



## Research

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# Terrestrial reproduction as an adaptation to steep terrain in African toads

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How evolutionary novelties evolve is a major question in evolutionary biology. It is widely accepted that changes in environmental conditions shift the position of selective optima, and advancements in phylogenetic comparative approaches allow the rigorous testing of such correlated transitions. A longstanding question in vertebrate biology has been the evolution of terrestrial life histories in amphibians and here, by investigating African bufonids, we test whether terrestrial modes of reproduction have evolved as adaptations to particular abiotic habitat parameters. We reconstruct and date the most complete species-level molecular phylogeny and estimate ancestral states for reproductive modes. By correlating continuous habitat measurements from remote sensing data and locality records with life-history transitions, we discover that terrestrial modes of reproduction, including viviparity evolved multiple times in this group, most often directly from fully aquatic modes. Terrestrial modes of reproduction are strongly correlated with steep terrain and low availability of accumulated water sources. Evolutionary transitions to terrestrial modes of reproduction occurred synchronously with or after transitions in habitat, and we, therefore, interpret terrestrial breeding as an adaptation to these abiotic conditions, rather than an exaptation that facilitated the colonization of montane habitats.

## 1. Introduction

Adaptation, the positive selection of particular traits given a specific environment, is a central concept in evolutionary biology. Adaptation has generated an impressive disparity in phenotypes, and has been implicated in the diversification of lineages (adaptive radiations; [1]). The development of comparative phylogenetic models for trait evolution has allowed explicit testing of macroevolutionary hypotheses of how environments have shaped character history. Questions that have been addressed include whether traits evolve randomly (Brownian motion; [2]) or towards a single or multiple selective optima (Ornstein Uhlenbeck; [3]), whether transitions between states of a trait are dependent on the state of a second trait [4], and whether changes in trait states have influenced speciation or extinction rates [5]. Particularly, strong cases for adaptation tend to be made in instances of trait convergence or where multiple independent evolutionary origins correlate with particular environmental parameters in a predicted way [6,7].

A question less frequently addressed is whether trait–environment correlations are the result of an evolutionary response to hypothesized selective regimes (i.e. adaptation) or whether the trait of interest, or a variant of it, was pre-existing and has provided the organism with a fitness advantage under new conditions (i.e. exaptation; [8]).

Amphibians are an interesting taxon to investigate evolutionary processes by virtue of the range of biological traits they display [9], convergences in form, and the diversity of habitats they occur in, including fossorial, terrestrial and arboreal lifestyles (e.g. [10]). Amphibians also display a remarkable array of reproductive strategies, ranging from biphasic life cycles consisting of larval and adult forms separated by metamorphosis, to strategies where either the larval or adult stage is entirely absent. Overall, amphibians are arguably the most reproductively diverse group of terrestrial vertebrates, and in anurans alone, more than 40 different reproductive modes have been recognized [9]. Many of these strategies have arisen multiple times independently [11,12], and particularly terrestriation of reproductive modes is often cited as an example of repeated adaptation to the same specific sets of environmental conditions [13–16]. Terrestrial breeding in particular may in itself be an evolutionary key character that, by opening up new niche space, promoted the radiation of some taxa [17,18].

On a global scale, anuran species with terrestrial reproductive modes occur most frequently in tropical climates characterized by high annual precipitation, temperature and humidity [11], but a consensus is lacking for the exact factors driving the evolution and maintenance of terrestrial breeding strategies in these areas. Hypothesized abiotic causal factors include the complete absence of water sources needed for aquatic eggs or larvae [19], montane environments characterized by fast-flowing streams [13,16,20,21], or humid, forested micro-habitats that prevent desiccation of terrestrial eggs [14,15]. However, no previous study has investigated whether terrestrial modes of reproduction have evolved in response to particular abiotic conditions or, whether once terrestrial breeding strategies evolved *ex situ*, lineages subsequently dispersed into (and proliferated in) such environments where terrestrial breeding bestows a fitness advantage.

A number of anuran reproductive modes resembling intermediate stages of an evolutionary sequence from complete aquatic to complete terrestrial development, and have indeed been viewed as such [22,23]. A broad-scale study by Gomez-Mestre *et al.* [11] found few examples of such a stepping stone model but instead recovered fully terrestrial breeding strategies arising from fully aquatic strategies with a surprisingly high frequency. Other studies focusing on single lineages however have found evidence for a more gradual evolution of fully terrestrial strategies [21,24,25].

African toads (Bufonidae) present an ideal group of amphibians for examining the evolution of terrestrial breeding strategies and their correlation with abiotic factors because (like anurans as a whole) they show a plethora of reproductive traits and strategies [26,27] and a wide range of habitat preferences [28]. By compiling the most complete phylogeny to date and analysed with an extensive geographical occurrence dataset, we test whether specific environmental conditions explain the evolution and distribution of terrestrial reproduction in African bufonids. Specifically, we test whether terrestrial reproduction correlates with: (i) steep slopes (surface gradient) as a proxy for swift flowing water or the absence of water bodies (topographic wetness index (TWI)), (ii) degree of

forest cover and humidity, (iii) areas of high precipitation and temperature. Furthermore, we examine whether ancestors evolved terrestrial reproductive modes in parallel to transitions in habitat, as adaptations to these conditions or whether terrestrial reproduction served as an exaptation for the colonization of habitats less suitable to species with complete aquatic reproduction. Lastly, we also investigate how many times terrestrial breeding evolved in African bufonids and determine whether intermediate stages acted as precursors for the evolution of complete terrestrial reproduction.

## 2. Material and methods

### (a) Phylogenetic reconstruction

A detailed version of the phylogenetic reconstruction is provided as electronic supplementary material 1 and all R scripts used are available from the Dryad Digital Repository [29]. In brief, a time calibrated phylogeny of African bufonids with a selection of Eurasian and New World outgroups was generated for this study based on approximately 5784 base pairs from three nuclear (CXCR4, NCX1, RAG1) and four mitochondrial markers (12S, 16S, CO1, ND2). A single representative per described species was used, totalling 125 species, of which 79 are African taxa. This sampling covers approximately 78% of all described African bufonid species and all genera but *Laurentophryne*, a monotypic genus for which no genetic data are available. We follow the taxonomy of Frost [30]. The resulting concatenated sequence matrix had the following gene coverage: 12S = 98.4%, 16S = 99.2%, COI = 81.6%, ND2 = 54.4%, CXCR4 = 84.8%, NCX1 = 18.4%, RAG1 = 88.0%.

Joint posterior distribution of model parameters was estimated using Bayesian Markov chain Monte Carlo (MCMC) searches in BEAST v. 1.8.2 [31]. Molecular clock models were estimated separately for mitochondrial and nuclear markers using uncorrelated lognormal relaxed clock (ucl) priors, a birth–death speciation tree prior was used and four fossil calibration constraints. A total of six MCMC searches with 100 million generations and sampling every 20 000 iterations were conducted to assess convergence and stability of parameters. Resampled post burn-in posterior trees were summarized as a maximum clade credibility (MCC) tree with median node heights.

### (b) Occurrence records and environmental parameters

Occurrence data for all African bufonid species included in the phylogeny were compiled from the open access databases of Global Biodiversity Information Facility (GBIF, [www.gbif.org](http://www.gbif.org), accessed February 2013) and HerpNet ([www.herpnet.org](http://www.herpnet.org), accessed February 2013) and from non-open access sources: including records from The Natural History Museum, London (UK), South African National Biodiversity Institute (South Africa), Trento Museum of Natural History (Italy) and the Museum für Naturkunde, Berlin (Germany), as well as published, non-digitized sources (electronic supplementary material 2). Duplicate records across data sources and duplicate records per species falling into the same grid cell for climatic layers were removed. Anecdotal records were geo-referenced where possible with the help of GeoNames (<http://www.geonames.org/>, Unxos GmbH, Switzerland), and Google Earth (<http://www.google.com/earth/>, Google Inc., USA). Anecdotal records that could not accurately be assigned to a taxon or location were not included. Occurrence records per species were vetted by visual inspection aided by overlaying IUCN red list v. 2013.2 range maps ([www.iucnredlist.org](http://www.iucnredlist.org), IUCN, Switzerland) in ArcGIS v. 10.0 (ESRI, USA) and questionable records were removed (e.g. records present outside of the currently hypothesized range).

Measures for forest cover, slope, topographic wetness, temperature and precipitation per occurrence record were extracted from Global Information System layers, all at the same spatial resolution

(30s). Forest cover, as a percentage of woody vegetation per grid cell, was measured using the Terra MODerate-resolution Imaging Spectroradiometer (MODIS) Vegetation Continuous Field layer for woody vegetation (2010 dataset; [www.landcover.org](http://www.landcover.org), University of Maryland, USA). Climate information was extracted from the WorldClim database and derived BioClim layers ([www.worldclim.org](http://www.worldclim.org), University of California, Berkeley, USA). Because seasonal anuran activity is determined by precipitation, measures for precipitation and temperature of the wettest quarter were taken (BioClim layers BIO8 and BIO16). As a measure of humidity, the aridity index  $Q$  outlined in Tieleman *et al.* [32] was adopted, using mean annual precipitation (BioClim layer BIO12), and maximum and minimum temperature records (BioClim layers BIO5 and BIO6) so that  $Q = \text{BIO12}/(\text{BIO5} + \text{BIO6})(\text{BIO5} - \text{BIO6}) \times 1000$ . Slope was calculated in degrees using the built-in function in QGIS v. 2.8.2, from the WorldClim digital elevation model reprojected to the Alberts Africa Equal Area Conic coordinate system, as was the TWI using the SAGA plugin in QGIS, using local slope and specific catchment area. TWI is calculated by combining effective drainage area information with slope so that  $\text{TWI} = \ln(\text{specific catchment area}/\tan(\text{slope}))$  [33], and gives a measure of how susceptible land surfaces are to ground saturation and the potential for overland flow. High index values are recovered for zones where contributing runoff area is high and slope is low and are predicted to be areas where bodies of water form most readily.

### (c) Reproductive modes

We assigned five discrete reproductive modes to species of African bufonids: (i) aquatic oviparity with generalized tadpoles developing in open bodies of water, including permanent and seasonal ponds, swamps, ditches and large, slow flowing streams, (ii) aquatic oviparity with specialized tadpoles developing in extremely small, ephemeral water bodies, such as water-filled tree holes, snail shells or hollow coconuts, (iii) aquatic oviparity with specialized tadpoles developing in fast-flowing water on steep gradients, (iv) terrestrial oviparity with either complete or partial pre-metamorphic development undergone in the egg, (v) viviparity, both lecithotrophic and matrotrophic, defined as the retention of eggs in the oviduct of females where development is completed and fetuses nourished only by the yolk of the ovum or supplemented by additional nutrients provided by the mother. Information on breeding biology was compiled from the IUCN Red List database ([www.iucnredlist.org](http://www.iucnredlist.org), accessed in October 2013). Three important species for which breeding biology has not yet been confirmed are *Didynamipus sjostedti*, *Churamiti maridadi* and *Wolterstorffina chirioi*. A recent report of a terrestrial clutch [34] conforms to previous assumptions that *D. sjostedti* deposits terrestrial eggs that possibly undergo direct development [35,36]. *Churamiti maridadi* has been scored as breeding in open water bodies, based on ovarian clutch characteristics [26,37]. *Wolterstorffina chirioi* has been recorded only from the summit of a single mountain at altitudes higher than any water body and is assumed to possess some form of terrestrial reproduction [38,39].

### (d) Phylogenetic signal and ancestral state reconstruction

We approximated whether there is phylogenetic signal in reproductive modes, by estimating discrete character evolution with and without a phylogenetic error structure and comparing model fit (AICc scores). This was done by comparing a lambda tree transformation (phylogenetic non-independence) with a model where lambda was fixed to 0 (complete phylogenetic independence) using the fitDiscrete function in the R package, geiger v. 2.06 [40].

Owing to the paraphyly of African bufonids (see results and [27]) ancestral state reconstructions on the most credible clade

topology were restricted to the larger of the two African clades (Clade A in electronic supplementary material 3; comprising 88.6% of sampled African bufonids). Ancestral states of discrete reproductive modes for all internal nodes of this clade were reconstructed using stochastic character mapping [41] with the R package phytools v. 0.5-38 [42]. A continuous-time reversible Markov model for the evolution of the reproductive modes was fitted to the data and then used to simulate stochastic character histories [43]. We performed 9999 simulations using the pruned MCC tree with an equal rates empirical transition matrix used for fitting the Markov model and equal root node prior probabilities. Posterior probabilities at each node were then summarized as pie charts. In addition, the MCMC method in BayesTraits v. 2 [4] was used to estimate the evolutionary transition rates that lead to the derived modes of reproduction in order to test whether terrestrial modes of reproduction have evolved directly from generalized tadpole ancestors or through 'evolutionary intermediate' specialized modes.

### (e) Environmental correlates with terrestrial reproduction

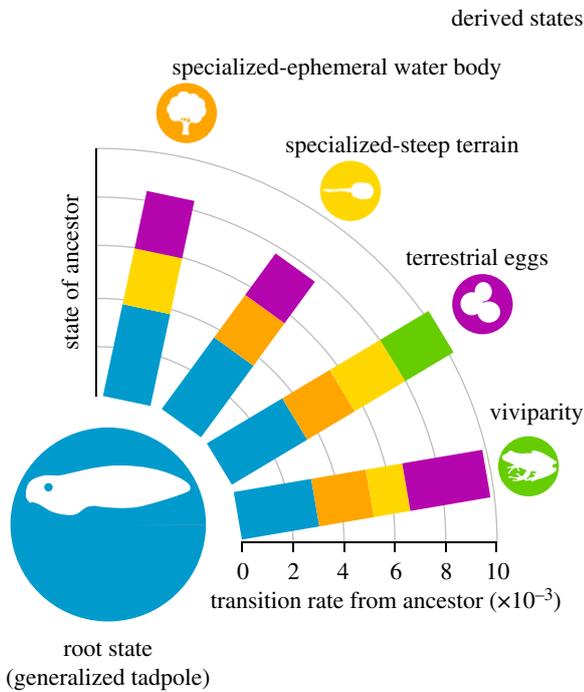
To visualize whether species with different reproductive modes occupy unique areas in environmental space, species medians of the six habitat measurements (forest cover, slope, topographic wetness, humidity and mean temperature and precipitation of the wettest quarter) were subjected to a rigid rotation via a PCA. Euclidean distances of the first two components were then used to construct a UPGMA dendrogram overlaid on the PCA biplot to visualize clustering of species and to categorize environmental guilds based on habitat and climate preferences. The PCA was performed using the prcomp function in R and the hclust function of the vegan package v. 2.4-0 [44] was used to construct the dendrogram.

Logistic link regressions were used to test the effect of the environmental variables on reproductive modes as a binary response. Because we were interested in the evolution of terrestrial reproduction, we recoded the five reproductive modes into an aquatic (categories i to iii) versus terrestrial (categories iv and v) binary variable and performed a generalized linear mixed model using both the MCMCglmm function in R package MCMCglmm v. 2.22.21 [45] and the binaryPGLMM function in the ape v. 4.0 package [46]. Both functions incorporate a phylogenetic variance-covariance matrix as a random effects variable to correct for phylogenetic non-independence and whereas the ape method alternates between penalized quasi-likelihood to estimate the mean components and restricted maximum-likelihood to estimate the variance components of the model, the MCMCglmm approach uses a MCMC sampler. For the MCMC models, two independent chains were run for 5 010 000 iterations, sampling every 500 iterations after a 10 000 burn-in. Chains were checked for convergence and each was checked for stationarity and lack of autocorrelation using the coda package v. 0.18-1 [47]. All variables were scaled and centred to have mean = 0 and variance = 1, and due to the intercorrelation of predictor variables, separate models for each of the six variables were run, with the addition of a seventh model on the first two principal component scores. Variables with significant effects were visualized using 'traitgrams' [48] with the phytools package in R using the maximum-likelihood approach of Schluter *et al.* [49], overlaid with the predicted MCMCglmm response curve at the tips.

## 3. Results

### (a) Phylogenetic inference

We present the most complete phylogeny for African species of Bufonidae to date. This includes 78% of all described species, based on a near-complete seven-gene dataset (electronic



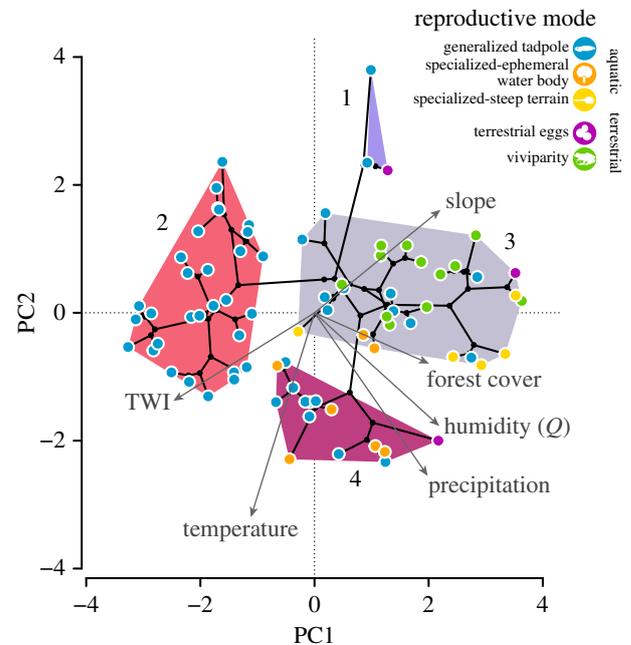
**Figure 1.** Ancestral state reconstructions for reproductive modes in the major of the two African clades of Bufonidae using BayesTraits. The pie chart shows the state probabilities for the root of the tree and the bar chart shows the transition rates to each of the four derived states from immediate ancestral states.

supplementary material 3; as Nexus file in the Dryad repository). In congruence with previous tree inferences [27,50–52], African bufonids are recovered as paraphyletic, with the crown ages of the two clades dating to 26.5 Ma (95% HPD = 22.5–41.4) and 17.5 Ma (95% HPD = 13.7–21.5).

## (b) Ancestral state reconstruction and evolution of reproductive modes

Reproductive modes show phylogenetic signal. A model of trait evolution evolving under a lambda transformation model (estimated lambda = 0.991; AICc = 97.426) is a better fit ( $\Delta$ AICc = 80.991) than one with no phylogenetic component (lambda fixed to 0; AICc = 178.417). Reproduction via aquatic oviparity and generalized tadpoles is the most common form of reproduction (figure 1; albeit also arguably the broadest category). As a special case, oviparity and tadpole development in small, ephemeral water bodies (e.g. tree holes, snail shells) evolved at least twice independently: once in *Nectophryne* and once in *Mertensophryne*. The evolution of specialized tadpoles adapted to torrential streams occurred twice: in *Werneria* and *Sclerophrys perreti* [53]. *Wolterstorffina parvipalmata* and *W. mirei* also occur near fast-flowing streams, but for both species the tadpole habitat remains unclear, although for the former this is thought to be confined to small side-pools, with tadpoles showing no signs of anatomical adaptations to living in fast-flowing water (discussed in [54]) and in one instance having been found in a discarded tin can [55].

Terrestrial forms of reproduction occur in a number of closely related species, all belonging to a well-supported clade comprising *Nectophrynoidea*, *Churamiti*, *Altiphrynoidea*, *Didynamipus*, *Nimbaphrynoidea* and *Schismaderma*. Within this clade, the lecithotrophic (*Nectophrynoidea*) and matrotrophic (*Nimbaphrynoidea*) viviparous genera are not sister taxa and neither are the terrestrial-egg-laying species (*Didynamipus*, *A. malcolmi* and, if confirmed, *W. chirioi*). This suggests that



**Figure 2.** PCA biplot on the species medians of the six investigated environmental variables: slope, topographic wetness index (TWI), temperature (BIO8), precipitation (BIO16), humidity ( $Q$ ) and forest cover. Overlaid is a UPGMA dendrogram and polygons delimiting four environmental clusters (habitat preferences), derived from the euclidean distances between species positions in the PC1 and PC2 space. Arrows show direction and relative contribution of variables to the PCA scores and colours of points represent reproductive modes.

no derived reproductive mode is without homoplasy, all having evolved more than once.

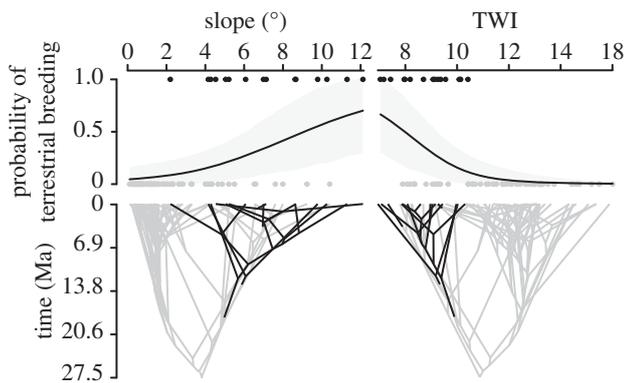
Stochastic character mapping on Clade A (electronic supplementary material 3) suggests that the most recent common ancestor for the major of the two African bufonid radiations had generalized tadpoles and that specialized breeding in ephemeral water bodies evolved twice, steep terrain adapted tadpoles once, terrestrial-egg-laying twice and viviparity twice independently. This is the case even when scoring the reproductive mode for *C. maridadi* as unknown (i.e. giving each of the five possible states an equal probability in the tip matrix of the stochastic character mapping input), as laying terrestrial eggs, or as viviparous (data not shown). Furthermore, the BayesTraits analysis shows that all derived modes most likely transitioned directly from generalized tadpole ancestors, with the possible exception of viviparity, where transition from a terrestrial-egg layer shows comparable rates (figure 1). The stochastic character mapping (see below) shows that one of the viviparous lineages (the matrotrophic viviparous *N. occidentalis*) may have evolved from a terrestrial-egg-laying ancestor, whereas the other lineage (the lecithotrophic viviparous *Nectophrynoidea*) likely evolved from generalized tadpole breeding ancestors.

## (c) Environmental determinants for reproductive modes

We vetted 7925 locality records for 79 species (median: 17; range: 1–1491 localities per species) and median values per species per habitat variable are given in electronic supplementary material 4. The first two components of the PCA represent 78.7% of the cumulative proportion of variance. The strongest contributing variables to PC1 were the habitat descriptors (TWI slope, and forest cover) with the climatic variables (temperature, precipitation and humidity) contributing most to PC2 (figure 2). The

**Table 1.** MCMCglmm and binaryPGLMM model statistics for the effect of habitat variables on terrestrial reproductive as a binary response variable. Abbreviations refer to mean temperature (BI08) and mean precipitation (BI016) in the wettest quarter, humidity (*Q*), topographic wetness index (TWI) and effective sample size (EES). Variables with significant effects ( $p < 0.05$ ) are annotated with an asterisk.

	MCMCglmm				binaryPGLMM			
	posterior means	95% CIs	EES	pMCMC	coefficient	sd	Z-score	p-value
<i>intercept</i>	-9.736	-18.263; -2.657	965	<0.001	-4.091	1.703	-2.402	0.016
PC1	6.651	1.209; 13.072	1009	0.002*	2.873	1.398	2.055	0.040*
PC2	0.999	-1.868; 3.808	6214	0.447	0.686	0.773	0.887	0.375
<i>intercept</i>	-5.849	-10.986; -1.728	3993	0.001	-2.657	1.031	-2.578	0.010
slope	3.057	0.294; 6.132	4199	0.010*	1.439	0.724	1.988	0.047*
<i>intercept</i>	-8.507	-15.843; -2.310	1420	<0.001	-3.592	1.478	-2.430	0.015
TWI	-5.768	-11.658; -0.943	1389	0.002*	-2.443	1.268	-1.928	0.054
<i>intercept</i>	-5.249	-10.007; -1.418	5877	0.003	-2.356	1.010	-2.333	0.020
forest	2.781	-0.063; 5.954	5750	0.039*	1.288	0.760	1.694	0.090
<i>intercept</i>	-6.090	-11.489; -1.281	4213	0.003	-2.414	1.055	-2.289	0.022
<i>Q</i>	2.508	-0.511; 5.882	4394	0.080	0.918	0.742	1.236	0.216
<i>intercept</i>	-5.546	-10.425; -1.244	5937	0.003	-2.289	1.000	-2.289	0.022
BI08	-1.470	-4.106; 0.803	7467	0.208	-0.595	0.617	-0.964	0.335
<i>intercept</i>	-5.240	-10.054; -1.051	5686	0.006	-2.189	0.970	-2.256	0.024
BI016	1.441	-1.137; 4.312	7562	0.258	0.591	0.666	0.888	0.375



**Figure 3.** Slope (left) and topographic wetness index (TWI; right) were recovered as having a significant, opposing effect on the evolution of terrestrial reproduction in African bufonids. Terrestrially breeding species inhabit areas characterized by steeper slopes and where soil moisture is less likely to accumulate to form water bodies. Above: predicted MCMCglmm response curves with 95% CIs where 0 (grey) are aquatic breeders and 1 (black) are terrestrial breeders. Below: traitgrams showing evolution of environmental preference, based on a randomly sampled stochastic character map.

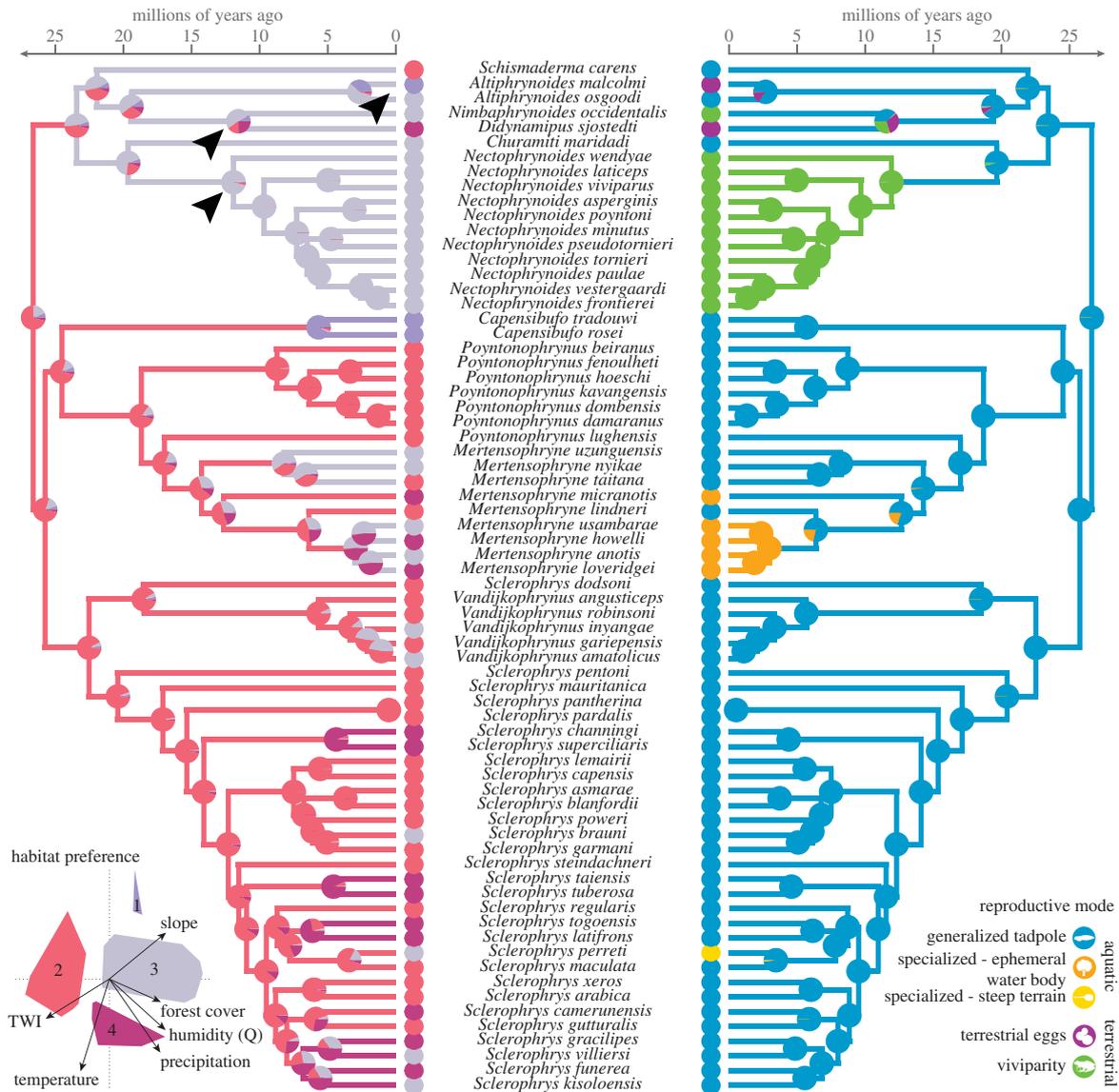
UPGMA dendrogram recovered four environmental clusters (figure 2), roughly corresponding to habitats of (i) steep terrain with little surface water, cool and dry, (ii) flat terrain with surface water, little tree cover and dry, (iii) steep terrain with little surface water, cool, wet and forested, and (iv) flat terrain with little surface water, warm, wet and forested. Only the second cluster shows homogeneity in reproductive modes with the others comprising mixed assemblages. This heterogeneity suggests that in areas where surface water is predicted to accumulate (high values of TWI and low values for slope) and thereby provide the hydrological conditions for likely

aquatic-breeding sites for anurans, with little tree cover and a generally dry climate (low values of *Q* and precipitation (BI016)), African toads all tend to breed via aquatic reproduction in open water bodies, but where habitats deviate from these conditions, alternative strategies are also present.

Both the likelihood and Bayesian logistic regressions recovered a significant ( $p < 0.05$ ) positive effect of PC1 on the evolution of terrestrial breeding. When testing environmental variables individually, slope was consistent in having a positive effect on terrestrial breeding using both methods, and TWI had a significant effect on terrestrial breeding using the Bayesian approach and marginally so using the likelihood approach ( $p = 0.054$ ; figure 3). To a lesser extent forest cover was also recovered as having a positive effect using the Bayesian approach (Bayesian model  $p = 0.039$ ; likelihood model  $p = 0.090$ ; table 1).

#### (d) Adaptation or exaptation?

The ancestral habitat for the major clade of African bufonids (Clade A; electronic supplementary material 3) is inferred as having been dry, flat, open habitat (cluster 2; figure 4). From there, multiple transitions to each of the other habitats occurred, with viviparous species transitioning to cluster 3 habitats, and the two terrestrial-egg-laying species transitioning to either cluster 1 or 4. Transitions to these habitats coincided in time with transitions in reproductive modes (reproductive mode transitions indicated by arrowheads in figure 4), occurring either synchronously (at the same nodes) or habitat preference transitions occurring at older nodes. In no case did the evolution of terrestrial breeding (in fact, any of the scored, derived reproductive mode) precede the transition in habitat preferences as would be expected under the exaptation hypothesis.



**Figure 4.** Ancestral state reconstruction based on stochastic character mapping of left: habitat preferences as defined by PCA habitat clusters (depicted as insert) and right: reproductive modes. Branches above nodes (closer to tips) are coloured according to the most probable ancestral state of that node. Arrowheads indicate nodes or branches at which terrestrial reproduction likely evolved.

## 4. Discussion

### (a) Evolution of terrestrial breeding and viviparity in African bufonids

The ancestral mode of reproduction for anurans is thought to be aquatic oviparity with an aquatic larval stage [11], which is also the case for bufonids [51]. Although most African bufonids have retained the plesiomorphic, generalized aquatic mode of reproduction, a number of taxa evolved different strategies, from adaptations to fast-flowing streams, breeding in tree holes, to semi and fully terrestrial development. With the exception of the matrotrophic viviparous *Nimbaphrynoides*, we find strong evidence that usually, derived reproductive modes present in African bufonids evolved from an aquatic-breeding ancestor with a generalized tadpole. *Nimbaphrynoides* is sister to the potentially direct developing *Didynamipus*, which leaves the possibility that viviparity in this species evolved from an ancestor with terrestrial oviposition. However, an aquatic ancestral state for *Nimbaphrynoides* and *Didynamipus* is equally likely based on our data.

In African bufonids, matrotrophic viviparity was speculated to be derived from lecithotrophic viviparity [56–58], echoing the long held belief of directional evolution towards terrestriality in amphibians [9]. Gomez-Mestre *et al.* [11] showed however that there need not be evolutionary ‘stepping stones’ for derived reproductive modes (see also [59]). We recovered the two viviparous lineages (matrotrophic *Nimbaphrynoides* and lecithotrophic *Nectophrynoides*) as representing independent evolutionary transitions to viviparity. If previous assumptions about the evolution of viviparity are instead correct, this would require that viviparity evolved once and was subsequently lost in *Altiophrynoides*, *Churamiti*, *Didynamipus* and *Schismaderma*. While some lineages of direct developing amphibians have likely reverted to having aquatic larvae (e.g. [60,61]) and a reversal from viviparity to egg-laying has been inferred for some squamates (e.g. [62,63]; previously thought unlikely [64]), no cases of reversal from viviparity are known in amphibians. The least inclusive clade containing *Nectophrynoides* and *Nimbaphrynoides* diverged relatively early in the history of bufonids in Africa and the two viviparous lineages, although relatively closely related, are separated by *ca* 23.3 million years of evolution. The origins of viviparity

however, occurred more recently, at *ca* 11–12 million years ago, and approximately at the same time in both lineages, coinciding with substantial climatic and tectonic changes in Africa [65]. The two modes of viviparity furthermore involve fundamentally different patterns of fetal development and morphology [66] possibly requiring independent evolutionary pathways without one representing an ‘intermediate’ form of the other.

Viviparity is otherwise exceedingly rare in anurans. Apart from African bufonids, the only confirmed species giving birth to fully developed young is the now possibly extinct, ovoviviparous *Eleutherodactylus jasperi*, and some evidence points to a similar mode of reproduction in *Craugastor laticeps* [67]. These two species are not closely related but form part of the South American Terrarana (Brachycephaloidea), a clade containing over 1050 species that otherwise are all known or thought to be direct developing. *Eleutherodactylus jasperi* and *C. laticeps* are known from predominantly montane areas of Puerto Rico, and northern Central America and southern Mexico, respectively. In addition, the recently described South East Asian microglossid *Limnonectes larvaepartus* gives birth to fully developed tadpoles [68] and occurs in the forested hills and mountains of northern Sulawesi and appears to be associated mostly with forest seeps and side pools of smaller streams [69]. Whether there is a causal link between viviparity in these species and their seemingly montane habitats requires further investigation.

## (b) Abiotic determinants of the evolution of reproductive modes

Based on our analyses we find that in African bufonids, generalized aquatic breeders occur in all habitat types, whereas terrestrial (or otherwise ‘specialized’) breeders are more restricted. Specifically, we recover steep terrain, the absence of water bodies and to a lesser degree forest cover to be strong predictors of terrestrial breeding. This confirms the predictions made by Goin & Goin [13] and supports the hypothesis that unsuitability, or sub-optimality, of breeding sites in steep terrain (where water is fast flowing) for aquatic breeding has imposed selective pressure for the evolution of terrestrial modes of reproduction. The same observations have been made for Neotropical anurans [16,70] and also in *Salamandra salamandra*, where populations restricted to areas of karstic limestone during Pleistocene fragmentation events have evolved the retention of eggs and development of embryos in the oviduct [71] as a response to lack of surface water. Conversely, Portik & Blackburn [21] found no connection between terrestrial oviposition and lotic systems in Afrobatrachia, but they discuss the potential role of montane over lowland lotic systems in the evolution of specialized reproduction. Continuous measurement of slope and topographic wetness as a more direct predictor of both stream flow rate and absence of standing water may help to elucidate such differences hidden in categorical habitat classifications. However, coarse spatial resolution of the environmental layers (approx. 1 km) leads to geographically restricted species such as many of the terrestrial breeders (e.g. *N. poyntoni*) being represented by only few data points. Similarly, potential sampling efforts may have been biased towards more accessible, flatter terrain.

We find no support for the hypothesis that ambient humidity or precipitation promotes the evolution of terrestrial breeding in African bufonids, as was also found not to be the

case for leptodactylids [59]. Although important for explaining the global distribution of reproductive modes [11], these parameters appear to play less of a role at finer spatial scales. Furthermore, the importance of forest cover [14,15] remains ambiguous. Poynton [14] stressed the importance of forests for preventing the desiccation of terrestrial eggs, which in the case of bufonids may be less relevant because two of the four confirmed terrestrial breeding lineages are viviparous and therefore eggs are not directly exposed to environmental conditions. A hypothesis worth testing in future is that other substrate or even behavioural (nest construction, burrowing, etc.) factors may provide access to micro-climates similarly humid to those found in forest undergrowth, that prevent the desiccation of eggs and small-bodied amphibians

## (c) *In situ* versus *ex situ* evolution of terrestrial breeding strategies

This study and others (e.g. [11,15,71]) have recovered a strong association between particular habitat types and terrestrial breeding strategies in amphibians. Yet, biotic factors including aquatic predation on eggs and larvae, parasites or interspecific competition have also been proposed as imposing a selective pressure in favour of terrestrial reproduction [14]. This therefore raises the question whether terrestrial reproductive strategies evolve as an adaptation to local environmental conditions or are examples of exaptations that under some other selective regime *ex situ* (e.g. biotic factors) confer a selective advantage for the colonization of habitats that seem less suitable for species with aquatic-breeding strategies. Here, we show via ancestral state reconstructions of habitat preferences and reproductive modes that in African bufonids, aquatic-breeding ancestors moved into habitats characterized by steep slopes and scarce availability of standing water and only then or at a later stage did terrestrial breeding evolve. In the light of this evidence, we therefore view the evolution of terrestrial breeding modes as indeed being an adaptation (not an exaptation) to these environmental conditions.

## 5. Conclusion

In African bufonids, terrestrial reproduction evolved in multiple forms and most often directly from aquatic ancestors. We find that terrestrial and aquatic-breeding species are distributed asymmetrically across environmental space, with terrestrial species limited to areas with particular conditions, whereas aquatic breeders are distributed more generally. Steep terrain and low accumulation of water bodies are strong predictors of the occurrence of terrestrial reproduction whereas climatic variables such as humidity, precipitation and temperature less so. Whether forest cover is a crucial habitat component for terrestrial reproduction [14,15] remains unclear. Furthermore, our analyses show that terrestrial modes have evolved as adaptations, not exaptations, after ancestors transitioned to habitats characterized by steep slopes with little accumulation of surface water.

**Data accessibility.** Data are available from the Dryad Digital Repository at: <http://dx.doi.org/10.5061/dryad.k9n2h> [29].

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laboratory work and data analysis; J.H., J.P., T.M., D.J.G. and M.-O.R. participated in data collection and all authors contributed to drafting the manuscript.

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