasolomiovazo et al. 2011; Hardy and Crnkovic 2006; Kosuch et al. 2001; Surendran and Vasudevan 2013). Out of the two only *H. tigerinus* was successfully sequenced, showing a non-resistant, but unique, Na<sup>+</sup>,K<sup>+</sup>-ATPase. This lack of resistance may be mitigated by their more recently shared evolutionary history with Asia (Padhye et al. 2008; Vences et al. 2004b; Vences et al. 2004c). As *H. tigerinus* is still known to occur in areas where bufonids are present they must have a solution even if it is as simple as niche partitioning (Padhye et al. 2008). They may have developed behaviours that mean they will avoid bufonid prey. The impacts on *H. tigerinus* are likely to be considered low priority due to its status as an introduced species (Kosuch et al. 2001).

It is hoped that whatever learnt or evolutionary avoidance demonstrated by sympatric non-resistant amphibians can be swiftly translated to natives of Madagascar. Looking to Australia again there is evidence that even originally naïve species can learn to avoid toxic prey (Shine et al. 2009).

*Heterixalus madagascariensis* (Madagascar reed frog) and *Dyscophus guineti* belong to non-endemic families that are also sympatric with bufonids. Not much information exists for *Heterixalus madagascariensis*' ecology but they are thought to

be a generalist and active at night (Vences and Glaw 2004b). *Dyscophus guineti* has garnered more attention, perhaps due to its close relation to the near-threatened, and flagship species, *Dyscophus antongilii* (Raxworthy et al. 2008). This additionally makes their lack of resistance an important consideration for Malagasy amphibian conservation. *Dyscophus guineti*'s tadpoles take a long time to metamorphose and therefore get very large (Segev et al. 2012), potentially allowing them to predate the toxic spawn of *Duttaphrynus melanostictus*. Fortunately for *Dyscophus guineti*, they are largely restricted to rainforest ponds (Tessa et al. 2011), unlike its near-threatened relation (Andreone et al. 2005a; Andreone et al. 2006; Raxworthy et al. 2008). The results suggest that if *Duttaphrynus melanostictus* expands to areas where *Dyscophus antongilii* are still present they could become an unwelcome addition to the pressures already on this threatened endemic.

This is also true for *Mantella baroni*, whose lack of resistance suggests the same for the closely related, endangered, *Mantella cowanii* (Cowan's mantella) (IUCN SSC Amphibian Specialist Group 2014; Chiari et al. 2005; Rabemananjara et al. 2007). *Mantella cowanii*'s restricted range and preference for open areas will likely make it additionally vulnerable to the pressures presented by a generalist toad (Andreone et al. 2005a). Both of these genera have species that have already experienced declines due to the pet trade and remain at risk from further habitat loss (Andreone et al. 2005a; Andreone et al. 2006; Rabemananjara et al. 2008).

The relative timing of egg laying, tadpole development, and metamorphosis could be critical to predator prey balance between natives and invader species (Crossland et al. 2009; Shine 2012). This is something that greatly obscures any predictions of amphibian impact given their non-resistant to bufotoxins. Where, when and how frequently eggs are laid could all influence the outcome. Some of the species mentioned so far lay eggs on leaves (Vences and Glaw 2005; Vences et al. 2015), that could relieve them of any elevated predation from *D. melanostictus*. Others may be vulnerable due to breeding later into the wet season (Schulze et al. 2016); or conversely suppress an invading toad's breeding capabilities (Haramura et al. 2016). All of them likely have the capacity to learn or adapt to avoid toxic prey as may be occurring in Australia (Shine et al. 2009). While others, such as those belonging to Microhylidae whose tadpoles are filter feeders (Wu 2004), may have no need to adapt at all. More so than any other order examined, amphibians have the greatest

potential for a complex interaction with the introduced *D. melanostictus* because of the greater number of potential interaction pathways (Kraus 2015).

The results presented here suggest widespread vulnerability to the toxins of *Duttaphrynus melanostictus* among Malagasy amphibians. *Duttaphrynus melanostictus* presents an additional pressure to Malagasy amphibians and should be considered in future conservation scenarios, with special attention paid to calculating niche overlap and potential predation situations.

### Chelonians

Samples were obtained for two species of Chelonian – *Pelomedusa subrufa* (African helmeted turtle) and *Pelusios castanoides* (yellow-bellied mud turtle) – that occur across Africa (Fritz et al. 2013; Wong et al. 2010). While both failed to be amplified with the primers available notes on their natural history may still provide some insight into their vulnerability.

Both are largely generalists in terms of diet and will eat frogs, tadpoles and frog eggs (Kuchling and Garcia 2004; Luiselli et al. 2004; Luiselli et al. 2011; Rödel 1999). *Pelomedusa subrufa* especially will eat large quantities of tadpoles and range between multiple ponds to do so (Rödel 1999). They are capable of eating large quantities of tadpoles in one sitting, between 13 to 31% of the turtle's body weight (Rödel 1999). This foraging strategy could greatly increase the dosage of bufotoxin ingested at one time and reduce any chance of survival if the species does not have an innate resistance. *Pelomedusa subrufa* also favour more ephemeral pools, as opposed to *Pelusios castanoides*, which is found more readily in permanent water bodies (Luiselli et al. 2000; Luiselli et al. 2004; Rödel 1999). This would suggest that *Pelomedusa subrufa* is going to encounter *D. melanostictus* more frequently. An additional factor that may alter how frequently *P. subrufa* predate *D. melanostictus* tadpoles is how fast and active the tadpoles are relative to any sympatric tadpoles. Feeding studies have shown they are more keen to eat tadpoles that are slower moving and spend more time near the base of pools (Rödel 1999). No sufficient data was found in the literature to estimate this, a study looking at activity patterns of *D. melanostictus* tadpoles and native anurans would be required. Such a study would be complex and difficult to translate to a natural environment given that tadpoles are

known to act and grow differently depending on the conditions of ponds and predation risk (Mogali et al. 2011).

Both of these species range across Africa (Fritz et al. 2013; Wong et al. 2010). Their arrival on Madagascar has occurred very recently in evolutionary terms, perhaps even due to humans (Fritz et al. 2013; Wong et al. 2010). Therefore, they would be expected to be extremely similar to their African counterparts in terms of toxin resistance and behaviour. Either behavioural or physiological adaptations must be responsible for their continued co-existence with bufonids. A study explicitly studying this family would be required to shed light on their evolutionary history in relation to the toxin resistance they may have. Further work could be aimed at assessing the vulnerability of the critically endangered *Erymnochelys madagascariensis* (Madagascar big-headed turtle) (Leuteritz et al. 2008).

### Crocodiles

Only one crocodile species is found on Madagascar and was unfortunately could not be amplified. The status of *Crocodylus niloticus* has been debated, whether it is a distinct sub-species or not (Schmitz et al. 2003). It appears to be closely related to those *C. niloticus* found in Mozambique and Zimbabwe (Hekkala et al. 2010). These populations live side by side with bufonids so there is a chance that the Malagasy populations have not diverged sufficiently with them to have lost whatever method they have for dealing with or avoiding bufonid poisoning. All other examined crocodilians have what appear to be resistant  $\text{Na}^+, \text{K}^+$ -ATPase, with charged amino acids at the start and end of the alpha 1 isoform's H1-H2 domain (see Appendix 10). The only record of the alpha 3 isoform is from *Crocodylus porosus* and would appear to be a non-resistant form. There are records of *C. porosus* eating *R. marina* in Australia without ill effect (Smith and Phillips 2006). However, there is no sequence data available for crocodilians that are confirmed to be incapable of eating bufonids to verify against. *Gavialis gangeticus* are widely considered to rely on fish and not other aquatic life, but still seem to harbor an apparent resistance  $\text{Na}^+, \text{K}^+$ -ATPase (Stevenson 2015; Whitaker 2007). A comprehensive investigation into where *Crocodylus porosus*' resistance originates is required before confident prediction of other crocodilians resistance can be made. Especially in light of other crocodiles being capable of consuming bufonids (Beaty and Beaty 2012).



The  $\text{Na}^+, \text{K}^+$ -ATPase has also been found to be connected with salt concentration maintenance in crocodiles (Cramp et al. 2010). So the apparent results of toxin resistance in the  $\text{Na}^+, \text{K}^+$ -ATPase could be circumstantial. For example, looking into the Australian situation we see a clear distinction with *Crocodylus porosus*, a crocodile species that continues to be sympatric with bufonids, and *Crocodylus johnstoni*, one that is restricted to the more isolated island. But equally *C. porosus* occupies salt water environments whereas *C. johnstoni* are freshwater (Smith and Phillips 2006). Unlike terrestrial predators there are a greater number of factors in the crocodilian scenario that require further study. It would be informative if future work discovered whether this pattern applies to Madagascar, especially considering young Malagasy crocodiles are known to eat aquatic vertebrates along with their mainland cousins (Corbet 1960; Kuchling et al. 2004; Wallace and Leslie 2008). Although there are no confirmed reports of either taking any toxic species.

## Mammals

While amphibians may be the most threatened group globally (Alford 2011; Hoffmann et al. 2010); on Madagascar the mammals probably represent the more threatened group. 63 native mammals are classified as endangered, with 22 as critical (IUCN 2017). That is 36% of native mammal species, compared to 20% of reptiles and 31% of amphibians. However, reptiles have nearly twice as many species ranked as data deficient (IUCN 2017). It is critical for their survival that new emerging threats are identified as quickly as possible.

### Carnivora

Madagascar is home to its own endemic assortment of carnivores. They diverged on Madagascar around 24-18Myr ago (Yoder et al. 2003). The diets of the Malagasy carnivora immediately make them more vulnerable than other mammals (Brown et al. 2016; Dollar et al. 2007; Dunham 1998; Goodman et al. 1997; Rasoloarison et al. 1995). *Eupleres goudotii* (eastern falanouc), *Fossa fossana* (Malagasy civet) and *Cryptoprocta ferox* are all also active nocturnally (Albignac 1972; Brown et al. 2016; Dollar 1999; Farris et al. 2015; Gerber et al. 2012), unlike *Galidia elegans* (ring-tailed mongoose) that forages diurnally and therefore may be less likely to encounter *D. melanostictus* (Albignac 1972; Britt and Virkaitis 2003; Farris et al. 2015; Gerber et

al. 2012). However, *G. elegans* has been known to use more anthropogenically altered land, which hosts *D. melanostictus* in higher densities (Brown et al. 2016; Moore et al. 2015)

These species have already gained some attention in relation to the incipient toad invasion. Brown et al. (2016) examined the likely niche overlap between *D. melanostictus* and the native predators, and found it to be considerable. They caveat their finding with the fact that their modelling does not take into account small-scale interactions and, like all of Malagasy fauna, more work gathering natural history information is required (Brown et al. 2016). Furthermore, their niche model could likely be improved with the refined knowledge supplied by Vences et al. (2017) on the invading population native climatic conditions. As opposed to using the entire south east Asian range. Although a fuller understanding of the species and species complex of *D. melanostictus* would be beneficial before further range modelling efforts (Wogan et al. 2016; Vences et al. 2017). The flexibility of the introduced *D. melanostictus* remains unknown. Out of the species they examined, *E. goudotii* and *S. concolor* were found to be the most vulnerable due to their specialisation and reduced ranges (Brown et al. 2016). Whereas *F. fossana* and *G. elegans* have a wider distribution, more generalist diets and can inhabit a variety of habitats likely increasing their ability to weather further pressures (Brown et al. 2016). The Genbank records suggest that these carnivores lack resistance to bufotoxins (see Appendix 11).

No sequenced African carnivore harbours any resistance. Unfortunately, the records cover Felidae among others and fail to include any species from the more closely related family Viverridae (Yoder et al. 2003). Given the lack of sequence data for viverrids, dietary studies must be relied upon. They suggest viverrids largely avoid eating amphibians (Colon 1999; Colon and Sugau 2012; Habtamu et al. 2017; Guy 1977; Mullu and Balakrishnan 2014; Mudappa et al. 2010). Or though some do eat amphibian species the species was unconfirmed and the it could easily not be a toad (Chuang and Lee 1997).

Given the albeit limited genetic investigation into carnivore resistance to bufotoxins the likely scenario is that Malagasy fauna will indeed be vulnerable. The niche overlap, paired with small-scale interactions and behavioural flexibility, are the

crucial factors if invasion impacts are to be estimated now that toxic resistance is known not to exist.

## Lemurs

Perhaps the most famous and best-loved of Malagasy fauna are the lemurs; they arrived in Madagascar 38Myr earlier than the carnivores producing an earlier divergence date from their mainland ancestors (Yoder et al. 2003; Yoder and Yang 2004). All species examined here have no resistance to bufotoxins. The sequences cover three out of the five families of lemur (Martin 2000). Except for *Daubentonia madagascariensis* they show identical forms of the Na<sup>+</sup>,K<sup>+</sup>–ATPase. A form consistent across all primates (see Appendix 9). *Daubentonia madagascariensis*’ substitutions produce no change in residual charge and therefore make no functional difference.

Despite the non-resistance, compared to other mammals they are likely to be less at risk due to their diet consisting largely of leaves, fruit and invertebrates (Simmen et al. 2003). The Aye-aye (*Daubentonia madagascariensis*), renowned for its bizarre grub targeting finger, eats mainly grubs, nectar, fungus and other plant matter (Andriamasimanana 1994; Sterling 1994; Sterling et al. 1994). Members of Indriidae are also quite uniquely equipped, suited to consuming leaf matter more effectively than other foods (Boyer 2008; Simmen et al. 2003). The lack of a resistant Na<sup>+</sup>,K<sup>+</sup>–ATPase indicates that these lemurs must also avoid toxic plants found on Madagascar such as *Bryophyllum* spp. (Oufir et al. 2015).

While most lemurs are primarily herbivorous there are reports of vertebrate predation by lemurs; ranging from reptiles and birds, to other lemur species (Glander et al. 1985; Ichino and Rambeloarivony 2011; Jolly and Oliver 1985; Jolly et al. 2000; Mizuta 2002; Oda 1996; Pitts 1995; Simmen et al. 2003). Oda (1996) postulates that these instances are more common than observations suggest, citing the efficacy of a *Lemur catta* (ring-tailed lemur) subdual of a chameleon. This is supported by the consistent manner of chameleon dispatch described by Ichino and Rambeloarivony (2011). They go on to suggest that the reason that chameleons seem to be the most predated vertebrate of lemurs is their lack of mobility. This would also help explain *Eulemur fulvus*’ (common brown lemur) targeting of nestling birds and eggs (Mizuta 2002; Nakamura 2004). If mobility is the limiting factor then fears of lemurs taking

slow toads may be well placed. Captive lemur observations also support the idea that lemurs will opportunistically take vertebrate prey if they can catch it (Glander et al. 1985; Jolly and Oliver 1985).

It is difficult to judge which of the two lemur species will be more at risk. *Lemur catta* are less likely than *Eulemur fulvus* to take vertebrate prey, but the observations of *L. catta* predation occur more frequently in the rainy season (Glander et al. 1985; Ichino and Rambeloarivony 2011; Jolly and Oliver 1985). It is thought these seasonal patterns reflect timing in lemur breeding cycles as well as how easily prey is identified (Ichino and Rambeloarivony 2011). Additionally, compared to other primates, the teeth of *L. catta* and *E. fulvus* are less specialised to an insectivore or folivore diets (Boyer 2008). Even in these better studied Malagasy faunas much is unknown and there is considerable uncertainty surrounding their reaction to an invasive toad.

This overview has focused on the instances of vertebrate predation and so may over-state their importance in lemur life histories. Overall lemurs are expected to be considerably less vulnerable to *Duttaphrynus melanostictus*' introduction than other vertebrate consuming mammals. Furthermore, there are no reports of them taking the other introduced anuran *Hoplobatrachus tigerinus*.

## Tenrecs

One tenrec has sequence data for the Na<sup>+</sup>,K<sup>+</sup>-ATPase, *Echinops telfairi* (lesser hedgehog tenrec). While this Malagasy mammal shares many characteristics with its doppelganger *Erinaceus europaeus* (European Hedgehog) it does not share *E. europaeus*' resistance to bufotoxins. *Erinaceus europaeus* is one of the few mammals known to be resistant to bufotoxins (see Appendix 8), going so far as to use the toxins in their own defense (Brodie Jr. 1977; Ujvari et al. 2015).

However, *Echinops telfairi* and other tenrecs share some natural history traits that will likely put them in contact with *D. melanostictus*. They are largely terrestrial, nocturnal and have wide but insect-focused diets (Gould and Eisenberg 1966; Lovegrove and Génin 2008; Nicoll 2004; Peveling et al. 2003; Somrimalala and Goodman 2004). There are reports of some tenrec species predating amphibians and tadpoles (Somrimalala and Goodman 2004; Benstead and Olson 2004; Jenkins 2004). Tenrecs are not restricted to pristine forest either, *E. telfairi* and *Hemicentetes*

*nigriceps* are found in more disturbed areas (Gould and Eisenberg 1966; Stephenson et al. 2016).

Tenrec are an additional potential predator that will not be able to combat the toxins of that prey. Perhaps the most at risk would be *Limnogale mergulus* otherwise known as the aquatic tenrec that not only eats frogs but lives, as the name suggests, near water bodies (Benstead and Olson 2004; Gould and Eisenberg 1966). They would of considerably higher conservation priority than other tenrecs due to their uniqueness among tenrecs and already elevated conservation status (Benstead et al. 2001; Stephenson 1994).

### Implications and Behavioural Adaptations

Unfortunately, while this natural history can be used to suggest differential levels of impact on species (Feit and Letnic 2015), there is no guarantee that any of it will translate in reality. In Australia, non-resistant species have shown a diverse reaction to *R. marina* (references throughout). Traits that may have played a key role in their survival are simply not known for many Malagasy species yet. The ability to differentiate between toads and frogs for example likely has saved *Boiga* and *Dendrelaphis spp.* (Pearson et al. 2014). Or other compounding effects may benefit some populations while harming others, as seen with two populations of *Acanthophis praelongus* (Brown et al. 2011b; Phillips et al. 2010). The results presented here provide a strong case for attention to be paid to this recent introduction of a toxic toad. Widespread vulnerability creates the potential for widespread impacts. Future work should aim to elucidate the potential niche overlap of the native and invasive species.

Beyond the immediate natural history traits modifying the impacts of species lack of resistance, there are learning and behavioural considerations. Some lizard species have demonstrated remarkable flexibility and cognitive ability on par with some birds (Leal and Powell 2012). While there have been mixed results in Australia regarding varanid's ability to learn avoidance (Doody et al. 2013; Jolly et al. 2016; Llewelyn et al. 2014); *Tiliqua scincoides* has demonstrated its ability to learn via taste aversion in laboratory tests (Price-Rees et al. 2011). Also some frogs are learning avoidance (Greenlees et al. 2010). However, there is a distinct possibility that bufonid toxin will be too potent to allow any taste aversion learning, supported by the fact that some of

the lab-based studies have failed to reflect the reality of population declines (Price-Rees et al. 2011). When paired with Groves and Groves (1978) observations of *Leioheterodon madagascariensis* rapid demise this may well be the case for even large bodied Malagasy herpetofauna. This does not mean the existence of taste aversion, if found, is useless. Price-Rees et al (2011) entertain the possibility of pre-exposing *Tiliqua scincoides* populations to lower potency toad bait, to allow species to learn the aversion while not receiving a lethal dose of toxin. O'Donnell et al. (2010) show similar possibilities for Quolls. Before such a solution was to be implemented in Madagascar much more work would be required to discern exactly how much toxin species can ingest and whether they are capable of aversion learning. Furthermore, *Cryptoprocta ferox* exists at low densities (Hawkins and Racey 2005), and may make such an endeavour incredibly labour intensive and expensive before a significant proportion of the population is successfully 'inoculated'. It is expected that lemurs would be more than capable of recognising and learning to avoid toads. Making use of their social nature, learning and ability to take visual cues concerning food (Botting et al. 2011; Hosey et al. 1997; Kendal et al. 2010; O'Mara and Hickey 2012; Sandel et al. 2011). They have shown a level of self-control in reverse-reward contingency tasks, not as significant as the great apes, but not unsubstantial (Genty et al. 2011; Genty et al. 2004). Genty et al. (2011) suggest these cognitive abilities may be further heightened in the wild where pressures are greater. Regardless of the conscious efforts of conservationists there is no reason to expect Malagasy fauna are incapable of the aversion learning seen in other places (Greenlees et al. 2010; Jolly et al. 2016; O'Donnell et al. 2010; Price-Rees et al. 2011).

Understanding the behavioural and natural histories of species may prove crucial to fully understanding the impacts of *D. melanostictus* on Madagascar given the widespread toxic vulnerability. The species on Madagascar have either lost or never gained resistance to bufotoxins. This could be because the mainland ancestors never developed it or the isolation of Madagascar for millions of years has relaxed selection and species have shifted to a more catalytically effective non-resistant Na<sup>+</sup>,K<sup>+</sup>-ATPase. The latter would be in keeping with the findings surrounding varanids in Australia (Ujvari et al. 2013). Their Asian common ancestor, that was in contact with bufonids, was likely resistant. After millennia in an environment devoid of bufonids, and the pressure to combat their toxins, the Australian varanids lost that

adaptation. This is in contrast to the Asian varanids who remain resistant, maintained by their continued sympatry with bufonids (Ujvari et al. 2013). This is a logical conclusion given the studies indicating the advantages of a non-resistant Na<sup>+</sup>,K<sup>+</sup>-ATPase (Dalla and Dobler 2016). However, there are examples of snakes that are non-bufofagous but continue to harbour resistance suggesting little or no cost (Mohammadi et al. 2016b). How or why this has occurred is still unknown, more recent studies indicate this may have been achieved via gene duplication (Mohammadi et al. 2017b).

The results presented here do not speak too much of why the Malagasy fauna are vulnerable. Future studies investigating the evolution of bufotoxin or more widely cardiac glycoside resistance would need to sample species closely related to Malagasy species to discover whether resistance ever existed and if so when it was lost. Did the isolation of Madagascar from bufonids for tens of millions of years cause the inability to combat bufotoxin? Or were the ancestors of Malagasy fauna never resistant and avoided bufonid prey? Regardless of the reasons behind the lack of resistance, it is present and appears to be extremely widespread. Promoting serious consideration of *D. melanostictus*' future impacts and the conservation actions required.

The results here confirm the suspicions that the introduced toxic toad *D. melanostictus* is capable of poisoning the most prominent reptile and amphibian anuran predators. This vulnerability is likely not restricted to herpetofauna and tentative results garnered from mammals begin to confirm this. *Duttaphrynus melanostictus* is a threat to this diverse biodiversity hotspot via direct predator poisoning. This supports efforts to control their spread and limit their incursions into protected areas. It adds to the base of knowledge concerning how island ecosystems can be vulnerable to non-native species and identifies a clear mechanism which these negative impacts could manifest. The confirmation of the mechanism supports a robust and immediate response, as well as aiding future assessments of the risk invasives pose to island species (Bellard et al. 2017).

Given the paucity of successful eradications of island invasives (Wingate 2011), it appears unlikely that *D. melanostictus* will be removed from Madagascar. The incursion may have already reached critical mass. Eradication plans from 2015 stated that 1.5 million toads needed to be removed per year to keep numbers stable

(McClelland et al. 2015). Now in light of a political standstill during the subsequent years (M. Vences pers. comm.), eradication efforts may need to be abandoned in favour of exclusion zones or more inventive bio-controls (Simberloff et al. 2013). The protected areas across the east coast will likely need to expressly monitor for *D. melanostictus*, and counter any colonisation. Fortunately, *D. melanostictus*' affinity for disturbed areas may slow their invasion of protected primary forest.

Bio-control measures are a possible solution, if there was a native species found to be resistant and capable of consuming toads en masse. The results here make that seem unlikely to be the case in vertebrates. Invertebrates may be an option. Ants have been shown to consume recently metamorphosed toads in Australia (Ward-Fear et al. 2010b), and similar initiatives could be explored in Madagascar. Regardless of the actions taken to limit *D. melanostictus*' invasion, tough biosecurity measures need to be implemented to prevent further toads making the journey or other species making their way into Madagascar (Simberloff et al. 2013). Ultimately, it is likely that a lack of finance and political coordination will stall efforts sufficiently to ensure that *D. melanostictus* will be Madagascar's newest permanent resident.



# Conclusions

There are real and serious implications for ecosystems that are exposed to invasive species (Chornesky and Randall 2003; Kraus 2015; Vitousek et al. 1997). Invading species that harbour a novel toxin not otherwise experienced by the native fauna can have a dramatic impact and cause cascading impacts (references throughout). Case studies like Australia demonstrate the impacts can be severe and that the occurrence of invasive species requires close attention, especially in the light of heightened extinction rates (Ceballos et al. 2017; Shine 2010).

The recent introduction of *Duttaphrynus melanostictus* to the island of Madagascar presents new risks for the fauna of the island (McClelland et al. 2015). Direct competition is one way *D. melanostictus* may alter the Malagasy ecosystems, but as the Australian case study suggests the main impacts will be felt via predator poisoning (Shine 2010). This study aimed to elucidate this possibility by examining native fauna's  $\text{Na}^+, \text{K}^+$ -ATPase that is known to be the target of bufotoxins. It aimed to demonstrate which, if any, native species would be vulnerable to ingesting *D. melanostictus*.

The results show a widespread and serious vulnerability to the toxins of the invading *D. melanostictus*. The breadth and diversity of species examined suggests that unique evolutionary history of Madagascar has rendered it especially vulnerable to the introduction of a novel toxin.

This adds to the case for the rapid and comprehensive eradication called for by McClelland et al. (2015). While there may be considerable issues with using Australia's situation with *R. marina* as an allegory, it does highlight how serious the impacts of a novel toxic invader on naïve predators can be. Madagascar's clear vulnerability, along with its status as one of the richest and most valuable biodiversity hotspots, warrants the full support of current measures to quantify, monitor and limit *D. melanostictus*' incursion.

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# Appendix

Appendix 1 - Full list of samples and associated numbers.

Sample #	Species	Sample #	Species
1818	<i>Psammophylax tritaeniatus</i>	5076	<i>Furcifer pardalis</i>
2919	<i>Psammophylax rhombeatus</i>	5077	<i>Calumma parsonii cristifer</i>
2725	<i>Psammophylax variabilis</i>	5078	<i>Dyscophus guineti</i>
2501	<i>Rhamphiophis oxyrhynchus</i>	5079	<i>Calumma brevicorne</i>
2782	<i>Psammophis crucifer</i>	5080	<i>Boophis tephraeomystax</i>
4936	<i>Ithycyphus miniatus (cf.)</i>	5081	<i>Boophis madagascariensis</i>
4937	<i>Geodipsas infralineata</i>	5082	<i>Aglyptodactylus sp. ("inguinalis")</i>
4938	<i>Thamnosophis lateralis</i>	5083	<i>Amphiglossus astrolabi</i>
4939	<i>Dromicodryas quadrilineatus</i>	5084	<i>Pelomedusa subrufa</i>
4940	<i>Madagascarophis meridionalis</i>	5085	<i>Pelusios castanoides</i>
4941	<i>Sanzinia madagascariensis madagascariensis</i>	5086	<i>Leioheterodon geayi</i>
4942	<i>Langaha madagascariensis</i>	5087	<i>Leioheterodon modestus</i>
4943	<i>Pseudoxyrhopus heterurus</i>	5088	<i>Mimophis mahfalensis</i>
4944	<i>Liopholidophis grandidieri</i>	5089	<i>Hoplobatrachus tigerinus</i>
4945	<i>Lycodryas granuliceps</i>	5090	<i>Laliostoma labrosum</i>
4946	<i>Acrantophis dumerili</i>	5091	<i>Boophis goudotii</i>
4947	<i>Dromicodryas bernieri</i>	5092	<i>Mantidactylus femoralis</i>
4948	<i>Zonosaurus madagascariensis/haraldmeieri (cf.)</i>	5093	<i>Zonosaurus brygooi</i>
4949	<i>Liophidium torquatum</i>	5094	<i>Thamnosophis epistibes</i>
4950	<i>Alluaudina bellyi</i>	5095	<i>Thamnosophis infrasignatus</i>
4951	<i>Lycodryas citrinus</i>	M1	<i>Eupleres goudoti</i>
4952	<i>Acrantophis madagascariensis</i>	M2	<i>Setifer setosus</i>
4953	<i>Liopholidophis sexlineatus</i>	M3	<i>Fossa fossana Fierenana</i>
4954	<i>Compsophis boulengeri</i>	M4	<i>Echinops telfairii</i>
4955	<i>Geodipsas laphystia</i>	M5	<i>Hemicentetes nigriceps</i>
4956	<i>Leioheterodon madagascariensis</i>	M6	<i>Hemicentetes semispinosus</i>
4957	<i>Pseudoxyrhopus microps</i>	M7	<i>Cheirogaleus medius</i>
4958	<i>Ithycyphus goudoti</i>	M8	<i>Galidia elegans</i>
5066	<i>Ptychadena mascareniensis</i>	M9	<i>Eulemur sanfordi</i>
5067	<i>Oplurus cyclurus</i>	M10	<i>Cryptoprocta ferox</i>
5068	<i>Oplurus quadrimaculatus</i>	M11	<i>Oryzoryctes hova</i>
5069	<i>Mantidactylus ulcerosus</i>	M12	<i>Suncus murinus</i>
5070	<i>Mantidactylus guttulatus</i>	M13	<i>Microgale sp.</i>
5071	<i>Mantella baroni</i>	M14	<i>Limnogale mergulus</i>
5072	<i>Heterixalus madagascariensis</i>	M15	<i>Brachytarsomys albicauda</i>
5073	<i>Guibemantis timidus</i>	M16	<i>Microgale longicaudata</i>
5074	<i>Gephyromantis redimitus</i>	M17	<i>Hemicentetes semispinosus</i>
5075	<i>Geckolepis maculata</i>		



#### *Appendix 2 - Qiagen DNeasy Protocol Summary*

Initially, the samples were split in half using a scalpel sterilised by alcohol and flame, to a size of approximately 2mm<sup>3</sup>. Equipment was re-sterilised between samples to avoid cross-contamination. Half of the tissue was returned to alcohol storage, ready for further extractions if the first attempt failed. Tissue that was to be used in the extraction was transferred to 1.5 ml tube and had 180 µl of ATL buffer and 20 µl of proteinase K added. Tubes were then vortexed to ensure good mixing. They were then incubated at 56°C for at least 3 hours. For many of the samples they were left to incubate overnight. Upon completion, the tubes were vortexed and spun down in a centrifuge, 8000 rpm for 10 seconds (Jenson-PLS Spectrafuge 24D). All samples then had 200 µl of buffer AL and 200 µl ethanol added, then vortexed and spun down, before moving all the liquid contents into spin columns. The spin columns were centrifuged for 1 minute at 8000 rpm. Once complete the spin column was transferred to a new collection tube and had 500 µl of buffer AW1 added. Again, centrifuged for 1 minute at 8000 rpm. After 500 µl of buffer AW2 was added and centrifuging for 3.5 minutes at 13000 rpm. To complete extraction the spin columns were transferred to 1.5 ml tubes and had 60 µl of buffer AE. Tubes were left to incubate at room temperature for 10 minutes then centrifuged for 1 minute at 8000 rpm. 2° elutions were completed for some samples by adding 50 µl of buffer AE, incubating for 5 minutes at room temperature, and centrifuging for 1 minute at 8000 rpm.

#### *Appendix 3 - Results of the DNA extractions.*

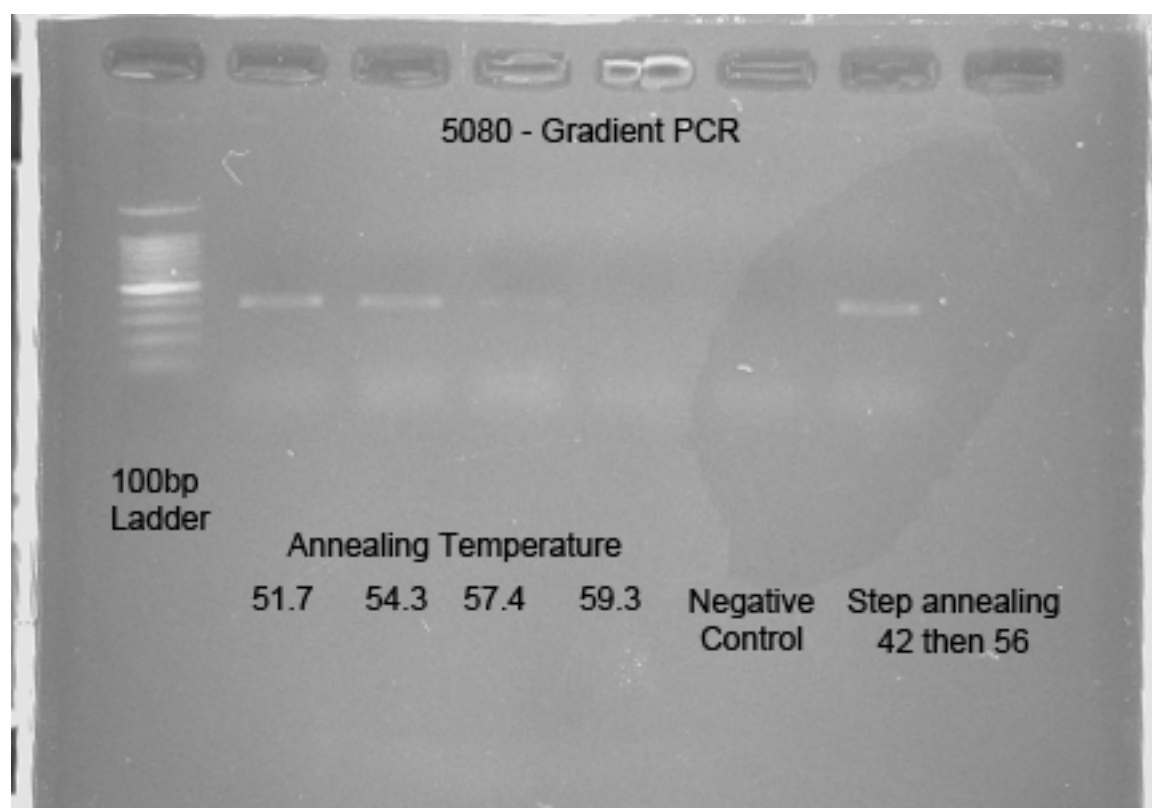
Sample ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
1818	18/05/2017	15:04	16.37	0.327	0.17	1.93	-2.82	50	230	-0.116	0.085
2501	07/04/2017	09:33	42.39	0.848	0.457	1.85	3.16	50	230	0.268	0.189
2725	18/05/2017	15:04	7.52	0.15	0.11	1.37	5.25	50	230	0.029	0.003
2782	07/04/2017	09:35	73.24	1.465	0.717	2.04	2.17	50	230	0.675	-0.036
2919	07/04/2017	09:36	40.92	0.818	0.373	2.19	5.18	50	230	0.158	-0.068
4936	08/11/2016	12:41	38.68	0.774	0.35	2.21	1.3	50	230	0.594	0.021
4937	08/11/2016	12:43	22.43	0.449	0.177	2.53	2.72	50	230	0.165	0.02
4938	08/11/2016	12:44	20.34	0.407	0.175	2.33	-11.45	50	230	-0.036	-0.022
4939	08/11/2016	12:46	65.07	1.301	0.59	2.2	2.77	50	230	0.47	0.024
4940	08/11/2016	12:48	92.87	1.857	0.875	2.12	2.78	50	230	0.667	-0.009
4941	08/11/2016	12:49	39.21	0.784	0.357	2.2	4.49	50	230	0.174	0.519
4942	16/03/2017	19:00	15.96	0.319	0.121	2.64	-56.71	50	230	-0.006	-0.013
4943	09/11/2016	12:50	34.78	0.696	0.341	2.04	5.09	50	230	0.137	0.081
4944	09/11/2016	12:51	54.67	1.093	0.497	2.2	2.44	50	230	0.448	-0.015
4945	09/11/2016	12:53	23.82	0.476	0.186	2.57	1.72	50	230	0.277	-0.036
4946	09/11/2016	12:54	31.88	0.638	0.272	2.35	5.79	50	230	0.11	-0.023
4947	16/03/2017	11:21	16.59	0.332	0.134	2.47	-3.32	50	230	-0.1	-0.098
4948	09/11/2016	12:55	68.19	1.364	0.63	2.16	2.53	50	230	0.54	-0.002
4949	16/03/2017	11:24	9.31	0.186	0.065	2.88	-2.08	50	230	-0.09	0.036
4950	09/11/2016	12:56	10.03	0.201	0.051	3.91	-1.41	50	230	-0.142	-0.017
4951	16/03/2017	11:25	9.04	0.181	0.068	2.67	1.18	50	230	0.153	0.033
4952	10/11/2016	10:08	307.76	6.155	3.046	2.02	2.26	50	230	2.725	0.005
4953	10/11/2016	10:09	49.27	0.985	0.443	2.23	1.72	50	230	0.573	0.068
4954	10/11/2016	10:10	257.72	5.154	2.722	1.89	2.37	50	230	2.172	0.043
4955	10/11/2016	10:11	36.71	0.734	0.317	2.32	2.72	50	230	0.27	0.017
4956	16/03/2017	11:26	18.39	0.368	0.134	2.75	-1.86	50	230	-0.198	7.847
4957	10/11/2016	10:12	120.89	2.418	1.111	2.18	2.76	50	230	0.876	0
4958	10/11/2016	10:13	64.88	1.298	0.58	2.24	2.67	50	230	0.486	0.031
5066	22/03/2017	12:17	191.48	3.83	1.796	2.13	2.36	50	230	1.626	0.049
5067	16/03/2017	15:47	39.31	0.786	0.4	1.96	0.67	50	230	1.168	0.303
5068	16/03/2017	15:48	113.17	2.263	1.107	2.05	1.23	50	230	1.836	0.271
5069	22/03/2017	12:18	24.52	0.49	0.217	2.26	2.13	50	230	0.23	0.061
5070	22/03/2017	12:19	43.36	0.867	0.461	1.88	1.37	50	230	0.633	1.8
5071	20/03/2017	11:45	57.56	1.151	0.512	2.25	1.99	50	230	0.58	0.659
5072	20/03/2017	11:46	30.31	0.606	0.27	2.25	7.22	50	230	0.084	0.015
5073	20/03/2017	11:48	9.95	0.199	0.046	4.32	12.89	50	230	0.015	0.028
5074	20/03/2017	11:49	6.29	0.126	0.017	7.26	-1.92	50	230	-0.065	0.037
5075	16/03/2017	15:49	118.75	2.375	1.378	1.72	0.75	50	230	3.182	1.555
5076	16/03/2017	15:50	67.35	1.347	0.733	1.84	0.83	50	230	1.628	2.655
5077	16/03/2017	15:51	67.42	1.348	0.785	1.72	0.6	50	230	2.245	0.962
5078	20/03/2017	11:49	8.24	0.165	0.056	2.96	3.11	50	230	0.053	0.095
5079	16/03/2017	15:52	69.9	1.398	0.817	1.71	0.65	50	230	2.161	1.728
5080	20/03/2017	11:50	64.28	1.286	0.516	2.49	3.73	50	230	0.345	0.05
5081	20/03/2017	11:51	3.29	0.066	0.011	5.81	-0.33	50	230	-0.197	0.02
5082	20/03/2017	11:52	33.01	0.66	0.277	2.38	4.76	50	230	0.139	0.046
5083	16/03/2017	15:53	26.72	0.534	0.26	2.05	0.49	50	230	1.082	0.065
5084	16/03/2017	15:54	57.84	1.157	0.645	1.79	0.59	50	230	1.968	0.812
5085	16/03/2017	15:55	80.59	1.612	0.906	1.78	0.59	50	230	2.719	1.085
5086	16/03/2017	11:27	180.57	3.611	2.113	1.71	0.92	50	230	3.929	25.021
5087	16/03/2017	11:28	13.91	0.278	0.133	2.09	1.39	50	230	0.2	-0.024
5088	16/03/2017	11:29	7.25	0.145	0.054	2.67	11.66	50	230	0.012	0.032
5089	22/03/2017	12:13	38.17	0.763	0.45	1.7	0.92	50	230	0.831	0.822
5090	22/03/2017	12:14	96.32	1.926	0.885	2.18	1.99	50	230	0.969	0.026
5091	22/03/2017	12:15	31.14	0.623	0.285	2.19	1.93	50	230	0.322	0.087
5092	22/03/2017	12:16	9.32	0.186	0.077	2.43	1.21	50	230	0.154	0.093
5093	16/03/2017	15:46	84.16	1.683	0.863	1.95	0.96	50	230	1.762	0.607
5094	16/03/2017	11:30	125.34	2.507	1.522	1.65	0.73	50	230	3.428	6.82
5095	16/03/2017	11:31	53.1	1.062	0.68	1.56	0.85	50	230	1.246	2.758
M1	17/05/2017	14:40	35.2	0.704	0.349	2.02	4.85	50	230	0.145	-0.007
M2	17/05/2017	14:40	36.78	0.736	0.351	2.09	2.61	50	230	0.282	-0.017
M3	17/05/2017	14:41	61.66	1.233	0.599	2.06	2.1	50	230	0.587	-0.001
M4	17/05/2017	14:42	38.41	0.768	0.367	2.09	2.08	50	230	0.369	-0.016
M5	17/05/2017	14:43	59.37	1.187	0.549	2.16	5.04	50	230	0.236	-0.002
M6	17/05/2017	14:43	36.47	0.729	0.363	2.01	2.47	50	230	0.295	0.482
M7	18/05/2017	09:22	300.88	6.018	2.926	2.06	2.32	50	230	2.596	0.096
M8	18/05/2017	09:22	31.7	0.634	0.283	2.24	5.14	50	230	0.123	-0.002
M9	18/05/2017	09:23	41.66	0.833	0.367	2.27	6.43	50	230	0.13	-0.015
M10	18/05/2017	09:24	57.1	1.142	0.56	2.04	1.51	50	230	0.755	0.007
M11	18/05/2017	09:25	42.56	0.851	0.359	2.37	2.17	50	230	0.393	0.002
M12	18/05/2017	09:26	28.6	0.572	0.271	2.11	5.49	50	230	0.104	-0.011
M13	18/05/2017	15:01	33.87	0.677	0.31	2.19	-20.99	50	230	-0.032	-0.035
M14	18/05/2017	15:01	14.57	0.291	0.133	2.19	-1.79	50	230	-0.163	0.014
M15	18/05/2017	15:02	18.51	0.37	0.167	2.22	-4.74	50	230	-0.078	-0.009
M16	18/05/2017	15:03	12.52	0.25	0.088	2.84	-1.2	50	230	-0.208	-0.017

Second Elutions											
4936_2	08/11/2016	12:52	18.35	0.367	0.163	2.25	-3.68	50	230	-0.1	-0.012
4937_2	08/11/2016	12:53	-0.11	-0.002	-0.031	0.07	0.01	50	230	-0.24	-0.013
4938_2	08/11/2016	12:54	1.15	0.023	-0.011	-2.07	-0.09	50	230	-0.264	-0.013
4939_2	08/11/2016	12:55	1.59	0.032	-0.026	-1.24	-0.13	50	230	-0.248	-0.014
4940_2	08/11/2016	12:56	23.03	0.461	0.2	2.3	-7.81	50	230	-0.059	0.006
4941_2	08/11/2016	12:57	23.43	0.469	0.227	2.06	-4.41	50	230	-0.106	-0.031
4943_2	09/11/2016	12:59	11.29	0.226	0.114	1.98	-1.07	50	230	-0.211	-0.045
4944_2	09/11/2016	13:00	11.2	0.224	0.094	2.37	114.09	50	230	0.002	-0.057
4945_2	09/11/2016	13:01	-0.09	-0.002	-0.043	0.04	0.01	50	230	-0.14	-0.037
4946_2	09/11/2016	13:02	4.92	0.098	0.028	3.47	-0.45	50	230	-0.218	-0.04
4948_2	09/11/2016	13:03	15.74	0.315	0.148	2.13	-2.04	50	230	-0.155	-0.039
4950_2	09/11/2016	13:04	-1.05	-0.021	-0.029	0.72	0.07	50	230	-0.307	-0.043
4952_2	10/11/2016	10:14	13.33	0.267	0.098	2.72	-2.72	50	230	-0.098	-0.003
4953_2	10/11/2016	10:17	2.48	0.05	-0.008	-6.3	-0.32	50	230	-0.156	-0.01
4954_2	10/11/2016	10:18	17.57	0.351	0.158	2.22	-18.81	50	230	-0.019	0.02
4955_2	10/11/2016	10:19	0.46	0.009	-0.033	-0.27	-0.04	50	230	-0.222	-0.028
4957_2	10/11/2016	10:20	6.06	0.121	0.034	3.53	-0.62	50	230	-0.195	-0.015
4958_2	10/11/2016	10:16	66.42	1.328	0.656	2.03	2.94	50	230	0.452	-0.018
M7.2	18/05/2017	11:02	33.12	0.662	0.303	2.19	3.43	50	230	0.193	0.085
M10.2	18/05/2017	11:03	41.26	0.825	0.387	2.13	2.03	50	230	0.406	0.101

Appendix 4 - GenBank records used during the primer design process.

ATP1a1 Mammals			
ACCESSION #	Genus	Species	Details
XM_004714862.2	<i>Echinops</i>	<i>telfairi</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) transcript variant X1 mRNA
XM_012658471.1	<i>Propithecus</i>	<i>coquereli</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) mRNA
XM_012761812.1	<i>Microcebus</i>	<i>murinus</i>	ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) mRNA
XM_019457963.1	<i>Panthera</i>	<i>pardus</i>	ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) transcript variant X1 mRNA
XM_015066161.1	<i>Acinonyx</i>	<i>jubatus</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) mRNA
XM_012807645.1	<i>Otolemur</i>	<i>garnettii</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) mRNA
XM_004771080.2	<i>Mustela</i>	<i>putorius furo</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) transcript variant X1 mRNA
XM_005334918.1	<i>Ictidomys</i>	<i>tridecemlineatus</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (Atp1a1) mRNA
XM_012730082.1	<i>Condylura</i>	<i>cristata</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) mRNA
NM_012504.1	<i>Rattus</i>	<i>norvegicus</i>	ATPase Na+/K+ transporting subunit alpha 1 (Atp1a1) mRNA
XM_006989093.2	<i>Peromyscus</i>	<i>maniculatus bairdii</i>	ATPase Na+/K+ transporting subunit alpha 1 (Atp1a1) mRNA
XM_017650322.1	<i>Manis</i>	<i>javanica</i>	ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) mRNA
XM_005357106.2	<i>Microtus</i>	<i>ochrogaster</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (Atp1a1) mRNA
NM_001163074.1	<i>Oryctolagus</i>	<i>cuniculus</i>	ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) mRNA
XM_013024679.1	<i>Dipodomys</i>	<i>ordii</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (Atp1a1) mRNA
XM_012519582.1	<i>Dasyus</i>	<i>novemcinctus</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) mRNA
XM_021195628.1	<i>Mus</i>	<i>pahari</i>	ATPase Na+/K+ transporting subunit alpha 1 (Atp1a1) transcript variant X1 mRNA
NM_144900.2	<i>Mus</i>	<i>musculus</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (Atp1a1) mRNA
XM_021157466.1	<i>Mus</i>	<i>caroli</i>	ATPase Na+/K+ transporting subunit alpha 1 (Atp1a1) mRNA
XM_01550228.1	<i>Marmota</i>	<i>marmota</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (Atp1a1) mRNA
XM_004667522.1	<i>Jaculus</i>	<i>jaculus</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (Atp1a1) transcript variant X1 mRNA
XM_021002622.1	<i>Phascolarctos</i>	<i>cinereus</i>	ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) mRNA
XM_007525504.1	<i>Erinaceus</i>	<i>europaeus</i>	ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) transcript variant X1 mRNA
XM_005076521.3	<i>Mesocricetus</i>	<i>auratus</i>	ATPase Na+/K+ transporting subunit alpha 1 (Atp1a1) mRNA
XM_006874800.1	<i>Chrysochloris</i>	<i>asiatica</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) transcript variant X1 mRNA
XM_012934427.1	<i>Sorex</i>	<i>araneus</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) transcript variant X1 mRNA
XM_007613637.2	<i>Cricetulus</i>	<i>griseus</i>	ATPase Na+/K+ transporting subunit alpha 1 (Atp1a1) mRNA
XM_004581872.2	<i>Ochotona</i>	<i>princeps</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) transcript variant X1 mRNA
XM_007948679.1	<i>Orycteropus</i>	<i>afer</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) transcript variant X1 mRNA
XM_017524196.1	<i>Cebus</i>	<i>capucinus imitator</i>	ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) mRNA
XM_011736937.1	<i>Macaca</i>	<i>nemestrina</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) transcript variant X1 mRNA
XM_012083235.1	<i>Cercocebus</i>	<i>atys</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) transcript variant X1 mRNA
XM_017869807.1	<i>Rhinopithecus</i>	<i>bieti</i>	ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) mRNA
NM_001266673.1	<i>Macaca</i>	<i>mulatta</i>	ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) mRNA
NM_001133855.1	<i>Pongo</i>	<i>abelii</i>	ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) mRNA
XM_008073520.1	<i>Tarsius</i>	<i>syrichta</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) mRNA
XM_003268035.2	<i>Nomascus</i>	<i>leucogenys</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) transcript variant X1 mRNA
XM_012458873.1	<i>Aotus</i>	<i>nancymaeae</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) transcript variant X1 mRNA
NM_000701.7	<i>Homo</i>	<i>sapiens</i>	ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) transcript variant 1 mRNA
XM_007977438.1	<i>Chlorocebus</i>	<i>sabaeus</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) transcript variant X1 mRNA
XM_004026412.2	<i>Gorilla</i>	<i>gorilla</i>	ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) transcript variant X1 mRNA
XM_010367906.1	<i>Rhinopithecus</i>	<i>roxellana</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) mRNA
XM_012000827.1	<i>Mandrillus</i>	<i>leucophaeus</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) transcript variant X1 mRNA
XM_005542208.2	<i>Macaca</i>	<i>fascicularis</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) transcript variant X1 mRNA
XM_016924989.1	<i>Pan</i>	<i>trogodytes</i>	ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) transcript variant X1 mRNA
XM_002751282.4	<i>Callithrix</i>	<i>jacchus</i>	ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) mRNA
XM_008973418.1	<i>Pan</i>	<i>paniscus</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) mRNA
XM_010344064.1	<i>Saimiri</i>	<i>boliviensis</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) mRNA
XM_017946509.1	<i>Papio</i>	<i>anubis</i>	ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) mRNA
XM_011933086.1	<i>Colobus</i>	<i>angolensis palliatus</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) transcript variant X1 mRNA
XM_003769822.2	<i>Sarcophilus</i>	<i>harrisii</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) mRNA
XM_007073456.2	<i>Panthera</i>	<i>tigris altaica</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) mRNA
XM_008697735.1	<i>Ursus</i>	<i>maritimus</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) mRNA
XM_019804162.1	<i>Ailuropoda</i>	<i>melanoleuca</i>	ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) transcript variant X1 mRNA
NM_001003306.2	<i>Canis</i>	<i>lupus familiaris</i>	ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) mRNA
XM_011285086.2	<i>Felis</i>	<i>catus</i>	ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) mRNA
ATP1a1 Crocodiles			
ACCESSION #	Genus	Species	Details
XM_019548736.1	<i>Crocodylus</i>	<i>porosus</i>	ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) mRNA XM19548736.1
XM_019526774.1	<i>Gavialis</i>	<i>gangeticus</i>	ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) mRNA XM19526774.1
XM_006030170.2	<i>Alligator</i>	<i>sinensis</i>	ATPase Na+/K+ transporting alpha 1 polypeptide (ATP1A1) mRNA XM6030170.2
XM_006261470.3	<i>Alligator</i>	<i>mississippiensis</i>	ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1) mRNA XM6261470.3

Appendix 5 - Example gel gradient results.



Appendix 6 - Genbank records accession numbers for squamate species as seen in Ujvari et al. 2013; 2015 and Mohammadi et al 2016b.

ATP1a3 Squamates			
ACCESSION #	Genus	Species	Details
KP238131.1	<i>Hydrodynastes</i>	<i>gigas</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238132.1	<i>Natrix</i>	<i>natrix</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238133.1	<i>Natrix</i>	<i>tessellata</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238134.1	<i>Nerodia</i>	<i>clarkii</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238135.1	<i>Nerodia</i>	<i>rhombifer</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238136.1	<i>Rhabdophis</i>	<i>leonardi</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238137.1	<i>Rhabdophis</i>	<i>subminiatus</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238138.1	<i>Stegonotus</i>	<i>cucullatus</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238139.1	<i>Thamnophis</i>	<i>brachystoma</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238140.1	<i>Thamnophis</i>	<i>melanogaster</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238141.1	<i>Thamnophis</i>	<i>sauritus</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238142.1	<i>Tropidonophis</i>	<i>mairii</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238143.1	<i>Xenochrophis</i>	<i>piscator</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238144.1	<i>Naja</i>	<i>melanoleuca</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238145.1	<i>Naja</i>	<i>naja</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238146.1	<i>Bitis</i>	<i>arietans</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238147.1	<i>Bitis</i>	<i>nasicornis</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238148.1	<i>Varanus</i>	<i>bengalensis</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238149.1	<i>Varanus</i>	<i>dumerilii</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238150.1	<i>Varanus</i>	<i>exanthematicus</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238151.1	<i>Varanus</i>	<i>albigularis</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238152.1	<i>Varanus</i>	<i>niloticus</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238153.1	<i>Varanus</i>	<i>rudicollis</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238154.1	<i>Varanus</i>	<i>salvator</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238155.1	<i>Morelia</i>	<i>spilota</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238156.1	<i>Liasis</i>	<i>fuscus</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238157.1	<i>Acrochordus</i>	<i>arafurae</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238158.1	<i>Boiga</i>	<i>irregularis</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238159.1	<i>Dendrelaphis</i>	<i>punctulatus</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238160.1	<i>Dendrelaphis</i>	<i>subocularis</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238161.1	<i>Acanthophis</i>	<i>praelongus</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238162.1	<i>Hemiaspis</i>	<i>signata</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238163.1	<i>Pseudechis</i>	<i>australis</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238164.1	<i>Vipera</i>	<i>berus</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238165.1	<i>Tiliqua</i>	<i>scincoides</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238166.1	<i>Varanus</i>	<i>acanthurus</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238167.1	<i>Varanus</i>	<i>eremius</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238168.1	<i>Varanus</i>	<i>giganteus</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238169.1	<i>Varanus</i>	<i>gouldii</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238170.1	<i>Varanus</i>	<i>mertensi</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238171.1	<i>Varanus</i>	<i>mitchelli</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238172.1	<i>Varanus</i>	<i>panoptes</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238173.1	<i>Varanus</i>	<i>scalaris</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238174.1	<i>Varanus</i>	<i>storri</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds
KP238175.1	<i>Varanus</i>	<i>tristis</i>	sodium/potassium-ATPase alpha-subunit isoform 3 (ATP1A3) mRNA cds

ATP1a3 Squamates			
ACCESSION #	Genus	Species	Details
KU738063.1	<i>Agkistrodon</i>	<i>contortrix</i>	isolate LSUMZH15993 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738064.1	<i>Amphisma</i>	<i>stolatum</i>	isolate 38857 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738065.1	<i>Atractus</i>	<i>flammigerus</i>	isolate LSUMZH14430 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738066.1	<i>Carphophis</i>	<i>amoenus</i>	isolate LSUMZH2484 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738067.1	<i>Causus</i>	<i>maculatus</i>	isolate LSUMZH20189 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738068.1	<i>Cerastes</i>	<i>cerastes</i>	isolate LSUMZH1878 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738069.1	<i>Clelia</i>	<i>clelia</i>	isolate LSUMZH13985 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738070.1	<i>Coluber</i>	<i>constrictor</i>	isolate Misc6 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738071.1	<i>Conophis</i>	<i>vittatus</i>	isolate LSUMZH6321 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738072.1	<i>Contia</i>	<i>tenuis</i>	isolate LSUMZH8250 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738073.1	<i>Crotalus</i>	<i>atrox</i>	isolate Misc1 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738074.1	<i>Cyclophiops</i>	<i>semicarinatus</i>	isolate 537 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738075.1	<i>Dasypeltis</i>	<i>scabra</i>	isolate Misc4 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738076.1	<i>Dendroaspis</i>	<i>angusticeps</i>	isolate LSUMZH2781 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738077.1	<i>Dendroaspis</i>	<i>polylepis</i>	isolate LSUMZH2734 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738078.1	<i>Diadophis</i>	<i>punctatus</i>	isolate LSUMZH9127 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738079.1	<i>Dipsas</i>	<i>catesbyi</i>	isolate LSUMZH17671 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738080.1	<i>Drymarchon</i>	<i>corais</i>	isolate LSUMZH3164 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738081.1	<i>Elaphe</i>	<i>quadrivirgata</i>	isolate Misc7 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738082.1	<i>Erythrolamprus</i>	<i>aesculapii</i>	isolate LSUMZH13997 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738083.1	<i>Erythrolamprus</i>	<i>mimus</i>	isolate LSUMZH6398 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738084.1	<i>Euprepophis</i>	<i>conspicillata</i>	isolate 626 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738085.1	<i>Farancia</i>	<i>abacura</i>	isolate SM33 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738086.1	<i>Helicops</i>	<i>angulatus</i>	isolate LSUMZH13999 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738087.1	<i>Hemachatus</i>	<i>haemachatus</i>	isolate LSUMZH2730 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738088.1	<i>Heterodon</i>	<i>nasicus</i>	isolate SM57 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738089.1	<i>Heterodon</i>	<i>platirhinos</i>	isolate LSUMZH16105 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738090.1	<i>Heterodon</i>	<i>simus</i>	isolate LSUMZH8006 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738091.1	<i>Hypsiglena</i>	<i>torquata</i>	isolate LSUMZH2672 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738092.1	<i>Enhydryis</i>	<i>plumbea</i>	isolate LSUMZH35673 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738093.1	<i>Lampropeltis</i>	<i>calligaster</i>	isolate LSUMZH21269 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738094.1	<i>Lampropeltis</i>	<i>getula</i>	isolate LSUMZH16031 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738095.1	<i>Leptodeira</i>	<i>septentrionalis</i>	isolate LSUMZH6250 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738096.1	<i>Lycodon</i>	<i>rufozonatus</i>	isolate 833 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738097.1	<i>Macropisthodon</i>	<i>rudis</i>	isolate 658 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738098.1	<i>Naja</i>	<i>atra</i>	isolate LSUMZH2384 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738099.1	<i>Naja</i>	<i>melanoleuca</i>	isolate LSUMZH8630 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738100.1	<i>Naja</i>	<i>nigricollis</i>	isolate LSUMZH20316 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738101.1	<i>Nerodia</i>	<i>cyclopion</i>	isolate SM28 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738102.1	<i>Nerodia</i>	<i>fasciata</i>	isolate SM16 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738103.1	<i>Nerodia</i>	<i>rhombifer</i>	isolate SM50 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738104.1	<i>Nerodia</i>	<i>sipidon</i>	isolate LSUMZH8783 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738105.1	<i>Oxyrhopus</i>	<i>formosus</i>	isolate LSUMZH14021 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738106.1	<i>Pantherophis</i>	<i>alleganiensis</i>	isolate AL-1 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738107.1	<i>Pantherophis</i>	<i>obsoletus</i>	isolate SM11 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738108.1	<i>Philodryas</i>	<i>olfersii</i>	isolate LSUMZH6355 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738109.1	<i>Pituophis</i>	<i>catenifer</i>	isolate SM58 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738110.1	<i>Python</i>	<i>regius</i>	isolate 9933 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738111.1	<i>Regina</i>	<i>alleni</i>	isolate LSUMZH8565 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738112.1	<i>Regina</i>	<i>grahami</i>	isolate LSUMZH8306 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738113.1	<i>Regina</i>	<i>rigida</i>	isolate LSUMZH3366 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738114.1	<i>Regina</i>	<i>septemvittata</i>	isolate LSUMZH8166 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738115.1	<i>Rhabdophis</i>	<i>subminiatus</i>	isolate 68838 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738116.1	<i>Rhabdophis</i>	<i>tigrinus</i>	isolate 2005-36 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738117.1	<i>Sinonatrix</i>	<i>annularis</i>	isolate LSUMZH4834 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738118.1	<i>Sinonatrix</i>	<i>percarinata</i>	isolate LSUMZH4836 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738119.1	<i>Sistrurus</i>	<i>catenatus</i>	isolate SSM191 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738120.1	<i>Storeria</i>	<i>dekayi</i>	isolate LSUMZH14767 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738121.1	<i>Storeria</i>	<i>occipitomaculata</i>	isolate LSUMZH1995 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738122.1	<i>Thamnophis</i>	<i>atratus</i>	isolate LSUMZH8476 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738123.1	<i>Thamnophis</i>	<i>butleri</i>	isolate LSUMZH1787 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738124.1	<i>Thamnophis</i>	<i>couchii</i>	isolate LSUMZH8292 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738125.1	<i>Thamnophis</i>	<i>cyrtopsis</i>	isolate LSUMZH2667 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738126.1	<i>Thamnophis</i>	<i>elegans</i>	isolate 597 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738127.1	<i>Thamnophis</i>	<i>marcianus</i>	isolate LSUMZH7748 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738128.1	<i>Thamnophis</i>	<i>ordinoides</i>	isolate LSUMZH8158 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738129.1	<i>Thamnophis</i>	<i>proximus</i>	isolate SM55 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738130.1	<i>Thamnophis</i>	<i>radix</i>	isolate SM59 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738131.1	<i>Thamnophis</i>	<i>sauritus</i>	isolate LSUMZH9243 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738132.1	<i>Thamnophis</i>	<i>sirtalis</i>	isolate 587 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738133.1	<i>Vipera</i>	<i>aspis</i>	isolate LSUMZH6688 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738134.1	<i>Trimeresurus</i>	<i>stejnegeri</i>	isolate LSUMZH4811 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738135.1	<i>Xenochrophis</i>	<i>piscator</i>	isolate LSUMZH735 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738136.1	<i>Xenodon</i>	<i>merremi</i>	isolate LSUMZH6461 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738137.1	<i>Xenodon</i>	<i>neuwiedii</i>	isolate LSUMZH6567 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738138.1	<i>Xenodon</i>	<i>rabdoocephalus</i>	isolate LSUMZH6449 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU738139.1	<i>Xenodon</i>	<i>semicinctus</i>	isolate LSUMZH6363 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds
KU93521.1	<i>Natrix</i>	<i>maura</i>	isolate LSUMZH6816 sodium/potassium-ATPase alpha-subunit isoform 3 mRNA partial cds

African snake samples had repeatedly failed to amplify during the PCR process. Several attempts were made to correct this. First was the extensive testing of various PCR conditions; varying annealing and extension temperature, decreases and increases in PCR cycles, change in master mixes used (Reddymix to TaqGreen), and using fresh supplies of water and reagents. All these tests were performed alongside a positive control of either sample 4939 or 4956. Repeatedly the gel electrophoresis showed a positive amplification for the positive control and nothing for the African snake samples. To protect against primer contamination or errors in synthesis, primers were re-ordered and the process was repeated. Again, showing positive control success but African snake sample failure. Furthermore, the primers based on Mohammadi et al. (2016b) were re-ordered a third time but this instance had three base pairs removed from the 3' end. The 3' end of primers can be extremely sensitive to mismatches, the removal of three pairs was hoped to avoid any mismatches in this critical area. These primers succeeded in amplifying some DNA but the removal of those base pairs meant they produced an amplicon far outside the predicted size. This pattern was seen across all tested temperatures and with differing combinations of the trimmed primers and originals.

The mammalian attempts followed a similar pattern. All appropriate primer sets were tested systematically under different annealing temperatures, PCR conditions and with different master mix solutions. The first batch were tested using ATP1\_178, primers used by Shine et al. (2016) to investigate bats. While these primers worked with the amphibians they were originally designed for (Moore et al. 2009), they completely failed to amplify the correct region for mammals. They did amplify a region of similar length but only at extremely low annealing temperatures. Upon sequencing it was clear that the amplicon was incorrect.

The next attempt involved the design of new primers. Overall four sets of primers based on existing mammal sequences were tested. One based on lemurs, one on tenrecs and two on carnivores. The difficulty that greatly limited primer choice was the conserved nature of the Na<sup>+</sup>/K<sup>+</sup>-ATPase. Many of the primers, that had preferable attributes, were suggest by National Center for Biotechnology Information's Primer-Blast to co-amplify different isoforms of the Na<sup>+</sup>/K<sup>+</sup>-ATPase.



Any co-amplification possibilities were unacceptable as there would be no way of confirming the correct isoform was sequenced. All these primers were tested in various mammals, starting with those most closely related to the species used as a template. Gradient PCR reactions were performed for all, along with tested using different cycles and extension conditions. These tests were repeated using both Reddymix and TaqGreen master mixes, as well as 'new' water and reagents. The same process was also performed for the crocodile sample and primers. Ultimately, these tested exhausted all feasible avenues of investigation available within the time and cost of the Masters program.

Appendix 8 - Genbank results for other mammals covering the H1-H2 domain of the alpha 1 subunit of the Na<sup>+</sup>/K<sup>+</sup>-ATPase.

Common Name	Species	Codon - alpha 1													# of negatively charged residues (Asp/D +)	# of positively charged residues (Arg/R + Lys/K)
		111	112	113	114	115	116	117	118	119	120	121	122	123		
Lesser Hedgehog Tenrec	<i>Echinops telfairi</i>	Q	A	G	T	E	E	D	P	Q	N	D	N		4	0
Aardvark	<i>Orycteropus afer</i>	Q	A	G	T	E	E	D	P	Q	N	D	N		4	0
Alpine Marmot	<i>Marmota marmota</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
American Pika	<i>Ochotona princeps</i>	R	A	A	T	E	E	D	F	D	N	D	N		4	0
Cape Golden Mole	<i>Chryschloris asiatica</i>	Q	A	G	T	E	E	D	P	Q	N	D	N		4	0
Chinese Hamster	<i>Cricetulus griseus</i>	R	S	A	T	E	E	E	P	P	N	D	D		4	0
Common Marmoset	<i>Callithrix jacchus</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Common Rat	<i>Rattus norvegicus</i>	R	S	A	T	E	E	E	P	P	N	D	D		4	0
Common Shrew	<i>Sorex araneus</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Damaraland Mole-Rat	<i>Fukomys damarensis</i>	E	T	L	S	M	E	E	A	P	S	N	D	N	4	0
Deer Mouse	<i>Peromyscus maniculatus bairdii</i>	R	S	A	T	E	E	E	P	P	N	D	D		4	0
Degu	<i>Octodon degus</i>	E	S	L	S	M	E	E	G	P	S	N	D	N	4	0
European Hedgehog	<i>Erinaceus europaeus</i>	R	A	A	T	E	E	E	V	D	N	D	N		4	0
European Rabbit	<i>Oryctolagus cuniculus</i>	L	A	A	T	E	E	D	F	D	N	D	N		4	0
Ferret	<i>Mustela putorius</i>	L	A	A	T	E	E	E	L	Q	N	D	N		4	0
Gairdner's shrewmouse	<i>Mus pahari</i>	R	S	A	T	E	E	E	P	P	N	D	D		4	0
Golden Hamster	<i>Mesocricetus auratus</i>	R	S	A	T	E	E	E	P	P	N	D	D		4	0
Guinea Pig	<i>Cavia porcellus</i>	E	S	L	S	M	E	E	G	P	S	N	D	N	4	0
House Mouse	<i>Mus musculus</i>	R	S	A	T	E	E	E	P	P	N	D	D		4	0
Koala	<i>Phascolarctos cinereus</i>	L	A	A	T	E	E	E	P	Q	N	D	N		4	0
Lesser Egyptian Jerboa	<i>Jaculus jaculus</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Long-Tailed Chinchilla	<i>Chinchilla lanigera</i>	E	S	L	S	M	E	E	G	P	S	N	D	N	4	0
Naked Mole-Rat	<i>Heterocephalus glaber</i>	E	T	L	S	M	E	E	M	P	S	N	D	N	4	0
Nine-Banded Armadillo	<i>Dasybus novemcinctus</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Northern Israeli blind subterranean mole-rat	<i>Nannospalax galili</i>	R	S	A	T	E	E	E	P	P	N	D	D		4	0
Ord's Kangaroo Rat	<i>Dipodomys ordii</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Prairie Vole	<i>Microtus ochrogaster</i>	R	S	A	T	E	E	E	P	P	N	D	D		4	0
Ryukyu mouse	<i>Mus caroli</i>	R	S	A	T	E	E	E	P	P	N	D	D		4	0
Star-Nosed Mole	<i>Condylura cristata</i>	Q	A	A	T	E	E	D	P	Q	N	D	N		4	0
Thirteen-Lined Ground Squirrel	<i>Ictidomys tridecimlineatus</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0

Appendix 9 - Genbank results for primates covering the H1-H2 domain of the alpha 1 subunit of the Na<sup>+</sup>/K<sup>+</sup>-ATPase.

Common Name	Species	Codon - alpha 1													# of negatively charged residues (Asp/D +)	# of positively charged residues (Arg/R +)
		111	112	113	114	115	116	117	118	119	120	121	122	123		
Human	<i>Homo sapiens</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Aye-aye	<i>Daubentonia madagascariensis</i>	Q	A	A	T	D	E	E	S	Q	N	D	N		4	0
Coquerel's Sifaka	<i>Propithecus coquereli</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Gray Mouse Lemur	<i>Microcebus murinus</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Angolan Colobus	<i>Colobus angolensis</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Black Snub-Nosed Monkey	<i>Rhinopithecus bieti</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Black-Capped Squirrel Monkey	<i>Saimiri boliviensis</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Bonobo	<i>Pan paniscus</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Common Chimpanzee	<i>Pan troglodytes</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Crab-Eating Macaque	<i>Macaca fascicularis</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Drill	<i>Mandrillus leucophaeus</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Golden Snub-Nosed Monkey	<i>Rhinopithecus roxellana</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Green Monkey	<i>Chlorocebus sabaues</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Nacy Ma's Night Monkey	<i>Aotus nancymaae</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Norther White-Cheeked Gibbon	<i>Nomascus leucogenys</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Northern Greater Galago	<i>Otolemur garnetti</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Olive Baboon	<i>Papio anubis</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Philippine Tarsier	<i>Carlito syrichta</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Rhesus Monkey	<i>Macaca mulatta</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Sooty Mangabey	<i>Cercocebus atys</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Southern Pig-Tailed Macaque	<i>Macaca nemestrina</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Sumatran Orangutan	<i>Pongo abelii</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Sunda Pangolin	<i>Manis javanica</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
Western Lowland Gorilla	<i>Gorilla gorilla</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0
White-Headed Capuchin	<i>Cebus capucinus imitator</i>	Q	A	A	T	E	E	E	P	Q	N	D	N		4	0

Appendix 10 - Genbank results for crocodilians covering the H1-H2 domain of the alpha 1 subunit of the Na<sup>+</sup>/K<sup>+</sup>-ATPase. Along with the alpha 3 isoform for *Crocodylus porosus*.

Common Name	Species	Codon - alpha 1												# of negatively charged residues (Asp/D)	# of positively charged residues (Arg/R)
		111	112	113	114	115	116	117	118	119	120	121	122	123	
American Alligator	<i>Alligator mississippiensis</i>	K	A	A	L	E	E	E	V	D	N	D	N	5	1
Chinese Alligator	<i>Crocodylus sinensis</i>	K	T	A	L	E	E	E	V	D	N	D	N	5	1
Gharial	<i>Gavialis gangeticus</i>	R	A	A	M	E	E	E	A	D	N	D	N	5	1
Saltwater Crocodile	<i>Crocodylus porosus</i>	R	T	A	M	E	E	E	A	D	N	D	N	5	1
Codon - alpha 3															
Saltwater Crocodile	<i>Crocodylus porosus</i>	L	A	G	T	E	D	E	P	S	N	D	N	4	0

Appendix 11 - Genbank results for carnivores covering the H1-H2 domain of the alpha 1 subunit of the Na<sup>+</sup>/K<sup>+</sup>-ATPase.

Common Name	Species	Codon - alpha 1												charged residues (Asp/D + Glu/E)	charged residues (Arg/R + Lys/K)
		111	112	113	114	115	116	117	118	119	120	121	122	123	
Cat	<i>Felis catus</i>	Q	A	A	T	E	E	E	P	Q	N	D	N	4	0
Giant Panda	<i>Ailuropoda melanoleuca</i>	Q	A	A	T	E	E	E	P	Q	N	D	N	4	0
Gray Wolf	<i>Canis lupus</i>	Q	A	A	T	E	E	E	P	Q	N	D	N	4	0
Leopard	<i>Panthera pardus</i>	Q	A	A	T	E	E	E	P	Q	N	D	N	4	0
Polar Bear	<i>Ursus maritimus</i>	Q	A	A	T	E	E	E	P	Q	N	D	N	4	0
Siberian Tiger	<i>Panthera tigris altaica</i>	Q	A	A	T	E	E	E	P	Q	N	D	N	4	0
South African Cheetah	<i>Acinonyx jubatus</i>	Q	A	A	T	E	E	E	P	Q	N	D	N	4	0
Tasmania Devil	<i>Sarcophilus harrisii</i>	Q	A	A	T	E	D	E	P	Q	N	D	N	4	0