Volume 28 (April 2018), 51-62

Herpetological Journal REVIEW

Published by the British Herpetological Society

Genetic contributions to herpetofauna conservation in the British Isles

Trevor J. C. Beebee[1,2]

[1]School of Life Sciences, University of Sussex, Falmer, Brighton BN1 9QG, UK

[2]Amphibian and Reptile Conservation Trust, 655A Christchurch Rd, Bournemouth, Dorset BH1 4AP, UK

The use of molecular genetic markers is considered in the context of their application to conservation of amphibians and reptiles in the British Isles. Aspects reviewed include population viability, connectivity and origins together with developments in molecular identification. Genetic diversity measures are not in themselves sufficient to identify risks of inbreeding or genetic erosion in the absence of direct measures of individual fitness. Neutral markers are however useful for defining populations, the extent of migration between them and the identification of permeable habitat corridors. Phylogeography has resolved previously uncertain origins of several populations and species found in the British Isles, including *Pelophylax lessonae*, *Bufo calamita* and *Triturus cristatus*. Molecular studies have clarified the species status of toads *Bufo bufo*, *B. spinosus*, and grass snakes *Natrix helvetica* in the British Isles and provided methods for species and clade identifications where this is difficult using morphology alone. DNA-based techniques have revealed the distributions of viral and fungal pathogens and environmental DNA (eDNA) has proved its worth as a technique for surveying pond-breeding amphibians.

Key words: DNA, genetics, amphibians, reptiles, conservation, British Isles

INTRODUCTION

Wildlife conservation has a long history in the British Isles and, until recently, was carried out in the virtual absence of genetic considerations. That situation has changed markedly in the past 25 years due to the development of genetic tools readily applicable to wild populations of plants and animals. A combination of DNA-based analyses and increasingly powerful computation, reviewed by Frankham et al. (2010) and Rowe et al. (2017) has brought genetics into the mainstream of conservation planning and application. Herpetofauna around the world have been among the groups attracting conservation genetic investigations, with those on amphibians reviewed recently by McCartney-Melsted & Shaffer (2015). This global analysis included case studies of several species, only one of which (the great crested newt *Triturus cristatus*) occurs in the British Isles. In this review, I consider the main contributions that genetic analysis has made to amphibian and reptile conservation specifically in the British Isles.

GENETIC ANALYSES AND THEIR IMPLICATIONS

The arrival of molecular markers

Population genetics came of age almost a century ago, at a time when variation was expected to be solely the result of natural selection. Early field studies were interpreted with this in mind and all morphological differences among individuals, however trivial, were considered to reflect variations in selection pressures over time and space. It came as a shock when allozymes, the first major family of molecular genetic markers, revealed vast amounts of variation that were impossible to explain by selection alone. Neutral theory was the result, proposing that many genetic differences were due to changes that had little or no effect on individual fitness. Subsequent development of DNA markers, primarily mitochondrial DNA (mtDNA) and nuclear microsatellites, showed that variation among these molecules too was usually neutral with respect to selection. Perhaps counter-intuitively, neutral markers are of great value in population studies but, as we shall see, the distinction between selection and neutrality is of more than academic interest to genetic applications in conservation. DNA analyses require only tiny amounts of tissue which can usually be obtained without sacrificing animals because the required sequences can be amplified by the polymerase chain reaction (PCR). The history of molecular ecology including both theoretical and practical developments is described in Rowe et al. (2017). Four main areas relevant to conservation biology have been informed by genetic analyses, notably: (1) population demography, including diversity, viability and inbreeding risk; (2) population size, structure and gene flow; (3) phylogeography, the history of populations in time and space; and (4) identification issues including those relating to species, individuals

Correspondence: Trevor Beebee (t.j.c.beebee@sussex.ac.uk)

and hybrids. All of these approaches have been used in studies of amphibians and reptiles in the British Isles, as described below.

Population demography

Assessing genetic diversity

Conservationists have long recognised the increased extinction risk implicit in small or declining population sizes. In addition to stochastic factors such as predation, disease, adverse weather and imbalanced sex ratios, reductions in genetic diversity in small populations could predispose inbreeding depression and thus further declines not relieved by habitat or species management methods. This concern has been, arguably, the commonest reason to use genetic tools in conservation. To this end molecular genetic markers, especially microsatellites, have been developed to investigate many amphibian and reptile species. Tables 1 and 2 summarise examples of genetic diversity estimates based on microsatellites for many of the species found in the British Isles. Comparisons with populations in mainland Europe are provided to demonstrate the extent to which diversity has been affected by isolation in the island archipelago. In the cases of *Lissotriton vulgaris* (smooth newt), *L. helveticus* (palate newt), *Zootoca vivipara* (viviparous lizard), *Anguis fragilis* (slow-worm) and *Natrix helvetica* (grass snake) there was no comparable information, at least for British Isles populations, at the time of writing. Sixteen *Vipera berus* (adder) populations in England and Wales were also analysed for genetic diversity at microsatellite loci and the results, not yet published (hence their absence from Table 2), were broadly similar to those from north-east France.

Expected heterozygosity (He) and allelic richness (Ar) are the two main statistics for estimating population genetic diversity. In practice either one will usually suffice. Across all the taxa listed in Tables 1 and 2, He and Ar (or allele number, An, where Ar was not calculated from An) correlated strongly, with $r_s = 0.925$, $P<0.0001$. Suites of microsatellite loci are unique to each species, so cross-species comparisons should be treated with caution. Nevertheless *R. temporaria* populations probably are, on average, individually more diverse than those of *B. bufo* perhaps for ecological reasons considered later (see gene flow section). Intraspecific comparisons are on safer ground, though even here different investigations have sometimes used different marker sets. Range-edge populations of native species, including those in the British Isles, generally had lower levels of diversity than those in central or southern Europe. This was apparent for *R. temporaria* (common frog), *B. bufo* (common toad), *B. calamita* (natterjack toad), *C. austriaca* (smooth snake) and *L. agilis* (sand lizard). Non-native introduced species in Britain also often showed reductions in diversity compared with their populations of origin. This was true for *P. ridibundus* (marsh frog) and especially for *P. muralis* (wall lizard), but the case was different for recently re-introduced *P. lessonae* (pool frog) as the source population in Sweden was itself genetically depauperate compared with central European populations. (see references in Table 2).

Factors influencing genetic diversity

A theoretical expectation is that genetic diversity at neutral loci should correlate with population size. Testing this hypothesis is usually impossible because most genetic studies on herpetofauna have been with populations of unknown size. *Bufo calamita* in Britain is an exception because multiple populations have been studied for several decades and spawn string counts used as a proxy for population size in many of them. Averaged over time, these counts have proved reliable as estimators of relative, though not absolute population sizes. Microsatellite He correlated significantly with *B. calamita* population sizes across 33 localities in Britain, but equally strongly, and independently, with the distance of the population from those at the northerly range edges on the east and west coasts, as summarised in Table 3 (Rowe et al., 1999). Range effects are even stronger at the full biogeographical range scale. Microsatellite diversity in *B. calamita* declines continuously from south-west Europe, the probable glacial refugium, eastwards to Poland and Estonia (Rowe et al., 2006) despite the persistence of large toad populations in eastern Europe. This, too, is a theoretical expectation. Postglacial colonisation is based on successive founder events, each involving only a few individuals and thus just a fraction of the genetic diversity present in the population of origin. The important consequence of two separate factors strongly affecting genetic diversity at neutral loci is that diversity estimates cannot be taken simply to reflect population size. Many European species in various taxonomic groups exhibit this pattern of genetic depauperisation following postglacial recolonisation (Ibrahim et al., 1996).

Genetic diversity and fitness

Particularly important for conservation are the implications of genetic measurements for assessing population viability. Amphibians are good subjects for studies on fitness because larval growth and development rates are important survival factors and are easily quantified. In many species, rapid growth predicates high survival to metamorphosis, especially where animals breed in small or temporary ponds. Laboratory investigations comparing larval growth and survival of *R. temporaria* and *B. bufo* from populations breeding in garden ponds with larger ones in rural habitats showed, for both species, elevated numbers of developmental abnormalities together with reduced survival and heterozygosity at multiple allozyme loci in the urban sites (Hitchings & Beebee, 1997; 1998). Growth rates of *B. calamita* larvae from six populations of widely differing microsatellite diversities also correlated with He (Rowe et al., 1999). However, a study using microsatellites found no differences in genetic diversity between urban and rural populations of *R. temporaria* and higher larval growth rates in urban relative to rural populations (Zeisset & Beebee, 2010). This discrepancy is rather typical of variable reports on the relationship between neutral loci and fitness attributes. Perhaps it is not too surprising that correlations are often weak or inconsistent. An alternative is to look at loci likely to

Table 1. Genetic diversity estimates for amphibian populations. He, expected heterozygosity; An, mean number of alleles per locus; Ar, allelic richness (= An adjusted for sample size. Not always estimated).

Species and sample location	Number of microsatellite loci analysed	He	An	Ar	References
Triturus cristatus:					Babik et al. (2009)
Gaddesby, UK	5	0.73		5.0	
Krefeld, Germany	5	0.76		4.5	
Rana temporaria					Brede & Beebee (2006a)
Ainsdale, UK	8	0.60		4.56	
Bonn, Germany	8	0.62		5.21	
Bufo bufo					Brede & Beebee (2006a)
Ainsdale, UK	8	0.57		3.27	
Bonn, Germany	8	0.66		4.05	
Bufo (Epidalea) calamita					Rowe et al. (2006)
Ainsdale, UK	8	0.31	3.0		
Coto Donana, Spain	8	0.80	7.2		
Parnu, Estonia	8	0.28	1.5		
Pelophylax lessonae					Zeisset & Beebee (2001)
Thetford, UK	6	0.00	1.0		
Uppsala, Sweden	6	0.00	1.0		
Paris, France	6	0.49	3.3		
Pelophylax ridibundus					Zeisset & Beebee (2003)
Romney, UK	5	0.48	2.2		
Balaton, Hungary	5	0.52	3.2		

Table 2. Genetic diversity estimates for reptile populations. He, expected heterozygosity; An, mean number of alleles per locus; Ar, allelic richness (= An adjusted for sample size. Not always estimated).

Species and sample location	Number of microsatellite loci analysed	He	An	Ar	References
Vipera berus					Ursenbacher et al. (2015)
NE France, Belgium & Netherlands	9	0.39	2.46		
Massif Central, France	9	0.61	3.62		
Coronella austriaca					
Arne, UK	8	0.53	2.60	2.50	Pernetta (2009)
Pieniny, Poland	14	0.68		4.06	Sztencel-Jablonka et al. (2015)
Lacerta agilis					
Dorset, UK	15	0.75		5.32	Russell (2012)
Asketunnan, Sweden	15	0.54	4.3		Schwartz & Olsson (2008)
'Continuum', Hungary	15	0.83	11.2		Schwartz & Olsson (2008)
Podarcis muralis					Michaelides et al. (2016)
23 populations, UK	16	0.62	3.9		
21 populations, Italy	16	0.77	6.5		
13 populations, France	16	0.69	5.0		

be under selection, and which might therefore better reflect population genetic health. Such loci are harder to find and to analyse than microsatellites, but one set that has attracted widespread attention is the Major Histocompatibility (MHC) Complex. These genes are vital components of the immune system and vertebrates usually have a highly diverse array of MHC alleles. MHC class II loci have been analysed at the population level for *T. cristatus*, *R. temporaria* and *B. calamita*. For those hoping these genes might provide a window into the genetics of population viability, the results have been somewhat disappointing. In both *T. cristatus* and *B. calamita*, MHC diversity varied at the biogeographical range level in a way that largely mirrored microsatellite diversity, declining as a function of distance from their glacial refugia. It seems that random genetic drift has been more important than selection for MHC diversity in these species, a result that implies disease resistance may not have played a major role in determining their current status (Babik et al., 2009; Zeisset & Beebee, 2014). Similarly, among urban and rural *R. temporaria* populations there were no indications that MHC alleles or diversity levels related substantially to larval fitness attributes (Zeisset & Beebee, 2010).

Is there, then, any reliable way of detecting genetic problems in herpetofauna populations? Events at Saltfleetby, in Lincolnshire, suggest that the task is not impossible. The small *B. calamita* population on this nature reserve is at the species' northerly range edge on England's east coast. In the early 1970s it was on the verge of extinction and subsequently responded only slowly to extensive conservation management. Diversity at microsatellite loci was very low in the 1990s, with average He = 0.189, though two other populations in Cumbria had similarly low diversities but were apparently thriving. In laboratory trials, Saltfleetby larvae had by far the slowest growth rate of any *B. calamita* population tested (Rowe et al., 1999). Given the unreliability of lab fitness trials described earlier, experiments were also carried out in the field at Saltfleetby comparing growth

Table 3. Genetic diversity and demography in *B. calamita*. Data were from 33 British natterjack toad populations and subpopulations. Population sizes were each the averages of spawn string counts over 5-10 years between 1986 and1995 (derived from Rowe et al., 1999).

Comparison	No. comparisons	Correlation coefficient (r)	Probability of no correlation
He x Mean population size	28	0.46	<0.02
He x distance to range edge	33	0.51	<0.01
Mean population size x distance to range edge	28	0.25	0.15

Table 4. Census (Nc) and effective breeding (Nb) population size estimates for two widespread anurans. Both populations of both species bred in the same ponds in Sussex (derived from Brede & Beebee, 2006b).

Species	Site name	Mean Nc estimate	Mean Nb estimate	Nb:Nc
Bufo bufo	Pells	1,200	49	0.04
	Whitelands	1,022	34	0.03
Rana temporaria	Pells	236	86	0.36
	Whitelands	36	12	0.33

Table 5. Comparative survey results for *T. cristatus* using conventional and eDNA methods.Results are from four surveys of each of 35 ponds, and are derived from Biggs et al. (2015).

Method	Mean no. of successful detections (range)	Mean percentages of successful detections
eDNA	34.75 (34-35)	>99
Bottle trapping	27.50 (26-30)	79
Torch searching	26.25 (20-34)	75
Bottle trapping + torch searching	34.00 (33-35)	>97

rates and survival of larvae from Saltfleetby with those from a large population at Ainsdale on the Merseyside coast. These trials confirmed the lab results; Saltfleetby larvae were much less fit for survival in an ephemeral pond environment than those from the large population, even on their home ground (Rowe & Beebee, 2003). However, the Fis statistic (an indicator of local inbreeding) of Saltfleetby toads did not differ significantly from zero. This implies that inbreeding was not the primary cause of the genetic problem. Fis varies from zero (no inbreeding) to one (highly inbred) so an alternative reason for the lack of fitness must be responsible. It seems that progressive fixation of deleterious alleles, consequent upon genetic drift in the rapid decline of this small population, led to a damaging genetic load that put the population's viability at risk.

Starting in 2003, Saltfleetby *B. calamita* were subject to an attempted genetic restoration by introducing larvae (accidentally, as escapes during a replicated pond experiment) from Ainsdale and, later, deliberately from East Anglia. Spawn string counts, metamorph success and genetic diversity subsequently increased at Saltfleetby, indicating that the restoration may have made a positive contribution to population viability (Beebee, 2014). Average numbers of spawn strings per year increased significantly (Median Test P = 0.046), by about 45%, between 2009 and 2017, compared with the preceding nine years for which data were available. 2009 was the first year in which substantial numbers of post-restoration adults would have been sexually mature, However, direct measurements of larval growth rates in recent years are lacking, but necessary, to confirm a fitness effect of the restoration.

Investigations into the genetics and viability of non-native *P. muralis* showed that diversity was lower in England than in the French and Italian source populations, and that embryonic mortality was highest in the introduced British populations (Michaelides et al., 2016). Inbreeding may put the long-term future of these lizards at risk. As with Saltfleetby *B. calamita*, there was no simple correlation between microsatellite diversity and any fitness measure at the population or individual level. A *V. berus* population in Sweden exhibited both low genetic diversity and evidence of inbreeding depression, manifest as high incidences of stillborn and malformed offspring. A successful genetic rescue was accomplished by the addition of several males from an outbred population, leading to both increased offspring survival and genetic diversity (Madsen et al., 1999). The unpublished British *V. berus* study mentioned earlier was also intended to assess risks from inbreeding in British populations but looked only at genetic diversity. Based on the experiences outlined above, molecular studies alone are unlikely to identify genetic risks to British adders in the absence of quantitative information about fitness characters.

Assessing population size

Another prediction from genetic theory is the '50/500 rule' (Franklin, 1980). This infers that populations averaging 50 or fewer individuals over several generations will become subject to inbreeding effects, while in those with fewer than 500 genetic diversity will gradually erode over time. These numbers seem unrealistically large for most amphibian and reptile populations in the British Isles, especially for the rare species in restricted habitat areas. Furthermore, the theory relates to 'effective' population size, Ne, and not census size, Nc. Ecologists typically measure Nc using methods such as capture-mark-recapture, but Ne approximates to the number of individuals that successfully reproduce and is of greater relevance to population viability. Ne is typically much smaller than Nc (Frankham, 1995).

Neutral genetic markers provide methods for estimating Ne, which is difficult to do by standard ecological approaches. Because neutral genetic diversity is affected by random genetic drift, allele frequencies change more quickly in small than in large populations. It is therefore possible to compare allele frequencies across generations and thence obtain estimates of Ne.

In amphibians, this has been attempted by genotyping adults and larvae in the same year. Because many amphibians survive to breed more than once, these estimates are referred to as Nb, the effective breeding population size, which will be smaller than Ne but often not by much.

Estimates of Nb and Nc in several British *B. bufo* populations resulted in very small Nb:Nc ratios, varying from around 0.01 to 0.04; by contrast, ratios for multiple *R. temporaria* populations averaged around 0.3 (Scribner et al., 1997; Brede & Beebee, 2006b; Table 4). More recently, methods for determining Nb based on a single generation sample have been developed and applied to multiple British *B. calamita* populations yielding an average Nb:Nc ratio of around 0.15 (Beebee, 2009). These estimates are typical of those found in many other species and imply a need for census population sizes in the hundreds to avoid inbreeding risks and an order of magnitude higher to minimise genetic deterioration. These unrealistic aspirations are moderated to some extent by the discovery of genetic compensation. In both *T. cristatus* and *B. calamita* the Nb:Nc ratio increases markedly when population sizes are low (Jehle et al., 2005; Beebee, 2009) but nevertheless very few amphibian and reptile populations will ever be large enough to comply with these theoretical requirements to maintain long-term genetic health. There seems no straightforward way of relating theory and practice in this situation, though a lot hinges on the definition of a population and whether high gene flow can be interpreted as reflecting large metapopulations. This certainly could apply to relatively widespread species, but hardly to rare and geographically isolated ones.

Neutral genetic markers also provide a method for determining recent trends in population size. This is based on the theoretical expectation that He will change more slowly than Ar when populations increase or decrease. The method should reveal bottlenecks or expansions that occurred within the past 50 or so years, depending on species generation time and current population size. For *B. calamita* this approach was tested by comparing genetic inferences with known demographic histories, based on spawn string counts, for 15 British populations. The bottlenecks indicated by genetic analysis from samples taken in the 1990s corresponded almost completely with those known to have happened during the 20th century (Beebee & Rowe, 2001). Vibrant populations showed no bottleneck effects whereas some others, including recent translocations yielded significant bottleneck signatures. No recent population increases were implied. The *R. ridibundus* population at Romney in Sussex, established from just 12 founding individuals in 1935, also showed genetic confirmation of a bottleneck (Zeisset & Beebee, 2003).

Population structure and gene flow

Detecting gene flow and migration between populations

It is often useful to know the extent to which populations are interconnected by migration. Even occasional movements of individuals can help maintain genetic diversity and reduce extinction risks. This is hard to determine by standard ecological methods, especially when migrants are infrequent, as is often the case. Molecular markers offer solutions to this problem. The Fst statistic (Rowe et al., 2017) is a measure of how distinct two populations are with respect to their genetic diversities, and varies from zero (populations identical) to one (totally distinct, with no recent migration). Intermediate Fst values indicate the degree of differentiation, and thus the number of migrants moving between populations averaged over recent time; the lower the Fst, the higher the number of migrants per generation. Fst values indicate 'effective' migration, meaning the numbers of individuals that reproduced successfully in their new location, but cannot easily distinguish the directions of movement. Wherever migration is possible, a theoretical expectation is that pairwise Fst estimates will increase as a function of distance between multiple population pairs. This is a useful basis for identifying barriers to movement, as shown when a pairwise Fst is higher than expected under this 'isolation by distance' model.

Allozyme-based Fst estimates among *R. temporaria* and *B. bufo* populations in Sussex were more than twice as high among urban compared with rural breeding ponds (Hitchings & Beebee, 1997; 1998) despite the fact that distances between urban ponds were much smaller, averaging just over 2 km, than those between rural sites averaging > 40 km. However, this distinction was not seen in a similar study with *R. temporaria* in the same area using microsatellites, but did occur at an MHC locus (Zeisset & Beebee, 2010). Possibly the allozyme and MHC results reflected the fact that these markers can be subject to local selection. There was no correlation between Fst and intersite distances for microsatellites or MHC markers, indicating the existence of substantial barriers to movement between the Sussex ponds in both rural and urban habitats. Further comparisons using microsatellites with rural Sussex populations separated by an average of just 8 km produced Fst estimates for *B. bufo* averaging five-fold higher than those for *R. temporaria* using the same ponds. In this instance, with relatively close ponds, *R. temporaria* showed significant isolation by distance but *B. bufo* did not (Brede & Beebee, 2004). These results imply that *R. temporaria* is genetically a more mobile species than *B. bufo*, probably because the frog typically utilises more ponds in a landscape than does the toad. Taken together the relatively high diversity, mobility and Nb/Nc ratios of *R. temporaria* populations relative to those of *B. bufo* are in accord with differences in densities of breeding sites and operational sex ratios between these species.

Recognition that many amphibians persist as metapopulations was the basis of migration estimates, based on microsatellite analyses, in three areas of Britain with semi-continuous coastal habitat occupied by *B. calamita* (Rowe et al., 2000). Mean Fst estimates between pond clusters, essentially sub-populations in each region increased as a function of distances within suitable habitat between them. It was possible from this study to infer barrier effects of a river and of an intervening urban development, and also which subpopulations were the main sources or recipients of intersite migrants. At the

level of entire biogeographical range both microsatellites and MHC markers exhibited isolation by distance among *B. calamita* populations across Europe, but the gradient was significantly steeper for MHC than microsatellites indicating that selection has operated on the functional gene even though random drift was the most important structuring force (Zeisset & Beebee, 2014).

Defining populations

Assessing migration rates between populations requires, in the first instance, defining the populations in question. Even for pond-breeding amphibians this is often problematic because many species exist as metapopulations, with individuals moving among groups of ponds. For reptiles, there is even greater uncertainty. Are animals on one hillside really quite separate from those on the next one along the ridge? Sophisticated methods, mostly based on assignment tests, have been developed to address this issue using genetic data. In a discrete, isolated population there is a theoretical expectation that the genotypes of all the individuals present should occur at frequencies predicted by the Hardy-Weinberg equilibrium (HWE). Computer programs can analyse all the genotypes from a sample of individuals and assess whether they fall into one HWE group, or into more, in which case several different populations are implied. These groups can then be examined to see whether they are constituted by animals sampled in (say) a corresponding set of ponds or hillsides.

Applying this approach to *B. calamita* microsatellite data from all the localities in Britain where the toad occurred resulted in 38 previously defined populations contracting to about 15 metapopulations, the final number varying slightly according the exact method used (Rowe & Beebee, 2007). This result provided more clarity about 'actual' population size, which could often be considered as the totals of several sub-populations, and demonstrated where it will be important to maintain connectivity by habitat management. Assignment methods can also identify individual migrants if an animal ascribed to one HWE group was actually found at a location where the population was defined by a different HWE group. This does not reveal whether the migrant bred successfully in its new home, but merely establishes its presence. An extended type of assignment analysis can identify larvae in a pond that have mixed parentage via individuals from two separate ponds (Jehle et al., 2005) but no evidence of that was found among the British *B. calamita* populations. Fst analysis therefore remains a useful method for identifying effective migration between populations defined by assignment approaches.

Investigations of *R. temporaria* in Scotland added another dimension to understanding population structure. Microsatellite analyses of frogs from sites in central Scotland up to tens of km apart and at altitudes from <80 m to 900 m above sea level mostly failed to resolve into discrete populations, and low Fst estimates indicated universally high levels of intersite migration (Muir et al., 2013). However, larval period and growth rates did vary, with larvae from populations at the highest altitudes growing faster and metamorphosing more quickly than those from lower down the mountains (Muir et al., 2014). Differentiation of these heritable quantitative traits, quantified as Qst, was fivefold stronger than microsatellite Fst differentiation. Evidently this adaptive variation was maintained in the face of high rates of intersite migration demonstrated by the microsatellite data. Neutral markers do not tell the whole of the story.

Coronella austriaca is the rarest reptile in Britain and of high conservation concern. This snake was the subject of a genetic investigation to compare gene flow among multiple sites across Dorset, the county in which most surviving British populations occur. Smooth snakes were genotyped at microsatellite loci but assignment methods failed to define specific populations associated with the sample sites, despite them being widely dispersed (at least several km apart). Fst estimates between sampling sites were low, with no indication of isolation by distance (Pernetta, 2009). It may be that the patterns of genetic diversity seen in this long-lived reptile reflect population structures that were present decades ago, before recent barriers to movement, especially the expansion of urban areas, fragmented the snake populations to their present state. However, a study within a single area of smooth snake habitat proved more informative. In Wareham Forest, an area of mixed heath and woodland, *C. austriaca* was sampled at 10 locations separated from one another by distances between about 0.5 and 5.5 km. There was a significant isolation by distance effect (Pernetta et al., 2011) and attempts to improve the Fst – distance correlation using hypothesised habitat corridors such as forest rides failed to improve on the correlation with direct-line distances, inferring that all the habitat was available for migration by *C. austriaca*. This microsatellite study made other interesting revelations, demonstrating that males tend to move further than females and that multiple paternity of clutches is commonplace.

Populations of Britain's other rare reptile, *L. agilis*, were the subject of an extensive genetic study based on diversity at multiple microsatellite loci (Russell, 2012). Assignment methods clearly identified distinct groups in the main British distribution zones of this species in Dorset, the Weald and Merseyside. Some clusters of populations within Dorset were also resolved. However, at the finest scale investigated, within Wareham Forest, there was no population differentiation indicating substantial mobility of lizards within the Forest. An important element of the *L. aglis* study within Dorset was progression from isolation-by-distance analysis to a landscape scale investigation identifying habitat features likely to influence the movement of individuals (Fig. 1). This approach revealed probable 'least cost paths' and 'resistance surfaces' for lizards around Bournemouth. Rivers were substantial barriers to sand lizard movement and major roads were probably similar, but because some roads were relatively new their impact on genetic differentiation was not yet strong. Some agricultural land looked permeable to lizards and might become increasingly suitable as climate warming continues. This kind of investigation is valuable for the identification

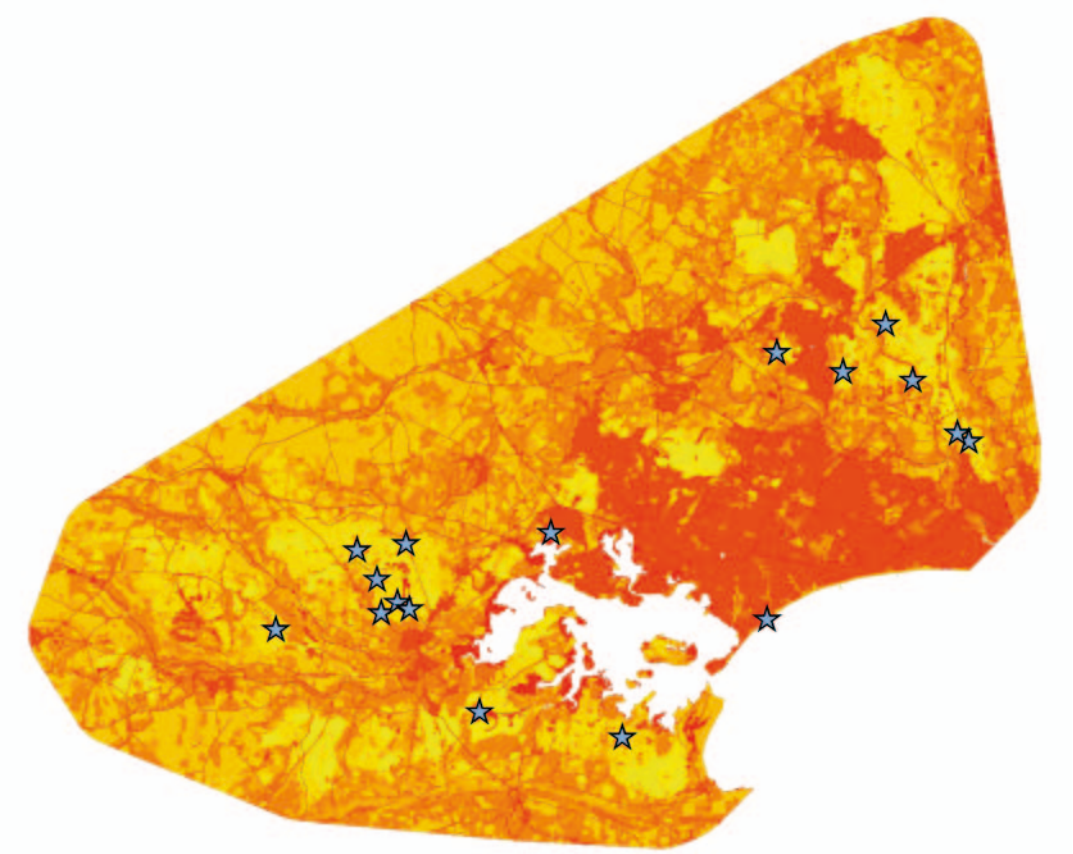

Figure 1. Landscape permeability surfaces for sand lizards in Dorset. The figure shows a resistance surface for *L. agilis* around the Bournemouth-Poole connurbation. Palest (yellow) areas are the most permeable, grading through to deep orange, the least permeable to sand lizard movement. Blue stars represent the lizard sampling sites. After Russell (2012).

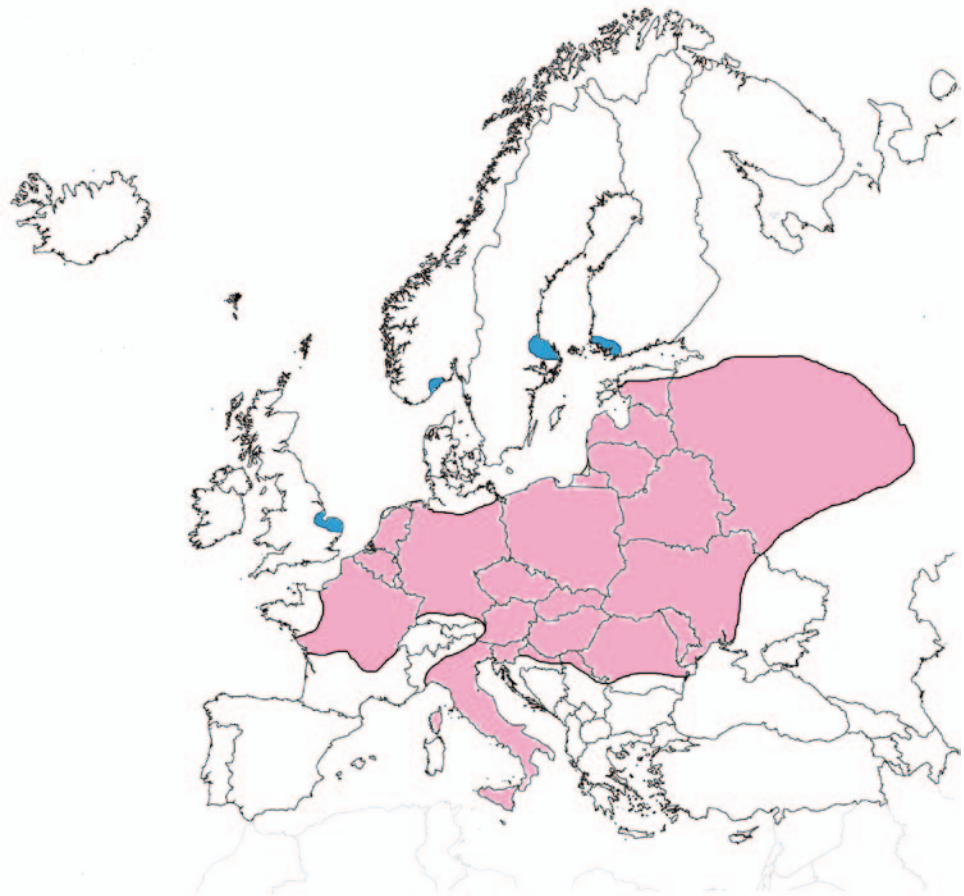

Figure 2. The pool frog in Europe. Blue = approximate distribution limits for northern clade frogs. Pink = approximate distribution of central European pool frogs.

and improvement of corridors to maintain connectivity between increasingly fragmented lizard populations and could usefully be extended to other herpetofauna species in Britain.

Phylogeography

From a conservation perspective, it can be important to know the origins of specific populations, and in particular whether they are likely to be longstanding components of a natural range or recent introductions at the hand of man. This information can be critical at the policy level for determining whether a population should be conserved or, at worst, eliminated. Comparing the genetic profiles of reference populations can provide convincing evidence to distinguish these scenarios and, in the most favourable situations, indicate both the pathways of historical colonisation and likely timing of events.

Mitochondrial DNA is a popular marker for phylogeographical analysis but for herpetofauna in the British Isles it has usually shown too little variation to work well. Fortunately, microsatellites have frequently come to the rescue. A phylogeographic study of *B. calamita* indicated that populations on the north-west coast of England were genetically distinct from those in southern and eastern England, and that the postglacial colonisation of the British Isles by this toad from mainland Europe probably followed separate western and eastern routes. It also showed that *B. calamita* populations in Ireland were very similar genetically to those on the British west coast but must have separated from them during a common original colonisation event several thousand years ago (Rowe et al., 2006). This genetic evidence ran counter to previous speculations that natterjacks were introduced to Ireland by humans, and thus confirmed the importance of conserving these isolated toad populations. A finer-scale study attempted to determine whether recently discovered large populations of *B. calamita* in a geographically separate dune system in Ireland were longstanding, or a result of recent translocation from the well-known colonies. On balance the results indicated that the newly found populations were probably not of recent origin, but this inference was weakly supported and highlighted difficulties of using genetic methods at the limits of distribution where diversity is low (May & Beebee, 2010). A similar picture to that of *B. calamita* emerged for the history of *R. temporaria* in Ireland. Populations in much of the country had west European mtDNA haplotypes like those found in mainland Britain but some in the south-west had unique sequences suggesting that, like natterjack toads, they may have survived in and colonised Ireland from a distinct and separate glacial refugium (Teacher et al., 2009).

Phylogeographical analysis with microsatellite and 'RAPD' genetic markers provided contributory evidence that *P. lessonae* was native to eastern England until the last population went extinct there in the 1990s (Beebee et al., 2005). The East Anglian frogs formed part of a distinct 'northern clade' of *P. lessonae*, only otherwise found in Scandinavia, and distinctly different from introduced populations of this frog in England originating from elsewhere in Europe (Fig 2). The genetic evidence together with that of archaeological remains and male call signatures led to formal recognition of northern clade pool frogs as a native British species and thence to a so-far successful attempt to reintroduce them from Sweden.

Great crested newts *T. cristatus* are widespread in Britain but there was speculation that an isolated group of populations in the far north of Scotland may have originated via human translocation. However, microsatellite-based analyses of genetic diversity patterns indicated that the northern Scottish populations are most probably of natural origin (O'Brien et al., 2015). There were no signs of recent genetic bottlenecks and the observed diversity patterns would have required multiple separate translocations to generate the situation seen today. Once gain the conclusion was that the populations merit continued conservation measures.

An exception to confirmation of native status followed microsatellite analysis of a *L. agilis* population at Aberffraw dunes in Anglesey (Russell, 2012). This study pointed very clearly to a recent introduction, mostly with animals originating from Merseyside but possibly 'contaminated' with some input from Dorset lizards, perhaps following captive breeding. Nevertheless, because this is a rare species that has declined severely in the UK the sand lizards certainly warrant protection both there and at nearby Newborough Warren where another illicit translocation must have taken place.

Podarcis muralis is by far the most successful non-native reptile in Britain. Using a combination of mtDNA and microsatellite markers, Michaelides et al. (2015) sought to identify the origins of 23 English populations of this lizard by comparison with potential sources in France and Italy. For at least nine of the British populations the results suggested separate introduction events from mainland Europe, while eleven probably originated as secondary translocations from the primary colonies. Some of the British wall lizard sites probably have animals hailing from more than one European locality while most British populations apparently originated from Tuscany. English wall lizard colonies are thriving and adapting to the country's relatively cool temperatures, and in some areas, could pose a threat to native *L. agilis*. However, eradication of *P. muralis* from Britain is hardly feasible and, probably not sufficiently warranted to be desirable. Wall lizards are surely in Britain to stay.

Identification issues

Species

Among the most striking recent developments have been the recognition of two new species identities in the British Isles. The taxonomic separation of toads *B. bufo* and *B. spinosus* (previously designated as the subspecies *B. bufo spinosus*) was achieved primarily on the basis of mtDNA and a nuclear DNA marker. *Bufo spinosus* occurs in Iberia and much of western France, while *B. bufo* is widespread in other parts of France, and further east, as well as in mainland Britain. However, it turns out that bufonid toads on the Channel Island of Jersey are

B. spinosus (Arntzen et al., 2014). This discovery added a new species to the British list (Fig 3). Then came a revelation about grass snakes (*Natrix* species), long known to exhibit substantial phenotypic variation across their wide European range. Based on results from both mtDNA and microsatellite studies, a strong morphological and genetic divide between grass snake populations runs north-south, more or less along the Rhine valley, in western Germany. Snakes east of this divide are now classified as *Natrix natrix* while those in France and Britain have been elevated from subspecies to full species status, becoming *N. helvetica* instead of *N. natrix helvetica* (Kindler et al., 2017). Contrary to some press reports this is a reclassification and not a 'new' species in Britain, but in a few areas, there are populations of non-native grass snakes originating in mainland Europe, and which are not *N. helvetica* (e.g. Nash, 2011).

For the most part, the few species of amphibians and reptiles found in the British Isle are easy to distinguish morphologically, though there are a few exceptions. In these cases diagnostic molecular markers can come to the rescue. Larvae of *B. bufo* and *B. calamita* are morphologically indistinguishable when small but can be separated by either protein or species-specific microsatellite analysis. Visual identification of some water frogs (*Pelophylax* species) is difficult and often unreliable. Larvae cannot be separated morphologically and the hybrid edible frog *P. esculentus* can be hard to distinguish from *P. lessonae*. Even more problematic is differentiating between central European and northern clade *P. lessonae*. Microsatellite markers are available for separating *P. lessonae* from *P. esculentus*, and from the marsh frog *P. ridibundus*, providing reference material is available from frogs of known provenance for assignment tests (Holsbeek et al., 2009). There are also RAPD primers that can distinguish northern clade *P. lesssonae* from non-native central European pool frogs (Snell et al., 2005).

Figure 3. *Bufo spinosus*, a new species for the British Isles. Photo: John Wilkinson

The newts *L. vulgaris* and *L. helveticus* occasionally hybridise in the wild. Male hybrids are readily recognised by their intermediate morphology but, probably because females of these species look very similar, no female hybrids have yet come to light. A mixture of RAPD and mtDNA sequences can not only identify hybrids but, because mtDNA has an exclusively female inheritance, can also reveal the direction of the cross (Beebee et al., 1999). Evidently hybrids can result from pairings of either type, i.e. with either *L. vulgaris* or *L. helveticus* mothers. Larvae of these two newts are morphologically indistinguishable but can also be identified non-lethally using the molecular tools appropriate for hybrids.

Disease diagnosis

Infectious diseases have caused mass mortalities of amphibians around the world in recent decades. In Britain, ranavirus outbreaks regularly decimate populations of *R. temporaria* and, to a lesser extent, *B. bufo* (Teacher et al., 2010). Although the pathology of ranavirus in dead or dying frogs is usually obvious, it may sometimes be important to make a definitive identification. This can be achieved by PCR amplification of part of the major viral capsid protein gene in DNA extracted from infected tissue. The fungus *Batrachochytrium dendrobatidis* (Bd) has caused widespread declines and extinctions of amphibians, mostly in tropical regions, and in an unpublished citizen science project (Cunningham & Minting, 2008) was found to be widespread in several British species. Fortunately, this infection seems to be benign in the UK and the fungus was only detectable by PCR amplification of a short section of chytrid-specific DNA in swabs obtained from skin surfaces. In *B. calamita* some mortality due to Bd was seen in captivity but there were no detectable consequences even for heavily infected wild populations (Minting, 2012). Essentially similar molecular analyses have been used to identify the newly emerged *B. salamandrivorans*, the causative agent of a usually fatal disease of some newts and salamanders in parts of north-west Europe (Martel et al., 2014). The effects of this fungus, with the potential to kill *T. cristatus*, look set to be anything but benign if it spreads extensively in Britain.

A fungal snake pathogen, *Ophidiomyces ophiodiicola*, has recently been found in British snakes. First detected in North America, this organism is of a different variety in Europe and can be detected in carcasses and skin sloughs by a specific PCR amplification of part of a ribosomal RNA gene. Skin lesions caused by *O. Ophiodiicola* can be fatal but so far, at least in Britain, seem not to be. In the UK Grass snakes were the species most commonly infected, although the fungus was detected on one adder (Franklinos et al., 2017).

Environmental DNA

The discovery that ponds contain DNA released into the water by animals living in them has the potential to revolutionise survey methods for amphibians. This environmental DNA, 'eDNA', can be amplified from water samples using the PCR and its subsequent analysis can identify the presence of particular species without ever seeing them. In Britain, the method has been extensively characterised for *T. cristatus* and is as or more reliable than conventional techniques such as night torching and bottle trapping for determining whether great crested

newts are present (Table 5, Biggs et al., 2015). False negatives were rare and false positives non-existent in that study. Newts were very rarely found at sites where no eDNA was detected and newts were located at every site containing their eDNA. These controls were essential to validate widespread use of the eDNA method. Because *T. cristatus* is widespread but strictly protected in Britain there is a need for a wide survey efforts to find as many newt populations as possible. Environmental DNA sampling might prove effective for achieving this goal and thus improve the long-term conservation prospects of this charismatic species. The next step in eDNA-based surveys has already been taken. Using universal primers for amplifying part of amphibian mtDNA, it proved possible to identify every species of amphibian present in a pond from a single water sample. This was achieved by 'next generation' simultaneous sequencing of all the DNA molecules produced in the PCR, generating detection probabilities close to one for all species in all the ponds sampled (Valentini et al., 2016). Environmental DNA surveys have advantages of minimal disturbance and a need for fewer site visits than standard surveys, but there are disadvantages too. DNA analyses are expensive and require dedicated lab facilities to minimise cross-contamination risks among samples. Even so, eDNA seems set to become increasingly important as a survey method in future though like any other survey method, DNA concentrations can be affected by environmental factors including in this case UV exposure, temperature and pond substrate.

DISCUSSION

Genetic investigations of amphibians and reptiles in the British Isles have generated some insights relevant to their ongoing conservation. Specifically:

(1) Simply measuring genetic diversity with neutral markers is inadequate as a reliable indicator of population genetic health and viability. This has been known for many years, but not always appreciated by conservationists. A wide-ranging meta-analysis showed that there was no significant correlation between neutral marker and adaptive variation in life history traits (Reed & Frankham, 2001). Low diversity at neutral loci can be a consequence of factors other than reduced fitness, and in particular can occur in large populations near range edges. Investigation of suspected genetic problems such as inbreeding or loss of adaptive variation requires measurements of fitness attributes directly, as shown for natterjack toads and wall lizards in Britain, and for adders in Sweden.

(2) Neutral markers are valuable for defining genetically discrete populations, and for assessing the extent of migration between them. This approach becomes all the more interesting if, as with the studies of Scottish common frogs, it can be related to selective effects on adaptive variation. More applications of landscape genetics to species other than sand lizards will surely be a valuable next step, identifying habitat permeability and putative corridors facilitating movement between populations. Climate change may make the identification of such corridors ever more important.

(3) Phylogeography has proved successful in confirming native status, in various sites, of natterjack toads, pool frogs and great crested newts. These results have informed decisions about conservation priorities and, in one case (sand lizards in Anglesey) brought to light a recent, unlicensed introduction.

(4) Molecular analysis has convincingly split two species that were previously one (each with subspecies) into two. *Bufo spinosus* as well as *B. bufo*, and *Natrix helvetica* rather than *N.n. helvetica* are, as we now know, native to the British Isles. Molecular tools are also available to aid identification in the rather few instances where this can be problematic with British species though it seems likely that, in practice, they will rarely be needed.

(5) Molecular identification is standard practise for the viral and fungal causative agents of disease in British herpetofauna. The technology involved in this work is impressive but has not, and perhaps cannot lead to ways of controlling disease outbreaks. Its value lies in demonstrating the origins, extent and spread of these unwelcome pathogens.

(6) Environmental DNA is an important new tool in the armoury of amphibian surveyors. Its reliability has been well validated as an alternative to standard survey protocols but it remains to be seen as to what degree the costs and demanding lab facilities required will limit its future use.

Inevitably there will be further developments of genetic methods in ecology and their applications to conservation are bound to increase concordantly. High throughput DNA sequencing and the ability to compare rapidly large sections of genomes will surely increase our understanding of genetic diversity and the role of selection on wild populations. DNA editing protocols may make it possible to engineer disease resistance in the face of increasing threats from the spread of novel pathogens. The future is notoriously difficult to predict but certain to be interesting.

ACNOWLEDGEMENTS

Thanks to John Buckley and Yvette Martin for Saltfleetby natterjack data, Liam Russell for Figure 3, John Wilkinson for the photograph of *B. spinosus* and Richard Griffiths and Inga Zeisset for constructive comments on the text.

REFERENCES

Arntzen, J.W., Wilkinson, J.W., Butôt, R. & Martinez-Solano, I. (2014). A new vertebrate species native to the British Isles: *Bufo spinosus Daudin*, 1803, in Jersey. *Herpetological Journal* 24, 209-216.

Babik, W., Pabijan, M., Arntzen, J.W., Cogălniceanu, D. et al. (2009). Long-term survival of a urodele amphibian despite depleted major histocompatibility complex variation. *Molecular Ecology* 18, 769-781.

Beebee, T.J.C. (2009). A comparison of single-sample effective population size estimators using empirical toad (*Bufo calamita*) population data: genetic compensation and size-genetic diversity correlations. *Molecular Ecology* 18, 4790-4797.

Beebee, T.J.C. (2014). Amphibian conservation in Britain: a 40-year history. *Journal of Herpetology* 48, 2-12.

Beebee, T.J.C., Rowe, G. & Arntzen, J.W. (1999). Identification of newts (*Triturus* species) and their hybrids using molecular methods. *Journal of Zoology (London)* 249, 43-47.

Beebee, T.J.C., Buckley, J., Evans, I., Foster, J.P. et al. (2005). Neglected native or undesirable alien? Resolution of a conservation dilemma concerning the pool frog *Rana lessonae*. *Biodiversity & Conservation* 14, 1607-1626.

Beebee, T.J.C. & Rowe, G. (2001). Application of genetic bottleneck testing to the investigation of amphibian declines: a case study with natterjack toads. *Conservation Biology* 15, 266-270.

Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C. et al. (2015). Using eDNA to develop a national citizen-science based monitoring programme for the great crested newt (*Triturus cristatus*). *Biological Conservation* 183, 19-28.

Brede, E.G. & Beebee, T.J.C. (2004). Contrasting population structure in two sympatric anurans: implications for species conservation. *Heredity* 92, 110-117.

Brede, E.G. & Beebee, T.J.C. (2006a). Consistently different levels of genetic variation across the European ranges of two anurans, *Bufo bufo* and *Rana temporaria*. *Herpetological Journal* 16, 265-271.

Brede, E.G. & Beebee, T.J.C. (2006b). Large variations in the ratio of effective breeding and census population sizes between two species of pond-breeding anurans. *Biological Journal of the Linnaean Society* 89, 365-372.

Cunningham, A.A. & Minting, P. (2008) *National survey of Batrachochytrium dendrobatidis infection in UK amphibians, 2008*. Institute of Zoology and Natural England.

Frankham, R. (1995) Effective population size/adult population size ratios in wildlife: a review. *Genetical Research* 66, 95-107.

Frankham, R,, Ballou, J.D. & Briscoe, D.A. (2010). *Introduction to Conservation Genetics (2nd edition)*. Cambridge: Cambridge University Press, Franklin, I.R. (1980) Evolutionary change in small populations. In *Conservation Biology: an evolutionary/ecological perspective*. Eds Soulé, M.E. & Wilcox, B.A. Sinauer Associates.

Franklin, I.R. (1980). Evolutionary change in small populations. In *Conservation Biology: an evolutionary/ecological perspective*. Eds Soulé, M.E. & Wilcox, B.A. Sinauer Associates.

Franklinos, L.H.V., Lorch, J.M., Bohuski, E., Rodriguez-Ramos, J. et al. (2017). Emerging fungal pathogen *Ophidiomyces ophiodiicola* in wild European snakes. *Science Reports* 7: 3844 DOI:10.1038/s41598-017-03352-1.

Hitchings, S.P. & Beebee, T.J.C. (1997). Genetic substructuring as a result of barriers to gene flow in urban common frog (*Rana temporaria*) populations: implications for biodiversity conservation. *Heredity* 79, 117-127.

Hitchings, S.P. & Beebee, T.J.C. (1998). Loss of genetic diversity and fitness in common toad (*Bufo bufo*) populations isolated by inimical habitat. *Journal of Evolutionary Biology* 11, 269-283.

Holsbeek, G., Maes, G.E., De Meester, L. & Volckaert, F.A.M. (2009). Conservation of the introgressed European water frog complex using molecular tools. *Molecular Ecology* 18, 1071-1087.

Ibrahim, K.M., Nichols, R.A. & Hewitt, G.M. (1996) Spatial patterns of genetic variation generated by different forms of dispersal during range expansion. *Heredity* 77, 282-291.

Jehle, R., Wilson, G.A., Arntzen, J.W. & Burke, T. (2005). Contemporary gene flow and the spatio-temporal genetic structure of subdivided newt populations (*Triturus cristatus*, *T. marmoratus*). *Journal of Evolutionary Biology* 18, 619-628..

Kindler, C., Chèvre, M., Ursenbacher, S., Böhme, W. et al. (2017). Hybridisation patterns in two contact zones of grass snakes reveal a new central European snake species. *Scientific Reports* 7:7378 DOI:10.1038/s41598-017-07847-9.

Madsen, T, Shine, R., Olsson, M. & Witttzell, H. (1999) Restoration of an inbred adder population. *Nature* 402, 34-35.

Martel, A., Blooi, M., Adriaensen, C., Van Rooij, P. et al. (2014). Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. *Science* 346, 630-631.

May, S. & Beebee, T.J.C. (2010). Recent introduction or ancient ancestry? Use of genetic evidence to investigate the origin of range edge populations in natterjack toads (*Bufo calamita*). *Conservation Genetics* 11, 293-300.

McCartney-Melsted, E. & Shaffer, H.B. (2015). Amphibian molecular ecology and how it has informed conservation. *Molecular Ecology* 24, 5084-5109.

Michaelides, S.N., While, G.M., Zajac, N., Aubret, F. et al. (2016). Loss of genetic diversity and increased embryonic mortality in non-native lizard populations. *Molecular Ecology* 25, 4113-4125.

Michaelides, S.N., While, G.M., Zajac, N. & Uller, T. (2015). Widespread primary but geographically restricted secondary human introduction of wall lizards *Podarcis muralis*. *Molecular Ecology* 24, 2702-2714.

Minting, P.J. (2012). *An investigation into the effects of Batrachochytrium dendrobatidis (Bd) on natterjack toad (Bufo calaita) populations in the UK*. University of Sussex: PhD thesis.

Muir, A.P., Biek, R., Thomas, R. & Mable, B.K. (2014). Local adaptation with high gene flow: temperature parameters drive adaptation to altitude in the common frog *Rana temporaria*. *Molecular Ecology* 23, 561-574.

Muir, A.P., Thomas, R., Biek, R. & Mable, B.K. (2013). Using genetic variation to infer associations with climate in the common frog, *Rana temporaria*. *Molecular Ecology* 22, 3737-3751.

Nash, D.J. (2011) Assessment of an established population of atypical grass snakes *Natrix natrix* in the Aire Valley, UK. *Herpetological Bulletin* 115, 12-16.

O'Brien, C.D, Hall, J.E., Orchard, D., Barratt, C.D. et al. (2015). Extending the natural range of a declining species: genetic evidence for native great crested newt (*Triturus cristatus*) populations in the Scottish highlands. *European Journal of Wildlife Research* 61, 27-33.

Pernetta, A.P. (2009). *Population ecology and conservation genetics of the smooth snake (Coronella austriaca) in a fragmented heath landscape*. University of Southampton: PhD thesis.

Pernetta, A.P., Allen, J.A., Beebee, T.J.C. & Reading, C.J. (2011). Fine-scale population genetic structure and sex-biased dispersal in the smooth snake (*Coronella austriaca*) in southern England. *Heredity* 107, 231-238.

Reed, D.H. & Frankham, R. (2001). How closely correlated are molecular and quantitative measures of genetic variation? A meta-analysis. *Evolution* 55, 1095-1103.

Rowe, G. & Beebee, T.J.C. (2003). Population on the verge of a mutational meltdown? Fitness costs of genetic load for an amphibian in the wild. *Evolution* 57, 177-181.

Rowe, G. & Beebee, T.J.C. (2007). Defining population boundaries: use of three Bayesian approaches with microsatellite data from British natterjack toads (*Bufo calamita*). *Molecular Ecology* 16, 785-796.

Rowe, G., Beebee, T.J.C. & Burke, T. (1999). Microsatellite heterozygosity, fitness and demography in natterjack toads *Bufo calamita*. *Animal Conservation* 2, 85-92.

Rowe, G., Beebee, T.J.C. & Burke, T. (2000). A microsatellite analysis of natterjack toad (*Bufo calamita*) metapopulations. *Oikos* 88, 641-651.

Rowe, G., Harris, D.J. & Beebee, T.J.C. (2006). Lusitania revisited: a phylogeographic analysis of the natterjack toad *Bufo calamita* across its entire biogeographical range. *Molecular Phylogenetics and Evolution* 39, 335-346.

Rowe, G,, Sweet, M. & Beebee, T.J.C. (2017). *An Introduction to Molecular Ecology (3rd edition)*. Oxford: Oxford University Press.

Russell, L. (2012). *The conservation and landscape genetics of the sand lizard Lacerta agilis*. University of Sussex: PhD thesis.

Schwartz, T.S. & Olsson, M. (2008) Microsatellite markers devised for a Swedish population of sand lizard (*Lacerta agilis*). *Conservation Genetics* 9, 715-717.

Scribner, K.T., Arntzen, J.W. & Burke, T. (1997) Effective numbers of breeding adults in *Bufo bufo* estimated from age-specific variation at minisatellite loci. *Molecular Ecology* 6, 701-712.

Snell, C., Tetteh, J. & Evans, I.H. (2005). Phylogeogrphy of the pool frog (*Rana lessonae Camerano*) in Europe: evidence for native status in Great Britain and for an unusual postglacial colonisation route. *Biological Journal of the Linnean Society* 85, 41-51.

Sztencel-Jablonka, A., Mazgajski, T.D., Bury, S., Najbar, B. et al. (2015). Phylogeography of the smooth snake *Coronella austriaca* (Serpented: Colubridae): evidence for a reduced gene pool and a genetic discontinuity in central Europe. *Biological Journal of the Linnean Society* 115, 195-210.

Teacher, A.G.F., Garner, T.W.J. & Nichols, R.A. (2009). European phylogeography of the common frog (*Rana temporaria*): routes of postglacial colonisation into the British Isles, and evidence for an Irish glacial refugium. *Heredity* 102, 490-496.

Teacher, A.G.F., Cunningham, A.A. & Garner, T.W.J. (2010). Assessing the long-term impact of ranavirus infection in wild common frog populations. *Animal Conservation* 13, 514-522.

Ursenbacher, S., Guillon, M, Cubizolle, H., Dupoué, A. et al. (2015). Postglacial recolonisation in a cold climate specialist in western Europe: patterns of genetic diversity in the adder (*Vipera berus*) support the central-marginal hypothesis. *Molecular Ecology* 24, 3639-3651.

Valentini, A., Taberlet, P., Miaud, C., Civade, R. et al. (2016). Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Molecular Ecology* 25, 929-942.

Zeisset, I. & Beebee, T.J.C. (2001). Determination of biogeographical range: an application of molecular phylogeography to the European pool frog *Rana lessonae*. *Proceedings of the Royal Society of London B* 268, 933-938.

Zeisset, I. & Beebee, T.J.C. (2003). Population genetics of a successful invader: the marsh frog *Rana ridibunda* in Britain. *Molecular Ecology* 12, 639-646.

Zeisset, I. & Beebee, T.J.C. (2010). Fitness attributes, microsatellite diversity and MHC class II diversity in common frog (*Rana temporaria*) populations. *Heredity*104, 423-430.

Zeisset, I. & Beebee, T.J.C. (2014). Drift rather than selection dominates MHC class II allelic diversity patterns at the biogeographical range scale in natterjack toads *Bufo calamita*. *PLoSONE* 9(6): e100176. doi:10.1371. pone.0100176.

Accepted: 2 December 2017

Volume 28 (April 2018), 63-72

Herpetological Journal FULL PAPER

Published by the British Herpetological Society

A reassessment of the biogeographic range of northern clade pool frogs (*Pelophylax lessonae*)

Inga Zeisset[1] & Tom Hoogesteger[2]

[1] School of Pharmacy and Biomolecular Sciences, College of Life, Health and Physical Sciences, University of Brighton, Brighton, UK

[2] Myllytie 10 B 31, 04410 Järvenpää, Finland

Distinguishing between native and introduced species can be difficult, particularly at range borders where patchily distributed populations may occur away from a species' natural core range. The case of native pool frog (*Pelophylax lessonae*) populations at their northern range limit in Europe is particularly interesting. These are morphologically and genetically distinct populations that are patchily distributed and have been reported from the UK, Sweden and Norway, but up until 2013 were thought to be absent from Finland. When pool frog populations were discovered in south-western Finland they were morphologically classified as belonging to this northern clade. However, the origin of these populations has been unclear and it is possible that the Finnish populations originated through human aided introductions, established themselves recently through natural migration, or are indeed previously undiscovered relic populations. To establish the origin and relationship of these frogs to other populations across Europe we used phylogeographical analysis based on microsatellite and mitochondrial DNA markers. Our results indicate that the Finnish, Norwegian, Swedish, UK, as well as Estonian populations belong to the northern clade. The Finnish frogs are most closely related to Swedish northern pool frogs, but are genetically more diverse. This suggests that the Finnish pool frogs are most likely a relic from postglacial migration, though we could not entirely rule out the possibility of a recent natural or human aided colonisation from Sweden. This has implications for the conservation status of the pool frog in Finland, where it thus far has been considered an invasive alien species.

Key words: Pelophylax lessonae, microsatellites, phylogeography, northern clade, pool frogs

INTRODUCTION

Determining natural biogeographic ranges, particularly at range borders, is central to conservation biology and important for establishing local conservation priorities, often deciding between conservation and eradication (Simberloff, 2003). Where populations occur away from a species' continuous range and beyond their natural dispersal abilities, their origin can be particularly difficult to establish. Such populations may be the result of natural demographic processes, e.g. relics from a previously larger range, or the result of human activities that have caused the translocation and global movement of many species. For conservation purposes, it is important to distinguish between native and introduced populations, as the former is usually deemed more valuable in terms of conservation. Today, genetic tools can be used to investigate the origin of such populations, distinguish between native and introduced populations, and sometimes even pinpoint the source of introductions. Genetic markers have, for example, been used successfully to establish the origin and species identity of water frogs on Cyprus (Plötner et al., 2015), as well as the likely origin of a non-native population of crested newts in Geneva (Arntzen, 2001). They have also been used to determine that potentially native populations of water frogs in Switzerland stem from human introductions (Dubey et al., 2014), as well as establishing the likely number of translocations giving rise to non-native populations in the case of American bull frogs in Europe (Ficetola et al., 2008). Recently, molecular tools have also been used to establish that great crested newt (*Triturus cristatus*) populations in Scotland were native to the Scottish Highlands and not the result of a human translocation, as previously assumed (O'Brien et al., 2015).

Pool frogs (*Pelophylax lessonae*; previously *Rana lessonae*) are another good example of the importance of distinguishing between native and introduced populations. These members of the western Palearctic water frog complex have a European-wide distribution and for most of their range they are found in a hybridogenetic complex with the hybrid edible frogs (*Pelophylax kl. esculentus*). Edible frogs are hybrids between the pool frog and the marsh frog (*Pelophylax ridibundus*) and reproduce by hybridogenesis with either parental species, discarding one parental genome during gametogenesis (Graf & Polls-Pelaz, 1989). This system allows the hybrids to coexist with only one parental species, which in most cases is the pool frog (Berger,

Correspondence: Inga Zeisset (i.zeisset@brighton.ac.uk)

1973; Ragghianti et al., 2007). Hybrid edible frogs can also reproduce without either of the parental species if there are triploid individuals in the population. Such all-hybrid populations are common in northern Germany, Denmark and southern Sweden (Fog et al., 2001; Christiansen & Reyer, 2011). The distribution of pool frogs and the hybrid edible frog extends from France and Italy to Estonia and the western parts of Russia (Sillero et al., 2014) and partially overlaps with that of other water frogs, such as the marsh frog.

Only at the northernmost edge of the species' range do isolated populations of *P. lessonae* occur without any other water frog species or hybrids present (e.g. Sjögren, 1991a). These northern pool frogs form a distinct clade, which differs genetically, morphologically and behaviourally from central European populations (Zeisset & Beebee, 2001; Buckley & Foster, 2005; Snell et al., 2005; Snell, 1994; Sjögren, 1991b, Fog et al., 2001). The northern pool frog is currently known to naturally occur along to the Baltic coast of Uppland, central Sweden (Sjögren, 1991a; Edenhamn & Sjögren-Gulve, 2000; Nilsson, 2013; Lindgren et al., 2014) and in three ponds in southern Norway, which were first discovered in 1986 (Dolmen, 1997; Dolmen, 2012). It was present in the UK until the 1990s, when it went extinct due to habitat degradation and lack of conservation efforts. At the time it was tragically assumed to be an introduced species, and only after its disappearance genetic data established native species status for this species in the UK (Zeisset & Beebee, 2001; Snell et al., 2005; Beebee et al., 2005). A species recovery program has since helped to re-establish this species in the UK through the translocation of individuals from Sweden (Buckley & Foster, 2005). In Estonia pool frogs are often found in mixed populations, together with the hybrid edible frog, but pure pool frog populations have been recorded in the northern parts of the country (Talvi, 1992; Kuzmin, 1995). A study considering vocalisation patterns in pool frogs across Europe indicated that the Estonian frogs also belong to the northern clade of pool frogs (Wycherley et al., 2002), but so far genetic evidence has been lacking.

The northern clade populations are now recognised as being distinct conservation units of evolutionary importance and the pool frog is now a UK priority species (Joint Nature Conservation Committee, 2010), red-listed as vulnerable in Sweden (Nilsson, 2013), critically endangered in Norway (Direktoratet for naturforvaltning, 2006) and protected under EU legislation as a European Protected Species (EPS, schedule 2 of the Conservation of Habitats and Species Regulations, 2010).

In Finland, water frogs are not known to occur naturally. Apparently introduced populations of marsh frogs (*P. ridibundus*) occurred in the estuaries of the rivers Vantaa and Porvoo in the 1930s-1950s but had gone extinct by the 1960s (Terhivuo, 1993). However, since 2008 water frogs have been reported from several locations near Turku in south-western Finland. On the basis of their morphology these have been identified as hybrid edible frogs (*P. kl. esculentus*) in most locations. No parental species (e.g. pool frogs or marsh frogs) have been found in these populations, indicating the likely presence of polyploid edible frogs. However, populations of pool frogs were more recently reported from at least two locations (Hoogesteger et al., 2013, 2014). These pool frogs closely resemble the northern clade pool frogs in Sweden and have been assumed to belong the northern clade based on morphology (Hoogesteger et al., 2013, 2014). The Finnish pool frog populations are within the natural range of northern clade pool frogs, but the edible frogs are outside of their normal range, indicating possibly different origins of these two species in Finland.

Considering the rarity and precarious status of the few isolated populations of northern clade pool frogs in Europe, the question of whether the Finnish populations present a valuable addition to the northern clade is of great importance. Like many temperate species, *P. lessonae* survived the last glacial maximum in warmer refugia, such as in Italy, where climatic conditions were less extreme (Hewitt, 1999; Zeisset & Beebee, 2001; Snell et al., 2005). As a result of postglacial recolonisation processes pool frog populations at northern range edges have reduced genetic diversity but also carry distinct microsatellite alleles in some populations (Hewitt, 1996; Zeisset & Beebee, 2001). Mitochondrial DNA, the protein-coding gene for cytochrome b in particular, is frequently used to establish phylogeographic relationships and a number of partial cytochrome b haplotypes have been identified in pool frogs (e.g. Canestrelli & Nascetti, 2008; Hofman et al., 2012; Dufresnes et al., 2017). Here we present data on the phylogeography and genetic diversity of pool frog populations across Europe (using mtDNA and microsatellites), with a particular focus on the northern clade populations. We were particularly interested in the question of whether the Finnish pool frogs belong to the northern clade and if their presence in Finland can be explained by recent human translocations, natural colonisation from nearby populations, or whether they may indeed be previously undiscovered, relic populations, which reached Finland through postglacial colonisation processes. We also collected, for the first time, genetic data on Estonian pool frogs to establish their genetic relationship to the northern clade.

MATERIALS AND METHODS

Samples

Tissue samples from adult and juvenile frogs were taken from eight Finnish pool frogs (three from Kaarina and five from Raisio), as well as from nine edible frogs from nearby locations (six from Piikkiö, two from Rusko and one from Kaarina; all museum specimens collected between 2008 and 2015 and preserved in 95% ethanol). Morphological characteristics that distinguish pool frogs and edible frogs, as described in Hoogesteger et al. (2013), were used to separate the two. DNA extraction was subsequently carried out using a Qiagen DNeasy Blood and Tissue kit according to the manufacturer's instructions.

We verified morphological species identification using molecular markers as follows: the hybrid edible frogs (*P. kl. esculentus*) contain the marsh frog (*P. ridibundus*) genome and *P. ridibundus* specific markers can be expected to amplify in hybrids. We

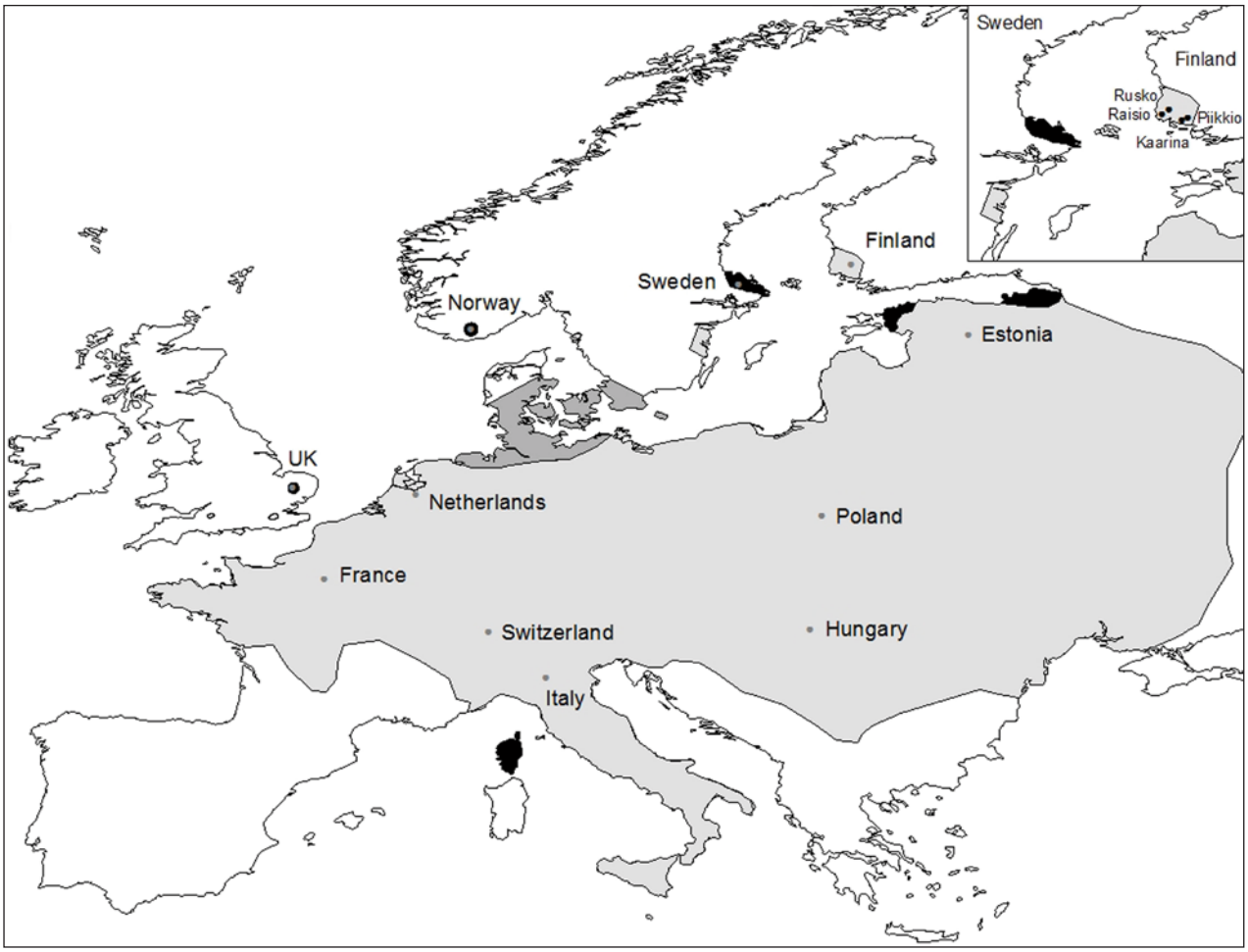

Figure 1. Sampling sites and approximate distribution of *P. lessonae* and *P. kl. esculentus* in Europe. Light grey shading indicates the approximate extent of the European range of both species, dark grey shading indicates pure *P. kl. esculentus* populations and pure *P. lessonae* populations are indicated in black (based on Sillero et al., 2014 & Arioli et al., 2010). Sampling sites are indicated as grey dots; the UK sample was based on historic (native) samples. Details of Finnish populations sampled are presented in map insert.

used two molecular markers to test for the presence of *P. ridibundus* DNA in all Finnish individuals: one *P. ridibundus* specific microsatellite marker, res22 (Zeisset et al., 2000) and a primer pair designed to amplify part of the serum albumin intron in *P. ridibundus* (458bp; Psai1F: TGTGCTAAGTAGGTTTGAGTGT (as in Hauswaldt et al., 2012); Psai1ridR: GTTTTAGTGAGTGGCCCGTG based on GenBank sequence MF667646). PCR reaction mixtures (20µl total) contained 1x standard reaction buffer (NEB), 0.2µM of each primer, 100µM of each dNTP and 0.5 units of Taq DNA polymerase (NEB). All PCRs started with 3 min denaturation at 94 ˚C, then 35 cycles of denaturation at 94 ˚C for 30 sec, annealing (with a touchdown protocol starting with two cycles at 62 ˚C reducing in 2 ˚C steps to 50 ˚C) for 30 sec and elongation at 72 ˚C for 30 sec, with a final elongation of 3 min. We used two *P. ridibundus* and two *P. lessonae* DNA samples as controls. PCR products were visualised using agarose gel electrophoresis.

Pool frog samples from across Europe stemmed from an earlier microsatellite study (Figure 1; Zeisset & Beebee, 2001). The following European countries represented in that study were used: France (N=19), Switzerland (N=40), Netherlands (N=34), Italy (N=18), Hungary (N=31), Poland (N=41), Sweden (N=12), Norway (N=5), UK native (prior to re-introduction), including museum specimens (N=5). Additionally, tissue samples from eight Estonian pool frogs (from Karula national park) were collected and analysed in 2003 (unpublished data).

Microsatellite DNA analysis

Microsatellite genotype data for populations across Europe were obtained from a previous study (Zeisset & Beebee, 2001). Individuals from Estonia and Finland were genotyped at five microsatellite loci using PCR conditions as in Zeisset et al. (2000) for res3, res5, res16 and res20 and Garner et al. (2000) for RlCA18 and RlCA19, and fluorescently labelled primers. Fragments were genotyped using an ABI Prism 377 sequencer (Applied Biosystems) and GENESCAN 3.1.2 software (Estonian samples) or using an automated capillary DNA sequencer (Applied Biosystems, model 3730) at DNA Sequencing and Services (University of Dundee) and scored using Peak Scanner 1v.0 software (Finnish samples). Due to the different allele sizing methods in the current study, we adjusted the sizing of alleles to previously obtained genotypes by including DNA samples with known genotypes from Zeisset & Beebee (2001). One locus (res3) failed to amplify consistently and was excluded from this study. As the hybrid *P. kl. esculentus* contains the marsh frog and pool frog genome, some

Table 1. Microsatellite diversity and mtDNA haplotypes of *P. lessonae* populations (and *P. kl. esculentus*). N= number of individuals, N_l= number of polymorphic loci, N_a= mean number of alleles, N_p= number of private alleles, R=allelic richness based on three individuals, H_o= observed heterozygosity; H_e=expected heterozygosity, N_{mt}= number of individuals sequenced for mtDNA haplotype, hap= name of haplotype obtained.

	N	N_l	N_a	N_p	R	H_o	H_e	N_{mt}	hap
Netherlands	34	5	2.6	1	1.9	0.33	0.36		
France	19	5	3.4	1	2.2	0.30	0.47	3	CC-01 (2x) CC-03 (1x)
Switzerland	40	5	3	0	2.0	0.29	0.39		
Italy	18	4	5.6	6	3.1	0.40	0.58	1	NC-01
Hungary	31	5	8	15	3.1	0.47	0.58	1	CC-02
Poland	41	3	5	1	2.3	0.24	0.36		
UK	5	0	1	0	1.0	0.00	0.00		
Estonia	8	1	1.2	1	1.1	0.05	0.05		
Norway	5	0	1	0	1.0	0.00	0.00	1	NC-01
Sweden	12	0	1	0	1.0	0.00	0.00	4	NC-01
Finland	8	3	1.6	0	1.4	0.05	0.20	8	NC-01
(*P.kl.esculentus*)	9	3	1.6	0	1.5	0.18	0.22		

Table 2. Northern clade pool frog microsatellite genotypes and frequencies. The numbers represent allele sizes in base pairs, with allele frequency given in brackets where more than one allele was present; N=number of individuals genotyped from each country.

Origin	res16	res5	res20	RICA18	RICA19
UK (native) (N=5)	108	131	92	168	92
Norway (N=5)	108	131	92	174	92
Sweden (N=12)	108	131	92	176	92
Estonia (N=8)	108	131	92	172 (0.875) 186 (0.125)	92
Finland (N=8)	108 (0.937) 114 (0.063)	131	92 (0.750) 102 (0.250)	172 (0.313) 176 (0.687)	92
Finland *P.kl. esculentus* (N=9)	108 (0.556) 114 (0.444)	131	92 (0.111) 102 (0.889)	172 (0.778) 176 (0.222)	92

of the microsatellite loci can be amplified from both genomes (e.g. res16) whilst others cannot (Zeisset et al., 2000, Garner et al., 2000). Therefore the data from *P. kl. esculentus* do not represent the actual genetic makeup of these individuals, but do provide some information on the *P. lessonae* part of their genome.

Microsatellite polymorphism was quantified by the mean number of alleles per locus (N_a), number of private alleles (N_p), allelic richness R, observed (H_o) and expected (H_e) heterozygosities for each of the populations studied using GenAlEx vs. 6.4 (Peakall & Smouse, 2006; 2012), Genepop on the web (Raymond & Rousset, 1995; Rousset, 2008) and FSTAT vs. 2.93 (Goudet, 2001). Calculation of genetic distance estimates (D_A, Nei et al., 1983) and neighbour-joining tree construction were carried out in POPTREE2 (Takezaki et al., 2010).

The population affinities of the Finnish *P. lessonae* and *P. kl. esculentus* to other European populations were also tested using STRUCTURE v2.3.4 (Pritchard et al., 2000) with a model that assumed admixture and correlated allele frequencies. We tested from 1 to 12 groups (K), with five replicate runs per K, a 50 000 burn-in period and 10 0000 iterations. The ΔK test was used to determine the most likely number of groups (Evanno et al., 2005) and CLUMPAK (Kopelman et al., 2015) was used to generate a consensus solution and compare the clustering results across different K values.

Mitochondrial DNA

To investigate mtDNA sequence diversity in pool frogs we sequenced 552bp (excluding primer sequences) of the cytochrome *b* gene corresponding to positions 129-680, encoding amino acids 44 to 226 (based on *P. lessonae* mtDNA sequence JN627426) from a total of 18 *P. lessonae* individuals from Finland (N=8), Sweden (N=4), Norway (N=1), France (N=3), Italy (N=1) and Hungary (N=1), using primer cytbPelophylax F1 (CTCCTGGGAGTCTGCCTAAT) and cytbPelophylaxR1 (CGAAGCCTAGAAGATCTTTG) as described in Dubey et al. (2014). This represents a larger section of the cytochrome *b* gene sequenced in a previous cross-European study (based on 410bp, corresponding to positions 129-539; Zeisset & Beebee, 2007). Sequencing was carried out at DNA Sequencing and Services, University of Dundee (https://www.dnaseq.co.uk). Native British and Estonian samples were no longer available. However, we additionally used the haplotypes we obtained to carry out a nucleotide BLAST search on the genetic sequence database GenBank

in order to identify further pool frog cytochrome *b* sequences from across Europe.

RESULTS

Verification of species identification

The marsh frog (*P. ridibundus*) specific markers (res22 and the serum albumin intron marker) verified that all pool frog (*P. lessonae*) individuals had been correctly identified using morphological characters. In the hybrid edible frogs (*P. kl. esculentus*) the two markers failed to amplify any marsh frog DNA in one sample (a female from Piikkiö) and this is most likely a case of a misidentified pool frog. This result was supported by the microsatellite genotype for this individual, which was identical to that of the Swedish pool frogs.

Microsatellite DNA

Microsatellite diversity estimates for *P. lessonae* are presented in Table 1. Central and southern populations (e.g. Italy, Hungary, France, Poland, Netherlands and Switzerland) were all more polymorphic as measured by allelic richness (R), mean number of alleles (N_a), number of polymorphic loci as well as having higher observed and expected heterozygosities, than the northern populations (UK, Norway, Sweden, Finland and Estonia). All individuals from Norway, Sweden and UK (native) were invariant at all five loci and fixed for the same allele at four loci. Only the allele at locus RlCA18 distinguished these three populations (see also Zeisset & Beebee, 2001). Six of the Finnish pool frogs, as well as two edible frogs, had the same allele at this locus as the Swedish population. However, the Estonian and Finnish pool frogs also showed slightly more variation, RlCA18 was polymorphic in Estonia and three loci (RlCA18, res16 and res20) were polymorphic in Finland (in individuals from Raisio). The alleles found in the Finnish pool frogs generally also occurred in the Finnish *P. kl. esculentus*. For details of allele sizes and frequencies in northern clade pool frogs see Table 2.

We used allele frequencies from the five microsatellite loci to construct a phylogeographical tree for *P. lessonae*. The neighbour-joining tree (Saitou & Nei, 1987) in Figure 2 is based on Nei's D_A distance (Nei et al., 1983). D_A is generally considered best for correct tree topology (Takezaki & Nei, 2008), but similar tree topologies were obtained using Nei's standard genetic distance (D_{ST}), with or without sample size bias correction (Nei, 1972), as well as when using the UPGMA method for tree construction (Sneath & Sokal, 1973). There was strong support for the 'northern clade' group, previously consisting of UK, Sweden and Norway (see also Zeisset & Beebee, 2001) and now including the populations from Estonia and Finland with a bootstrap value of 83%. Due to the relatively small number of loci used not all relationships could be resolved, but there was strong support for two larger groups, a 'north-eastern' group consisting of UK, Sweden, Norway, Estonia, Finland, Hungary, Poland and Italy as well as another 'western' group, consisting Switzerland, the Netherlands and France (bootstrap value of 92%).

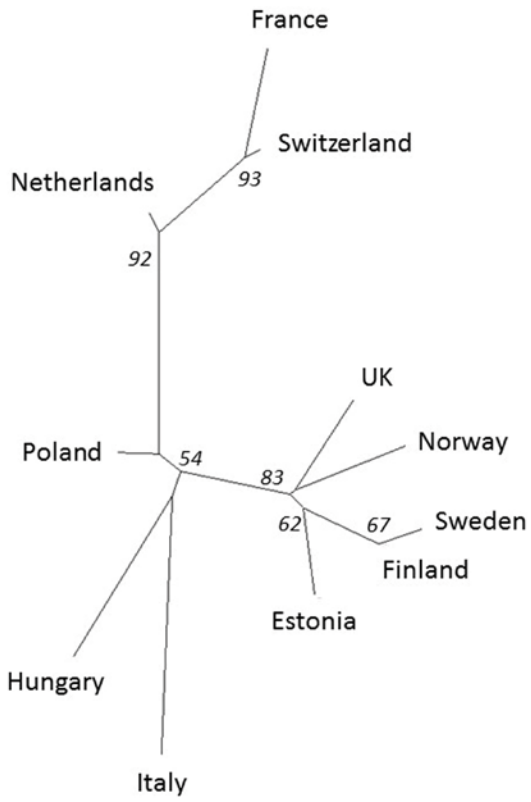

Figure 2. Phylogeographical tree of European *P. lessonae* populations based on D_A distances and the neighbour-joining method. The numbers are percentages of bootstraps (out of 1000); only bootstrap values >50% are shown.

Clustering of microsatellite genotypes

Bayesian clustering assignment of all European *P. lessonae* populations, including the Finnish *P. kl. esculentus*, using STRUCTURE, indicated highest support for the same two larger groups as the phylogeographical tree (ΔK= 429.4; Figure 3). Group one consisted of Norway, Sweden, native UK (prior to re-introduction), Estonia, Finland (*P. lessonae*; PL), Italy, Hungary and Poland (the 'north-eastern' group); group two encompassed the Netherlands, France and Switzerland (the 'western' group), as well as the Finnish *P. kl. esculentus* (PE). There were two individuals within the Finnish *P. lessonae* (both from Raisio) which were assigned to group two and two *P. kl. esculentus* (one from Rusko and one from Piikkiö), which were assigned to group one. One of these was most likely a misidentified pool frog (see 'verification of species identification' above).

Mitochondrial DNA sequences

We sequenced a fragment of 552bp of the mitochondrial DNA (mtDNA) cytochrome *b* gene of 18 individuals. We identified four haplotypes, NC-01 (MG214959), CC-01 (MG214960) and CC-02 (MG214961), which all differed between 1 and 3bp, and CC-03 (MG214962), which differed in 28 to 30bp from the other three. All individuals from northern clade populations (Finland (N=8), Sweden (N=4) and Norway (N=1)), as well as the Italian sample consisted of one genetic lineage (haplotype NC-01). In

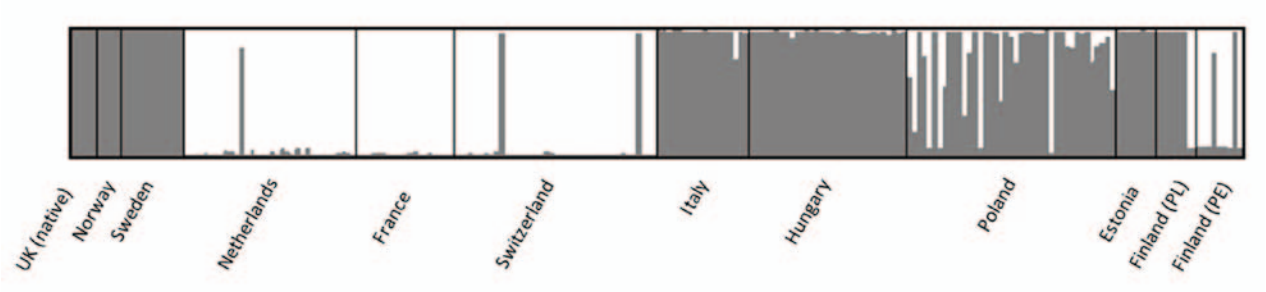

Figure 3. Assignment of European *P. lessonae* populations to genetic clusters using the STRUCTURE algorithm (K=2), assuming admixture and correlated allele frequencies. Vertical lines represent individuals, black lines separate different populations. Finland (PE) consist of *P. kl. esculentus* individuals.

France we detected two haplotypes (two individuals with haplotype CC-01 and one with haplotype CC-03; 534bp sequenced) and a fourth haplotype was found in the Hungarian sample (haplotype CC-02). A BLAST nucleotide search of GenBank revealed that the northern clade haplotype NC-01 is widely distributed in Europe and can be also found in central Sweden (LES17, MF094344), South Sweden (LES25, MF094352), Germany (LES25, MF094352), Italy (LES25, MF094352; LES21, MF094348), Ukraine (LES25, MF094352), Czech Republic (LES20, MF094347) , France (LES25, MF094352; LES23, MF094350), Austria (LES25, MF094352), and Switzerland (LES22, MF094349; LES20, MF094347) (Dufresnes et al., 2017). Haplotype CC-02, which we found in Hungary, can also be found in other central/eastern European countries, such as Poland (LES26, MF094353), the Czech Republic, Romania and the Ukraine (LES04, MF094331) and haplotype CC-01, which we detected in France, has been found in northern Germany (LES11, MF094338). Haplotype CC-03 differed greatly from the other three haplotypes, but only in 1 base pair to sequences identified by others as belonging to the Italian pool frog *P. bergeri* (e.g. BER21, MF94325; Dufresnes et al., 2017).

DISCUSSION

The molecular phylogeographical analysis based on five microsatellite loci strongly inferred that both the Finnish and Estonian *P. lessonae* belong to the distinct northern clade of pool frogs, which include populations in Norway, Sweden and the UK (Zeisset & Beebee, 2001). Although sample sizes were low, the similarity in the genetic profile between the Swedish and Finnish pool frog populations was notable. The five microsatellite loci are invariant within populations in Norway, Sweden and the UK and only one of the five (RlCA18) is polymorphic across these populations (Zeisset & Beebee, 2001). Many of the Finnish pool frogs, as well as some of the edible frogs, had an allele at locus RlCA18 which is found in Swedish pool frogs and only at very low frequency elsewhere (i.e. at a frequency of 0.013 in Poland). As per theoretical expectations the northern clade populations have markedly lower genetic diversity indices than other European populations. Although based on small sample sizes, the Estonian as well as the Finnish population both exhibited slightly higher diversity values than those from Norway, Sweden and UK (historic samples).

The phylogeographical analysis and the inclusion of the Estonian pool frogs in the northern clade supports the notion that the northern clade pool frogs originated from an easterly postglacial colonisation route (Zeisset & Beebee, 2001; Snell et al., 2005). Northward range expansion was generally faster along eastern routes than in western Europe for many species (Hewitt, 2000). There is also mounting evidence of more northerly 'cryptic' refugia for many species (Provan & Bennett, 2008; Schmitt & Varga, 2012; Stewart & Lister, 2001) and the moor frog *Rana arvalis*, for example, is thought to have survived several glacial cycles in a refugium in the Carpathian basin (Babik et al., 2004). The discovery of isolated pool frog populations in Romania, and higher genetic diversity in pool frogs in central and eastern Europe, also point towards the possibility of secondary glacial refugia in this geographic region, which may have been the main contributors during the northward colonisation after the last ice age (Covaciu-Marcov et al., 2008; Hoffmann et al., 2015; Dufresnes et al., 2017).

Intraspecific nucleotide polymorphism for mitochondrial DNA in pool frogs in the post-glacial expansion area of Europe is low, with for example four sequenced *P. lessonae* mtDNA genomes (15,376-78 bp without control region) differing on average by only 19 nucleotides in central and western Poland (Hofman et al., 2012). A recent study which sequenced 974bp of the cytochrome *b* in pool frogs across Europe identified 27 haplotypes, although many of these haplotypes differed in only 1bp and northern clade populations were not included (Dufresnes et al., 2017). In our study, all eastern and northern European *P. lessonae* had one of two haplotypes (NC-01 or CC-02) and all northern clade populations in this study had the same haplotype (NC-01), exhibiting no diversity at the section of cytochrome *b* we investigated. Haplotype NC-01 was the most wide-spread haplotype in Europe, found across much of central, eastern and northern Europe, as well as in northern Italy. Additionally we identified a haplotype (CC-03) in France which was highly similar to those commonly found in central Italy and markedly different from the other haplotypes. Pool frogs in Italy may in fact be comprised of two species or subspecies, the pool frog (*P. lessonae*) in the north, and the Italian pool frog (*P. bergeri*) in central and southern Italy. Central Italian mtDNA haplotypes have been documented by others in French and Swiss water frog populations (Dubey et al., 2014; Dufresnes et al., 2017).

There was clear evidence that the Finnish pool frog population was most closely related to the Swedish frogs and there are three possible explanations for this: the population could be (1) a longstanding relic of post-glacial migration patterns, (2) a recent natural colonisation from Sweden or (3) the result of a recent introduction.

Although there are no historical records of the species in Finland, the first explanation seems, according to our results and previously published work, the most likely. The Finnish population is within the natural range limit for northern clade pool frogs and the existence of a natural population in south-western Finland would concord with proposed postglacial recolonisation routes (see Zeisset & Beebee, 2001; Snell et al., 2005). The species is much harder to detect than other water frogs, and populations can easily remain unnoticed for long times. The population in Uppland, Sweden for example was only discovered in the 1940s (Gislén & Kauri 1959), the Norwegian population as late as 1986 (Dolmen, 1997; Dolmen, 2012) and the now extinct population near Norfolk, UK, had gone unnoticed for over a century before being rediscovered in the 1960s (Buckley & Foster, 2005). Moreover, according to some local residents in south-western Finland, water frogs had in fact been present in the area long before they were first reported in 2008 (Ari Karhilahti, pers. comm.). There has been a longstanding lack of records of the Finnish herpetofauna and for example the moor frog (*Rana arvalis*), which is a widespread and common species in Finland, was only known from very few locations before the 1960s (Haapanen & Salkio, 1966). The inland populations of the great crested newt (*Triturus cristatus*) also remained undiscovered until the 1990s (Terhivuo, 1993). The higher level of genetic variation in the Finnish and Estonian pool frogs, compared to the Swedish populations, indicates a possible expansion westwards from Finland and Estonia towards Sweden during postglacial recolonisation. The Swedish population would have lost diversity further during the postglacial colonisation process due to serial bottlenecking and random genetic drift, whilst the Finnish populations would have retained more.

However, a recent or indeed historic natural colonisation from Sweden is possible. We did not find any 'Finnish-specific' alleles at the microsatellite locus RlCA18, as can be found in other northern clade populations and the Swedish population is located on the coast of Uppland on the other side of the Baltic Sea at the same latitude as the Finnish population. The total distance between the populations is about 200 km, but the Åland islands and Turku archipelago form a continuum of islands between Sweden and Finland, with a maximum of about 12 km between islands. This has been a known colonisation route for several species, such as the adder (*Vipera berus*), smooth snake (*Coronella austriaca*) and grass snake (*Natrix natrix*) (Galarza et al., 2014; Kindler et al., 2014). Water frogs are known to be tolerant of brackish water (e.g. Milto, 2008; Litvinchuk et al., 2015) and pool frogs have been found even on distant islets off the Swedish coast in the Baltic Sea (Lindgren et al., 2014; Sjögren-Gulve, 1994). The possible natural expansion of the pool frog from Sweden to Finland via the archipelago has been suggested already by Kaisila (1949). There have been no records from the Åland islands and the Turku archipelago so far, but the presence of undiscovered populations here is possible.

A recent introduction by humans is another possible explanation that cannot be entirely eliminated. A couple of amphibian species traditionally not belonging to the Finnish fauna have been found near Turku in south-western Finland since 2008. Apart from *P. lessonae* and *P. kl. esculentus*, there is an established population of alpine newts (*Ichthyosaura alpestris*; Finnish Invasive Alien Species Portal, vieraslajit.fi) and one confirmed observation of yellow bellied toad (*Bombina variegata*; Ari Karhilahti, pers. comm.), which is strong evidence that introductions of amphibians have recently taken place in Finland. Human aided translocations of water frogs across Europe appear to have happened with some frequency, for example the existence of central Italian lineages of *P. bergeri* in Switzerland and France have been attributed to movement by humans (Dubey et al., 2014; Dufresnes et al., 2017) and a number of alien water frog species have been reported from Belgium (Holsbeek et al., 2010). The presence of the edible frog (*P. kl. esculentus*) in Finland can hardly be explained by other means than an introduction by humans, because there are no known populations within natural colonisation range and the occurrence of the edible frog at this latitude is highly unusual.

However, a recent natural or human aided colonisation should result in a loss of diversity, rather than a gain, and the higher diversity values, albeit based on a small sample size, indicate that the existence of relic populations may be a better explanation.

Our results also suggest that interbreeding between *P. lessonae* and *P. kl. esculentus* may have taken place in Finland, as northern clade specific alleles could be found in the edible frogs. Edible frogs are unlikely to have contributed to the genome and diversity of the Finnish pool frogs, as most matings between hybrids (or hybrid and pool frog) produce hybrid offspring. Matings between triploid hybrids can occasionally produce pool frogs, but these usually die during the larval stage (Christiansen et al., 2010; Christiansen & Reyer, 2011). However, there is a small possibility that there are some pool frogs, produced by hybrid matings, amongst the edible frogs in Finland, and they may have contributed to the genetic diversity of the northern clade pool frogs.

The origin of the Finnish hybrid water frogs *P. kl. esculentus* could not be established in this study and requires further investigation. In our study, their microsatellite genotypes clustered to the 'western' group along with those from the Netherlands, France and Switzerland, but further data from edible frog populations across Europe are needed to resolve this fully.

To conclude, the pool frog populations we investigated belong to the rare northern clade of this species. The Finnish populations appear to be most likely relicts of postglacial migration, but we cannot rule out a recent colonisation (natural or human aided) from Sweden, or indeed the possibility that more than one of the proposed scenarios acted together. The extent of interbreeding between the northern clade pool frogs and other water

frogs in Finland is currently unclear but warrants further investigation. Establishing the presence of northern clade pool frogs in Finland and Estonia presents a valuable addition to our efforts to preserve this unique clade of pool frogs. The protection of the species and its habitats in Finland is recommendable, whether these frogs are relic populations, or the result of a natural or human aided range expansion from Sweden, as currently both pool frogs and edible frogs are considered invasive alien species in Finland.

ACKNOWLEDGEMENTS

We would like to thank Ditte Guldager Christiansen for providing Estonian samples and water frog advice, Joel Rahkonen and Ari Karhilahi (Zoological museum, University of Turku) for providing Finnish samples, Maureen Berg for technical help and Trevor Beebee as well as two anonymous reviewers for advice on the manuscript.

REFERENCES

Arioli, M., Jakob, C. & Reyer, H-U. (2010). Genetic diversity in water frog hybrids (*Pelophylax esculentus*) varies with population structure and geographic location. *Molecular Ecology* 19, 1841-1828.

Arntzen, J. W. (2001). Genetic variation in the Italian crested newt, *Triturus carnifex*, and the origin of a non-native population north of the Alps. *Biodiversity & Conservation* 10, 971-987.

Babik, W., Branicki, W., Sandera, M., Litvinchuk, S., Borkin, L. J., Irwin, J. T. & Rafiski, J. (2004). Mitochondrial phylogeography of the moor frog, *Rana arvalis*. *Molecular Ecology* 13, 1469–1480.

Beebee, T. J. C., Buckley, J., Evans, I., Foster, J.P., Gent, A. H., Gleed-Owen, C. P., Kelly, G., Rowe, G., Snell, C., Wycherley, J. T. & Zeisset, I. (2005). Neglected native or undesirable alien? Resolution of a conservation dilemma concerning the pool frog *Rana lessonae*. *Biodiversity and Conservation* 14, 1607-1626.

Berger, L. (1973). Systematics and hybridization in European green frogs of *Rana esculenta* complex. *Journal of Herpetology* 7, 1-10.

Buckley, J. & Foster, J. (ed.) (2005) *Reintroduction strategy for the pool frog Rana lessonae in England*. English Nature Research Reports, no 642. English Nature, Peterborough, UK.

Canestrelli, D. & Nascetti, G. (2008). Phylogeography of the pool frog *Rana* (*Pelophylax*) *lessonae* in the Italian peninsula and Sicily: multiple refugia, glacial expansions and nuclear–mitochondrial discordance. *Journal of Biogeography* 35, 1923–1936. doi: 10.1111/j.1365-2699.2008.01946.x.

Christiansen, D. G., Jakob, C., Arioli, M., Roethlisberger, S. & Reyer, H-U. (2010). Coexistence of diploid and triploid hybrid water frogs: population differences persist in the apparent absence of differential survival. *BMC Ecology* 10, 14.

Christiansen, D. G. & Reyer, H-U. (2011). Effects of geographic distance, sea barriers and habitat on the genetic structure and diversity of all-hybrid water frog populations. *Heredity* 106, 25-36.

Covaciu-Marcov, S. D., Sas, I. & Cupsa, D. (2008). On the presence of *Rana* (*Pelophylax*) *lessonae* in south-western Romania: distribution, biogeographical signification and status. *North-Western Journal of Zoology* 4, 129-133.

Direktoratet for naturforvaltning (2006) Handlingsplan for damfrosk *Rana lessonae*. Rapport 2006-2.

Dolmen, D. (1997). The green frogs of Norway. (Proceedings of the Sixth Nordic Herpetological Symposium in Ry 10-15 June 1997). *Memoranda Societatis pro Fauna et Flora Fennica* 73, 125.

Dolmen, D. (2012). Damfrosk *Rana* (*Pelophylax*) *lessonae* – sluttrapport for arbeids- og framdriftsplanen for perioden 2006-2011. Fylkesmannen i Aust-Agder.

Dubey, S., Leuenberger, J., Perrin, N. (2014). Multiple origins of invasive and 'native' water frogs (*Pelophylax* spp.) in Switzerland. *Biological Journal of the Linnean Society* 112, 442–449. doi: 10.1111/bij.12283.

Dufresnes, C., Di Santo, L., Leuenberger, J., Schuerch, J., Mazepa, G., Grandjean, N., Canestrelli, D., Perrin, N. & Dubey, S. (2017). Cryptic invasion of Italian pool frogs (*Pelophylax bergeri*) across Western Europe unraveled by multilocus phylogeography. *Biological Invasions* 19, 1407-1420.

Edenhamn, P. & Sjögren-Gulve, P. (2000). Åtgärdsprogram för bevarende avgölgroda (*Rana lessonae*). Naturvårdsverket. Stockholm.

Evanno, G., Regnaut, S. & Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* 14, 2611–2620. doi: 10.1111/j.1365-294x.2005.02553.x.

Ficetola, G. F., Bonin, A. & Miaud, C. (2008). Population genetics reveals origin and number of founders in a biological invasion. *Molecular Ecology* 17, 773-782.

Fog, K., Schmedes, A. & de Lasson, D. R. (2001). *Nordens padder og krybdyr*. p365. Gads forlag, København.

Galarza, J. A., Mappes, J. & Valkonen, J. K. (2015). Biogeography of the smooth snake (*Coronella austriaca*): origin and conservation of the northernmost population. *Biological Journal of the Linnean Society* 114, 426-435.

Garner, T. W., Gautschi, B., Röthlisberger, S., Reyer, H. U. (2000). A set of CA repeat microsatellite markers derived from the pool frog, *Rana lessonae*. *Molecular Ecology* 12, 2173-2175.

Gislén, T. & Kauri, H. (1959). Zoogeography of the Swedish amphibians and reptiles, with notes on their growth and ecology. *Acta Vertebratica* 1(3), 191-397.

Goudet, J. (2001). FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9. 3). Lausanne University, Lausanne, Switzerland.

Graf, J. D., Polls-Pelaz, M. (1989). Evolutionary genetics of the *Rana esculenta* complex. In Evolution and ecology of unisexual vertebrates (ed. RM Dawley and JP Bograt) pp. 289-301. New York Sate Museum.

Haapanen, A. & Salkio, V. (1966). Lisätietoja viitasammakosta (*Rana arvalis Nilss*) Suomessa. *Luonnon tutkija* 70, 20-21.

Hauswaldt, J. S., Höer, M., Ogileska, M., Christiansen, D. G., Dziewulska-Szwajkowska, D., Czernicka, E., Vences, M. (2012). A simplified molecular method for distinguishing among species and ploidy levels in European water frogs (Pelophylax). *Molecular Ecology Resources*, 12: 797–805. doi:10.1111/j.1755-0998.2012.03160.x

Hewitt, G. M. (1996). Some genetic consequences of ice ages,

and their role in divergence and speciation. *Biological Journal of the Linnean Society* 58, 247–276.

Hewitt, G. M. (1999). Post-glacial re-colonization of European biota. *Biological Journal of the Linnean Society* 68, 87–112.

Hewitt, G. M. (2000). The genetic legacy of the Quaternary ice ages. *Nature* 405, 907-913.

Hoffman, A., Plötner, J., Pruvost, N. B. M., Christiansen, D. G., Röthlisberger, S., Choleva, L., Mikulíček, P., Cogălniceanu, D., Sas-Kovács, I., Shabanov, D., Morozov-Leonov, S. & Reyer, H-U. (2015). Genetic diversity and distribution patterns of diploid and polyploid hybrid water frog populations (*Pelophylax esculentus* complex) across Europe. *Molecular Ecology* 24, 4371–4391. doi:10.1111/mec.13325

Hofman, S., Pabijan, M., Dziewulska-Swajkowska, D. D. & Szymura, J. M. (2012). Mitochondrial genome organization and divergence in hybridizing central European waterfrogs of the *Pelophylax esculentus* complex (*Anura*, Ranidae). *Gene* 491, 71-80. doi: 10.1016/j.gene.2011.08.004.

Holsbeek, G., Mergeay, J., Volckaert, F. A. M & De Meester, L. (2010). Genetic detection of multiple exotic water frog species in Belgium illustrates the need for monitoring and immediate action. *Biological Invasions* 12, 1459-1463.

Hoogesteger, T., Rahkonen, J. & Karhilahti, A. (2013). Pool frog (*Pelophylax lessonae*) Camerano 1882 (Anura, Ranidae), an addition to the Finnish amphibian fauna. *Memoranda Societatis pro Fauna et Flora Fennica* 89, 25-31.

Hoogesteger, T., Rahkonen, J. & Karhilahti, A. (2014). Suomen vihersammakot. *Luonnon Tutkija* 118, 4-18.

Kaisila, J. (1949). Kirjallisuus- ja kokoelmatiedot maamme sammakkoeläinten ja matelijoiden levinneisyydestä. *Luonnon tutkija* 2/1949, 40-45.

Kindler, C., Bringsøe, H. & Fritz, U. (2014) Phylogeography of grass snakes (*Natrix natrix*) all around the Baltic Sea: implications for the Holocene colonization of Fennoscandia. *Amphibia-Reptilia* 35, 413-424.

Kuzmin, L. (1995). Die Amphibien Russlands und angrenzender Gebiete. Die Neue Brehm-Bücherei.

Kopelman, N. M., Mayzel, J., Jakobsson, M., Rosenberg, N. A. & Mayrose, I. (2015). CLUMPAK: a program for identifying clustering modes and packaging population structure inferences across K. *Molecular Ecology Resources* 15, 1179-1191.

Lindgren, B., Nilsson, J., Söderman, F. (2014). Åtgärdsprogram för gölgroda, 2014–2019 (*Pelophylax lessonae*). Naturvårdsverket, Länsstyrelsen Uppsala län.

Litvinchuck, S. N., Borkin, L. J., Litvinchuck, Y. S. & Rosanov, J. M. (2015). Distribution and Population Systems of Green Frogs (*Pelophylax esculentus* complex) in Kaliningrad Oblast', Russia (Baltic Sea Region). *Russian Journal of Herpetology* 22, 188-196.

Milto, K. D. (2008). Amphibians breed in the Baltic Sea. *Russian Journal of Herpetology* 15, 8–10.

Nei, M. (1972). Genetic distance between populations. *American Naturalist* 106, 283-291.

Nei, M., Tajima, F. & Tateno, Y. (1983). Accuracy of estimated phylogenetic trees from molecular data. *Journal of Molecular Evolution* 19, 153–170. doi: 10.1007/bf02300753 .

Nilsson, J. (2013). 2009 års inventering av gölgroda (*Rana lessonae*) I Norduppland. Länsstyrelsen I Uppsala län. Länsstyrelsens Meddelandeserie 2013, 02.

O'Brien, C. D., Hall, J. E., Orchard, D., Barratt, C. D., Arntzen, J. W. & Jehle, R. (2015). Extending the natural range of a declining species: genetic evidence for native great crested newt (*Triturus cristatus*) populations in the Scottish Highlands. *European Journal of Wildlife Research* 61, 27-33.

Peakall, R. & Smouse, P. E. (2006). GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6, 288–295.

Peakall, R. & Smouse, P. E. (2012). GenALEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics* 28, 2537–2539.

Plötner, J., Pekşen, Ç.A., Baier, F., Uzzell, T. & Bilgin, C.C. (2015). Genetic evidence for human-mediated introduction of Anatolian water frogs (*Pelophylax cf. bedriagae*) to Cyprus (Amphibia: Ranidae). *Zoology in the Middle East* 61, 125-132.

Pritchard, J. K., Stephens, M. & Donnelly, P. J. (2000). Inference of population structure using multilocus genotype data. *Genetics* 155, 945-959.

Provan, J. & Bennett, K. D. (2008). Phylogeographic insights into cryptic glacial refugia. *Trends in Ecology and Evolution* 23, 564-571.

Ragghianti, M., Bucci, S., Marracci, S., Casola, C., Mancino, G., Hotz, H., Guex, D. G., Plötner, J. & Uzell, T. (2007). Gemetogenesis of intergroup hybrids of hemiclonal frogs. *Genetic Research* 89, 39-45.

Raymond, M. & Rousset. F. (1995). GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86, 248-249.

Rousset, F. (2008). Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. *Molecular Ecology Resources* 8, 103-106.

Saitou, N., Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4, 406-425.

Schmitt, T., Varga, Z. (2012). Extra-Mediterranean refugia: The rule and not the exception? *Frontiers in Zoology* 9, 22.

Sillero, N., Campos, J., Bonardi, A., Corti, C., Creemers, R., Crochet, P. A., Isailović, J. C., Denoël, M., Ficetola, G. F., Gonçalves, J., Kuzmin, S., Lymberakis, P., de Pous, P., Rodríguez, A., Sindaco, R., Speybroeck, J., Toxopeus, B., Vieites, D. R. & Vences, M. (2014). Updated distribution and biogeography of amphibians and reptiles of Europe. *Amphibia-Reptilia* 35, 1-31.

Simberloff, D. (2003). How much information on population biology is needed to manage introduced species? *Conservation Biology* 17, 83-92.

Sjögren, P. (1991a). Extinction and isolation gradients in metapopulations: the case of the pool frog (*Rana lessonae*). *Biological Journal of the Linnean Society* 42, 135-147.

Sjögren, P. (1991b). Genetic variation in relation to demography of peripheral pool frog populations (*Rana lessonae*). *Evolutionary Ecology* 5, 248-271.

Sjögren Gulve, P. (1994). Distribution and Extinction Patterns within a Northern Metapopulation of the Pool Frog, *Rana lessonae*. *Ecology* 75, 1357-1367.

Sneath, P. H. A & Sokal, R. R. (1973) Numerical Taxonomy. W. H. Freeman, San Francisco.

Snell, C. (1994). The pool frog- a neglected native? *British Wildlife* 6, 1-4.

Snell, C., Tetteh, J. & Evans, I. H. (2005). Phylogeography of the pool frog (*Rana lessonae* Camerano) in Europe: evidence for native status in Great Britain and for an unusual postglacial

colonization route. *Biological Journal of the Linnean Society* 85, 41-51.

Stewart, J. R. & Lister, A. M. (2001). Cryptic northern refugia and the origins of the modern biota. *Trends in Ecology and Evolution* 16, 608-613.

Takezaki, N. & Nei, M. (2008). Empirical tests of the reliability of phylogenetic trees constructed with microsatellite DNA. *Genetics* 1, 385-392.

Takezaki, N., Nei, M. & Tamura, K. (2010) POPTREE2: Software for constructing population trees from allele frequency data and computing other population statistics with Windows interface. *Molecular Biology and Evolution* 27, 747–752. doi: 10.1093/molbev/msp312.

Talvi, T. (1992). Amphibians and reptiles of Estonia: list, geographic relationships and current situation. Proceedings of the sixth Ordinary General Meeting of the Societas Europaea Herpetologica 19-23 August 1991 Budapest, Hungary. Hungarian Natural History Museum.

Terhivuo, J. (1993). Provisional atlas and status of populations for the herpetofauna of Finland in 1980-1992. *Annales Zoologici Fennici* 30, 55-69.

Wycherley, J., Doran, S., Beebee, T. J. C. (2002). Frog calls echo microsatellite phylogeography in the European pool frog (*Rana lessonae*). *Journal of Zoology* 258, 479-484.

Zeisset, I. (2000). Molecular Ecology of North European water frogs. D.Phil Thesis, University of Sussex.

Zeisset, I. & Beebee, T. J. C. (2001). Determination of biogeographical range: an application of molecular phylogeography to the European pool frog *Rana lessonae*. *Proceedings of the Royal Society B-Biological Sciences* 268, 933-938.

Zeisset, I. & Beebee, T. J. C. (2007). Two clades of north European pool frogs *Rana lessonae* identified by cytochrome *b* sequence analysis. *Herpetological Journal* 17, 255-260.

Zeisset, I., Rowe, G. & Beebee, T. J. C. (2000). Polymerase chain reaction primers for microsatellite loci in the north European water frogs *Rana ridibunda* and *R. lessonae*. *Molecular Ecology* 9, 1173-1174.

Accepted: 29 January 2018

Volume 28 (April 2018), 73-86

Herpetological Journal FULL PAPER

Published by the British Herpetological Society

Re-examination of the giant fossil tortoise *Hesperotestudo* from near the Illinois glacial-sangamon interglacial boundary in North America with commentary on zoogeography

Lauren E. Brown[1], Don Moll[2] & Evan S. Brown[3]

[1]School of Biological Sciences, Illinois State University, Campus Box 4120, Normal, Illinois 61790-4120, USA

[2]Department of Biology, Missouri State University, Springfield, Missouri 65897, USA

[3]Colorado Technical University, Schaumburg, Illinois 60173, USA

The Illinois Episode was the most extensive Quaternary glaciation in North America and extended deep into the central USA, further south than any other glacial episodes. It was followed by a period of mild climate termed the Sangamon Interglacial Episode. Relatively few reptile fossil sites have been found along the Illinois-Sangamon boundary. Thus, the 1986 report of isolated fossil remains of a giant tortoise (*Hesperotestudo*) near the boundary is of particular significance. We re-examined this important fossil because of inconsistencies and misinterpretations of prior researchers. The three morphological characters used for prior species identification of the tortoise are faulty and unreliable. Lack of additional, pertinent, diagnostic fossil elements presently prevents positive species identification. We critically appraised the pollen-based analysis of climate and environment at the tortoise strata by prior researchers. Their data suggest a transitional area between forest and prairie, or savannah, but the prior researchers misinterpreted their own data, concluding the vegetation was "relatively xeric grassland". Consequently, the climate and environment at the tortoise stratum are yet to be determined. We present several zoogeographical scenarios pertaining to the origin and movement of the tortoise to the collection site. The most likely is perhaps northward movement from the central Gulf Coast along the Mississippi River floodplain before the major meltdown of the Illinois glaciation. East of St. Louis, the glacier met the Mississippi River floodplain and as the meltdown progressed, the tortoise could have travelled on the till plain north-east to the collection site. *Hesperotestudo* likely had considerable cold adaptation and thus may have tracked the Illinois glacier relatively closely as it melted northward.

Key words: giant fossil tortoises, *Hesperotestudo*, climate, zoogeography

INTRODUCTION

The Quaternary of North America (ca. 2.6 million years before present [YBP]) was a period of repeated climatic changes, alternating between cold glacial episodes and warmer interglacial episodes (Hansel & McKay, 2010). During every major glaciation (at least six), the southern margin of the ice sheet extended into Illinois in the central USA. The most recent was the Wisconsin Glacial Episode, which was preceded by the Sangamon Interglacial Episode, which followed the Illinois Glacial Episode. The geology of the Wisconsin Episode in Illinois has been heavily researched (Willman & Frye, 1970; Hansel & Johnson, 1996; Hansel & McKay, 2010), but the Illinois Glacial Episode is not as well-known.

Among the most prominent reptiles of the Quaternary in North America were the giant fossil tortoises of the genus *Hesperotestudo* Williams (1950). These magnificent animals became extinct by the Late Wisconsin Glacial - Early Holocene Interglacial Episodes (Moodie & Van Devender, 1979; Holman & Clausen, 1984). The consensus as to the causes (Turtle Extinction Working Group, 2015) are primarily human exploitation for consumption as well as habitat and climate change.

The genus *Hesperotestudo* (Figure 1) has been found at many localities across North America from Pennsylvania (*H. percrassa*) and Delaware (*H. ducatelli* [probably]) in the east, to California (*H. dehiscus*) on the west coast, and from Texas (*H. turgida*), Baja California Sur, Mexico (*H. turgida* group), and Florida (*H. incisa*) in the south, to Saskatchewan, Canada (*H. exornata*) in the north (Lambe, 1906; Des Lauriers, 1965; Bramble, 1971; Auffenberg, 1974; Miller, 1980; Parris & Daeschler, 1995; Holman, 1998).

Meylan & Sterrer (2000) suggested the existence of 32 species in the genus *Hesperotestudo* in North America based on Auffenberg's (1974) checklist. However, since 1974 there have been a number of new species described. Comparison of species is difficult because: 1. the sample size for many of the species is quite small (often only one individual); 2. it is rare to find a specimen with a complete skeleton (often there are only a few or sometimes one plate or fragment of the shell available); 3. sexually dimorphic characters are often unknown; 4. geographic and ontogenetic variation of characters are unknown; and 5. no indication of reproductive isolating

Correspondence: Don Moll (donmoll@missouristate.edu)

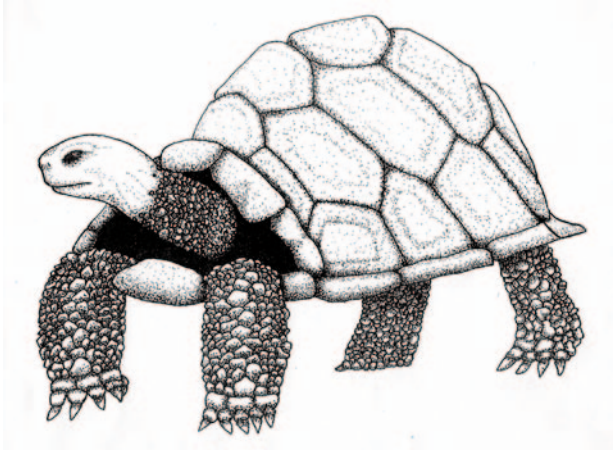

Figure 1. Reconstructed image of a fossil giant North American tortoise (*Hesperotestudo*). Drawing by C. Spahn. Originally published in *The Herpetological Journal* Volume 27, Page 279 (July 2017). (Permission granted by Rachael Antwis, Editor.)

Figure 2. Terminal boundary of the Illinois Glacial Episode (heavy dark line) in central North America. MR designates the Mississippi River, the Great Lakes are dark grey, and the states are outlined in white. Cartography by M. Maher. Source: 2014 Encyclopedia Britannica.

mechanisms has been discovered (except for body size in a few cases). Thus, in the future we can expect the lumping of some species and the continuing description of new species as more specimens are acquired and more research conducted. Consequently, it may be some time before a comprehensive revision of *Hesperotestudo* is undertaken because of the problems heretofore mentioned, and the true number of biologically valid species may remain an enigma for the foreseeable future.

Research on *Hesperotestudo* has focused primarily on taxonomy and phylogeny. An exception was that of Hibbard (1960). He used imported extant Galápagos tortoises as a proxy to create a theory that suggested adaptations of Cenozoic giant fossil tortoises were for subtropical or tropical climates across North America. Subsequently, Hibbard (1960) was widely cited and his interpretation became perhaps the most prominent paleoclimatic theory for many groups of organisms (Moll & Brown, 2017).

Moll & Brown (2017) recently provided evidence that Hibbard's (1960) theory was invalid. Seven alternative concepts were presented by Moll & Brown (2017) that suggest North American giant fossil tortoises could have evolved the necessary adaptations to survive northern winters and in montane areas that could have been colder than Hibbard (1960) envisioned: 1. cold-adaptive morphology; 2. behavioural thermoregulation; 3. burrowing; 4. use of caves as shelters; 5. tolerance of prolonged cessation of food consumption; 6. cryoprotection and supercooling; and 7. gigantothermy.

Over 30 years ago, an isolated locality record of a giant fossil tortoise thought to be *Hesperotestudo crassiscutata* (Leidy, 1889) was reported from southern Illinois in the Sangamon not far from the glacial boundary of the Illinois (King & Saunders, 1986; Saunders & King, 1986). This record is thus of considerable interest.

Hesperotestudo crassiscutata is known from the Middle Pleistocene to the Early Holocene (Turtle Extinctions Working Group, 2015). Its known distribution is primarily in the south-eastern USA from Texas eastward through Louisiana, Mississippi, and Alabama to Florida, and to the north-east through Georgia and South Carolina to North Carolina (Slaughter, 1966; Auffenberg, 1974; Holman, 1995; Russell et al., 2009; Turtle Extinctions Working Group, 2015). Extra-marginal records occur in Illinois and Pennsylvania (King & Saunders, 1986; Holman, 1995). The known distribution of *H. crassiscutata* may be one of the largest in the genus. Because of its wide distribution, it clearly evolved adaptations to live in numerous environments and climates. Conant & Collins (1998), Stebbins (2003), and Moll & Brown (2017) noted this for many extant North American chelonians. In some areas (e.g., Florida), locality records for *H. crassiscutata* are relatively common, whereas in other areas they are very rare (only one locality in Illinois).

There are two records of *Hesperotestudo* from Missouri caves reported by Hawksley (1986). Holman (1995) indicated that these specimens need to be confirmed because they do not fit his theory of stability of the herpetofauna in the Ozark region. Moreover, they may have considerable zoogeographical significance to the origin of *Hesperotestudo* in the central USA.

Hesperotestudo contains both giant and moderate to smaller species. Among the largest is *H. crassiscutata*. Leidy (1889) estimated that the shell of the type specimen was about five feet (1.52 m) in length and had a very thick shell (the plastron varied from 46 - 90 mm in thickness). Bentley & Knight (1998) found shell fragments of *H. crassiscutata* 40 mm thick. A more recent carapace length estimate was 120 - 125 cm (Turtle Extinctions Working Group, 2015). However, the plastrons of two specimens of giant "*Geochelone* sp." (= *Hesperotestudo*) from Texas were reported by Hibbard & Dalquest (1966) to reach six feet (1.83 m). Holman (1969) indicated that these fossils are similar to *H. crassiscutata*. Due to its huge size, *H. crassiscutata* may have been a keystone herbivore in optimal habitats where the species' densities could have been moderate to high.

Because of inconsistencies, omissions, and misinterpretations in King & Saunders (1986) and Saunders & King (1986), and the questioned status of the Hawksley (1986) specimens, we re-examined the fossil fragments of *Hesperotestudo* from Illinois and Missouri and the results of the aforementioned studies. The objectives of this paper are to: i) review the geology

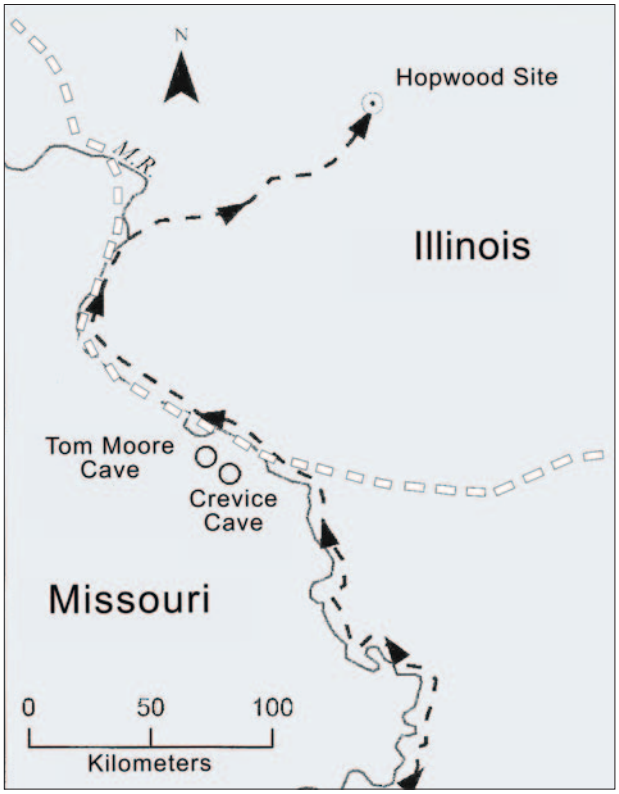

Figure 3. Collection locality (circle with dark center) for xiphiplastron of giant fossil tortoise (*Hesperotestudo*) in southern Illinois, USA showing relative closeness to the terminal boundary of the Illinois glacier (dashed white line). Arrows indicate suggested route the Illinois *Hesperotestudo* may have taken to enter the state. See text for other possible routes. Cartography by M. Maher.

of the Illinois and Sangamon Episodes; characterise the fossil collection localities; ii) describe pertinent fossil remains; iii) evaluate species identification of the fossils (various fragments may indicate two or more specimens or species could be present); iv) critically appraise the proposed former climate and environment at the tortoise strata at the Illinois locality; and v) discuss the zoogeography of the tortoise.

MATERIALS AND METHODS

Fossil fragments of *Hesperotestudo* (11 from Illinois and 6 from Missouri) were examined at the Illinois State Museum, Springfield (Appendix 1). Measurements were taken with a dial Vernier caliper (Scienceware, Bel-Art Products). The xiphiplastron, hypoplastron, and other fragments were examined in detail with a dissecting microscope (Bausch and Lomb, zoom lens) from 10.5-45X magnification. Three cameras were used: Apple iPhone Model A1533, Nikon Coolpix S3100, and Plugable USB 2.0 Digital Microscope.

An in-depth literature survey following the methodology of Brown et al. (2008) was carried out that retrieved hundreds of references. We used four search engines: Google; Google Scholar (Advanced Scholar Search); JSTOR Advanced Search; and Metacrawler Advanced. Numerous combinations of search words were used. Milner Library at Illinois State University, Normal was used for traditional searching of paper sources (including many on microfilm and others in deep storage), as well as LEB's and DM's extensive herpetological - paleontological libraries. Interlibrary loan and I-Share were used extensively to obtain copies of references not in the libraries heretofore mentioned.

Scientific names of extinct and subfossil tortoises follow Bramble (1971), Auffenberg (1974), and Turtle Extinctions Working Group (2015). Scientific and common names for extant tortoises and other turtles follow Turtle Taxonomy Working Group (2014), for other fossil vertebrates, Carroll (1988), for extant snakes, Moriarty (2012), for extant frogs, Fouquette & Dubois (2014), and for extant trees and other plants, Mohlenbrock (1986).

RESULTS AND DISCUSSION

Illinois Glacial Episode

The Illinois Glacial Episode lasted from ca. 190,000 to 130,000 YBP (Hansel & McKay, 2010; Grimley & Phillips, 2011). The ice originated from an accumulation in the Canadian Shield east of Hudson Bay in Labrador. Possibly two ice lobes entered Illinois: mainly the Lake Michigan Lobe from the north-east and possibly the Erie Lobe from the east (Willman & Frye, 1970; Hansel & McKay, 2010). The extensive ice sheet covered nearly 90% of Illinois (Figures 2 and 3), moving deep into the southern part of the state (37˚ 35′ N latitude). At its maximum, it extended further south than any other continental glaciation in the Northern Hemisphere (Willman & Frye, 1970, 1980; Taylor et al., 2009; Hansel & McKay, 2010).

The Illinois Glacial Episode had a widespread effect on the landscape by the formation of the Illinois Till Plain covering much of Illinois (Hansel & McKay, 2010). This till plain is unique for its extreme flatness (Figure 4). Near the southern glacial extent, the till is thin but becomes thicker northward (Leverett, 1899; Willman & Frye, 1980; Taylor et al., 2009). Landforms such as eskers, drumlins, kames, and moraine segments were formed by silt, sand, clay, gravel, and rocks left by the melting glacier (Hansel & McKay, 2010; Grimley & Phillips, 2011). The ice also temporarily diverted the course of the Mississippi River in south-eastern Iowa (Hansel & McKay, 2010).

The southern portion of the melting Illinois glacier displayed several declines and re-advances (Willman & Frye, 1970; Hansel & McKay, 2010). The melting produced an abundance of water which formed large slackwater lakes as well as eroded valleys, and diverted many water channels.

Pollen analysis (Grüger, 1972; Zhu & Baker, 1995; Teed, 2000) suggested that boreal coniferous forest was predominate in the Late Illinois (but see Climate and environment at the Hopwood Farm tortoise stratum). Thus, the Illinois Glacial Episode had a tremendous, lasting impact on the geology and landscape of the central Midwestern USA.

Sangamon Interglacial Episode

The Sangamon Interglacial Episode extended from the end of the Illinois Glacial Episode ca. 130,000 YBP to the beginning of the Wisconsin Glacial Episode ca. 60,000

Figure 4. Detailed location (darkened circle) where xiphiplastron of the giant fossil tortoise *Hesperotestudo* was found on the Hopwood Farm north of Fillmore, Montgomery County, Illinois, USA. Roads are in white. Note relatively flat topography (except for eroded creeks) characteristic of the Illinois Till Plain. Cartography by M. Maher. Roads and rivers after ESRI, Inc.

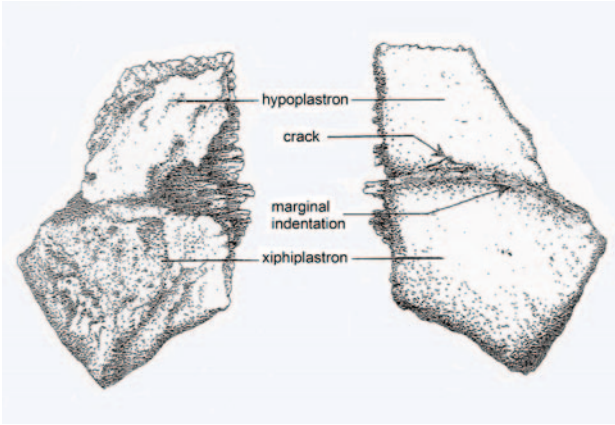

Figure 5. Left xiphiplastron attached to part of hypoplastron of giant fossil tortoise (*Hesperotestudo*) ISM 490,015 from southern Illinois, USA. Ventral view is on the right; dorsal view is to the left. Anterior is at top. Note marginal indentation between xiphiplastron and hypoplastron in ventral view. Maximum length of xiphiplastron is 63.3 mm (parallel to longitudinal axis of plastron). Drawings by C. Spahn after photographs of the fossil.

YBP (Willman & Frye, 1970; Curry & Follmer, 1992; Hansel & Johnson, 1996; Taylor et al., 2009; Hansel & McKay, 2010; Grimley & Phillips, 2011). The transitions from the Illinois to the Sangamon and the Sangamon to the Wisconsin were not marked by singular mega-events, and the gradual changes varied geographically. Lack of glaciation during the Sangamon implies a mild climate but the beginning and end were probably considerably cooler.

In the Early Sangamon, remnants of large slackwater lakes probably still remained. The primary paleosol was Sangamon Geosol (Curry & Follmer, 1992; Hansel & McKay, 2010), which was mostly poorly drained (Willman & Frye, 1970). Eventually the Mississippi River resumed its ancient course (Willman & Frye, 1970; Taylor et al., 2009). Many of the geological landforms of the Late Illinois still remained but were often weathered, eroded, or otherwise modified with passage of time. Pollen analysis for the Early Sangamon (Grüger, 1972; Curry & Follmer, 1992; Zhu & Baker, 1995; Curry & Baker, 2000; Teed, 2000) suggested that deciduous forest predominated (but see Climate and environment at the Hopwood Farm tortoise stratum).

Characterisation of the fossil collection locality in southern Illinois

The giant fossil tortoise *H. crassiscutata* was collected (King & Saunders, 1986; Saunders & King, 1986) on the Curtis Hopwood Farm, SE, NE, SW (sic), Section 23, T8N, R2W, just north of the town of Fillmore, Montgomery County, in southern Illinois, USA (Figure 4). The farm is on the Illinois Till Plain on a ridged drift complex (Willman & Frye, 1970; King & Saunders, 1986). The terminal southern rim of the Illinois glacial lobe was located ca. 184 km SSE of Hopwood Farm. The western terminal rim was much closer to Hopwood Farm (ca. 79 km SW).

The fossil remains were found in what was interpreted as a filled "kettle" of Late Illinois (Jubileen) age (Saunders & King, 1986). The supposed kettle is about 1.5 km wide (Blackwell et al., 2016). Kettles result from the melting of a large block of glacial ice that becomes detached from the rim of a glacial lobe to form a depression. There are no rivers or streams that flow into or out of a kettle. The Hopwood Farm kettle was filled (surface to glacial till) with Wisconsin loess overlying alluvial silty clay, marl, clayey marl, hard peat, silty muck, organic silt, and sandy silt over the glacial till (King & Saunders, 1986). The creek Lanes Branch bisected the kettle resulting in the displacement of some of the fossils to the bottom of the creek (King & Saunders, 1986).

The fossil remains of the tortoise were from a depth of ca. 3 m in marl of upper stratum 3 and lower stratum

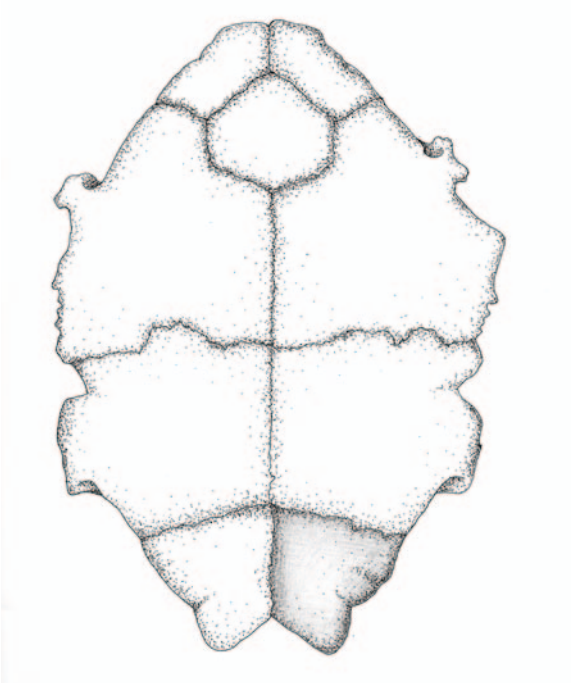

Figure 6. Ventral view of left xiphiplastron (shaded) in plastron of subadult giant fossil tortoise (*Hesperotestudo crassiscutata*) from Florida, USA. Anterior is at top. Drawing by C. Spahn after part of figure 13 in Auffenberg (1963).

2 (King & Saunders, 1986), which is not far from the Illinois/Sangamon boundary. The tortoise fragments found in lag gravel in the bottom of the creek contained marl in the exposed internal spaces indicating they were from stratum 3 (King & Sanders, 1986). Blackwell et al. (2016) reported tortoise remains at a depth of 4 m in peat of upper stratum 4 in the kettle. (Saunders & King [1986] placed the Illinois – Sangamon boundary at the "stratum 3b/stratum 4 contact.") An abundance of fossils (Saunders & King, 1986) of other animals occurred throughout the sequence from lower stratum 2 through stratum 6: invertebrates (e.g., mollusks), fishes (e.g., large pike, *Esox*), amphibians, other reptiles, birds (e.g., goose, *Branta*) and mammals (e.g., mastodon, *Mammut*).

The presence of a large northern pike ("*Esox cf. lucius*") as well as other smaller fishes reported by Saunders & King (1986) indicates that the Hopwood site had to have been connected to a river or stream (predecessor of Lanes Branch?) allowing access to the kettle. Also, the report of gar scales (*Lepisosteus* or *Atractosteus*) by Blackwell et al. (2016) at the Hopwood site supports the same scenario. Both pikes and gars typically inhabit rivers, streams, and lakes (Hubbs & Lagler, 1964; Page & Burr, 1991). Consequently, the Hopwood site was likely a slackwater lake or other type of lake rather than a kettle.

Quaternary sites older than 50,000 YBP are difficult to reliably date (Blackwell et al., 2016). At Hopwood Farm, nine different methods have been used to date the fossil site over the last 25+ years but no consensus has been reached. Curry et al. (2011) pointed out that it was clear that the various dating methodologies showed considerable conflict. The most recent study (Blackwell et al., 2016) dated gastropods of "Unit 3" in the kettle using the proxy electron spin resonance to get an age of

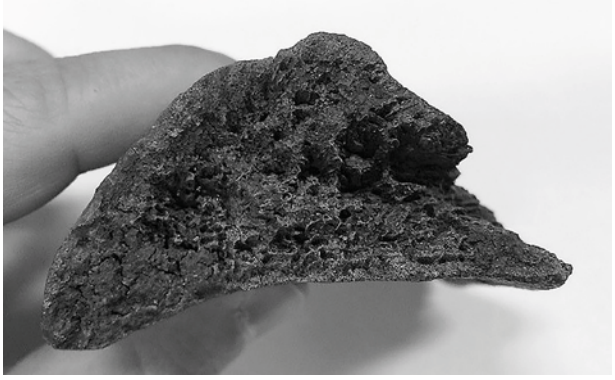

Figure 7. Marginal fragment of carapace of giant fossil tortoise (*Hesperotestudo*) from Hopwood Farm in southern Illinois, USA (ISM 498,602). Note thickness of fossil (maximum depth = 30.0 mm) which may indicate a different individual from the collection locality. Photograph by E. Brown.

102 ± 7 ka to 90 ± 6 ka. Blackwell et al. (2016) thought this was a period of cool dry climate. However, evidence for the authenticity of their proxy for predicting real time at the Hopwood site was not given, as is generally true with use of other proxies. Furthermore, the gastropods could have been washed into the site via a river. As previously mentioned, the presence of two large fish, pike (*Esox*) and gar (*Lepisosteus* or *Atractosteus*) as well as smaller fish at the Hopwood Lake indicates it had to have been connected to a river or stream.

Fossil remains of *Hesperotestudo* from Hopwood Farm

A left xiphiplastron (ISM 490,015, Figure 5) which is a posterior plate of the plastron plus two small fragments (ISM 490,016 and 490,017) thought to be part of the plastron were the only fossil bones reported by King & Saunders (1986) and Saunders & King (1986). Figure 6 shows a xiphiplastron in its typical position in the plastron of *H. crassiscutata*. When we examined the fossils (16 November 2016 and 24 April 2017), an additional eight fragments of *Hesperotestudo* from the site had been deposited in the Illinois State Museum (total = 11, Appendix 1). Another fragment (ISM 497,443) was out on loan, and two others (ISM 498,554 and 498,601) may not be *Hesperotestudo* as they are very thin.

King & Saunders (1986) did not recognise that the xiphiplastron was still attached anteriorly to a relatively large portion of the hypoplastron. The two plates can be easily recognised by a marginal indentation between them (Figure 5). Thus, King & Saunders (1986) measurement of length (110.5 mm) incorrectly included the xiphiplastron plus a significant portion of the hypoplastron.

The ventral side of the xiphiplastron shows very little damage (Figure 5). However, the dorsal side has large areas flaked off and considerable weathering and demineralisation. A long crack occurs along and near the margin of the xiphiplastron and hypoplastron. The maximal length and width of the xiphiplastron (parallel to and perpendicular to the longitudinal axis of the plastron) are 63.3 mm and 69.8 mm, respectively.

A peripheral fragment (ISM 498,602, Figure 7) of the carapace is quite thick (maximum = 30.0 mm). In larger

chelonians, the peripherals can be thicker than other carapace bones, but the exceptional thickness of ISM 498,602 suggests that it may be from another individual tortoise at Hopwood Farm. However, we do not know for sure. Additional support for more than one individual is that some of the fossil fragments were found in the creek bed "7 m north (upstream)" (our emphasis) from the principal fossil site (King & Saunders, 1986). Moreover, the finding of tortoise remains at two different depths (3 m in marl and 4 m in peat) in the Hopwood site (King & Saunders, 1986; Blackwell et al., 2016) provides further evidence. Thus, there may be up to four individuals of *Hesperotestudo* from the Hopwood site.

King & Saunders (1986) distinguished the xiphiplastron (ISM 490,015) from that of another species, *Hesperotestudo incisa* (to date, only found in Florida and Georgia; Turtle Extinctions Working Group, 2015), by only three characteristics: 1. extrapolated larger carapace length; 2. ratio of length to posterior notch depth; and 3. inferred obtuse angle between fused xiphiplastra. King & Saunders (1986) used their length for the "xiphiplastron" (110.5 mm) to infer the carapace length of the Illinois specimen as 680 mm by comparison to the carapace of Florida *H. crassiscutata* (after Auffenberg, 1963). However, since King & Saunders' (1986) original measurement of the Illinois "xiphiplastron" was erroneous (because it included part of the hypoplastron), the carapace length extrapolation (680 mm) was incorrect, and distinguishing characters 1 and 2 are invalid. The Illinois tortoise is thus considerably smaller than projected by King & Saunders (1986). Moreover, it is circular reasoning to use specimens of Florida *H. crassiscutata* to extrapolate carapace length for the Illinois specimen, and then conversely use longer carapace length as identification character 1 for the Illinois specimen as *H. crassiscutata*. Furthermore, characters 2 and 3 were clumped into one of nine characters in Auffenberg's (1963) diagnosis. However, that diagnosis was only for comparison with *H. incisa*. Auffenberg (1963) also indicated that *H. crassiscutata* exhibits considerable variation in many diagnostic characters. In conclusion, the characters used to identify the Illinois specimen are faulty, and there is not enough evidence that the specimen actually represents the species *H. crassiscutata*.

The Turtle Extinctions Working Group (2015) recognised 12 species of *Hesperotestudo* that became extinct during the Pleistocene and Holocene. Four of these closest to Hopwood Farm are: *H. campester* (Hay, 1908), Kansas and Texas; *H. equicomes* (Hay, 1917), Kansas and Nebraska; *H. oelrichi* (Holman, 1972), Nebraska; and *H. turgida* (Cope, 1892), Kansas, Nebraska, Oklahoma, and Texas. All of these species were described long before King & Saunders' (1986) paper, and all species were found in the Great Plains which is immediately west of the Prairie Peninsula where Hopwood Farm is located. Nonetheless, King & Saunders (1986) did not (or were unable to) compare their Illinois tortoise to the four preceding species. Fossil tortoise taxonomy and identification is often quite difficult, particularly if material is limited. When or if more Pleistocene fossil tortoise material from southern Illinois becomes available, it may provide definitive evidence concerning the correct species nomen for King & Saunders (1986) tortoise.

King & Saunders (1986) counted "growth areas" on the xiphiplastron and hypoplastron and estimated an age of 9-18 years, thus concluding the tortoise was immature. We examined the xiphiplastron and hypoplastron microscopically. Most of the growth areas are visible as fissures on the latter. Microscopic examination revealed that the many fissures on the fossil are short, discontinuous, and irregular in alignment. Width and length are quite variable. It is possible they may represent the process of taphonomy or subsequent demineralisation and weathering.

Growth rings, which mark areas of major growth cessation in intermittent growth patterns characteristic of chelonians, are commonly represented on the scutes and/or underlying skeletal elements as elevated ridges on many extant, and subfossil forms, and some fossil forms. This includes specimens of *Hesperotestudo* (e.g., Meylan, 1995 [Florida]; Morgan et al., 2000 [New Mexico]; Sullivan et al., 2011 [Arizona and New Mexico]). Research on extant chelonians has shown that use of growth rings of scutes for age determination can be problematic. Wilson et al. (2003) presented a critical evaluation of the growth ring controversy and its value and difficulties when used to age chelonians. For instance, Moll & Klemens (1996) found a "double ring" pattern in scutes of the extant pancake tortoise (*Malacochersus tornieri*) in northern Tanzania, Africa correlated with two growth periods in two chronologically separated rainy seasons in one year. In the Tarangire National Park in northern Tanzania, three individuals accumulated 2, 3 and 4 growth rings, respectively, over 17 months. In central Tanzania there is only a single wet season and single dry season with only one growth ring per year. In the case of the fossil *Hesperotestudo* from Hopwood Farm, it is unclear if there is a relationship between scute ring counts and the fossil "growth areas," or if the latter actually represent areas of growth. However, on the basis of the small size of the xiphiplastron of the Illinois *Hesperotestudo* we agree that the specimen was probably immature or perhaps another smaller species.

Climate and environment at the Hopwood Farm tortoise stratum

In continental areas, the most widely used proxy for predicting Quaternary climatic change is fossil pollen (Zhu & Baker, 1995), which is in turn used to infer type of environment. A number of such studies have been carried out in south-western Illinois (e.g., Grüger, 1972; King & Saunders, 1986; Saunders & King, 1986; Curry & Follmer, 1992; Zhu & Baker, 1995; Curry & Baker, 2000; Teed, 2000; Curry et al., 2010). The best known sites (Pittsburg Basin, Raymond Basin, Bald Knob Basin, and Hopwood Farm) were all thought to be kettles. Curry & Baker (2000) showed that the biostratigraphic pollen zones differed markedly in depth and in other respects at the four sites, particularly Hopwood Farm. We will focus primarily on that location, as it is the site of the fossil *Hesperotestudo*.

The Hopwood Farm site was cored with a hollow

rotary drill almost to the underlying glacial till (King & Saunders, 1986). The pollen record revealed a dominance of conifers at the base followed by deciduous trees, then mainly grasses and herbs, and finally deciduous trees. There was no tundra stratum uncovered but the core did not quite reach glacial till. However, till was exposed at the edges of the kettle. At Pittsburg Basin, Raymond Basin, and Bald Knob Basin the drilling reached Illinois till (Grüger, 1972; Zhu & Baker, 1995) but no indication of tundra was revealed.

At Hopwood Farm, King & Saunders (1986) and Saunders & King (1986) indicated that the environment at the core strata (lower level 2 and upper level 3) containing the Illinois *Hesperotestudo* was "a relatively xeric grassland". However, their pollen record diagram presents a different picture at that level. Pollen from 13 genera of trees were present: *Quercus* (oak) high abundance; *Carya* (hickory), *Ulmus* (elm), *Pinus* (pine), *Ostrya* (hophornbeam), and *Carpinus* (ironwood), moderate abundance; and *Betula* (birch), *Salix* (willow), *Fraxinus* (ash), *Tilia* (basswood), *Picea* (spruce), *Juglans* (walnut), and *Acer* (maple) low abundance. The abundance of *Quercus* (oak) pollen approached that of the Gramineae (grasses).

The abundance of tree pollen at 4 m depth where *Hesperotestudo* was reported by Blackwell et al. (2016) showed increases in *Picea*, *Pinus*, *Betula*, and *Salix*, by reference to the pollen diagram of King & Saunders (1986).

King & Saunders' data suggest the presence of a savannah environment or a transitional stage between deciduous forest and prairie or savannah, quite in contrast to their concluding statement in the Discussion (e.g., the presence of *Salix* suggests a more mesic environment). After this conclusion, King & Saunders (1986) discussed as support Hibbard's (1960) theory concerning the climate adaptations of giant fossil tortoises of North America. This theory was shown by Moll & Brown (2017) to be invalid. Thus, an erroneous theory may have negatively impacted King & Saunders' (1986) interpretation of their own data.

The use of fossil pollen as a proxy for prediction of past climate and environment is fraught with serious problems. For instance, the presence of pollen of an oak tree at a given site does not necessarily mean that an oak tree grew at that site. Pollen contain male gametes and they will not grow into a tree unless they participate in fertilisation of an ovule. Trees and other flowering plants produce enormous quantities of pollen every year (for most species). This pollen is spread through the air by wind for great distances (Stanley & Linskens, 1974) and can also be transported by water, mammals, birds, insects, and probably many other animals. Even glacial ice and coprolites (fossil faeces) can contain pollen (Williams, 2016). Moreover, turbulence of water and bottom sediments in bodies of water could alter deposition of pollen. Numerous fossil species of animals found in the Hopwood lake could have caused considerable turbulence (mastodon *Mammut*, beaver *Castor*, giant beaver *Castoroides*, muskrat *Ondatra*, goose *Branta*, giant tortoise *Hesperotestudo*, amphibians, pike *Esox*, gar *Lepisosteus* or *Atractosteus*, other smaller fish

Figure 8. Fragment of fossil *Hesperotestudo* (CM 6.1) from Tom Moore Cave in Perry County, south-east Missouri, USA. This is the largest Missouri fossil fragment (maximum length = 65.4 mm, maximum thickness = 22.1 mm). Photograph by E. Brown.

[Sanders & King, 1986; Blackwell et. al., 2016]). Wind also causes water turbulence. Furthermore, pollen would have been transported into the lake by the stream/river that ran into it or by erosion.

In a review of the use of pollen as climate proxies, Smith (2012) indicated that pollen can be identified only to the genus level in many cases. The consequence of this is that inter- and intraspecies variation in adaptation to climate and environment is ignored. Such variation is often extreme. For example, Moll & Brown (2017) pointed out that 19 of 45 species of extant North American chelonians have distributions (based on the maps of Conant & Collins [1998] and Stebbins [2003]) that extend from the southern USA into the northern USA and Canada, and have evolved adaptations to live at both climatic extremes.

The pollen diagram in King & Saunders (1986) and Saunders & King (1986) lists species for only two genera (*Fraxinus*, *Typha*). Thirty-eight genera have no listing of species, and one subfamily and nine families have no listing of genera or species. Thus, a very large number of species were not listed. This has serious consequences for interpretation of the pollen data. For example, pine and spruce pollen composed an important portion in the lower strata which were interpreted as the coldest zones. However, five extant pine species now occur in Illinois (Critchfield & Little, 1966; Mohlenbrock, 1986). Three species (*strobus*, *resinosa*, *banksiana*) are cold-adapted and occur in the northern USA, Canada, and Appalachian Mountains of the eastern USA. The other two species (*taeda*, *echinata*) are primarily warm-adapted and occur mostly in the south. Furthermore, it is possible that one or more now extinct undescribed species of *Pinus* occurred in southern Illinois during the Late Illinois or Early Sangamon Episodes, or that 2 – 3 other species were present. The reality is we do not know which species of pine(s) existed at the Hopwood site during the Illinois and Sangamon Episodes. Thus, the pollen data of King & Saunders (1986) and Saunders

& King (1986) do not resolve this problem, which exemplifies the great difficulties with their climatic and environmental interpretations. Furthermore, organisms from the past may have had habits, behaviours, ecological requirements, and other characteristics that their extant relatives lack (Cassiliano, 1997). Also, there may have been past geological and climatic processes that operated on a completely different scale than those at present (Chambers, 2012). Consequently, we regard King & Saunders' (1986) use of pollen as proxies as inadequate to determine that the climate and environment for *Hesperotestudo* at Hopwood Farm was "relatively xeric grassland".

Fossil remains of *Hesperotestudo* from Missouri caves

There are two records thought to be *Hesperotestudo* ("cf. *Geochelone* sp.") from Perry County in south-eastern Missouri (Figure 3) near the Mississippi River (Hawksley, 1986). Five fragments of shells (3 from the plastron, 2 from the carapace) were found in Crevice Cave, and a fragment of the left side of the "plastron" was found in Tom Moore Cave (Appendix 1). These localities are the closest records of *Hesperotestudo* to Hopwood Farm. They are only ca. 162 km south-southwest of Hopwood Farm, and close to the maximum south-western boundary of the Illinois glaciation (Crevice Cave ca. 12.5 km, Tom Moore Cave ca 12.0 km; Figure 3).

Fragment CM 6.1 from Tom Moore Cave is the largest of all of the Missouri fragments, having a maximum length of 65.4 mm and a maximum thickness of 22.1 mm (Figure 8). The thickness of CM 6.1 indicates it probably is from a fairly sizable tortoise. A note in the specimen box (possibly written by Hawksley) indicates "*Geochelone* prob." (= *Hesperotestudo*). We concur with Hawksley's identification. However, not enough material is available to allow species identification.

The five fragments (CMS 619.1-619.5) from Crevice Cave vary in size. Although they may be *Hesperotestudo*, we reserve judgement on their identification because of their smaller size. Again, not enough material is available for species identification of the Crevice Cave fossils.

Zoogeography

Blair (1958, 1965) developed an influential zoogeographical hypothesis concerning North American terrestrial vertebrates which was an expansion of less documented earlier proposals by Adams (1902) and Deevey (1949). This hypothesis suggested that southward movements of Pleistocene glaciations were accompanied by cold climates in front of the glacial rims which extended far into the southern USA. This supposedly forced many temperate-adapted and warm-adapted species to withdraw into refugia in Florida and Mexico. After the melting of the glaciation, the formerly conspecific Floridian and Mexican isolates returned northward. In some cases, speciation had occurred, and in others it is probable that sub-speciation occurred. Evidence was primarily from then-available Pleistocene vertebrate fossils, pollen profiles, vegetation, and extant animal distributions (particularly east-west species and subspecies pairs). Apparently Blair (1958, 1965) was referring primarily to the Wisconsin glaciation and the Holocene, although he mentioned briefly the "Illinoian", "Sangamon", Late Pleistocene, Pleistocene, pre-Pleistocene, and Early Pliocene. Blair's papers were widely cited and his hypothesis became well established and long-lasting. It convinced many that the periods of Pleistocene glaciation were very cold across much of North America.

Hibbard (1960) put forth another hypothesis (now invalid [Moll & Brown, 2017]) that maintained that giant fossil tortoises "*Geochelone*" (= *Hesperotestudo*) were adapted to living in subtropical or tropical areas in North America from the Cenozoic through the Pleistocene. Blair (1965) did not cite Hibbard (1960).

The first major challenges to Blair's hypothesis were from Graham (1985) and Graham & Mead (1987). Their model suggested that in North America, glaciers displaced arctic, boreal, and other northern species southward. However, resident species south of the terminal glacial rim were not affected because the climate was not as severe. Thus, south of the glaciation there was an intermingling of boreal and temperate taxa. Evidence was from the Late Pleistocene (Wisconsin and Holocene) mammals, reptiles, amphibians, and terrestrial invertebrate faunas as well as vegetation. Holman (1995) summarised additional evidence for fossil reptiles and amphibians for the model mainly from the Wisconsin and Holocene Episodes. Many of the reptile and amphibian species are still extant.

Thus, there is inferred evidence that many terrestrial vertebrates during the Wisconsin Glacial Episode evolved adaptations for withstanding moderately cold climates in central North America, and that Blair's (1958, 1965) model of intolerance to glaciations is incorrect. However, Blair's (1958, 1965) strongest evidence of east-west geographical separation of species and subspecies pairs from his original hypothesis is still valid because it is based on extant distributions. At present, the western taxa tend to be more xeric adapted such as grassland and desert, whereas in the east the trend is toward more mesic forest adaptation (Blair, 1958, 1965; Conant & Collins, 1998; Stebbins, 2003). Glacial cycles and tectonic processes can provide other alternatives that may resolve this conundrum.

During glacial meltdowns, massive amounts of water were produced around the earth resulting in substantial rises in ocean levels. There were 119 rises and declines of sea levels from the Triassic through the Quaternary (Haq et al., 1987) that could have affected resident species in numerous ways. For instance, Moll (1983) suggested that during Pleistocene glacial periods when sea levels dropped, the continental shelf feeding areas of the extant green sea turtle (*Chelonia mydas*) on the Brazilian coast were altered in size and position, and shifts in the locations of feeding and nesting sites occurred as a result. The subsequent return of higher sea levels due to glacial meltdown made extensive continental shelf feeding areas available along the Brazilian coast once again, and individuals from now divergent nesting sites (some recently established) were able to mingle on the feeding grounds. However, at nesting time these individuals segregated themselves and returned to the nesting areas from which they hatched.

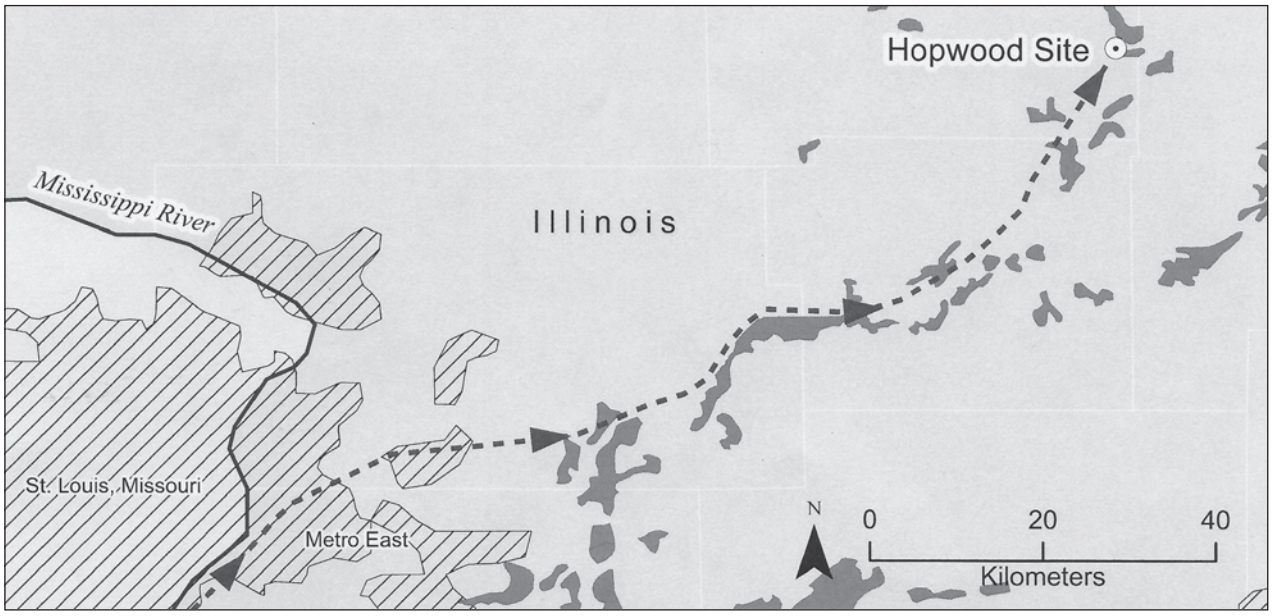

Figure 9. Tunnel valleys (dark grey) in south-eastern Illinois, USA which originated as water channels under the Illinois glaciation. The giant fossil tortoises (*Hesperotestudo*) may have travelled (arrows) in or adjacent to these valleys as they moved north-eastward. Cartography by M. Maher after Stiff (2000).

During the Cretaceous and Tertiary, periodic sea level rises due to tectonic processes and glacial meltdown resulted in inundation that extended up the Mississippi River Embayment far north to southern Illinois and south-eastern Missouri (Reed et al., 2005; Frankie et al., 2008). The salt water inundation which is toxic to most amphibians led Lemmon et al. (2007) to suggest that it caused geographical isolation and subsequent speciation of an east-west species pair of extant frogs (southern chorus frog *Pseudacris nigrita* and Cajun chorus frog *P. fouquettei*) during the Miocene-Early Pliocene. We propose that inundation of the Mississippi River Embayment was the cause of the geographical isolation of many of the east-west pairs of species and subspecies of terrestrial vertebrates during the Pleistocene and/or earlier mentioned by Blair (1958, 1965).

The zoogeographical position of the Illinois *Hesperotestudo* does not correspond to the Blair model or the Graham-Mead-Holman model in several respects. First, there are no extant representatives of *Hesperotestudo*. The last record for *Hesperotestudo* was in the Early Holocene 11,465 YBP in Florida (Turtle Extinctions Working Group, 2015). Second, there was a long-time span (ca. 70,000 years) between the Illinois-Sangamon boundary and the beginning of the Wisconsin Episode. Thus, there could have been many changes in geology, climate, and animals present and their habitats occupied as well as distributions, and evolution of characteristics. Third, the closeness of the Illinois *Hesperotestudo* record to the Illinois terminal glacial rim and the extreme southern location of the latter imply different climate and habitats available to the tortoise. Fourth, the arrival of *Hesperotestudo* at the terminal glacial rim could have been affected by potential new competitor species migrating from the north, especially with the possible intermingling of temperate and boreal habitats here.

There are suggestions of very different climates near the Illinois-Sangamon boundary. The thinness of the Illinois glacial till in southern Illinois (Leverett, 1899; William & Frye, 1980; Taylor et al., 2009) indicates that the glacial rim was present there for a much shorter time span than further north. The absence of a tundra stratum at four sites in southern Illinois (Grüger, 1972; King & Saunders, 1986; Zhu & Baker, 1995) substantiates the same thing. Furthermore, the Illinois glacier was so far south there may have been nearly constant diurnal melting which would have resulted in considerable ponded meltwater and erosion. South of the terminal glacial rim, the vegetation may have been much the same as it was prior to the advancement of glaciation. We suggest that the climate and habitat available immediately south of the glacial rim may have been quite hospitable to *Hesperotestudo*. Also, the wide range of *Hesperotestudo* and variety of habitats occupied suggest sufficient plasticity to allow the tortoise to follow the glacier as it melted northward.

The extant plains leopard frog (*Rana blairi*) has a distribution that extends far northward in the Great Plains to southern South Dakota (Dunlap & Kruse, 1976; Brown, 1992; Ballinger et al., 2000; Davis et al., 2017a, 2017b) indicating it has considerable cold-adaptation. This area is close to the edge of the southern rim of the Wisconsin glaciation in South Dakota. Thus, it is possible that the range of *R. blairi* retracted little, if at all, during the Wisconsin Episode (Brown et al., 1993).

The extant red-bellied snake (*Storeria occipitomaculata*) is one of the most cold-adapted snake species in eastern North America with a range extending well into Canada (see map in Conant & Collins, 1998). Brown & Phillips (2012) suggested that this species closely followed or occupied tundra, boreal forest, and other areas near the glacial rim during the melting of the Wisconsin glaciation.

Dr. Craig Gatto and Dr. Robert Nelson (pers. comms.) observed the rims of melting glaciers at different locations and observed that vegetation grew close to the rim. Thus there likely was vegetation available for consumption by *Hesperotestudo* at the glacial rim in southern Illinois. The probable cold-adaptations of *Hesperotestudo* (Moll & Brown, 2017), variety of habitats adapted to throughout its extensive range, and implied plasticity suggest that it should have been able to follow the glacial melt northward without difficulty. All of the extant giant tortoises (African spurred tortoise *Centrochelys sulcata*, Aldabra giant tortoise *Aldabrachelys gigantea*, Galápagos giant tortoises *Chelonoidis nigra* species complex) are well known as being capable of traveling moderate to long distances (Coe et al., 1979; Swingland & Lessells, 1979; Gibson & Hamilton, 1983; Swingland et al., 1989; Kaplan, 1996; Pritchard, 1996; Blake et al., 2013; Blake et al., 2015). Moreover, many species of extant reptiles are widely adapted and not restricted to a single type of habitat (Conant & Collins, 1998; Stebbins, 2003).

There has been no prior attempt to explain the zoogeographical origin of the extramarginal record of *Hesperotestudo* in southern Illinois. We propose that the tortoise could have migrated northward from the Gulf Coast region. The most likely route would have been along the Mississippi River Embayment because of its relative flatness. This may have occurred prior to the great meltdown of the Illinois glaciation when great quantities of meltwater flushed down the embayment. Before the meltdown, the Mississippi River would have occupied much less of the embayment although marshes, swamps, ponds, and lakes could have been scattered along the floodplain. The tortoise could have entered the southern tip of Illinois and proceeded northward. However, there are many hills and valleys present in the Shawnee Hills south of the Illinois glacial maximum. Extant Galápagos tortoises (*C. nigra* species complex) are quite adept at climbing volcanoes (Pritchard, 1996; Blake et al., 2013; Blake et al., 2015), but progress is slow. A number of other fossil species of *Hesperotestudo* have been found in high altitude areas of the western USA (Auffenberg, 1974; Auffenberg & Iverson, 1989). Furthermore, the Réunion giant tortoise *Cylindraspis indica* (extinct by ca. 1840 [Turtle Extinctions Working Group, 2015]) inhabited the mountains on the island of Réunion in the western Indian Ocean (Cheke & Bour, 2014). Thus it is possible *Hesperotestudo* may have traversed northward through the upland Shawnee Hills south of the Illinois glacial maximum, but at a relatively slow pace. An alternate perhaps more likely route may have involved following the Mississippi River floodplain further north-west to east of the current Metro East area east of St. Louis where the Illinois glaciation was near the floodplain (Figure 3). (From the Late Illinois to the present there were no major changes in the course of the Mississippi River between Metro East and extreme southern Illinois except for possible minor changes along the floodplain [Dr. Dave Malone, pers. comm.].) The tortoises then could have travelled to the north-east a much shorter distance over flat till, following the melting glacier to Hopwood Farm (Figure 3). They may have travelled (Figure 9) in or adjacent to tunnel valleys (water channels that originate under glaciers [Dott & Attig, 2004]). These were often quite wide and could have provided water and vegetation for consumption by travelling tortoises. The tortoises could have also exited the Mississippi River floodplain further south if the exit site was not glaciated or over-flowing with glacial meltwater.

The topography of Perry County, Missouri (near Tom Moore and Crevice caves) immediately south-west of the Mississippi River is flat broad floodplain. Further westward the landscape is low upland of variable elevation that is dissected by streams and ravines that lead into the floodplain. Presumably *Hesperotestudo* would have had little difficulty entering this type of landscape, whether they came from the north or south along the Mississippi River floodplain/Embayment. However, there is no clear-cut indication of the species identification of either the Illinois or Missouri tortoises and hence they may have been different taxa. Moreover, the Missouri tortoises may have arrived in a different time frame than those in Illinois.

There is another Midwestern record (Farlow et al., 2001) for *Hesperotestudo* sp. from the Pipe Creek Sinkhole in Grant County of east-central Indiana (the state east of Illinois). However, it cannot be assumed that this record indicates that the Illinois *Hesperotestudo* sp. was a direct descendant of the Indiana *Hesperotestudo* sp. or the reverse. The mammalian fossil fauna of Pipe Creek Sinkhole was considered by Farlow et al. (2001) to be Early Pliocene in age, long before (nearly 2.5 million years) the end of the Illinois Glacial Episode. Thus, there was considerable time for evolution to have occurred (e.g., speciation). If the genus is monophyletic, the Illinois, Indiana, and Missouri tortoises probably shared a common ancestor, but the location(s) and date(s) are unknown.

As mentioned previously, future fossil discoveries may reveal that the Illinois *Herperotestudo* is not *H. crassiscutata* but rather another species, potentially one of the western Pleistocene species (e.g., *H. campester*, *H. equicomes*, *H. oelrichi*, or *H. turgida*) of the Great Plains. Should that be the case, the Illinois *Hesperotestudo* may have originated from the eastward migration of the Great Plains species into the Prairie Peninsula which extends on till plain across much of Illinois (Transeau, 1935). A number of extant terrestrial vertebrate species have distributions in the Great Plains that extend into the Prairie Peninsula (Schmidt, 1938; Smith, 1957). The tortoises may have travelled eastward adjacent to western rivers (e.g., Missouri River, Arkansas River) and their tributaries that flow eastward into the Mississippi River. This would allow them access to herbaceous food and water. Brown & Brown (2014) proposed a model for speciation involving riverine-adapted terrestrial vertebrates moving along rivers in the Great Plains during the Miocene.

ACKNOWLEDGEMENTS

We thank Jill Brown, Angelo Capparella, Leslie Fay, Dave Malone, Ed Mockford, Janice Moll, Robert Nelson, Chris Phillips, and Don Shepard for critically reading the manuscript; Craig Gatto, Meredith Mahoney, Dave

Malone, Ed Mockford, Robert Nelson, and Eric Peterson for discussion; Carleen Spahn for drawing Figs. 1, 5, and 6; Rachael Antwis for granting permission to publish Figure 1 which originally appeared in *The Herpetological Journal*; Megan Maher for cartography (Figs. 2-4, 11); Janice Moll for typing the manuscript; Alan Resetar for facilitating and Bill Simpson for performing a data base search in the Paleontology Collection of the Field Museum of Natural History; Meredith Mahoney (Illinois State Museum) for completing a database search, for allowing us to examine fossils under her care, and other assistance; Kurt Dean, Stephen Wilson, Jay Raveill and particularly Steve Mills who directed DM to the location of O. Hawksley's fossil *Hesperotestudo* in the Illinois State Museum; and Sam Heads for directing LEB to the location of a *Hesperotestudo* reported by staff of the Illinois State Geological Survey and deposited at the Illinois State Museum.

REFERENCES

Adams, C. C. (1902). Southeastern United States as a center of geographical distribution of flora and fauna. *The Biological Bulletin* 3, 115-131.

Auffenberg, W. (1963). Fossil testudinine turtles of Florida genera *Geochelone* and *Floridemys*. *Bulletin of the Florida State Museum, Biological Sciences* 7, 53-97.

Auffenberg, W. (1974). Checklist of fossil land tortoises (*Testudinidae*). *Bulletin of the Florida State Museum, Biological Sciences* 18, 121-251.

Auffenberg, W. & Iverson, J. B. (1989). Demography of terrestrial turtles. In *Turtles, Perspectives and Research*, 541-569. Harless, M. & Morlock, H. (eds.). Malabar, Florida: Robert E. Krieger Publishing Company.

Ballinger, R. E., Meeker, J. W. & Thies, M. (2000). A checklist and distribuion maps of the amphibians and reptiles of South Dakota. *Transactions of the Nebraska Academy of Sciences* 26, 29-46.

Bentley, C. C. & Knight, J. L. (1998). Turtles (Reptilia: *Testudines*) of the Ardis Local Fauna Late Pleistocene (*Rancholabrean*) of South Carolina. *Brimleyana* 25, 3-33.

Blackwell, B. A. B., Kim, D. M. K., Curry, B. B., Grimley, D. A., Blickstein, J. I. B., & Skinner, A. R. (2016). Shell we date? ESR dating Sangamon Interglacial Episode deposits at Hopwood Farm, IL. *Radiation Protection Dosimetry* 172, 283-295.

Blair, W. F. (1958). Distributional patterns of vertebrates in the southern United States in relation to past and present environments. In *Zoogeography*, 433-468. Hubbs, C. L. (ed.). Publication No. 51, Washington, D.C.: *American Association for the Advancement of Science.*

Blair, W. F. (1965). Amphibian speciation. In *The Quaternary of the United States, A review volume for the VII Congress of the International Association for Quaternary Research*, 543-556. Wright, H. E., Jr. & Frey, D. G. (eds.). Princeton, New Jersey: Princeton University Press.

Blake, S., Yackulic, C. B., Cabrera, F., Tapia, W., Gibbs, J. P., Kümmeth, F. & Wikelski, M. (2013). Vegetation dynamics drive segregation by body size in Galapagos tortoises migrating across altitudinal gradients. *Journal of Animal Ecology* 82, 310-321.

Blake, S., Yackulic, C. B., Wikelski, M., Tapia, W., Gibbs, J. P., Deem, S., Villamar, F. & Cabrera, F. (2015). Migration by Galapagos giant tortoises requires landscape-scale conservation efforts. In: *Biodiversity and Ecosystem Restoration, Galapagos Report* 2013-2014, 144-150.

Bramble, D. M. (1971). Functional morphology, evolution, and paleoecology of gopher tortoises. Ph.D. Dissertation. Berkeley: University of California.

Brown, L. E. (1992). *Rana blairi* Mecham, Littlejohn, Oldham, Brown, & Brown, Plains Leopard Frog. *Catalogue of American Amphibians and Reptiles* 536.1-536.6.

Brown, L. E. & Brown, E. S. (2014). A new genus of fossil frogs (Amphibia: Anura: Hylidae) from the Miocene of the northern Great Plains (USA), with a commentary on vertebrate diversity. *Life: The Excitement of Biology* 2, 136-146.

Brown, L. E., Dubois, A. & Shepard, D. B. (2008). Inefficiency and bias of search engines in retrieving references containing scientific names of fossil amphibians. *Bulletin of Science, Technology & Society* 28, 279-288.

Brown, L. E., Morris, M. A. & Johnston, T. R. (1993). Zoogeography of the Plains Leopard Frog (*Rana blairi*). *Bulletin of the Chicago Academy of Sciences* 15, 1-13.

Brown, L. E. & Phillips, C. A. (2012). Distribution, habitat, and zoogeography of the semifossorial Red-bellied snake *Storeria occipitomaculata* (Storer) in Illinois. *Illinois Natural History Survey Bulletin* 39, 297-322.

Carroll, R. L. (1988). Vertebrate paleontology and evolution. New York: W. H. Freeman and Company.

Cassiliano, M. L. (1997). Crocodiles, tortoises, and climate: a shibboleth re-examined. *Paleoclimates* 2, 47-69.

Chambers, F. M. (2012). Reconstructing and inferring past environmental change. In *The SAGE handbook of environmental change*, 67-91. Volume 1. Mathews, J. A., Bartlein, P. J., Briffa, K. R., Dawson, A. G., De Vernal, A., Denham, T., Fritz, S. C. & Oldfield, F. (eds.). London: SAGE Publications, Ltd.

Cheke, A. S. & Bour, R. (2014). Unequal struggle - how humans displaced the dominance of tortoises in island ecosystems. In *Western Indian Ocean Tortoises, Ecology, Diversity, Evolution, Conservation, Paleontology*, 31-120. Gerlach, J. (ed.). Manchester, UK: Siri Scientific Press.

Coe, M. J., Bourn, D. & Swingland, I. R. (1979). The biomass, production and carrying capacity of giant tortoises on Aldabra. *Philosophical Transactions of the Royal Society of London Series B, Biological Sciences* 286, 163-176.

Conant, R. & Collins, J. T. (1998). *A field guide to reptiles & amphibians, eastern and central North America*, The Peterson Field Guide Series, Third Edition. Boston & New York: Houghton Mifflin Company.

Cope, E. D. (1892). A contribution to the vertebrate paleontology of Texas. *Proceedings of the American Philosophical Society* 30, 123-131.

Critchfield, W. B. & Little, E. L., Jr. (1966). *Geographic distribution of the pines of the world*. Washington, D. C.: U.S. Department of Agriculture, Forest Service, Miscellaneous Publication 991.

Curry, B. B. & Baker, R. G. (2000). Palaeohydrology, vegetation, and climate since the late Illinois Episode (~130 ka) in south-central Illinois. *Palaeogeography, Palaeoclimatology, Palaeoecology* 155, 59-81.

Curry, B. B. & Follmer, L. R. (1992). The last interglacial-glacial transition in Illinois: 123-25 ka. *Geological Society of America Special Paper* 270, 71-88.

Curry, B., Wang, H. Dorale, J., Blackwell, B. & Lundstrom, C. (2011). Stop 9: Pittsburg Basin. Paleoenvironmental history from fossil pollen and ostracode records in south-central Illinois. In *Ridges, mounds, and valleys: Glacial-interglacial history of the Kaskaskia Basin, southwestern Illinois, 55th Midwest Friends of the Pleistocene Field Conference*, 112-123, Open File Series 2011-1. Grimley, D. A. & Phillips, A. C. (eds.). Champaign, Illinois: Illinois State Geological Survey. Prairie Research Institute.

Curry, B. B., Wang, H., Panno, S. V. & Hackley, K. C. (2010). Quaternary paleoclimate. In *Geology of Illinois*, 248-260. Kolata, D. R. & Nimz, C. K. (eds.). Champaign, Illinois: University of Illinois at Urbana-Champaign, Institute of Natural Resource Sustainability, & Illinois State Geological Survey.

Davis, D. R., Farkas, J. K., Johannsen, R. E., Leonard, K. M. & Kerby, J. L. (2017a). Distributional records of amphibians and reptiles from South Dakota, USA. *Herpetological Review* 48, 133-137.

Davis, D. R., Farkas, J. K., Johannsen, R. E. & Maltaverne, G. A. (2017b). Historic amphibian and reptile county records from South Dakota, USA. *Herpetological Review* 48, 394-406.

Deevey, E. S., Jr. (1949). 3. Biogeography of the Pleistocene. Part I: Europe and North America. *Bulletin of the Geological Society of America* 60, 1315-1416.

Des Lauriers, J. R. (1965). A new Miocene tortoise from Southern California. *Bulletin of the Southern California Academy of Sciences* 64, 1-10.

Dott, R. H., Jr. & Attig, J. W. (2004). *Roadside geology of Wisconsin*. Missoula, Montana: Mountain Press Publishing Company.

Dunlap, D. G. & Kruse, K. C. (1976). Frogs of the *Rana pipiens* complex in the northern and central plains states. *The Southwestern Naturalist 20*, 559-571.

Farlow, J. O., Sunderman, J. A., Havens, J. J., Swinehart, A. L., Holman, J. A., Richards, R. L., Miller, N. G. Martin, R. A., Hunt, R. M., Jr., Storrs, G. W., Curry, B. B., Fluegeman, R. H., Dawson, M. R. & Flint, M. E. T. (2001). The Pipe Creek Sinkhole Biota, a diverse Late Tertiary continental fossil assemblage from Grant County, Indiana. *The American Midland Naturalist* 145, 367-378.

Fouquette, M. J., Jr. & Dubois, A. (2014). A checklist of North American amphibians and reptiles, The United States and Canada, Volume 1 - Amphibians, Seventh Edition. Xlibris LLC.

Frankie, W. T., Devera, J. A. & Seid, M. (2008). Guide to the geology of the Horseshoe Lake State Conservation Area and surrounding area, Alexander County, Illinois, 1-48. Geological science field trip guidebook 2008A. Champaign, Illinois: Illinois Department of Natural Resources & Illinois State Geological Survey.

Gibson, C. W. D. & Hamilton, J. (1983). Feeding ecology and seasonal movements of giant tortoises on Aldabra Atoll. *Oecologia* 56, 84-92.

Graham, R. W. (1985). Diversity and community structure of the late Pleistocene mammal fauna of North America. *Acta Zoologica Fennica* 170, 181-192.

Graham, R. W. & Mead, J. I. (1987). Environmental fluctuations and evolution of mammalian faunas during the last deglaciation in North America. In *North America and adjacent oceans during the last deglaciation*, K-3, 371-402. Ruddiman, W. F. & Wright, H. E., Jr. (eds.). The Geology of North America. Boulder, Colorado: The Geological Society of America.

Grimley, D. A. & Phillips, A. C. (2011). Part I: Quaternary geologic overview of the lower Kaskaskia Basin. In *Ridges, Mounds, and Valleys: Glacial-interglacial History of the Kaskaskia Basin, Southwestern Illinois*. 55th Midwest Friends of the Pleistocene Field Conference, 14-37, Open File Series 2011-1. Grimley, D. A. & Phillips, A. C. (eds.). Champaign, Illinois: Illinois State Geological Survey. Prairie Research Institute.

Grüger, E. (1972). Late Quaternary vegetation development in south-central Illinois. *Quaternary Research* 2, 217-231.

Hansel, A. K. & Johnson, W. H. (1996). Wedron and Mason groups: lithostratigraphic reclassification of deposits of the Wisconsin Episode, Lake Michigan lobe area. Illinois State *Geological Survey Bulletin* 104, 1-116.

Hansel, A. K. & McKay, E. D., III (2010). Quaternary Period. In *Geology of Illinois*, 216-247. Kolata, D. R. & Nimz, C. K. (eds.). Champaign, Illinois: University of Illinois at Urbana-Champaign, Institute of Natural Resource Sustainability, & Illinois State Geological Survey.

Haq, B. U., Hardenbol, J. & Vail, P. R. (1987). Chronology of fluctuating sea levels since the Triassic. *Science* 235, 1156-1167.

Hawksley, O. (1986). Remains of Quaternary vertebrates from Ozark caves and other miscellaneous sites. *Missouri Speleology* 26, 1-67.

Hay, O. P. (1908). *The Fossil Turtles of North America*. Carnegie Institution of Washington, Publication No. 75, Washington, D.C.

Hay, O. P. (1917). On a collection of fossil vertebrates made by Dr. F. W. Cragin in the Equus (sic) beds of Kansas. *The Kansas University Science Bulletin* 10, 39-51.

Hibbard, C. W. (1960). The President's Address. An interpretation of Pliocene and Pleistocene climates in North America. *Annual Report of the Michigan Academy of Sciences, Arts and Letters* 62, 5-30.

Hibbard, C. W. & Dalquest, W. W. (1966). Fossils from the Seymour Formation of Knox and Baylor counties, Texas, and their bearing on the late Kansan climate of that region. *Contributions from the Museum of Paleontology, University of Michigan* 21, 1-66.

Holman, J. A. (1969). The Pleistocene amphibians and reptiles of Texas. *Publications of the Museum, Michigan State University, Biological Series* 4, 161-192.

Holman, J. A. (1972). Amphibians and reptiles. In Early Pleistocene pre-glacial and glacial rocks and faunas of north-central Nebraska, 55-71. Skinner, M. F., Hibbard, C. W., Gutentag, E. D., Smith, G. R., Lundberg, J. G., Holman, J. A., Feduccia, J. A. & Rich, P. V. *Bulletin of the American Museum of Natural History* 148, 1-148.

Holman, J. A. (1995). *Pleistocene amphibians and reptiles in North America*. New York & Oxford: Oxford University Press & Clarendon Press.

Holman, J. A. (1998). Reptiles of the Lower Miocene (Hemingfordian) Pollack Farm fossil site, Delaware. In *Geology and paleontology of the Lower Miocene Pollack Farm fossil site, Delaware*, 21, 141-147. Benson, R. N. (ed.), Newark: Delaware Geological Survey Special Publication.

Holman, J. A. & Clausen, C. J. (1984). Fossil vertebrates associated with paleo-Indian artifact at Little Salt Spring, Florida. *Journal of Vertebrate Paleontology* 4, 146-154.

Hubbs, C. L. & Lagler, K. F. (1964). *Fishes of the Great Lakes region*. Ann Arbor: The University of Michigan Press.

Kaplan, M. (1996). African Spurred Tortoises, *Geochelone sulcata*. *Reptile & Amphibian Magazine* (42), 32-45.

King, J. E. & Saunders, J. J. (1986). Geochelone in Illinois and the Illinois-Sangamon vegetation of the type region. *Quaternary Research* 25, 89-99.

Lambe, L. M. (1906). Descriptions of new species of Testudo (*sic*) and Baëna (*sic*), with remarks on some Cretaceous forms. *The Ottawa Naturalist* 19, 187-196.

Leidy, J. (1889). Description of vertebrate remains from Peace Creek, Florida. *Transactions of the Wagner Free Institute of Science of Philadelphia* 2, 19-31.

Lemmon, E. M., Lemmon, A. R. & Cannatella, D. C. (2007). Geological and climatic forces driving speciation in the continentally distributed trilling chorus frogs (*Pseudacris*). *Evolution* 61 2086-2103.

Leverett, F. (1899). The Illinois glacial lobe. Washington, D. C.: *Monographs of the United States Geological Survey* 38.

Meylan, P. A. (1995). Pleistocene amphibians and reptiles from the Leisey Shell Pit, Hillsborough County, Florida. *Bulletin of the Florida Museum of Natural History* 37 Pt. I(9): 273-297.

Meylan, P. A. & Sterrer, W. (2000). *Hesperotestudo* (Testudines: Testudinidae) from the Pleistocene of Bermuda, with comments on the phylogenetic position of the genus. *Zoological Journal of the Linnean Society* 128, 51-76.

Miller, W. E. (1980). The Late Pliocene Las Tunas Local Fauna from southernmost Baja California, Mexico. *Journal of Paleontology* 54, 762-805.

Mohlenbrock, R. H. (1986). *Guide to the vascular flora of Illinois*. Second Edition. Carbondale & Edwardsville, Illinois: Southern Illinois University Press.

Moll, D. (1983). A proposed origin of nesting location divergence and a shared feeding range in Brazilian Green Turtle populations. *Copeia* 1983, 121-125.

Moll, D. & Brown, L. E. (2017). Reinterpretation of the climatic adaptation of giant fossil tortoises in North America. *The Herpetological Journal* 27, 276-286.

Moll, D. & Klemens, M. W. (1996). Ecological characteristics of the Pancake Tortoise, *Malacochersus tornieri*, in Tanzania. *Chelonian Conservation and Biology* 2, 26-35.

Moodie, K. B. & Van Devender, T. R. (1979). Extinction and extirpation in the herpetofauna of the southern High Plains with emphasis on *Geochelone wilsoni* (Testudinidae). *Herpetologica* 35, 198-206.

Morgan, G. S. & Lucas, S. G. (2000). Biostratigraphic significance of Late Neogene vertebrate fossils from the Gila Group, Pearson Mesa, southwestern New mexico (sic) and southeastern Arizona. In *Southwest passage: a trip through the Phanerozoic*, New Mexico Geological Society 51st annual fall field conference guidebook, 211-220. Lawton, T. F., McMillian, N. J. & McLemore, V. T. (eds.). Albuquerque: New Mexico Geological Society. Available from http://nmgs.nmt.edu/publications/guidebooks/51.

Moriarty, J. J. (ed.) (2012). *Scientific and standard English names of amphibians and reptiles of North America north of Mexico, with comments regarding confidence in our understanding*. Herpetological Circular No. 39, Seventh Edition. Salt Lake City, Utah: Society for the Study of Amphibians and Reptiles.

Page, L. M. & Burr, B. M. (1991). A field guide to freshwater fishes, North America north of Mexico. *Peterson Field Guide Series*. Boston: Houghton Mifflin Company.

Parris, D. C. & Daeschler, E. (1995). Pleistocene turtles of Port Kennedy Cave (Late Irvingtonian), Montgomery County, Pennsylvania. *Journal of Paleontology* 69, 563-568.

Pritchard, P. C. H. (1996). The Galapagos Tortoises: nomenclatural and survival status. *Chelonian Research Monographs No. 1*.

Reed, J. C., Jr., Wheeler, J. O. & Tucholke, B. E. Compilers. (2005). *Decade of North American geology, geologic map of North America: continental-scale map 001, scale 1:5,000,000*. Boulder, Colorado: The Geological Society of America.

Russell, D. A., Rich, F. J., Schneider, V. & Lynch-Stieglitz, J. (2009). A warm thermal enclave in the Late Pleistocene of the south-eastern United States. *Biological Reviews* 84, 173-202.

Saunders, J. J. & King, J. E. (1986). Stratified Illinois-Sangamon pollen, plant macrofossil, invertebrate and vertebrate record at Hopwood Farm. In *Quaternary records of southwestern Illinois and adjacent Missouri AMQUA*, 86, field guide, 86, 1-6. Graham, R. W., Styles, B. W., Saunders, J. J., Wiant, M. D., Mckay, E. D., Styles, T. R. & Hajic, E. R. (eds.). Urbana, Illinois: American Quaternary Association, Illinois State Geological Survey, Illinois State Museum, Illinois State Water Survey & University of Illinois.

Schmidt, K. P. (1938). Herpetological evidence for the postglacial eastward extension of the steppe in North America. *Ecology* 19, 396-407.

Slaughter, B. H. (1966). The Moore Pit Local Fauna; Pleistocene of Texas. *Journal of Paleontology* 40, 78-91.

Smith, A. J. (2012). Evidence of environmental change from terrestrial and freshwater palaeoecology. In *The SAGE Handbook of Environmental Change*, 254-283, Volume 1. Matthews, J. A., Bartlein, P. J., Briffa, K. R., Dawson, A. G., De Vernal, A., Denham, T., Fritz, S. C. & Oldfield, F. (eds.). London: SAGE Publications Ltd.

Smith, P. W. (1957). An analysis of post-Wisconsin biogeography of the Prairie Peninsula region based on distributional phenomena among terrestrial vertebrate populations. *Ecology* 38, 205-218.

Stanley, R. G. & Linskens, H. F. (1974). Pollen biology biochemistry management (sic). New York, Heidelberg & Berlin: Springer-Verlag.

Stebbins, R. C. (2003). A field guide to western reptiles and amphibians, *The Peterson Field Guide Series*, Third Edition. Boston & New York: Houghton Mifflin Company.

Stiff, B. J. (2000). Surficial geologic map of Illinois. Scale 1:500,000. Champaign, Illinois: Illinois State Geological Survey. Available online: http://isgs.illinois.edu/sites/isgs/files/maps/statewide/ofs2000-07.pdf.

Sullivan, R. M., Lucas, S. G. & Spielmann, J. A. (eds.). (2011). Fossil Record 3. New Mexico Museum of Natural History & Science Bulletin 53, 676-678.

Swingland, I. R. & Lessells, C. M. (1979). The natural regulation of giant tortoise populations on Aldabra Atoll. Movement polymorphism, reproductive success and mortality. *Journal of Animal Ecology* 48, 639-654.

Swingland, I. R., North, P. M., Dennis, A. & Parker, M. J. (1989). Movement patterns and morphometrics in giant tortoises. *Journal of Animal Ecology* 58, 971-985.

Taylor, S. J., Phillips, A. C., Post, S. L. & Brown, S. E. (2009). An overview of Illinois' geological history and landscape. In

Canaries in the catbird seat; the past, present, and future of biological resources in a changing environment, 30, 7-23. Taylor, C. A., Taft, J. B. & Warwick, C. E. (eds.). Champaign, Illinois: Illinois Natural History Survey.

Teed, R. (2000). A >130,000-year-long pollen record from Pittsburg Basin, Illinois. *Quaternary Research* 54, 264-274.

Transeau, E. N. (1935). The Prairie Peninsula. *Ecology* 16, 423-437.

Turtle Extinctions Working Group [Rhodin, A. G. J., Thomson, S., Georgalis, G., Karl, H.-V., Danilov, I. G., Takahashi, A., de la Fuente, M. S., Bourque, J. R., Delfino, M., Bour, R., Iverson, J. B., Shaffer, H. B. & van Dijk, P. P.] (2015). Turtles and tortoises of the world during the rise and global spread of humanity: first checklist and review of extinct Pleistocene and Holocene chelonians. In *Conservation biology of freshwater turtles and tortoises: a compilation project of the IUCN/SSC Tortoise and Freshwater Turtle Specialist Group*, 1-66. Rhodin, A. G. J., Pritchard, P. C. H., Van Dijk, P. P., Saumure, R. A., Buhlmann, K. A., Iverson, J. B. & Mittermeier, R. A. (eds.). Chelonian Research Monographs, 5(8):000e.

Turtle Taxonomy Working Group [van Dijk, P. P., Iverson, J. B., Rhodin, A. G. J., Shaffer, H. B. & Bour, R.] (2014). Turtles of the world, 7th edition: annotated checklist of taxonomy, synonymy, distribution with maps, and conservation status. In *Conservation biology of freshwater turtles and tortoises: a compilation project of the IUCN/SSC Tortoise and Freshwater Turtle Specialist Group*, 329-479. Rhodin, A. G. J., Pritchard, P. C. H., Van Dijk, P. P., Saumure, R. A., Buhlmann, K. A., Iverson, J. B. & Mittermeier, R. A. (eds.). Chelonian Research Monographs 5(7):000. Available at: doi:10.3854/crm.5.000.checklist.v7.2014.

Williams, B. G. (2016). Global warming causes >> paleoclimatology. https://www.briangwilliams.us/paleoclimatology/the-basis-of-pollen-analysis.html. Updated 26 April 2016, read 7 July 2017.

Williams, E. (1950). *Testudo cubensis* and the evolution of Western Hemisphere tortoises. *Bulletin of the American Museum of Natural History* 95, 1-36.

Willman, H. B. & Frye, J. C. (1970). Pleistocene stratigraphy of Illinois. *Illinois State Geological Survey Bulletin* 94, 1-204.

Willman, H. B. & Frye, J. C. (1980). The glacial boundry in southern Illinois. Illinois State Geological Survey Circular 511, 1-23.

Wilson, D. S., Tracy, C. R. & Tracy, C. R. (2003). Estimating age of turtles from growth rings: A critical evaluation of the technique. *Herpetologica* 59, 178-194.

Zhu, H. & Baker, R. G. (1995). Vegetation and climate of the last glacial-interglacial cycle in Southern Illinois, USA. *Journal of Paleolimnology* 14, 337-354.

APPENDIX 1

List of fossil fragments of the giant tortoise *Hesperotestudo* examined at the Illinois State Museum. Each number represents one specimen:

Hesperotestudo, Montgomery County, Illinois, USA: ISM 490,015; 490,016; 490,017; 498,595; 498,596; 498,597; 498,598; 498,599; 498,600; 498,602; 498,603

Hesperotestudo, Perry County, Missouri, USA: CM 6.1 “cf. *Geochelone* sp.” (Hawksley, 1986), Perry County, Missouri, USA: CMS 619.1, 619.2, 619.3, 619.4, 619.5

Accepted: 28 February 2018

Volume 28 (April 2018), 87-92

Herpetological Journal

SHORT NOTE

Published by the British Herpetological Society

Batrachochytrium dendrobatidis infection and treatment in the salamanders *Ambystoma andersoni*, *A. dumerilii* and *A. mexicanum*

Christopher J. Michaels[1], Matthew Rendle[1], Cathy Gibault[2], Javier Lopez[3], Gerardo Garcia[3], Matthew W. Perkins[4], Suzetta Cameron[5] & Benjamin Tapley[1]

[1] Zoological Society of London (ZSL) London Zoo, Regent's Park, London, NW1 4RY, UK

[2] Parc de Thoiry, Rue du Pavillon de Montreuil, 78770, Thoiry, France

[3] Chester Zoo, Moston Rd, Upton-by-Chester, Upton, Chester, CH2 1EU, UK

[4] ZSL Institute of Zoology, Moston Rd, Upton-by-Chester, Upton, Chester, CH2 1EU, UK

[5] Birch Heath Veterinary Clinic, Birch Heath Road, Tarporley CW6 9UU, UK

In order to better understand the impacts and treatment of infection with *Batrachochytrium dendrobatidis* (Bd) and *Batrachochytrium salamandrivorans* (Bsal) it is important to document host species, the effect of infection and response to treatment protocols. Here we report asymptomatic Bd infection detected through duplex qPCR screening of three Mexican ambystomatid salamanders; *Ambystoma andersoni*, *Ambystoma dumerilii* and *Ambystoma mexicanum* at three zoo collections, and *A. andersoni* and *A. mexicanum* in a private collection. Bsal was tested for but not detected. We also report the effectiveness and side effects of five treatment protocols in these species. Using the antifungal agent itraconazole, *A. dumerilii* were cleared of infection without side-effects using the granulated preparation (Sporanox). Morbidity and mortality occurred when *A. dumerilii* and *A. andersoni* were treated using a liquid oral preparation of the itraconazole (Itrafungol); infection was successfully cleared in surviving specimens of the latter species. *Ambystoma mexicanum* was successfully cleared without any side-effects using Itrafungol. Mortality and morbidity were likely caused by toxic effects of some component on the liquid preparation of itraconazole, but aspects of water quality and husbandry cannot be ruled out.

Key words: Bd, axolotl, *Ambystoma*, chytridiomycosis, itraconazole

Batrachochytrium dendrobatidis (Bd; Longcore et al., 1999) is a major fungal pathogen known to infect all orders of amphibian (Gower et al., 2013) and is a driver of population declines (Bosch et al., 2001; Lips et al., 2006; Briggs et al., 2010; Scheele et al., 2017). The recently described congener *B. salamandrivorans* (Bsal; Martel et al., 2013) also causes disease and its spread is a cause for major concern (Martel et al., 2014; Richgels et al., 2016; Spitzen-van der Sluijs 2016; Laking et al., 2017). Bsal is thought to be less cosmopolitan than the globally distributed Bd (Fisher et al., 2009; Sabino-Pinto et al., 2016); however, surveys of captive and free-living populations are far from comprehensive.

In captivity, a variety of chemical treatments as well as heat therapy (27-37 ˚C) have been used to treat the chytrid infections caused by both agents (e.g. Berger et al., 2004; Bowerman et al., 2010; Chatfield and Richards-Zawacki 2011; Martel et al., 2011; Woodhams et al., 2003, 2012; Brannelly et al., 2012; Blooi et al., 2015a; b). However, urodelan amphibians tend to exhibit low tolerances to elevated temperatures (e.g. Liu et al., 2006; Bury, 2008), which often precludes heat treatment as a viable method to clear Bd infection. It is therefore imperative that the efficacy of other treatment options is tested.

Successful therapy using itraconazole has been documented in numerous amphibian species from all three orders (Forzan et al., 2008; Tamukai et al., 2011; Brannelly et al., 2012; Rendle et al., 2015), but different species respond differently to the drug. Itraconazole toxicosis has been reported in some amphibian species treated at 'routine' doses of the drug (Garner et al., 2009; Woodhams et al., 2012). It has therefore been recommended to record the effects of Bd on host animals and to develop and test protocols for treating this widespread pathogen in a variety of species of amphibian (Woodhams et al., 2012).

We present data following Bd diagnosis and subsequent treatments of 40 *Ambystoma dumerilii*, 26 *A. mexicanum* and 10 *A. andersoni* in captivity (see Supplementary Materials for details). Animals were maintained according to the protocols outlined in Table 1 at the Zoological Society of London (ZSL), Chester Zoo (CZ), Parc de Thoiry (PT) and a private breeder in the UK (PB). Salamanders were sampled initially and post treatment (see Table 1) for Bd/Bsal screening by swabbing dorsum,

Correspondence: Christopher Michaels (christopher.michaels@zsl.org)

Table 1. Husbandry and swabbing protocols for *Ambystoma* salamanders reported in this study

Species	*Ambystoma dumerilii*	*A. mexicanum*			*A. andersoni*
Collection	**ZSL London Zoo**	**Parc de Thoiry**	**Chester Zoo**	**Private collection, UK**	
Swab numbers	**Pre-treatment:** 3 individual swabs **Post-treatment (0, 30 and 180 days):** 11 individual swabs	**Pre-treatment:** 2 pooled swabs, 8 individuals each **Post-treatment (0 days):** 2 pooled swabs, eight individuals each **Post-treatment (40 days):** 5 pooled swabs, 3 individuals each; 1 individual swab	**Pre-treatment:** 13 individual swab **Post-treatment:** N/A	**Pre-treatment:** 1 pooled swab for 4 *A. mexicanum* **Post-treatment (30 days):** 1 pooled swab for 4 *A. mexicanum*	**Pre-treatment:** 1 pooled swab for 2 *A. andersoni* **Post-treatment (30 days):** 1 pooled swab for 2 *A. andersoni*
Bd/Bsal qPCR results	**Bd:** 3/3 +ve, **Bd infection load:** 6.48, 12.6, 2964.12 GE **Bsal:** 3/3 -ve	**Bd:** 1/2 two pooled swabs +ve. **Bsal:** 2/2 pooled swabs -ve.	**Bd:** 6/13 +ve, **Bd infection load:** 31, 41.64, 84.72, 97.44, 114.72, 704.76 GE **Bsal:** 13/13 -ve	**Bd:** +ve **Bsal:** -ve	**Bd:** +ve **Bsal:** -ve
Animal housing during treatment period	3-4 animals held in 100 x 30 x 30 cm aquaria Aquaria filtered using air-stream sponge filters.	5 animals in a 100 x 50 x 60 cm aquarium; 11 animals individually in 40 x 30 x 30 cm plastic boxes. Large aquarium filter with internal filter. Small boxes unfiltered; 100% water change performed daily.	4-5 animals held in 400L aquaria.	Large plastic boxes (varying capacity). No filtration. Daily 100% water changes and disinfection of enclosures.	
Water quality parameters during treatment period	**pH:** c. 8 **Ammonia (NH_3^+):** 0 - 0.03mg/L (with two brief instances of c. 0.5mg/L) **Nitrite (NO_2):** 0-0.04mg/L (with one instance of c. 0.5mg/L **Nitrate (NO_3):** <10 mg/l **Alkalinity:** 175-200mg/L **Temperature:** 15-17 ˚C	**pH:** 6.8 - 7.2 **Nitrite (NO_2):** 0mg/L **Nitrate (NO_3):** 50 - 75 mg/l **Conductivity:** 370 micro Siemens. **Temperature:** 18 ˚C	Water parameters not recorded.	**pH:** 7.9. **Temperature:** 16-20 ˚C.	

ventrum, cloaca, lips, tail base and plantar aspect of the feet using a sterile dry swab (see Table 1 for numbers of swabs collected and Supplementary Materials for swabbing methods). Duplex qPCR was used to test for the presence of Bd and Bsal DNA following protocols developed by Hyatt et al. (2007) and Blooi et al. (2013) (see Supplementary Materials). Itraconazole baths were used for treatment in all institutions using the protocols outlined in Table 2 and detailed in Supplementary Materials. Results of initial and post-treatment qPCR testing, including Genomic Equivalent (GE) values, are presented in Table 1; all species presented with at least some animals infected with Bd, but Bsal was not detected. All animals that survived treatment tested negative for both pathogens after treatment (Table 2). Identification of the lineage or strain of Bd infecting animals (Retallick & Miera, 2007) was beyond the scope of this work.

All surviving salamanders in all collections repeatedly tested negative for Bd post treatment (see Table 2). At ZSL and PT, there were no observed adverse side effects to treatment with Sporanox in *A. dumerilii*. Water quality was monitored at ZSL and PT, and remained good (see Table 1). At CZ, there was 100% mortality of animals shortly after exposure to the treatment regimen using Itrafungol. Water quality was not monitored at CZ. At CZ on day 7 of treatment with Itrafungol, six *A. dumerilii* were found dead. The remaining animals exhibited excessive mucus production, cloudy eyes, erratic movements and inappetence. At post mortem examination and subsequent histopathology, the salamanders were thin and presented acute dermatitis (sometimes ulcerative or necrotic) and branchitis. Some specimens showed hepatocyte vacuolation. Treatment was stopped but after two days, the condition of the remaining animals had continued to worsen and they were euthanased on welfare grounds. No CZ animals completed the treatment protocol.

At PB, *A. mexicanum* showed no clinical adverse effects of treatment with Itrafungol. In *A. andersoni*, 50% mortality was encountered when treated with Itrafungol.

Table 2. Protocols for and outcomes of itraconazole treatment in *Ambystoma* salamanders reported in this study.

Species	*Ambystoma dumerilii*	*A. mexicanum*	*A. andersoni*	
Collection	**ZSL London Zoo**	**Parc de Thoiry**	**Chester Zoo**	**Private collection, UK**
Therapeutic drug and preparation	Itraconazole (Sporanox; Janssen Pharamceutica N.V., Beerse B-2340, Belgium).	Itraconazole (Itrafungol; Elanco, Division Eli Lilly Canada Inc., 150 Research Lane, Suite 120, Guelph, ON, N1G 4T2, Canada)	Itraconazole (Itrafungol)	
Therapeutic itraconazole concentration, duration and temperature	0.01%. 15 minute baths daily for eleven days at c. 16 ˚C.	**Group 1 (n=8):** 0.01%. 7 minute baths daily for seven days. **Group 2 (n=8):** 0.005%. 15 minute baths daily for seven days Both versions at c. 18˚C.	0.01%. 5 minute baths daily for ten days, followed by 10 rest days and then a further ten days of 5 minute baths. Treatment course not completed due to mortality. Water temperature not recorded.	0.01% in buffered with one tsp NaHCl/5L tap water to maintain pH 7. 5 minutes per day, daily over six days. 16-20 ˚C.
Treatment protocol	Animals were moved to individual c. 1L containers of itraconazole solution. Filtered aquaria were not sterilised between treatments in order to preserve biological filtration.	Animals were to be bathed in 1 litre of solution in a clear plastic bag. Aquaria and filters sterilized with 1:500 F10 disinfectant after 5 and 10 days of treatment. Treatment was not completed.	Animals bathed in individual 1L containers. Enclosures sterilised between treatments.	
Mortality	0%	100% (animals either died from presumed toxicosis or were euthanased)	*A. mexicanum*: 0%	*A. andersoni:* 50%
Bd negative post treatment?	Y	Animals did not survive treatment	Y	Y

At PB, *A. mexicanum* showed no clinical adverse effects of the Itrafungol treatment. In *A. andersoni*, however, animals lost vigour during treatment and within one week of completion of treatment, five animals had died (50% mortality). Animals exhibited shrinking gill branches, loss of gill filaments both of which were noticeable in living and dead animals. Animals also showed reduced feeding behaviour. Ten days post treatment, surviving animals were removed from the established aquarium into which they had been placed and maintained in a 160L plastic box with 100% daily water changes. Following this intervention, mortality stopped and animals recovered to normal appearance and behaviour. No histological data are available from these animals.

Bd infection has not previously been reported from *A. dumerilii* or *A. andersoni*, but has been recorded in other neotenic Mexican Ambystoma (namely *A. altamirani*, *A. granulosum*, *A. mexicanum*, *A. rivulare*, *A. ordinarium* and *A. velasci*; Forzan et al., 2008; Frias-Alvarez et al., 2008; Galindo-Bustos et al., 2014; Spitzen-van der Sluijs et al., 2011). Animals of all three species in all four collections were apparently able to carry Bd infection without clinical disease. This has been reported in other ambystomatid salamanders (Spitzen-van der Sluijs et al., 2011; Davidson et al., 2003; Padgett-Flohr, 2008) and this may be linked to the production of skin peptides that inhibit the growth of Bd (Sheafor et al., 2008). Our data suggest that all three species investigated here may be able to act as reservoirs of Bd, at least within captive populations and, potentially, in nature.

In *A. dumerilii*, infection loads, in terms of GE per sample, were broadly similar overall between Chester Zoo and ZSL (Table 1), but variation within species was substantial. All but one swabbed animal (ZSL; 2964.12 GE) had very low loads. *Ambystoma mexicanum* in a Bd positive laboratory colony were reported to have loads of 0 - 1726.29 GE (Frias Alvarez et al., 2008). Although the highest infection load in *A. dumerilii* is approximately 40% higher than the maximum infection load reported in *A. mexicanum*, no measure of variation was given by Frias-Alverez et al. (2008) and so direct comparison with our data is not possible. The use of pooled swabbing at Parc de Thoiry for *A. dumerilii*, and for *A. andersoni* and *A. mexicanum* in the private collection precluded any estimation of infection intensity and so comparisons with the literature are not possible.

For unknown reasons, Bd was not detected on some *A. dumerilii* individuals within Bd positive groups; this can probably be regarded as representing the bottom end of the detected variation in infection loads between infected salamanders. This observation mirrors circumstances reported in colonies of *A. mexicanum* in both laboratory (Frias Alvarez et al., 2008) and zoo (Galindo-Bustos et al., 2014) settings. Labial swabs, alongside samples from other sites, were collected as some larval ambystomatid salamanders possess keratinized jaw sheaths that may act as infection foci for Bd (Venesky et al., 2010) as well as keratin elsewhere on the body (Bosch and Martinez-Solano, 2006), and so it is likely that swabbing was as efficient as possible for the collection of chytrid DNA. Although these results are likely to reflect real negatives, extremely low infection burdens below the detection threshold are also possible.

All animals in this study tested negative for Bsal infection, although the animals were maintained within the optimal temperature range for this fungus (Martel et al., 2013; Blooi et al., 2015a). Negative results are important in delineating the overall presence of Bsal

in captive populations. These results contribute to the current belief that Bsal infection is still relatively rare in captive urodelans (Sabino-Pinto et al., 2016).

Our results show that Bd infection can be eliminated using the established anti-fungal chemical itraconazole in neotenic *A. dumerilii*, *A. mexicanum* and *A. andersoni*. Treatment with itraconazole of confirmed Bd infection in neotenic *Ambystoma* has not been previously reported. Metamorphosed *A. tigrinum* were successfully cleared of Bd infection using a similar protocol to that described here (10 minute 0.01% itraconazole (Itrizole oral solution) baths every other day for seven treatments; Tamukai et al., 2011). The variations employed by Thoiry and the private keeper demonstrate that Bd infection can be treated, at least in this case, by using a lower itraconazole solution (0.005%) and shorter bath duration (5 minutes) than the 0.01% and 10-minute immersion time typically used for treating Bd infection (Jones et al., 2012). This is congruent with trials in the anurans *Litoria caerulea* and *Anaxyrus baxteri* (0.005% itraconazole baths in Sporanox form; Jones et al., 2012) and indicates that low dosage and short immersion time may be useful in a wide range of amphibian taxa, at least with low infection loads.

Different preparations of itraconazole appear to have different effects and efficacy in different *Ambystoma* species. The granule (Sporanox) preparation of itraconazole can apparently be used without deleterious side effects in *A. dumerilii* (this study) and *A. mexicanum* (Forzan et al., 2008), while liquid preparations are apparently safe for use in *A. mexicanum* (this study; Itrafungol) and in *A. tigrinum* (Itrizole Oral Solution 1%, Janssen Pharmaceutical K.K., Tokyo, Japan; Tamukai et al., 2011). However, our data suggest that use of the liquid preparation (Itrafungol) of itraconazole may be linked to rapid morbidity and mortality in *A. dumerilii* and *A. andersoni*. As water quality was not measured in collections using Itrafungol, and as other aspects of husbandry including disinfection of filter media co-varied with treatment regimen, it is possible that detrimental effects observed were caused by factors other than the drug. However, deleterious side-effects have also been recorded in anuran tadpoles treated with Itrafungol (Garner et al., 2009). We found no evidence in the literature suggesting either safe use or toxic effects of any non-itraconazole ingredient (see Supplementary Materials) of Itrafungol on amphibians. Although the active ingredient is the same in both compounds (itraconazole), there may also have been interactive effects between itraconazole and other compounds in the drug, for example through effects on bioavailability of the active compound. The Itrafungol solution was buffered at PB, but not at CZ. This may have an effect on its efficacy against Bd (e.g. pH affects Bd growth; Piotrowski et al., 2004) and any side-effects on the salamanders themselves, but this preparation led to morbidity and mortality in both collections. We recommend that the use of this preparation should probably be treated with caution in ambystomatid salamanders.

The deleterious side-effects reported here represent only impacts on health that can be detected in the short term. It is possible that other effects on health may be more subtle or require more longitudinal studies to detect. Furthermore, the treatment designs used here were based on previous reports of successful treatments and do not represent targeted or evidence-based approaches. The use of data from in vitro exposures of fungus to candidate treatment regimens could inform the selection of the lowest dose and shortest exposures possible to successfully eliminate the pathogen (e.g. Martel et al., 2011). Such an approach may avoid negative side-effects and reduce the chance of unforeseen negative outcomes of treatment attempts, although susceptibility of the fungus in vitro does not necessarily equate to successful therapy in vivo (Berger et al., 2010).

We also demonstrate that Bd infection can be successfully treated without sterilisation of biological filters. This is congruent with Rendle et al. (2015) and reinforces that a balance can be struck between effective therapy and the maintenance of appropriate environmental parameters. By disinfecting biological filters between itraconazole treatments, and unless other methods of dealing with nitrogenous waste (e.g. chemical filtration) are employed, the environment in which animals are kept may rapidly become toxic due to the accumulation of waste products. As Bd can, on the basis of these and other data, be eliminated without the disinfection of the environment, biological filters may be left intact during the treatment of aquatic amphibians for Bd. Bd can survive outside amphibian hosts (Johnson & Speare, 2003). We were unable to determine if infective colonies of Bd survived on the filter media post treatment, but repeated and long term negative Bd results suggest that such colonies were either absent or at least unable to re-infect salamanders.

ACKNOWLEDGEMENTS

The authors would like to extend our thanks to keeping and veterinary staff at ZSL (Iri Gill, Luke Harding, Daniel Kane, Joe-Smiley Capon-Doyle, Martin Franklin, Nic Masters, Heather Macintosh, Sophie Sparrow, Mary-Anne Jones and Jo Korn), at Chester Zoo and at Parc de Thoiry (Roseline Chambaud, Fanny Grados and Guillaume Vialaret). We would also like to thank Mark Pickstock, Debbie Moore and Pinmoore Animal Laboratory Services for providing us with data for *A. mexicanum* and *A. andersoni*.

REFERENCES

Berger, L., Speare, R., Hines, H.B., Marantelli, G., Hyatt, A.D., McDonald, K.R., Skerratt, L.F., Olsen, V., Clarke, J.M., Gillespie, G. & Mahony, M. (2004). Effect of season and temperature on mortality in amphibians due to chytridiomycosis. *Australian Veterinary Journal* 82, 434–439.

Berger, L., Speare, R., Pessier, A., Voyles, J. & Skerratt, L.F., (2010). Treatment of chytridiomycosis requires urgent clinical trials. Diseases of Aquatic Organisms 92, 165-174.

Blooi, M., Pasmans, F., Longcore, J.E., Spitzen-van der Sluijs, A., Vercammen, F. & Martel, A. (2013). Duplex real-time PCR for rapid simultaneous detection of *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* in

amphibian samples. *Journal of Clinical Microbiology* 51, 4173–4177.

Blooi, M., Martel, A., Haesebrouck, F., Vercammen, F., Bonte, D. & Pasmans, F. (2015a). Treatment of urodelans based on temperature dependent infection dynamics of *Batrachochytrium salamandrivorans*. Scientific Reports 5, 8037.

Blooi, M., Pasmans, F., Rouffaer, L., Haesebrouck, F,. Vercammen, F. & Martel, A. (2015b). Successful treatment of *Batrachochytrium salamandrivorans* infections in salamanders requires synergy between voriconazole, polymyxin E and temperature. *Scientific Reports* 5, 11788.

Bosch, J., Martínez-Solano, I. & García-París, M. (2001). Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biological Conservation* 206, 331–7.

Bosch, J. & Martínez-Solano, I. (2006). Chytrid fungus infection related to unusual mortalities of *Salamandra salamandra* and *Bufo bufo* in the Penalara Natural Park, Spain. *Oryx* 40, 84–89.

Bowerman, J., Rombough, C., Weinstock, S.R. & Padgett-Flohr, G.E. (2010). Terbinafine hydrochloride in ethanol effectively clears *Batrachochytrium dendrobatidis* in amphibians. *Journal of Herpetological Medicine and Surgery* 20, 24–28.

Brannelly, L.A., Richards-Zawacki, C.L. & Pessier, A.P. (2012). Clinical trials with itraconazole as a treatment for chytrid fungal infections in amphibians. *Diseases of Aquatic Organisms* 101, 95–104.

Briggs, C.J., Knapp, R.A. & Vredenburg V.T. (2010). Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proceedings of the National Academy of Sciences* 107, 9695–9700.

Bury, R.B. (2008). Low thermal tolerances of stream amphibians in the Pacific north-west, Implications for riparian and forest management. *Applied Herpetology* 5, 63–74.

Chatfield, M.W. & Richards-Zawacki, C.L. (2011). Elevated temperature as a treatment for *Batrachochytrium dendrobatidis* infection in captive frogs. *Diseases of Aquatic Organisms* 94, 235–238.

Davidson, E.W., Parris, M., Collins, J.P., Longcore, J.E, Pessier, A.P. & Brunner, J. (2003). Pathogenicity and transmission of chytridiomycosis in tiger salamanders (*Ambystoma tigrinum*). *Copeia* 2003, 601–7.

Fisher, M.C., Garner, T.W. & Walker, S.F. (2009). Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. *Annual Review of Microbiology* 63, 291–310.

Forzan, M., Gunn, H. & Scott, P. (2008). Chytridiomycosis in an aquarium collection of frogs, diagnosis, treatment, and control. *Journal of Zoo and Wildlife Medicine* 39, 406–411.

Frías-Alvarez, P., Vredenburg, V.T., Familiar-López, M., Longcore, J.E., González-Bernal, E., Santos-Barrera, G., Zambrano, L. & Parra-Olea, G. (2008). Chytridiomycosis survey in wild and captive Mexican amphibians. *EcoHealth* 5, 18–26.

Galindo-Bustos, M.A., Hernandez-Jauregui, D.M.B., Cheng, T., Vredenburg V. & Parra-Olea, G. (2014). Presence and prevalence of *Batrachochytrium dendrobatidis* in commercial amphibians in Mexico City. *Journal of Zoo and Wildlife Medicine* 45, 830–835.

Garner, T.W.J., Garcia, G., Carroll, B. & Fisher, M.C., (2009). Using itraconazole to clear *Batrachochytrium dendrobatidis* infection, and subsequent depigmentation of *Alytes muletensis* tadpoles. *Diseases of Aquatic Organisms* 83, 257–260.

Gower, D.J., Doherty-Bone, T., Loader, S.P., Wilkinson, M., Kouete, M.T., Tapley, B., Orton, F., Daniel, O.Z., Wynne, F., Flach, E. & Müller, H. (2013). *Batrachochytrium dendrobatidis* infection and lethal chytridiomycosis in caecilian amphibians (Gymnophiona). *EcoHealth* 10,173–83.

Hyatt, A.D., Boyle, D.G., Olsen, V., Boyle, D.B., Berger, L., Obendorf, D., Dalton, A., Kriger, K., Heros, M., Hines, H., Phillott, R., Campbell, R., Marantelli, G., Gleason, F. & Coiling, A. (2007). Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms* 73, 175–192.

Johnson, M.L., Berger, L., Philips, L. & Speare, R. (2003). Fungicidal effects of chemical disinfectants, UV light, desiccation and heat on the amphibian chytrid *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms* 57, 255–260.

Johnson, M.L. & Speare, R. (2003). Survival of *Batrachochytrium dendrobatidis* in water, quarantine and disease control implications. *Emerging Infectious Diseases* 9, 922–925.

Jones, M.E., Paddock, D., Bender, L., Allen, J.L., Schrenzel, M.D. & Pessier, A.P. (2012). Treatment of chytridiomycosis with reduced-dose itraconazole. *Diseases of Aquatic Organisms* 99, 243–249.

Laking, A.E., Ngo, H.N., Pasmans, F., Martel, A. & Nguyen, T.T. (2017). *Batrachochytrium salamandrivorans* is the predominant chytrid fungus in Vietnamese salamanders. *Scientific Reports* 7, 44443.

Lips, K.R., Brem, F., Brenes, R., Reeve, J.D., Alford, R.A., Voyles, J., Carey, C., Livo, L., Pessier, A.P. & Collins, J.P. (2006). Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings of the National Academy of Sciences* 103, 3165–70.

Liu, J., Tan ,Y., Tan, Q., He, X., Zhang, Y. & Liu, M. (2006). Research on Chinese giant salamander F2 adaptability and growth advantages. *Sichuan Journal of Zoology* 25, 387–390.

Longcore, J.E., Pessier, A.P. & Nichols, D.K. (1999). *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia* 91, 219–227.

Martel, A., Van Rooij, P., Vercauteren, G, Baert, K., Van Waeyenberghe, L., Debacker, P., Garner, T.W., Woeltjes, T., Ducatelle, R., Haesebrouck, F. & Pasmans, F. (2011). Developing a safe antifungal treatment protocol to eliminate *Batrachochytrium dendrobatidis* from amphibians. *Medical Mycology* 49, 143–149.

Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M.C., Woeltjes, A., Bosman, W., Chiers, K., Bossuyt, F. & Pasmans, F. (2013). *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. *Proceedings of the National Academy of Sciences* 110, 15325–15329.

Martel, A., Blooi, M., Adriaensen, C., Van Rooij, P., Beukema, W., Fisher, M.C., Farrer, R.A., Schmidt, B.R, Tobler, U., Goka, K,. & Lips, K.R. (2014). Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. *Science* 346, 630–631.

Padgett-Flohr, G.E. (2008). Pathogenicity of *Batrachochytrium dendrobatidis* in two threatened California amphibians,

Rana draytonii and *Ambystoma californiense*. *Herpetol Conservation Biology* 3,182–91.

Piotrowski, J.S., Annis, S.L. & Longcore, J.E., (2004). Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia* 96, 9-15.

Rendle, M.E., Tapley, B., Perkins, M., Bittencourt-Silva, G., Gower, D.J. & Wilkinson, M. (2015). Itraconazole treatment of *Batrachochytrium dendrobatidis* (Bd) infection in captive caecilians (Amphibia, Gymnophiona) and the first case of Bd in a wild neotropical caecilian. *Journal of Zoo and Aquarium Research* 3, 137–140.

Retallick, R.W. & Miera, V. (2007). Strain differences in the amphibian chytrid *Batrachochytrium dendrobatidis* and non-permanent, sub-lethal effects of infection. *Diseases of Aquatic Organisms* 75,201–207.

Richgels, K.L., Russell, R.E., Adams, M.J., White, C.L & Grant, E.H.C. (2016). Spatial variation in risk and consequence of *Batrachochytrium salamandrivorans* introduction in the USA. *Royal Society Open Science* 3, 150616.

Sabino-Pinto, J., Bletz, M.C., Islam, M.M., Shimizu, N., Bhuju, S., Geffers, R., Jarek, M., Kurabayashi, A. & Vences, M. (2016). Composition of the cutaneous bacterial community in Japanese amphibians, effects of captivity, host species, and body region. *Microbial Ecology* 72, 460–469.

Scheele, B.C., Skerratt, L.F., Grogan, L.F., Hunter, D.A, Clemann, N., McFadden, M., Newell, D., Hoskin, C.J., Gillespie, G.R., Heard, G.W. & Brannelly, L. (2017). After the epidemic, Ongoing declines, stabilizations and recoveries in amphibians afflicted by chytridiomycosis. Biological Conservation 206, 37–46.

Sheafor, B., Davidson, E.W., Parr, L. & Rollins-Smith, L. (2008). Antimicrobial peptide defenses in the salamander, *Ambystoma tigrinum*, against emerging amphibian pathogens. *Journal of Wildlife Diseases* 44, 226–36.

Spitzen-van der Sluijs, A., Martel, A., Wombwell, E., Van Rooij, P., Zollinger, R., Woeltjes, T., Rendle, M., Haesebrouck, F. & Pasmans, F. (2011). Clinically healthy amphibians in captive collections and at pet fairs, a reservoir of *Batrachochytrium dendrobatidis*. *Amphibia-reptilia* 32, 419–23.

Spitzen-van der Sluijs, A., Martel, A., Asselberghs, J., Bales, E.K., Beukema, W., Bletz, M.C., Dalbeck, L., Goverse, E., Kerres, A., Kinet, T. & Kirst, K. (2016). Expanding distribution of lethal amphibian fungus *Batrachochytrium salamandrivorans* in Europe. *Emerging Infectious Diseases* 22, 1286–1288.

Tamukai, K., Une, Y., Tominaga, A., Suzuki, K. & Goka, K. (2011). Treatment of spontaneous chytridiomycosis in captive amphibians using itraconazole. *The Journal of Veterinary Medical Science* 73, 155–159.

Venesky, M.D., Parris, M.J. & Altig, R. (2010). Pathogenicity of *Batrachochytrium dendrobatidis* in larval ambystomatid salamanders. *Herpetological Conservation Biology* 5, 174–182.

Woodhams, D.C., Alford, R.A. & Marantelli, G. (2003). Emerging disease of amphibians cured by elevated body temperature. *Diseases of Aquatic Organisms* 55, 65–67.

Woodhams, D.C., Geiger, C.C., Reinert, L.K., Rollins-Smith, L.A., Lam, B., Harris, R.N., Briggs, C.J., Vredenburg, V.T. & Voyles, J. (2012). Treatment of amphibians infected with chytrid fungus, learning from failed trials with itraconazole, antimicrobial peptides, bacteria, and heat therapy. *Diseases of Aquatic Organisms* 98, 11–25.

Accepted: 12 February 2018

Please note that the Supplementary Material is available online via the Herpetological Journal website: https://thebhs.org/publications/the-herpetological-journal/volume-28-2-april-2018

Volume 28 (April 2018), 93-95

Herpetological Journal

SHORT NOTE

Published by the British Herpetological Society

Intersexuality in *Helicops infrataeniatus* Jan, 1865 (Dipsadidae: Hydropsini)

Ruth A. Regnet[1], Fernando M. Quintela[1], Wolfgang Böhme[2] & Daniel Loebmann[1]

[1]Universidade Federal do Rio Grande, Instituto de Ciências Biológicas, Laboratório de Vertebrados. Av. Itália km 8, CEP: 96203-900, Vila Carreiros, Rio Grande, Rio Grande do Sul, Brazil

[2]Zoologisches Forschungsmuseum A. Koenig, Adenauerallee 160, D-53113 Bonn, Germany

Herein, we describe the first case of intersexuality in the Hydropsini tribe. After examination of 720 specimens of *Helicops infrataeniatus* Jan, 1865, we discovered one individual that presented feminine and masculine reproductive features. The specimen was 619 mm long, with seven follicles in secondary stage, of different shapes and sizes, and a hemipenis with 13.32 and 13.57 mm in length. The general shape of this organ is similar to that observed in males, although it is smaller and does not present conspicuous spines along its body. Deformities found in feminine and masculine structures suggest that this specimen might not be reproductively functional.

Key words: Follicles, hemipenis, hermaphroditism, water snake.

As all amniotes, lepidosaurian reptiles have internal fecundation and, with the exception of Tuataras, which have no copulatory organ, male specimens have hemipenes (Vitt & Caldwell, 2013). However, some squamate species evolved to break free of sexual reproduction, being the only vertebrates that truly reproduce by parthenogenesis (Kearney et al., 2009; Vitt & Caldwell, 2013). Such mechanism is predominant in lizards (e.g. Darevski, 1966; Hardy & Cole, 1981; Schmidtler, 1993; Schmidtler et al., 1994), but it also occurs in snakes (e.g. Wynn et al., 1987; Groot et al., 2003; Booth et al., 2012).

Another condition reported for reptiles is intersexuality, which is defined as a condition in which reproductive structures in a given sex are also found in the opposite sex (Goldschmidt, 1917). The condition may include both hermaphroditism (presence of both ovarian and testicular tissues) and pseudohermaphroditism (presence of gonadal tissue of one single sex) (Forbes, 1964). Intersexual individuals in squamates have been recorded for snakes only (Hardy, 1970). In lizards, hermaphroditic males are not viable and occur in hybridogenic rock lizards (genus *Darveskia*, see Darvesky 1966).

The Hydropsini tribe currently encompasses a total of 22 valid species allocated in the following genera: *Helicops*, *Hydrops* and *Pseudoeryx* (Uetz et al., 2017). While *Hydrops* and *Pseudoeryx* are oviparous, most species of *Helicops* are viviparous, and interestingly, *H. angulatus* exhibits both reproductive modes (Rossman, 1984; Aguiar & Di-Bernardo, 2005; Braz et al., 2016). *Helicops infrataeniatus* has a wide distribution that encompasses south-southeastern Brazil, southern Paraguay, North-eastern Argentina and Uruguay (Deiques & Cechin, 1991; Giraudo, 2001; Carreira & Maneyro, 2013). At the coastal zone of southernmost Brazil, *H. infrataeniatus* is among the most abundant species in many types of limnic and estuarine environments (Quintela & Loebmann, 2009; Regnet et al., 2017). In October 2015 at the Laranjal beach, municipality of Pelotas, state of Rio Grande do Sul, Brazil (31°46'S, 52°13'W), a remarkable aggregation of reptiles and caecilians occurred after a flood event associated to an El Niño event (Regnet et al., 2017). This event provided an outstanding opportunity to investigate aspects of the reproductive biology of several species with a large sample size. When such specimens were examined, one showed follicles in advanced vitellogenesis as well as a hemipenis, characterising a rare case of intersexuality, which is here described.

The intersexual specimen was deposited in the Herpetological Collection of the Universidade Federal do Rio Grande - FURG (CHFURG 3946). Although the individual presented female and male sexual characteristics, it presents a number of subcaudal scales corresponding to the females of the species (64) (Giraudo, 2001). For comparison purposes, we everted and inflated a total of four males hemipenes as well as follicles in secondary vitellogenesis from a female. For each specimen studied, we measured the hemipenis length as well as snout-vent length (SVL). For standardisation, we used only the length of the right hemipenis. Description of reproductive organs follows Zaher (1999) for hemipenis and Almeida-Santos et al. (2014) for follicles.

Snout-vent length of the intersexual specimen (Fig. 1) was 537 mm, tail length was 82 mm and weight was 103.87 g. The left ovary contained seven follicles in secondary stage, with different shapes and sizes, and follicles of the right ovary were all in the primary stage (Fig. 1). Right and left hemipenes were 13.32 and 13.57 mm long, respectively. Males had SVL ranging from 380 to 432 (mean = 410.5 mm) and right hemipenes length

Correspondence: Ruth A. Regnet (regnet_ruth@hotmail.com)

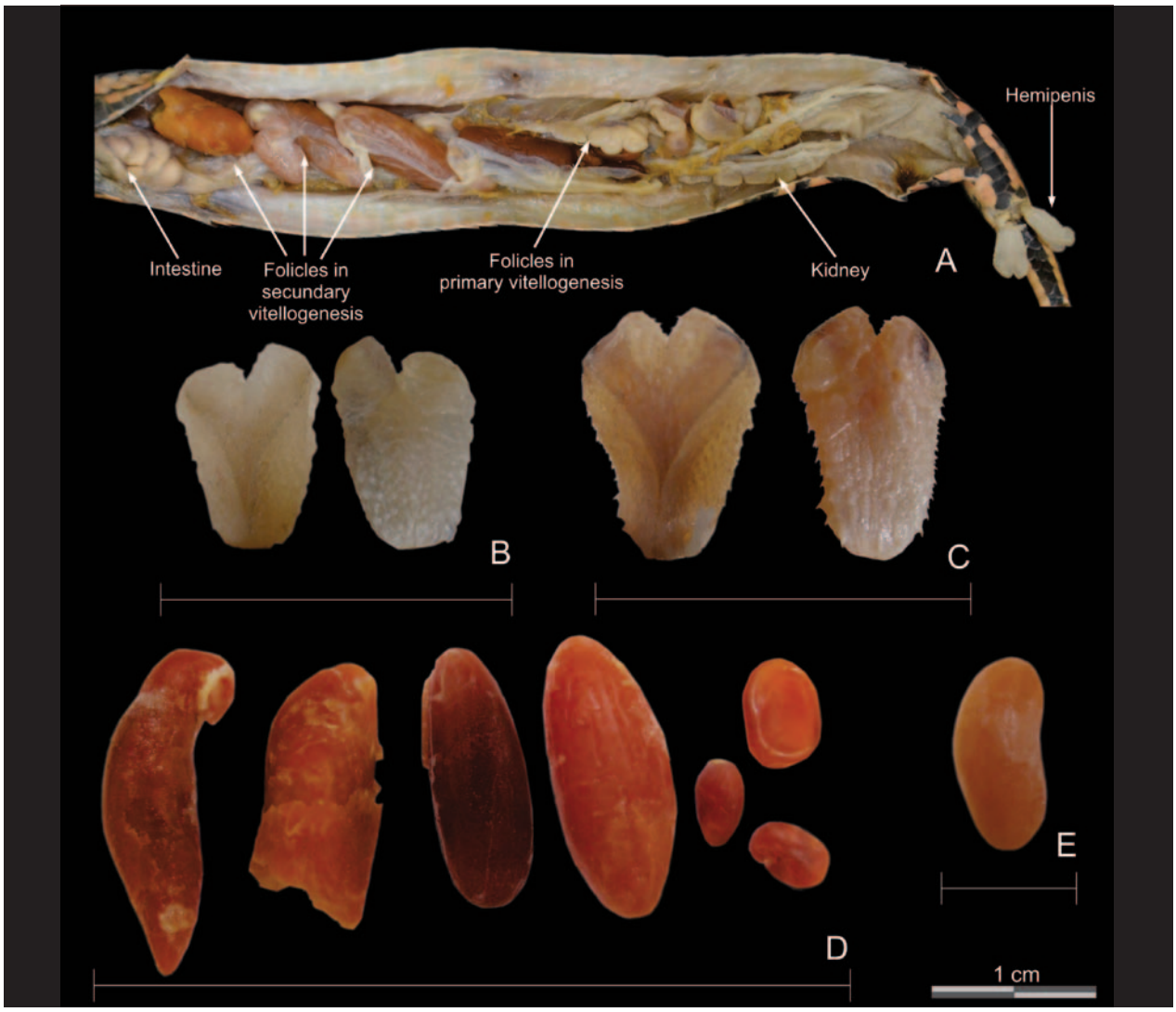

Figure 1. (A) General view of reproductive organs of the intersexual specimen of *H. infrataeniatus*; **(B)** intersexual hemipenis; **(C)** male hemipenis; (**D)** follicles of the intersexual specimen with different shapes and sizes and **(E)** follicle of a normal female.

ranging from 13.79 to 14.61 (mean = 14.17 mm). Relative measures (organ length/SVL) of right hemipenes were 0.025 and 0.035 on average, respectively. General shape of the intersexual hemipenis in both sides (assulcate and sulcate) is similar in both structures, although is smaller (ca. 71.43% in relation to the mean length of hemipenes measured). Both structures are bilobated, but only male hemipenes have conspicuous spines along their body (Fig. 1).

The intersexual specimen here described was detected in a large sample of 720 individuals (327 females and 376 males), that is, it represents only 0.14% of the studied population. Therefore, the condition should be considered rare for this species. Aguiar & Di-Bernardo (2005) provide biological data on the reproduction of *H. infrataeniatus* and, when comparing those data to the observation of the intersexual specimen, the number of follicles found is to be expected. However, follicles from the intersexual specimen were highly variable in size, shape and color as well, so it is possible that intersexual follicles may not be viable.

Intersexual condition in squamates is usually evidenced by the presence of erectile organs in females similar to the male hemipenis (Forbes, 1964). It is important to emphasise that this condition differs from individuals that exhibit hemiclitoris, a homologous structure to the hemipenis in females, but with smaller size (Böhme, 1995). Examples of females with hemiclitoris seem to be more frequent in squamates than intersexuality (e.g. Böhme, 1995; Ziegler & Böhme, 1997, Kasperoviczus et al., 2011), which has been reported only for snakes (e.g. *Bothrops insularis* (Hoge et al., 1954), *Bothrops moojeni* (Mcclean, 1968), *Pseudoficimia frontalis* (Hardy, 1970), and *Pareas stanleyi* (Pope, 1935). Interestingly, *B. insularis* females with hemipenes are frequently found in populations and individuals without this structure seem to be sterile (Hoge et al., 1959).

Seminiferous tubules or testicles were absent in the intersexual specimen and its hemipenis was smaller, more translucent and did not present spines along their body in comparison with males hemipenes. A notorious case regarding confusion in the interpretation of this structure can be found in the description of *Pseudoficimia pulcherrima*, where two females with smaller hemipenes and without spines were described as males of a new species (Taylor & Smith, 1942). After the mistake

was discovered, the species was symonymised with *Pseudoficimia frontalis* (Hardy, 1970).

This is the first report of an intersexual individual in the Hydropsini tribe. The presence of intersexual individuals emphasises the importance to check other sexual features concomitantly (Ziegler & Böhme, 1996) to avoid erroneous sex determination. Although cases of intersexuality are apparently rare in most snake species, we recommend that sexual determination based on the presence of a structure similar to a hemipenis might not be conclusive and, therefore, it should be used other morphological sexual features whenever is possible.

ACKNOWLEDGEMENTS

R. A. Regnet thanks CAPES for the Msc. scholarship and F. M. Quintela is grateful to CAPES for the postdoctoral fellowship.

REFERENCES

Aguiar, L. F. S. & Di-Bernardo, M. (2005). Reproduction of the water snake *Helicops infrataeniatus* (Colubridae) in southern Brazil. *Amphibia-Reptilia* 26, 527-533.

Almeida-Santos, S. M., Braz, H. B. P., Santos, L. C., Barros, V. A., Rojas, C. A. & Kasperoviczus, K. N. (2014). Biologia reprodutiva de serpentes: recomendações para a coleta e análise de dados. *Herpetologia Brasileira* 3, 14-24.

Böhme, W. (1995). Hemiclitoris discovered: a fully differentiated erectile structure in female monitor lizards (*Varanus* spp.) (Reptilia: Varanidae). *Journal of Zoological Systematics and Evolutionary Research* 33, 129-132.

Booth, W., Smith, C. F., Eskridge, P. H., Hoss, S. K., Mendelson, J. R. & Schuett, G. W. (2012). Facultative parthenogenesis discovered in wild vertebrates. *Biology letters* 8, 983-985.

Braz, H. B., Scartozzoni, R. R. & Almeida-Santos, S. M. (2016). Reproductive modes of the South American water snakes: A study system for the evolution of viviparity in squamate reptiles. *Zoologischer Anzeiger* 263, 33-44.

Carreira, S. & Maneyro, R. (2013). *Guía de reptiles del Uruguay*. Ediciones de la Fuga, Montevideo, Uruguay, 285p.

Darevski, I. S. (1966). Natural parthenogenesis in a polymorphic proup of caucasian rock lizards related to *Lacerta saxicola* Eversmann. *Journal of the Herpetological Society* 5, 115-152.

Deiques, C. H. & Cechin, S. Z. (1991). O status de *Helicops carinicaudus* (Wied, 1825) (Serpentes: Colubridae). *Acta biologica leopoldensia* 12, 313-326.

Forbes, T. R. (1964). Intersexuality in reptiles. In *Intersexuality in vertebrates including man*, 273-284. Armstrong, C. N. & Marshall, A. J. (Eds.). Academic Press, New York.

Giraudo, A. R. (2001). *Serpientes de la Selva Paranaense y del Chaco Húmedo*. Literature of Latin America, Buenos Aires, Argentina, 328p.

Groot, T. V. M., Bruins, E. & Breeuwer, J. A. J. (2003). Molecular genetic evidence for parthenogenesis in the Burmese python, *Python molurus bivittatus*. *Heredity* 90, 130-135.

Goldschmidt, R. (1917). Intersexuality and the endocrine aspect of sex. *From the Osborn Zoological Laboratory, Yale University* 1, 433-456.

Hardy, L. M. (1970). Intersexuality in a Mexican Colubrid snake (*Pseudoficimia*). *Herpetologica* 26, 336-343.

Hardy, L. M. & Cole, C. J. (1981). Parthenogenetic reproduction in lizards: histological evidence. *Journal of Morphology* 170, 215-237.

Hoge, A. R., Belluomini, H. E. & Schreiber, G. (1954). Intersexuality in a highly isolated population of snakes. *Atti del IX Congresso Internazionale di Genetica, Caryologica* 964-965.

Hoge, A. R., Belluomini, H. E., Schreiber, G. & Penha, A. M. (1959). Sexual abnormalities in *Bothrops insularis* (Amaral) 1921. *Memórias do Instituto Butantan* 29, 17-88.

Kaspervoviczus, K. N., Santos, L. C. & Almeida-Santos, S. (2011). First report of hemiclitores in a female of the amphisbaenian *Amphisbaena microcephala* (Wagler, 1824). *Herpetology notes* 4, 41-43.

Kearney, M., Fujita, M. K. & Ridenour, J. (2009). Lost sex in the reptiles: constraints and correlations. In *Lost Sex - The evolutionary biology of parthenogenesis*, 447-474. Schön, I., Martens, K. & van Dijk, P. (Eds.). Springer, Dordrecht, Heidelberg, London, New York.

Maclean, W. P. (1968). A case of intersexuality in *Bothrops moojeni* Hoge. *Copeia* 1968, 170.

Pope, C. H. (1935). *The reptiles of China*. American Museum of Natural History. 604 p.

Quintela, F.M. & Loebmann, D. (2009). *Guia Ilustrado: os répteis da região costeira do Rio Grande do Sul*. Pelotas, USEB, 84 p.

Rossman, D. A. (1984). *Helicops angulatus* (South American Water Snake). Reproduction. *Herpetological Review* 15, 50.

Regnet, R. A., Quintela, F. M., Entiauspe-Neto, O. M., Teixeira, V. H. S., Silva, F. L., Loebmann, D. (2017). Remarkable aggregation of squamates and caecilians associated with flood events during El Niño in southern Brazil. *Salamandra* 53, 537-540.

Schmidtler, J. F. (1993). Parthenogenetische Felseideshsen aus de Ost-Türkei. *Die Eidechse* 8, 3-5.

Schmidtler, J. F., Eiselt, J. & Darevsky, I. S. (1994). Untersuchungen an Felseidechsen (*Lacerta saxkola-Gruppe*) in der östlichen Türkei: 3. Zwei neue parthenogenetische Arten. *Salamandra* 30, 55-70.

Taylor, E. H. & Smith, H. M. (1942). Concerning the snake genus *Pseudoficimia Bocourt*. *The University of Kansas science Bulletin* 28, 241-251.

Uetz, P., Freed, P. & Hošek, J. (2017). The Reptile Database, http://www.reptile-database.org, accessed June 5th.

Vitt, L. J. & Caldwell, J. P. (2013). *Herpetology: an introductory biology of amphibians and reptiles*. Academic Press. 776 p.

Ziegler, T. & Böhme, W. (1996). Zur Hemiclitoris der squamaten Reptilien: Auswirkingen auf einige Methoden der Geschlechtsunterscheidung. *Herpetofauna (Weinstadt)* 18, 11-19.

Ziegler, T. & Böhme, W. (1997). Genitalstrukturen und Paarungsbiologie bei squamaten Reptilien, speziell den Platynota, mit Bemerkungen zur Systematik. *Mertensiella, Supplement zu Salamandra* 8, 1-207.

Zaher, H. (1999). Hemipenial morphology of the South American xenodontine snakes, with a proposal for a monophyletic Xenodontinae and a reappraisal of colubroid hemipenes. *Bulletin of the American Museum of Natural History* 240, 1-168.

Wynn, A., Charles, H., Cole, J. & Gardner. A. L. (1987). Apparent triploidy in the unisexual brahminy blind snake, *Ramphotyphlops braminus*. *American Museum Novitates* 2868, 1-7.

Accepted: 2 December 2017

Volume 28 (April 2018), 96-99

Herpetological Journal

SHORT NOTE

Published by the British Herpetological Society

New record of the introduced species *Eleutherodactylus planirostris* (Anura: Eleutherodactylidae) in the state of Veracruz, Mexico

Angel Ivan Contreras-Calvario[1,2], Abigail Mora-Reyes[2], Gabriela Parra-Olea[3] & Ángela María Mendoza[3,4]

[1] Universidad Veracruzana, Facultad de Ciencias Biológicas y Agropecuarias, Campus Peñuela. Camino Peñuela- Amatlán S/N Peñuela, Amatlán de los Reyes, Veracruz C. P. 94945, Mexico

[2] Herpetario Palancoatl. Av. 19 #5225 Col. Nueva Esperanza, Córdoba, Veracruz C.P. 94540, Mexico

[3] Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, PO 70-153, 04510 México City, Mexico

[4] Grupo de Investigación en Ecología y Conservación Neotropical, Samanea Foundation, 760046 Cali, Colombia

Numerous direct developing species of the genus *Eleutherodactylus* native to the Caribbean Islands have been introduced outside its natural range by human activities. The greenhouse frog, *Eleutherodactylus planirostris* is native to Cuba and the Bahamas and has been introduced to many parts of the world. Here, we report the rediscovery of *E. planirostris* in the Mexican Gulf. The species was not reported in the region since 1974. Molecular identification of the species was possible by comparing 16S and COI sequences with samples from the type locality, five introduced populations and 20 other *Eleutherodactylus* species. The species was also verified by morphological characters. By means of phylogenetic reconstruction we propose that its introduction in Veracruz is independent to the Mexican Caribbean event. This is the first record of the species in a small rural region from Veracruz, and thus a comprehensive evaluation of the distribution of the species in Mexico is needed.

Key words: Introduced species, DNA barcoding, Mexico, *Eleutherodactylus*

Introduced species have been considered among the main threats for the preservation of biodiversity worldwide (Bellard et al., 2016). Among the amphibians, several Antillean *Eleutherodactylus* species have been introduced to new areas mainly through the trade of ornamental plants (Kraus et al.,1999; Kaiser et al., 2002). For example, *Eleutherodactylus coqui* is listed as one of the world's worst invasive alien species by IUCN (Lowe et al., 2000), and *Eleutherodactylus johnstonei* has been widely introduced, currently present in the Caribbean countries and recently recorded in Brazil (Kaiser, 1997; Ernst et al., 2011; Melo et al., 2014).

The greenhouse frog (*Eleutherodactylus planirostris*) has been introduced to many regions throughout the world such as continental and island USA (including Hawaii), Hong Kong, Guam, Philippines, Jamaica, Honduras, Panama and Suriname (McCrane et al., 2008; Crawford et al., 2011; Heinicke et al., 2011; Olson et al., 2012; McCranie & Valdés-Orellana 2014; Lee et al., 2016). In Mexico, it was reported on one occasion for the state of Veracruz, 43 years ago (Schwartz, 1974) at the port of Veracruz (Flores-Villela & MCoy, 1993), and recently in the Yucatan Peninsula (Cedeño-Vázquez et al., 2014; García-Balderas et al., 2016; Pavón-Vázquez et al., 2016; Gómez-Salazar & Cedeño-Vázquez, 2017; Ortíz-Medina et al., 2017). Molecular data from specimens collected in the Mexican Caribbean showed they are closely related to populations from Philippines and Panama (Cedeño-Vázquez et al., 2014).

As shown by Crawford et al. (2011), "the identification of invasive species in new localities may be difficult, especially when local knowledge and comparative material of the invader may be limited". The identification of introduced Caribbean *Eleutherodactylus* species is a challenge, considering their huge diversity (191 species to date) and low morphological variation. In this case, DNA sequencing provided characters to assist in species identification. Here, we report the rediscovery of *E. planirostris* in the Mexican Gulf, and we discuss the application of molecular tools in the detection of introduced species.

During field surveys performed on Ejido La Laja (Cuichapa Municipality) in Veracruz, Mexico (18°45.17' N, 96°47.13' W, 423 m. Figure 1A), we recorded an *Eleutherodactylus* population in the leaf litter and under trunks in a house garden. The morphology of the individuals did not coincide with any of the species known for the region. The individuals were found in the same site by visual and acoustic records in April, September and November 2016. Six individuals were captured and voucher specimens were deposited in the Colección Nacional de Anfibios y Reptiles (CNAR), Instituto de

Correspondence: Angela M. Mendoza (am.mendozah@gmail.com)

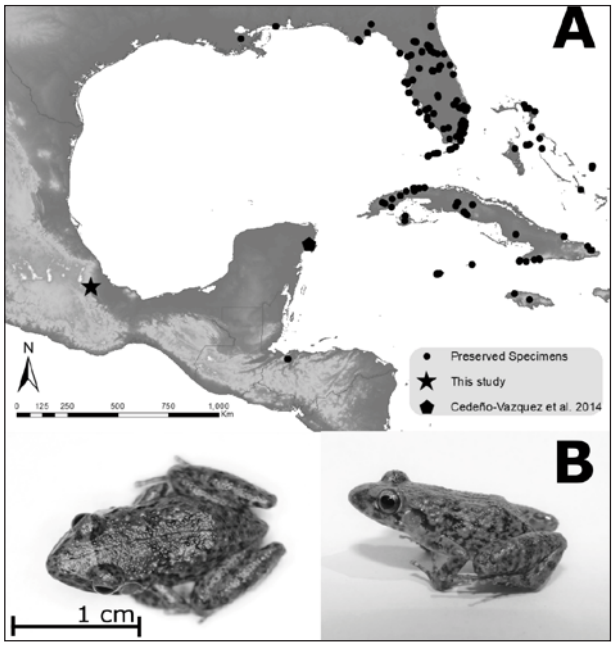

Figure 1. (A) Map with localities recorded for *E. planirostris* near the Gulf of Mexico. The record of Schwartz (1974) from Veracruz, Mexico has no locality details so it was omitted in the map. **(B)** Specimen of *E. planirostris* (IBH-31562) captured in Ejido La Laja, Municipality Cuichapa, Veracruz, México.

Biología, UNAM. Liver and muscle tissue were collected for two individuals (IBH-31562-63) and stored in RNA-laterTM Tissue Storage Reagent (Ambion).

We extracted DNA using the modified protocol of phenol-chloroform (Sambrook & Russell, 2006). For molecular identification, we amplified the mitochondrial genes COI and 16S. PCR amplifications were performed using the primers and procedures detailed in Mendoza et al. (2012) for 16S and Hebert et al. (2004) for COI. We included sequences from GenBank and BOLD databases for 21 species of *Eleutherodactylus* with available information for COI gene and/or species recorded for Mexico (*E. cystignathoides*, *E. marnockii*, *E. nitidus*, *E. pipilans* and *E. planirostris*). Those species are distributed in the states of Veracruz, Queretaro, Guerrero, Oaxaca and Chiapas (Flores-Villela & McCoy, 1993; Lemos-Espinal & Smith, 2007). The sequences obtained were aligned with Geneious using default settings and verified visually. We calculated pairwise distances using the Kimura 2 Parameter model in MEGA7 (Kumar et al., 2016) and performed a Bayesian phylogenetic analysis using the program MrBayes 3.2.2 (Ronquist & Huelsenbeck, 2003; Ronquist et al., 2012). The models of nucleotide substitution were defined following Crawford et al. (2010): one data partitions scheme for 16S (model GTR+I+Γ) and three data partitions for codon positions 1 through 3 in COI (models GTR+I, GTR+Γ, and GTR+I+Γ, respectively). Rates of evolution were allowed to vary across partitions using a rate multiplier. We ran two independent analyses for 20 million generations, each sampling trees and parameter values every 1000 generations. Burn-in was set to 25% and thus the first 5 million generations were discarded. A sequence of *Diasporus quitiddus* (AJC-1789) recovered from GenBank was used as outgroup. Considering that available COI and 16S sequences for *Eleutherodactylus* belong to different sets of species, we generated two analyses (one per gene). Sequences obtained were deposited in the Genbank repository (accession numbers MF374458-MF374461).

The BLAST of both genes matched with *E. planirostris* sequences, with a 99% identity, 0% gaps and an e-value of 0.0. Online BOLD identification generated a match with *E. planirostris* for COI sequences with 99.84% identity. Bayesian trees for both genes corroborate this result, placing the sequences obtained in the *E. planirostris* clade (Figure 2). The K2P estimated distances show a low divergence of our two sequences to all other *E. planirostris* sequences (0.000-0.016 for 16S and 0.002-0.017 for COI, minimum interspecific distance was 0.045 for 16S and 0.040 for COI). COI tree shows a closer relationship between our samples and those from Cuba than to samples than to samples reported by Cedeño-Vázquez et al. (2014) in the Mexican Caribbean (not available for 16S). To confirm the molecular results, we verified the morphological characters of the species, which coincided with the species description (snout-vent length less than 34 mm, finger discs slightly expanded and absence of interdigital webbing in toes; Schwartz, 1974; Köhler, 2010). *Eleutherodactylus planirostris* is native to the Caribbean islands including Bahamas, Cayman Islands Caicos Islands and Cuba (Lee et al., 2016). This species appears to be a generalist, occupying a diversity of habitats including mesic and xeric broadleaf forest as well as secondary forests, shrub land, agricultural fields, near fishponds, urban gardens and parks, and near human settlements (Lee et al., 2016; García-Balderas et al., 2016; Gómez-Salazar & Cedeño-Vázquez, 2017; Ortíz-Medina et al., 2017).

New records of *E. planirostris* have been achieved by help of molecular tools. The species was first reported in Panama (Crawford et al., 2011) and Hong Kong (Lee et al., 2016) through DNA barcoding. In Honduras, molecular data from three specimens showed them to be genetically identical to the Florida-western Cuba populations (McCrane et al., 2014). Cedeño-Vázquez et al. (2014) identified it on the Mexican Caribbean through molecular and morphological information. In the Mexican Gulf, there are no more records of the species after the record of Schwartz (1974) despite being a region constantly studied. It is possible that this previous record was a non-successful introduction. Here, we report the presence of a population 1 000 km west of the recent observation (Cedeño-Vasquez et al., 2014, Figure 1) and 83 km from the record by Schwartz (1974), and we may infer by the Bayesian tree that both populations are coming from independent introduction events. The invasion in Mexican Caribbean in the Yucatan Peninsula may be related to the same dispersal event that occurred for Philippines and Panama, while the Veracruz samples are more related to Cuban populations. Thus, *E. planirostris* is being introduced into Mexico from different source populations at different times.

In general, the introduced species of *Eleutherodactylus* have been reported in big cities or in localities with high commercial trade. *Eleutherodactylus planirostris* has not been recorded for the Veracruz state in the last 43 years. Thus, the presence of *E. planirostris* in Cuichapa is an

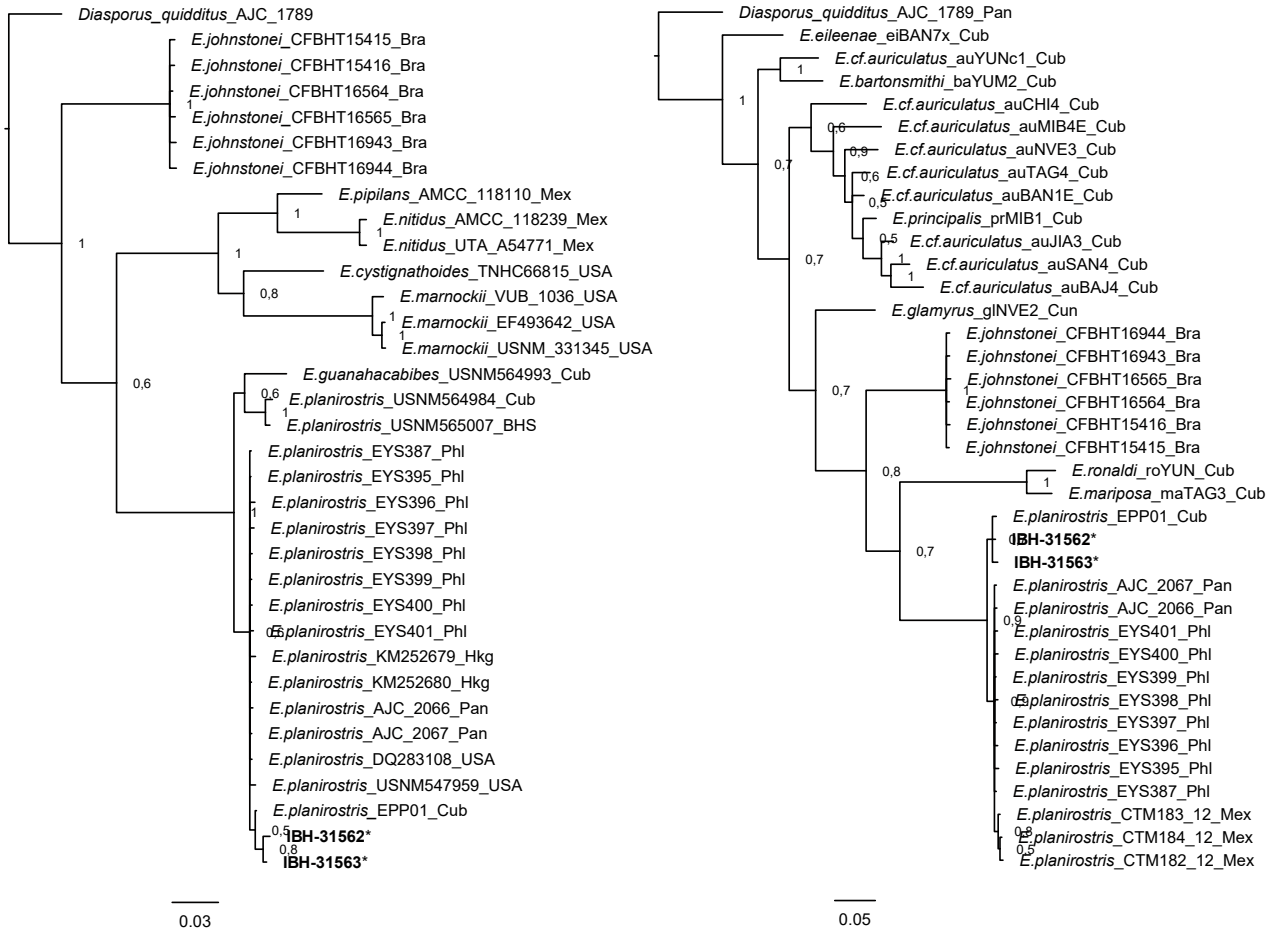

Figure 2. Phylogenetic position of two samples of *E. planirostris* from Veracruz, Mexico in a phylogenetic tree of this species as inferred from Bayesian Analyses (16S rRNA left and COI right). The samples with code IBH (in bold) were obtained for this study, while the other sequences were obtained from GenBank. Country of origin is by the corresponding ISO 3166-1 3-letter country code.

important record for expansion of this invasive species in Mexico. Cuichapa is a small rural municipality with 12,375 inhabitants according to the Instituto Nacional de Estadística y Geografía (INEGI) census (INEGI, 2009). The presence of the species in small rural regions (likely by ornamental plant trade) implies an extensive route of dispersal likely with multiple halfway localities where the species can also establish itself.

When introduced species become established, they feed, compete for food, transform and destroy the habitat, and carry transmissible diseases and parasites, capable of exterminating whole populations of native species (Williamson, 1996). Kraus et al. (1999) and Kraus & Campbell (2002) suggest that invasive populations of *E. planirostris* in Hawaii may be a serious threat to native arthropods, generating a new predation pressure primarily for insects and spiders. In Hawaii, specimens of *E. planirostris* with a population density of 12,522 frogs/ha were found to primarily consume leaf-litter invertebrates and were estimated to consume up to 129,000 invertebrates ha^{-1} $night^{-1}$ (Olson & Beard, 2012). The only population survey of this species in Mexico recorded a density of 20.3 individuals/km in Playa del Carmen, Quintana Roo (Gómez-Salazar & Cedeño-Vasquez, 2017), thus demonstrating that the species was effectively established in the sampled area. The reappearance of the species in the Mexican Gulf implies that an evaluation of the population status and the threats for biodiversity in Mexico is urgently needed, including population density, encounter frequency, spread to nearby localities, and arthropod species consumed.

ACKNOWLEDGEMENTS

This research was partially supported by UNAM PAPIIT: 203617. AMM was supported by a scholarship from Consejo Nacional de Ciencia y Tecnologia (CONACyT, Mexico), through Posgrado de Ciencias Biologicas of the Universidad Nacional Autónoma de Mexico (UNAM). Thanks to Mirna G. García by her support in the molecular analysis and two anonymous reviewers for their comment to earlier versions of the manuscript.

REFERENCES

Beard, K. H., Price, E. A., & Pitt, W. C. (2009). Biology and Impacts of Pacific Island Invasive Species. 5. *Eleutherodactylus coqui*, the Coqui Frog (Anura: Leptodactylidae) 1. *Pacific Science* 63, 297-316.

Bellard, C., Cassey, P., & Blackburn, T. M. (2016). Alien species as a driver of recent extinctions. *Biology Letters* 12, 20150623.

Cedeño-Vázquez, J. R., González-Vázquez, J., Martínez-Arce, A., & Canseco-Márquez, L. (2014). First record of the

invasive greenhouse frog (*Eleutherodactylus planirostris*) in the Mexican Caribbean. *Revista Mexicana de Biodiversidad* 85, 650-653.

Crawford, A. J., Lips, K. R., & Bermingham, E. (2010). Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in the highlands of central Panama. *Proceedings of the National Academy of Sciences* 107, 13777-13782.

Crawford, A. J., Alonso, R., Jaramillo, C.A., Sucre, S. & Ibáñez, R. (2011). DNA barcoding identifies a third invasive species of *Eleutherodactylus* (Anura: Eleutherodactylidae) in Panama City, Panama. *Zootaxa* 2890, 65-67.

Ernst, R., Massemin, D., & I. Kowarik. (2011). Non-invasive invaders from the Caribbean: the status of Johnstone's Whistling frog (*Eleutherodactylus johnstonei*) ten years after its introduction to Western French Guiana. *Biological Invasions* 13, 1767–1777.

Flores-Villela, O., & McCoy C. J. (1993) Herpetofauna Mexicana, Lista anotada de las especies de anfibios y reptiles de México, cambios taxonómicos recientes y nuevas especies. *Carnegie Museum of Natural* History, 17.

García-Balderas, C. M., Cedeño-Vázquez, J. R., & Mineros-Ramírez, R. (2016). *Eleutherodactylus planirostris*. Distribution Notes. *Mesoamerican Herpetology* 3, 173.

Gómez-Salazar, A. A., & Cedeño-Vázquez, J. R. (2017). Distribución y abundancia de la rana de invernadero, *Eleutherodactylus planirostris* (Cope, 1862), en Playa del Carmen, Quintana Roo, México. *Teoría y Praxis* 21, 55-66.

Hebert, P. D., Penton, E. H., Burns, J. M., Janzen, D. H., & Hallwachs, W. (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences of the United States of America* 101, 14812-14817.

Heinicke, M. P., Diaz, L. M., & Hedges, S. B. (2011). Origin of invasive Florida frogs traced to Cuba. *Biology Letters* 7, 407-410.

INEGI. (2009). Instituto Nacional de Estadística, Geografía e Informática. México. Http://www.inegi.org.mx/inegi/default.aspx [accessed 1 June 2017].

Kaiser, H. (1997). Origins and introductions of the Caribbean frog, *Eleutherodactylus johnstonei* (Leptodactylidae): management and conservation concerns. *Biodiversity and conservation* 6, 1391-1407.

Kaiser, H., Barrio-Amorós, C. L., Trujillo, J. D., & Lynch, J. D. (2002). Recent population changes-expansion of *Eleutherodactylus johnstonei* in Northern South America: Rapid Dispersal Through Human Interactions. *Herpetological Review* 33, 290-293.

Köhler, G. (2011). Amphibians of Central America, Offenbach: Herpeton.

Kraus, F., Campbell, E. W., Allison, A., & Pratt, T. (1999). *Eleutherodactylus* frog introduction to Hawaii. *Herpetological Review* 30, 21-25.

Kraus, F., & Campbell, E. W. (2002). Human-mediated escalation of a formerly eradicable problem: the invasion of Caribbean frogs in the Hawaiian Islands. *Biological Invasions* 4, 327-332.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33, 1870-1874.

Lee, W. H., Lau, M. W. N., Lau, A., Rao, D. Q., & Sung, Y. H. (2016). Introduction of *Eleutherodactylus planirostris* (Amphibia, Anura, Eleutherodactylidae) to Hong Kong. *Acta Herpetologica* 11, 85-89.

Lemos-Espinal, J. A., & H. M. Smith. (2007). Anfibios y Reptiles del Estado de Coahuila, México. México, D.F.: Comisión Nacional para El Conocimiento y Uso de la Biodiversidad, Universidad Nacional Autónoma de México.

Lowe, S., Browne, M., Boudjelas, S., & De Poorter, M. (2000). 100 of the world's worst invasive alien species: a selection from the global invasive species database (Vol. 12). Auckland: Invasive Species Specialist Group.

McCranie, J. R., & Valdés-Orellana, L. (2014). New island records and updated nomenclature of amphibians and reptiles from the Islas de la Bahía, Honduras. *Herpetology Notes* 7, 41-49.

McCrane, J. R., Collart, J. R., Castañeda, F. E., & Solis, J. M. (2008). Geographic distribution: *Eleutherodactylus* (Euhyas) planirostris. *Herpetological Review* 39, 362.

Melo, M. A., Lyra, M. L., Brischi, A. M., Geraldi, V. C., & Haddad, C. F. (2014). First record of the invasive frog *Eleutherodactylus johnstonei* (Anura: Eleutherodactylidae) in São Paulo, Brazil. *Salamandra* 50, 177-180.

Mendoza, A. M., García-Ramírez, J. C., & Cárdenas-Henao, H. (2012). Blood puncture as a nondestructive sampling tool to obtain DNA in frogs: comparison of protocols and survival analysis. *Molecular Ecology Resources* 12, 470-475.

Olson, C. A., & Beard, K. H. (2012). Diet of the introduced greenhouse frog in Hawaii. *Copeia* 2012, 121-129.

Ortiz-Medina, J. A., Nahuat-Cervera, P. E., Cabrera-Cen, D., Vega-Marcin, A. & Cedeño-Vázquez, J. R. (2017). First records for the invasive Greenhouse Frog, *Eleutherodactylus planirostris* (Cope, 1862) (Anura: Eleutherodactylidae), in the state of Yucatán, Mexico. Distribution Notes. *Mesoamerican Herpetology* 4, 191-193.

Pavón-Vázquez, C. J., Gray, L. N., White, B. A., García-Vázquez, U. O., & Harrison, A. S. (2016). First records for Cozumel Island, Quintana Roo, Mexico: *Eleutherodactylus planirostris* (Anura: Eleutherodactylidae), *Trachycephalus typhonius* (Anura: Hylidae), and *Indotyphlops braminus* (Squamata: Typhlopidae). Distribution Notes. *Mesoamerican Herpetology* 3, 531-533.

Ronquist, F., & Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, 19, 1572-1574.

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A. & Huelsenbeck, J.P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol*, 61: 539-542.

Sambrook, J., & Russell, D. W. (2006). Purification of nucleic acids by extraction with phenol: chloroform. Cold Spring Harbor Protocols, 2006, pdb-prot4455.

Schwartz, A. (1974). *Eleutherodactylus planirostris*. Catalogue of *American Amphibians and Reptiles*, 154.1-154,4.

Williamson, M. (1996) Biological invasions. Chapman and Hall, New York. 244 p.

Accepted: 23 January 2018