Geographic variation in the phenotypes of two sibling horseshoe bats *Rhinolophus simulator* and *R.swinnyi*

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To mum and dad:

Elizabeth and George Danayi Mutumi, you inspire me, you revive me. Your words have kept me strong “Ramba wakashinga – Keep going on”
GENERAL ABSTRACT

The study of geographic variation in the phenotypes of animals and its causes elucidates how evolutionary processes generate biodiversity. The basis of such diversity is the formation of new lineages often in response to local environments. Environmental factors, such as climate, habitat and geography may therefore be the main drivers of lineage diversification in animals as they adapt to local environments. Natural selection has therefore been suggested to play a key role in population divergence, despite other competing hypotheses which argue that divergence is largely through neutral processes of mutation and drift. This thesis attempts to uncouple the relative contributions of adaptive and neutral mechanisms to population divergence with a particular focus on acoustic signals in African horseshoe bats (genus *Rhinolophus*).

Two horseshoe bat species, *Rhinolophus simulator* and *R. swinnyi* were sampled from their distributional ranges within southern Africa and several morphometric and echolocation parameters were taken from each individual. The relative contributions of adaptation and drift were first tested (Chapter 2) using the Lande’s model. It was hypothesised in this chapter that adaptation would predominate in the diversification of the two horseshoe bats owing to the flight-echolocation and diet-echolocation adaptive complexes that intricately tie these two species to environmental conditions. Selection was also hypothesised to be stronger in *Rhinolophus swinnyi* because it uses higher frequency sound which is more sensitive to atmospheric conditions. The hypotheses were tested using a combination of soft tissue parameters (Chapter 2) and hard tissue parameters (Chapter 3), i.e., 3D scanned skulls analysed using 3D geometric morphometrics (Chapter 3). To reconstruct the selective forces, linear mixed-effects models were used to regress climatic variables against echolocation call signals (Chapter 4) based on two hypotheses, the Sensory Drive and the James’ Rule as a guide.
The Lande’s model (Chapter 2) showed that drift had a minimal effect to the variation of body parameters and echolocation and that selection was stronger on echolocation than on morphometric parameters. Only when echolocation was removed from the model, some traces of drift were shown. The two species also differed in the features that showed selection, manoeuvrability for Rhinolophus simulator and size for R. swinnyi. Additionally selection was differentially exerted across different localities in both species, making the relative roles of selection and drift context specific. The selection signal was further confirmed by the results of Lande’s model applied to the skulls of the two species (Chapter 3). Skulls collected from various museums allowed for an extended distribution of specimen analysed, further making the enquiry relatively more robust. Whereas there was no evidence of the influence of drift detected on skull variation in both species based on the results of this chapter, this finding was contrary to previous findings of other studies that have used a similar approach in primates – signals of drift were found in some structures of skulls of humans and monkeys. A stronger case for selection was therefore suggested by this finding.

Climatic variables (mean annual temperature and relative humidity) were inversely related to the variation in echolocation signals (Chapter 4) within each species but a good proportion of the variance was left unexplained. Body size was unrelated to the observed variation either, which provided evidence that echolocation signals did not vary as a result of the body size/climate relationship proposed by James’ Rule. Additionally, there still remained a latitudinal trend in echolocation signals across localities even after the above climatic variables had been accounted for. This implied that either another climatic/vegetation component influenced the variation in signals or genetic drift somewhat contributed to the phenotypic variation seen.

Bats rely on both flight and echolocation to survive and reproduce, systems that have to track local habitats closely to perform optimally. Adaptive complexes between body size,
echolocation and the structures of the skull are therefore necessary for bats to perform their
dife functions optimally. It is therefore not surprising that the results of this thesis indicate that
selection rather than drift plays a major role in the diversification of bats, which points
towards an increased sensitivity of echolocation to environmental conditions. Furthermore,
this thesis emphasizes that the relative effects of adaptation and drift are species specific and
associated with variations on a regional scale.
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DECLARATION

I, Gregory Lilgee Mutumi, affirm that the work presented in this thesis is based on my original work (except where acknowledgements indicate otherwise). Neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. I authorise the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

26 July 2016………………………………………………………..Signature: LGmutumi
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Chapter 2

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**Fig. S3** Spline correlograms of the residuals (with 95% confidence intervals) from a linear mixed effects model with study sites as random effects, including all predictor variables. *Rhinolophus simulator* and *Rhinolophus swinnyi* (bottom). The correlation is measured in Moran’s I spatial auto-correlation index.

**Table S1.** Means and standard deviations for phenotypic and environmental variables.
CHAPTER 1

General Introduction

Geographic variation is the dissimilarity amongst populations of the same species across its range, which is often seen as an adaptive response to spatial and temporal variability of environments. Geographic variation produces the medium on which natural selection acts to determine the ultimate occupants of available habitats (Hallgrimson and Hall 2005). Patterns of geographic variation in morphology and behaviour thus allow us to answer questions about adaptation to different environmental conditions with reference to biotic and abiotic changes over time (Rising 2001). For example, features such as wing dimensions, tooth row length, weight and tarsal lengths, and behavioural characteristics like sensory modalities (e.g., echolocation), prey preferences and foraging habitat may differ across the range of a species.

However, there are other factors besides adaptation that may also result in geographic variation. This is because both genetic (intrinsic) and environmental (extrinsic) factors influence geographic variation: Genotype + Environment = Phenotype. My research focuses on the extrinsic factors shaping phenotypes. Since environmental conditions vary in space and with time (Rising 2001; Endler 1977), it is plausible that the resultant geographic variation in phenotypes is caused by environmental conditions experienced across ecological and evolutionary timescales. Geographic variation can thus be caused either by dispersal and adaptation to novel environments, referred to as Adaptation Hypothesis (Magurran 1998; Lomolino et al. 2006; Morrone 2009), or by fragmentation of the species into subpopulations by vicariant events, such that each subpopulation becomes isolated from other populations of
the same species in terms of disruption of gene flow between them, which is referred to as
Drift Hypothesis (Malhotra and Thorpe 2000; Morrone 2009). Such disruption could be
caused by the emergence of a physical barrier (e.g. river, valley, mountains) or increased
distances between populations. The Adaptation Hypothesis also requires that gene flow
between populations, although not completely disrupted, be low enough so as not to dilute the
influence of selection. Failure to find adaptive explanations for geographic variation has been
traditionally used as evidence for drift. However, this study also applies the Lande’s model, a
null model of drift, against which the influence of adaptation can be weighed. Only a few
studies have applied this method.

Geographic variation in different organisms has been attributed to either
deterministic/predictable (e.g., adaptation) or stochastic (i.e., due to chance e.g., drift)
processes and in some instances, both. For example, sexual dimorphism of head size and tail
length in the grass snake Natrix tessellata varied between sites in relation to geography as an
adaptation to available prey (Werner and Shapira 2011). The body size of arctic sea birds
(Dovekie, Alle alle) increased with decreasing air temperature but showed no relationship
with inter-colony geographic distance (Wojczulanis-Jakubas et al. 2011), supporting an
adaptive response rather than chance processes such as drift (Solick and Barclay 2006).
Similarly, in frogs, variations in colour could not be explained by geographic distance and
was ascribed to local adaptation in response to selective pressures (Amezquita et al. 2006).
However, vicariant events through genetic subdivision were evinced in broad differences in
the echolocation signals of the Formosan lesser horseshoe bat, Rhinolophus monoceros
because the differences could be related to discontinuities in allele frequencies
(Chiang-Fan et al. 2009). In a similar way, speciation in Neotropic Woodcreepers,
Dendrocincla spp was suggested to have been initiated by the vicariant effects of the Andes
mountain range uplift (Weir and Price 2011). Vicariant barriers can be in any form that is
insurmountable to the organism involved. The Torres Strait land-bridge which lies between continental Australia and New Guinea once separated the Indian and Pacific Ocean taxa, persisted through the late Pleistocene, is suggested to have caused genetic partitioning of reef fishes, Apogon doederleini, Pomacentrus coelestis, Dascyllus trimaculatus, and Acanthurus triostegus (Mirams et al. 2011). Similarly, the Skyros wall lizard Podarcis gaigeae, a species which displays high phenotypic plasticity (inhabits an extensive island and relatively smaller islets in the Greek archipelago) showed genetic divergence among populations relating to geological times of islet separations (Runemark 2012).

Southern Africa presents an ideal opportunity for studies of geographic variation owing to its past and present landscape diversity. The subcontinent has been uplifted to 300 m in the east, promoting drainage to the west (Patridge 1997). Its geology has been influenced by the evolution of the East African Rift commonly known as the “Wall of Africa” (Gani and Gani 2007). It started to form 30 MYA as a result of tectonic plate movements (Gani and Gani 2007). The Wall of Africa is composed of a chain of mountain ranges and highlands from the Ethiopian Plateau in Ethiopia to the Karoo Plateau in South Africa. The valley formed by the Wall of Africa is the largest and oldest rift valley in the world (Gani and Gani 2007). The formation is characterised by freshwater lakes along the rift floors and dramatic relief that runs from 156 meters below sea level – Lake Assal in the Afar Depression, the lowest point in Africa – to 5,895 meters above sea level at Kilimanjaro, the highest point in Africa. Most of the uplifting of southern Africa started at about 7 MYA (Gani and Gani 2007), a time scale that coincides with estimated evolutionary times of many African bats (Eick et al. 2005).

**Phenotypic geographic variations in bats**

Bats are considered good model organisms for studies of geographic variation. Several species display distinct patterns of geographic variation (Burnett 1983; Chen et al.
2009; Dunbar and Brigham 2010; Jiang et al. 2010). For example, populations of *Chaerephon pumilus* from Tanzania’s Amani game reserve differed significantly in forearm length, wing-loading, echolocation and diet, from populations found elsewhere in Africa (Aspetsberger et al. 2003). Similarly, clear population structure were shown in echolocation call designs of *Rhinolophus monoceros* with differences among populations corresponding to allele discontinuities (Chen et al. 2009). These differences were attributed to vicariant events (e.g., volcanoes, plate tectonics and cyclones) that facilitated genetic subdivision (Chen et al. 2009). In *Hipposideros larvatus*, divergence in the resting frequency of echolocation calls was correlated to geographic distance and it was attributed to stochastic events viz random cultural drift (Tinglei et al. 2010). This species apparently adjusted its calls to enable precise colony mate identification and to facilitate individual communication. Geographic variation in echolocation was also reported for *Rhinolophus hipposideros* and *R. Euryale* in Sardinia and southern Italy (Russo et al. 2007). Here the variation was attributed to character displacement (species recognition and enhancement of intraspecific communication) possibly bearing implications for mate recognition (Russo et al. 2007). In addition bats are easy to handle, making them ideal for sampling many populations from as many localities as possible. Some species, especially the horseshoe bats (Family Rhinolophidae), are perch hunters and naturally echolocate from a resting position while scanning the environment for prey. This allows the use of hand-held rhinolophids to record these resting echolocation calls removing the potentially confounding changes in echolocation in response to different situations found in almost all other bat families.

The Rhinolophidae, consist of a single genus, *Rhinolophus*. It comprises 71 species that are distributed throughout Austral-Asia, Asia, Europe and Africa (Csorba et al. 2003). In Africa there are 26 species of Rhinolophids and about 15 of these occur in southern Africa (Monadjem et al. 2010). Recent phylogenies (Stoffberg et al. 2010; Dool et al. 2016) divides
the Rhinolophidae into two clades, the Oriental and African clades. The African clade is divided into four subclades with *R. hipposideros* basal to all four and *R. blasii* basal to three of the four subclades. The three subclades are (1) maclaudi subclade (*R. macluadi, R. hildebrandtii, R. darlingi* and *R. fumigatus*) with a predominant southern African distribution, (2) the clivosus subclade (*R. clivosus* and *R. ferrumequinum*) with *R. clivosus* found throughout Africa and *R. ferrumequinum* restricted to North Africa and (3) the capensis subclade (*R. capensis, R. swinnyi, R. denti* and *R. simulator*) which all have a largely southern African distribution (Csorba et al. 2003; Stoffberg et al. 2010). Many rhinolophids have wide geographic distributions which can cover several different biomes (Csorba et al. 2003; Monadjem et al. 2010). For example, *R. clivosus* has a distribution that covers the Western Cape, Kwazulu Natal and Gauteng provinces of South Africa, all the middle and highveld of Zimbabwe, and some parts of Malawi, Mozambique and Zambia. *Rhinolophus blasii* is distributed along the eastern half of South Africa, 75% of Zimbabwe, and some parts of Zambia, Malawi and Mozambique. Given their wide geographic distributions across several biomes, it is likely that considerable geographic variation in morphology and behaviour exists in rhinolophids.

Explanations advanced to describe geographic variation in rhinolophids are generally similar to those advanced for other organisms, mainly adaptation and/or drift. Under the *Adaptation Hypothesis*, bat characteristics follow ecogeographic gradients (Dietz et al. 2006) and the basic principles of allometry (Odendaal and Jacobs 2011) as well as sexual size dimorphism (Dietz et al. 2006; You et al. 2010). For example, *R. mehelyi* forearm length increased with latitude in south-east Europe conforming to the Bergmann’s Rule. Five species of European horseshoe bats (*R. hipposideros, R. mehelyi, R. blasii, R. euryale*, and *R. ferrumequinum*) all had bigger females than males (Russo et al. 2007). However, there are exceptions which involve deviations from allometry in some species including *Rhinolophus*.
clivosus (Jacobs et al. 2007). One of the reasons often suggested for deviation from allometry is highly localised selective forces (Jacobs et al. 2007). With respect to the Drift Hypothesis, geographic variation in several cases showing disjunction in character patterns has been suggested to result from processes facilitating genetic subdivision (Chen et al. 2009) i.e., ‘barriers to gene flow’. Thus physical, behavioural and chance events can disrupt gene flow. For example, the mitochondrial genetic differentiation of Rhinolophus ferrumequinum in southeastern Europe and Anatolia indicated glacial-mediated refugia and a suture zone in central Anatolia (Bilgin et al. 2009). The lineages diverged in allopatry dating back to the Pleistocene (Bilgin et al. 2009).

Echolocation and wing morphology have been central to studies of phenotypic adaptation in rhinolophids (Norberg and Rayner 1987; Odendaal and Jacobs 2011), because there is a direct correlation, not only between these two systems, but also between them and the habitat. Echolocation and wing morphology evolve in tandem forming an adaptive complex (Norberg and Rayner 1987). Wing morphology and echolocation are adapted to different habitats (i.e., open versus closed) and certain parameters of echolocation and wing morphology maybe combined to allow foraging efficiency. For example, short broad wings coupled with high frequency echolocation has been found to enable slow and manoeuvrable flight in cluttered habitats, detecting insects over relatively short ranges in confined spaces using high frequency calls (Norberg and Rayner 1987). Additionally, echolocation has a potential role in intraspecific communication (Duellman and Pyles 1983; Heller and von Helversen 1989; Jones 2008) and many studies have found acoustic character displacement as a plausible explanation of geographic variation in echolocation. For example, Rhinolophus. mehelyi showed higher frequency values when associated with R. euryale, possibly to avoid species misrecognition in south eastern Europe (Russo et al. 2007). Although much is known about how echolocation and wing morphology adapts a bat to its habitat, very few studies
have investigated how the evolutionary histories of landscapes, and the various activities they sustain, have influenced rhinolophid morphological and behavioural character variation.

The two species *Rhinolophus simulator* and *R. swinnyi*, both belonging to the *capensis* sub-clade (Csorba et al. 2003; Stoffberg et al. 2010), inhabit savannah woodlands and occur widely along the eastern half of southern Africa (Monadjem et al. 2010). *Rhinolophus simulator* roosts in mines and caves whereas *R. swinnyi* uses road culverts in addition to caves and mines (Monadjemn et al. 2010; Maree and Grant 1997). *Rhinolophus simulator* and *R. swinnyi* have similar distributions in southern Africa and their evolution should have been shaped by similar factors. Both species span a wide range of latitudes and a narrow range of longitudes. *Rhinolophus swinnyi*’s distribution extends from eastern South Africa through most parts of Zimbabwe to the eastern parts of Zambia and small portions of the Democratic Republic of Congo (DRC) and Tanzania. *Rhinolophus simulator* follows a similar distributional range to *R. swinnyi* but also occurs further north into central Ethiopia, western Kenya and central Tanzania (Monadjem et al. 2010). The two species diverged from their common ancestor (possibly *R. capensis*; Stoffberg et al. 2010; Dool et al. 2016) at about 14 MYA and 9 MYA, respectively (Stoffberg et al. 2010; Dool et al. 2016), i.e., 2 - 7 million years after the start of the major uplift of south-east Africa. However, because of the nature of southern Africa’s environments described earlier on, and the species’ high dispersal capacity, it is plausible that the two have moved into, and adapted to already existing biomes in southern Africa. Alternatively, *Drift* could have also occurred in some parts of the species distribution range, driven by the emergence of extensive mountain ranges.

Different populations of the two species could have exploited wide ranges with overlaps in habitat usage because of their dispersal capacity (*Adaptation hypothesis*). They interbred and exchanged genetic material especially across those populations that were close to each other. In this case, smoothly graded characteristics across the range of each species
with response to ecogeographic gradients would be evident. However, because individual characteristics may respond to habitat-specific selection pressures, which differ with location depending on prevailing environmental conditions, some characteristics (wing/echolocation or body mass/peak echolocation frequency) may deviate from allometry. Different biomes can impose divergent selection pressures and there are therefore many ways in which bats can adapt to different biomes. Consequently there are at least four adaptation hypotheses (Guillén et al. 2000; Jacobs et al. 2007) that could explain geographic variation in the two species of bats. The following paragraphs provide a summary of these hypotheses but only the James’ Rule and the Sensory Drive are analysed in this study.

*The Acoustic Communication Hypothesis* (Jacobs et al. 2007) also called *The Acoustic Resource Partitioning* by Duellman and Pyles (1983): Geographic variation in echolocation frequency in Rhinolophids results from selection to avoid using similar frequency bands as congeneric sympatric species. This would facilitate intraspecific communication (Neuweiller et al. 1987). If echolocation is used for social interaction (Kingston and Rossiter 2004), very similar calls among sympatric conspecifics might compromise the effectiveness of intraspecific communication. However, if echolocation frequency is different among sympatric conspecifics this could facilitate intraspecific communication conferring an advantage on individuals of a particular species that use different frequency calls to those used by conspecifics. Since different populations of bats are likely to share their habitat with different conspecifics, selection for non-overlapping frequency bands could result in geographic variation (Jacobs et al. 2007). This hypothesis therefore predicts that different populations will have different echolocation call parameters, if they are sympatric with different congeneric species.

However, other parameters have been shown in recent studies (describing call structure, e.g., duration and intensity), to also play a role in resource access and intra-specific
Communication (Schuchman and Siemers 2010). For example, *Rhinolophus hipposideros* females called at a higher frequency and intensity than males facilitating mate selection (Schuchman and Siemers 2010). High intensity calls also have high energy which can boost propagation against attenuation at high frequencies especially in humid habitats (Schuchman and Siemers 2010). Other examples include increases in sound duration to compensate for high absorption (Barclay 1986), and adjustments of call structure with atmospheric conditions such as atmospheric pressure, temperature and wind speed (Jiang *et al.* 2010). Environmental effects against sound propagation may lead signallers to evolve different signal structures to cope with signal absorption or receivers to evolve different receptive structures and these structures may be decoupled from other body appendage size changes. A robust test of this hypothesis would involve an analysis not only of patterns of allometry and sympatry of the two focal species but also of those of their congeners. Due to time limitations, this falls outside of the scope of this study.

*Sensory Drive Hypothesis* (Guillen *et al.* 2000): variations in humidity across the range of a species should select for a frequency that incurs minimised atmospheric attenuation due to humidity so as to optimise the detection range of echolocation. *Rhinolophus simulator* and *R. swinnyi* echolocate at relatively high frequencies compared to most other bat families and have resting frequencies (RF) of 80.1 and 106 kHz respectively (Aldridge and Rautenbach 1987; Schoeman and Jacobs 2008). Even though high frequency directional sounds are a pre-requisite for echolocation, this limits perception of unobstructed objects to a relatively short distance. Such sounds are attenuated more than low frequency ones. The detection distances of the two species should decrease as humidity increases. Therefore populations from locations of higher humidity and temperature should echolocate at a lower frequency compared to populations from cooler and less humid locations, to reduce atmospheric attenuation of their calls. The *Humidity Hypothesis* was tested in this study but
combining the interactions of temperature and atmospheric pressure together under the

*Sensory Drive Hypothesis.*

*James’ Rule* (James 1970), a modification of *Bergmans rule* (Bergman 1947; Blackburn *et al.* 1999) proposes that smaller bodied animals occur in hot humid environments and bigger bodied animals of the same species occur in cooler, humid areas, the largest are expected to occur in cool, dry areas. Thus geographic variation can also be the result of latitudinal and thermal clines in body size as different populations in different regions adapt to different temperature and humidity regimes. For example, delayed maturation in lizards living in colder areas was shown to lead to significant differences in morphology from their counterparts occurring in warmer areas (Angilletta *et al.* 2004). *James’ Rule* predicts a correlation between body size and humidity/temperature. It also predicts that any difference in other morphological features and perhaps also of echolocation between populations of the same species would be the result of differences in body size as allometry is maintained. For example, the larger the body size, the lower the echolocation call frequency because echolocation frequency and body size are correlated and in bats (Norberg and Rayner 1987). Thicker vocal chords and larger resonant chambers in larger bats produce lower frequency calls according to the physics of sound (Barclay and Brigham 1991). As an alternative explanation against the *Sensory Drive Hypothesis*, this study also tested the predictions of the *James’s Rule Hypothesis*.

*Foraging Habitat Hypothesis* (FHH; Jones and Barlow 2004). The FHH predicts that geographic differences between populations of the same species are due to differences in the physical structure of the habitats within which bats forage. Densely vegetated habitats have more obstacles that bats have to detect and avoid than open habitats i.e., they are more cluttered. Consequently, bats foraging in dense vegetation need echolocation calls that allow the resolution of small objects within dense vegetation and wing morphology that allow
increased manoeuvrability to avoid those obstacles. Some populations of a species foraging in more cluttered habitat may combine higher frequency echolocation calls with lower wing loading and aspect ratio to provide increased resolution and greater manoeuvrability, respectively, than populations of the same species foraging in less cluttered habitats (Jacobs et al. 2007). If so, difference in the degree of clutter at different geographic locations would lead to geographic variation in both echolocation and wing morphology in a co-ordinated way. However, at the high echolocation frequencies characteristic of R. simulator and R. swinnyi, big differences in frequency equate to very small differences in wavelength (e.g., 84 – 92 kHz gives a 0.4mm wavelength difference compared to 2.2mm given by a 32 – 40 kHz difference of vespertilionids; Jacobs et al. 2007). It is therefore unlikely that differences in echolocation calls or wing morphology between populations of the same species will be large enough to have consequences for habitat use. For this reason, this study did not analyse the foraging habitat hypothesis.

**Drift Hypothesis.** Through fragmentation of their habitats, the two species may have been subdivided into two or more populations separated by barriers that restrict gene flow. Geographic variation is therefore a result of fragmentation of the ancestral population into subpopulations due to the formation of environmental barriers which restricted gene flow between the subpopulations. Differences between populations would therefore be largely due to random genetic drift and founder effects. This could have been through vicariant events, e.g., climate and weather related events like cyclones, geologic events like earth processes (e.g., plate tectonics) leading to the formation of geographic barriers (e.g., mountains, lakes, and inhospitable biomes) and the fragmentation of biomes due to historical climate change. The subsequent disruption of gene flow between the separated populations, the associated evolutionary processes of genetic and cultural drift and the founder effect can then cause divergence in both the genotype and phenotype of the populations within each species. The
overall result is deviation of characters from that in the original population without the intervention of deterministic factors such as natural selection. Such phenotypic variation through vicariant events can be illustrated by disjunctions in character patterns. For example, some morphometric variables describing male tibiae of the damselfly *Platycnemis pennipes* (Battin 1992) showed disjunction and others, a gently stepped cline in the variation of maximum width and length of meso/metathoracic male tibiae. This suggested differentiation in allopatry followed by secondary reintegration (in some portions of the species’ range) which was linked to vicariant events (separation and re-joining of the Peloponnesus to the mainland) in the Lower Pliocene and early Pleistocene (Battin 1992). However other factors such as ecology, dispersal and human-induced changes may also have played a role because the operational efficiency of vicariant barriers depends on the dispersal ability of the organism considered (Battin 1992). To analyse drift, the study therefore used a mathematical model based on the quantitative theory of molecular evolution, the Lande’s model. The model predicts the effects of drift on phenotypic traits using patterns of phenotypic variation between and among populations.

**Thesis outline**

The primary objective was to test the two hypotheses, *Adaptation* and *Drift* by comparing the phenotypes of each species across their geographic ranges, linking their geographic variation to the geologic and climatic history of the region. Three fundamental questions were addressed: 1) Do these two closely-related species show the same kind of geographic variation? 2) Are the same factors responsible for their geographic variation? 3) Does variation in phenotypes relate to the geography and climate of the southern African region?

Objectives:
• To describe and compare the differences in phenotype (morphology and echolocation call parameters) among geographically separated populations of *Rhinolophus swinnyi* and *R. simulator* under varying environmental conditions.

• To test the relative contributions of adaptation and drift to the phenotypic diversification of two species of horseshoe bats, *R. simulator* and *R. swinnyi*.

Chapter 2 tested relative contributions of drift and adaptation to both linear morphometric measurements and echolocation by applying the principles of Lande’s model (Lande 1976; 1979) in the form of the β-test (Ackermann and Cheverud 2002). It was hypothesised that bat phenotype should diversify predominantly through adaptation owing to the echolocation-flight and echolocation-diet adaptive complex that ties these species relatively more intricately with environmental conditions.

As further confirmation (using hard tissue, relatively more sample coverage, more phenotypic parameters of the head and by incorporating museum collections) Chapter 3 used geometric morphometrics on 3D scanned skulls of the two species. Similar to Chapter 2, the relative contributions of adaptation and drift in the variation of the shapes and sizes of skulls was done using the Lande’s model. Here it was hypothesised that *Rhinolophus swinnyi* which uses higher RF than *R. simulator* would show stronger signals of selection.

To reconstruct selective forces involved in the geographic variation of the two species, Chapter 4 tested the *Adaptation Hypothesis* by analysing how climate (relative humidity, mean annual temperature and altitude – as a proxy for atmospheric pressure) could explain within species variation in resting frequency (RF) of the echolocation calls of two species of bats, *Rhinolophus simulator* and *R. swinnyi*. It was predicted that adaptive differences in RF were facilitated by habitat variation and may have led to the diversification
within *Rhinolophus simulator* and *R. swinnyi*. Variables that are known to attenuate sound (temperature, relative humidity and altitude) including their interactive effects (Luo *et al.* 2013) were analysed in models to test the *Sensory Drive Hypothesis* which predicts that geographic variation in atmospheric conditions selects for optimum sound frequencies within each habitat and that this could lead to diversification if the signals are used in communication. Lower frequency sounds undergo less attenuation and these should be favoured in conditions with high atmospheric attenuation, i.e., high humidity in combination with low temperature and high atmospheric pressure (Luo *et al.* 2013).

A comprehensive overview is given in the synthesis (Chapter 5), incorporating recommendations for areas of future research.

This study is the first to weigh the relative contributions of adaptation and drift in the diversification of horseshoe bat phenotypes using the Lande’s model. Currently most research derives evidence of drift by simply showing the absence of selection (e.g., Chen *et al.* 2009; Sun *et al.* 2013).

**Ethical Statement**

Sampling methods of this work followed the guidelines by the American Society of Mammalogists (Gannon and Sikes 2007), and other guidelines compiled by Aegerter *et al.* (2005) and Kunz and Parsons (2009). All methods were approved by the Science Faculty Animal Ethics Committee at the University of Cape Town (Clearance Number 2013/2011/V6/DJ). Every person handling bats was vaccinated for rabies and was provided with appropriate protective clothing/gear. Only non-protected species were sampled, from privately owned and/or protected areas with authority from: Zimbabwe (Parks and Wildlife Management Authority; Permit [23 (1) (C) (II) 25/2011; 19/2012 and 16/2013], South Africa (Northern Cape Province, Fauna 764/2010; Mpumalanga Tourism and Park Agency, MPB
5253; Cape Nature, 0035-AAA007-00081), Malawi (Department of Forestry Licence NO: 1/06/2013/1), Botswana (Ministry of Environment, Wildlife and Tourism, EWT 8/36/4 XVI – 78).
CHAPTER 2

The relative contributions of drift and selection to phenotypic divergence in the Horseshoe bats *Rhinolophus simulator* and *R. swinnyi*

**Abstract**

Natural selection and drift can act on populations individually, simultaneously or in tandem and our understanding of phenotypic divergence depends on our ability to recognise the contribution of each. According to the quantitative theory of evolution, if an organism has diversified through neutral evolutionary processes (mutation and drift), variation of phenotypic characteristics between different geographic localities (*B*) should be directly proportional to the variation within localities (*W*) such that \( B \propto W \). Significant variation from this null model shows that other non-neutral forces such as natural selection are acting on a phenotype. To analyse the relative contributions of drift and adaptation to within-species diversity, various morphological traits and echolocation resting frequency (RF) of horseshoe bats were measured based on 111 *Rhinolophus simulator* and 141 *R. swinnyi* individuals from various localities in southern Africa. It was evident that the geographic variation in both species was predominantly caused by natural selection (as \( B \) was not directly proportional to \( W \)). However, signals of drift were identified for some phenotypic characters, contributing to variation within both species. There were strong signals for selection on resting frequency (both species), manoeuvrability (*Rhinolophus simulator* only) and size (*R. swinnyi* only). Additionally, there was correlated selection (co-selection) amongst phenotypic characters confirming that drift was not substantial. Overall, selective forces were mostly disruptive with less variation shown between than within populations. The phenotypic traits under
selection differed between the two species, and between different localities, suggesting that
differential ecological selection had occurred. Because phenotypic divergence was also not
correlated to geographic distance, the most plausible explanation of the divergences in
phenotypes observed in this study is predominantly driven by adaptation to local habitats
with reduced gene flow among them as a result of vicariance and/or isolation by habitat.

Key Words
Micro-evolutionary forces, neutral evolution, speciation, vicariance, Lande’s model,
diversification, adaptation, natural selection

Introduction
Geographic variation in phenotypes can reveal the evolutionary effects of drift and
selection on population diversification within species. If a species is distributed over wide
geographic areas covering different habitats and biomes, animals from different geographic
localities may be subjected to a variety of selection pressures. As a result, phenotypic
divergence between localities ensues as different adaptations to different environmental
conditions like climate (e.g., rainfall and temperature), prey, and foraging habitat (Magurran
1998; Lomolino et al. 2006; Morrone 2009). Such divergence may be enhanced if gene flow
is restricted by physical or biological barriers that may limit dispersal (Malhotra and Thorpe
2000; Morrone 2009). Alternatively, random events like droughts, floods and disease may
decrease genetic variability in a population by eradicating a huge proportion of the population
and only leaving a few survivors carrying a subset of the original genetic variation (bottle-
neck effect). Similarly, new populations established by a small number of individuals would
also carry only a subset of the parent population’s genes (founder effect). Consequently,
chance fixation of certain alleles is enhanced and other traits may be lost completely leading
to genetic drift (Wright 1937; Millstein 2008). In both cases of adaptation and drift, if gene
flow is restricted, divergence will be enhanced especially when founder populations are
small. Even though phenotypic divergence may be driven both by natural selection and drift, most evolutionary explanations focus on adaptation (Weaver et al. 2007). Studies which investigate the relative roles of adaptation and drift to phenotypic divergence contribute to the understanding of how speciation initiates and proceeds in natural populations (Orr and Smith 1998; Coyne and Orr 2004).

Phenotypic diversity within species has been documented in several taxa, including animals that use acoustic signalling systems, such as insects, frogs and mammals (Claridge and Morgan 1993; Grant and Grant 1989; Morton 1977; Wilczynski et al. 1992). Earlier explanations of animal diversification were mostly based on natural selection (Schluter 2009), whereas explanations based on drift became only more numerous at a later stage when genetic drift was discovered (Wright 1929). However, there has been controversy on both the significance of drift to biological diversification and whether or not it can be distinguished from adaptation (Brandon and Carson 1996; Millstein 2002; Brandon 2005). Nevertheless evidence is accumulating for the role of drift (Lande 1976; Ackermann and Cheverud 2002; 2004; Weaver et al. 2007; Smith 2011; de Azevedo et al. 2015) and many studies are exploring various methods to account for the relative contributions of adaptation and drift e.g., the rate test (Turelli et al. 1988), genetic approaches (Leinonen et al. 2008; Rogell et al. 2010; Sun et al. 2013), and quantitative genetic models e.g., Lande’s model (Lande 1976; Ackermann and Cheverud 2002; 2004; Smith 2011; de Azevedo et al. 2015). The Lande’s model is easier to apply in this case because it has been adapted by Ackermann and Cheverud (2002) to use phenotypic parameters instead of genetic traits. Phenotypic traits that perform crucial functions form tight associations with environmental conditions to accrue fitness benefits. For example, sensory systems are crucial for survival, mate choice and reproduction (Puechmaille et al. 2014) and are sensitive to environmental conditions especially atmospheric conditions, such as climatic factors (Sun et al. 2013; Mutumi et al. 2016;
Kirschel *et al.* 2011). It is likely that adaptation predominantly shapes diversification of sensory traits more so than drift. Echolocation is a sensory trait that is used not only in obstacle avoidance and prey capture (Schnitzler and Kalko 2001) but also in mate choice (Puechmaille *et al.* 2014) and is therefore likely to show strong signals for adaptation rather than drift.

An adaptive complex exists between wings and acoustic signals in birds which use sound for orientation as observed in oil-birds and swiftlets (Griffin 1970; Konishi and Knudsen 1979; Fullard 1993; Iwaniuk 2006; Brinkløv *et al.* 2013) as well as in bats which echolocate (Aldridge and Rautenbach 1987; Jacobs *et al.* 2007). Because atmospheric attenuation increases with increase in sound frequency (Guillén *et al.* 2000; Mutumi *et al.* 2016) this adaptive complex should be tighter in bats than in birds because bats use higher sound frequencies. The adaptive complex however allows flying organisms to navigate their environments efficiently (Norberg and Rayner 1987) using tight coordination between flight manoeuvres and sonar call production (Falk *et al.* 2015). The need to resolve and avoid obstacles defines the sensitivity of flight and echolocation features to environmental conditions hence making them more likely to diversify through adaptation. The wing-sound adaptive complex also affects the evolution of the skull shape and size because it houses features for the production and reception of sensory signals while also functioning for handling and mastication of food. This is evident in both mammals and birds (Freeman and Lemen 2010; Jacobs *et al.* 2014). Nevertheless, several studies have also found roles for drift in acoustic signals that are used in reproduction rather than orientation e.g., in Neotropical singing mice (Campbell *et al.* 2010) and anurans (Ohmer 2009) despite evidence for selection too. The two processes may operate in tandem with their effects varying at different times and at different locations during the diversification of organisms (Orsini *et al.* 2013).
The quantitative theory of molecular evolution as described by the Lande’s model (Lande 1976) has been applied to show how random evolutionary processes can account for phenotypic divergence (Ackermann and Cheverud 2002; Ackermann and Cheverud 2004; Smith 2011; de Azevedo et al. 2015). The theory gives a null model against which the relative contributions of adaptation and drift can be weighed (Smith 2011). Diversification patterns based on variance/covariance (within and between localities) of phenotypes are used to assess the contributions of adaptation and drift. The model predicts the drivers of neutral evolutionary processes (mutation and drift) using the regression of between populations’ variance of phenotypic traits ($B$) against their variation within populations ($W$). It specifies that $B$ should be directly proportional to $W$ ($B \propto W$; Ackermann and Cheverud 2002) if the diversification is through drift. Significant deviations from proportionality imply non-neutral forces (natural selection) leading the diversification. For example, genetic drift was shown to be the primary force for diversification in facial features and skull structure of the genus *Homo* (Ackermann and Cheverud 2000; Smith 2011) and skull morphology in monkeys (Marroig et al. 2004) using this approach. Strong selective forces were also identified in some regions of the human skull using the same model (de Azevedo et al. 2015). Adaptive explanations may be over-represented if not weighed against a null model of drift (Marroig and Cheverud 2004; Betti et al. 2010). Few studies have used this approach, and none have used the Lande’s model to assess the underlying drivers of phenotypic divergence in mammals other than primates. This is mainly due to methodological limitations, the statistical procedures demanded by this method are complicated and time-consuming.

Bats offer an interesting test case. The use of an intricate sensory system (echolocation), for orientation (Schnitzler et al. 2003), prey detection (Neuweiler 1989) and communication (Knörnschild et al. 2012) in coordination with flight (Falk et al. 2015) in bats presents a unique opportunity to apply the Lande’s model. Bats exist in almost every known
biome on the globe with the majority of species having wide distributional ranges covering several habitats and even biomes (Csorba et al. 2003; Monadjem et al. 2010). Variations in habitat conditions likely pose an array of selective forces on a phenotype that is likely fine-tuned to specific habitats owing to the intricacies of the combined use of flight and echolocation (Norberg and Rayner 1987). This has been shown by adaptive trends in wing morphology which parallel those in echolocation call structure in several families of bats (Norberg and Rayner 1987). Despite being volant the dispersal ability of bats are limited and gene flow can be restricted (Moussy et al. 2012). Vicariance can split a population into smaller disparate groups e.g., barriers in the form of waterbodies, extensive human developments and mountain ranges. Small populations can therefore experience genetic drift. In one study that has tested the relative contributions of drift and adaptation using genetics in bat phenotypic diversification (Sun et al. 2013), predominant support for adaptation was shown. Wings and sensory modalities have to track environmental conditions (Norberg and Rayner 1987), it is therefore more likely that bat phenotypes are predominantly shaped by strong selective forces.

Horseshoe bats (Rhinolophidae) have wide geographic distributions across spatially heterogeneous environments in southern Africa (Csorba et al. 2003; Monadjem et al. 2010). Furthermore, they vary in population size (ranging from tens to thousands), body size, dispersal capabilities and the degree to which they are philopatric (Kunz and Parsons 2009). Within species divergence in the resting frequency of echolocation calls of many horseshoe bats has been shown to be mainly the result of adaptations to optimise sound propagation in habitats of varying atmospheric conditions e.g., humidity and temperature (Mutumi et al. 2016; Odendaal et al. 2014) and obstacles which have to be avoided during flight e.g., vegetation (Xu et al. 2008; Odendaal et al. 2014). It has been proposed that body size (e.g., wing dimensions) co-vary with resting frequency as a consequence of optimisation of flight
and echolocation in habitats of varying clutter and prey (Norberg and Rayner 1987; Jacobs et al. 2007). Despite evidence for genetic drift in anurans and Neotropical singing mice (Ohmer 2009; Campbell 2010) some horseshoe bats have shown predominant signals of selection (e.g., Sun et al. 2013).

This Chapter investigates the relative contributions of adaptation and drift in the phenotypic divergence of two horseshoe bats, Rhinolophus simulator and R. swinnyi against a null model of drift developed by Lande (1976) and adapted by Ackermann and Cheverud (2002). Three hypotheses were tested: 1) Adaptive Complex - that the signals of drift should be absent for phenotypic traits under selection pressure from the physics of sound and flight. 2) In two species of bats with similar sizes but different RF, there will be stronger signals of selection for species using higher frequency sound because atmospheric attenuation increases with increase in sound frequency. 3) There should be variation in the relative effects of selection and drift on specific phenotypic traits and across different populations. This kind of analysis has not been done before with small mammals and the results will elaborate on the relative importance of drift and selection in the phenotypic divergence in of bats.

Methods

Study sites and animals

Bats were captured from caves and disused mine-shafts across the distributional ranges of the two focal species R. simulator (10 localities) and R. swinnyi (9 localities) at different latitudes ranging from 16˚S to 32˚S (Fig 1). Rhinolophus simulator and R. swinnyi use high duty cycle echolocation calls characterised by a constant frequency component at means of 80 kHz and 107 kHz, respectively (See Fig S1 in Chapter 4). The two species occur across seven woodland types which allowed us to assess the effect of habitat variation. The study sites were located in the eastern half of southern Africa, ranging from Zambia in the
north, through Zimbabwe and Botswana into South Africa in the south. The northernmost locality was the Central Zambezian Miombo woodland in Zambia. The central localities include the Zambezian and Mopane woodlands, Southern Miombo woodlands, and the Eastern Zimbabwe Montane Forest-grassland Mosaic, in Zimbabwe. South African populations occur within Highveld grasslands. The Botswana populations occur within an ecotone of three woodlands; Kalahari Acacia-Baekiaea, Kalahari Xeric Savannah, and Southern Africa Bushveld. Climates differ between woodlands with Botswana site being the driest and the Eastern Zimbabwe Montane Forest-grassland Mosaic, the wettest (Olson et al. 2001). We used the same sampling methods as in Mutumi et al. (2016).

Fig 1. Sampling sites within southern Africa from where *R. simulator* and *R. swinnyi* were caught. Abbreviations: CC = Chinhoyi, Zimbabwe; DM = Dambanzara, Zimbabwe; GKC = Gatkop Cave, South Africa; JET = Jiri Estate Triangle, Zimbabwe; KL = Kalenda, Zambia; KP = Kapatamukombe, Zimbabwe; LOB = Lobatse Estate, Botswana; MC = Mabura Cave, Zimbabwe; MM = Monaci Mine, Zimbabwe; MT = Matobo Hills, Zimbabwe; OD = Odzi German Shafts, Zimbabwe; PA = Pafuri, South Africa; SH = Shimabala, Zambia; and SUD = Sudwala, South Africa.
Morphology and echolocation measurement

Several body, wing and head measurements (Table A1) were taken from captured live bats. These measurements were taken based on their ecological significance and the precision with which they could be measured on live bats in the field. Forearm length (FA), and other morphometric characters (Table A2) were measured using dial callipers to the nearest 0.1 mm and body mass using a portable electronic balance (to the nearest 0.5 g). After extending the right wing of each bat on graph paper, it was photographed using a digital camera (Saunders and Barclay 1992; Canon Powershot A540, Canon inc, Malaysia). The camera was positioned at an angle of 90° and a distance of 30cm above the wing and parallel to a flat table top to minimise angular distortion, and to allow length measurements and wing area using Sigma-Scan Pro 5 version 3.20 (SPSS Inc., Cary, NC, USA). The graph paper was used to calibrate Sigma Scan. From the images, wing area was calculated to include two wings, the tail membrane and the body between the wings i.e., excluding the head. Wingspan was taken as the distance between wingtips of fully extended wings (Norberg and Rayner 1987). Wing loading in Newtons per square meter (N.m⁻²) was calculated as the weight (mass in Kg X acceleration due to gravity in m.sec⁻¹) divided by the wing area (in m²) as in Norberg and Rayner (1987). Aspect ratio was calculated as specified by Norberg and Rayner (1987), i.e., the square of the wingspan divided by the wing area (Table A1).

Echolocation calls were recorded and analysed as described in Mutumi et al. (2016). Hand-held bats allow measurement of the resting frequency (RF) in Rhinolophid bats (Siemers et al. 2005) avoiding inherent differences in RF as a result of horseshoe bats compensating for Doppler shifts during flight (Schnitzler 1987).
Statistical Methods

Precision

Multiple scatter plots of 14 variables were used to identify potential outliers using STATISTICA (version 12, StatSoft Inc., 2013). The outliers were checked against field data and published literature; those deemed a result of measuring errors were re-measured where possible, and those not rectifiable were excluded. Additionally, a voucher specimen was measured 10 times to calculate the precision of each measurement (Table A3). Deviation statistics, including the coefficient of variation (CV) and standard error of the mean (S.E.) were calculated to rank the precision of the measurements. Using FA as the standard (easiest to measure under difficult field conditions), incidents where S.E. was higher than all possible population pairwise differences in FA versus other parameters were identified. To do this, a matrix of population pairwise simple differences of phenotypic measurements was computed. The number of differences that were less than the S.E. of the FA was counted. All phenotypic measurements with a greater number of occurrences than FA of population pair-wise differences lower than S.Es were discarded (Table A3).

For all subsequent analyses, data were first standardised using the z-transformation in STATISTICA (version 12) to equalise the scale of our variables as in Jacobs et al. (2013). As the majority of our variables satisfied normality and homogeneity of variances (among populations) only parametric tests for subsequent analyses were used (Ackermann and Cheverud 2002; 2004). Furthermore, central to the approach used are variance patterns rather than absolute sizes, so that minor violation of homogeneity of variances is generally not considered a major concern (see de Azevedo et al. 2015).
**Sexual dimorphism**

Sexual dimorphism was assessed using ANOVA (Siemers *et al.* 2005) with the phenotypic variables as multivariate response variables. Dependent variables, sex and site were specified as categorical predictors. Univariate results for each variable were used to analyse sexual dimorphism.

**Geographic variation**

To investigate the degree of geographic variation among samples from different localities, a discriminant function analysis (DFA) was used with the phenotypic variables as dependants and populations as independent variables. To avoid multicollinearity of independent predictors, phenotypic variables were first converted into principle component scores (PCs) using Principal Component Analysis (PCA). From the DFA using principal component scores (PCs) as input variables, Mahalanobis squared distances between populations ($D^2$) were extracted in bivariate space from the first two functions. To illustrate how localities separated in 2-dimensional phenotypic space, multi-dimensional scaling plots were applied to the Mahalanobis matrix of phenotypic distances ($D^2$). Additionally, a cluster diagram was generated for each species to gauge how the localities grouped based on their phenotype differences. The phenotypic distance matrix ($D^2$) was also regressed against the geographic distance matrix (calculated from geographic coordinates – straight-line distances) to determine whether the geographic patterning was driven by isolation by distance by a Mantel test in R statistics (R Development Team 2013), package Ade4 (Dray and Dufour 2007).

**Lande’s Model**

Signals of drift among populations of *Rhinolophus simulator* and *R. swinnyi* were tested using the null model of drift which is based on the Lande’s model (Lande 1976; 1979). To use phenotypic instead of genetic traits, for which this model was originally developed,
the version developed by Ackermann and Cheverud (2002), known as the beta-test ($\beta$-test; Ackermann and Cheverud 2002; de Azevedo 2015) was employed. Accordingly, phenotypic within-group covariance matrices ($P$) instead of genetic covariance matrices ($G$) were used. A known relationship has been established between the two kinds of matrices ($P$ and $G$; Cheverud 1988 and Roff 1996), so that $P$ can be used to approximate $G$ (de Azevedo et al. 2015). The model specifies that if diversification across populations is through neutral evolutionary processes (mutation and drift), variation of phenotypic characteristics between populations ($B$) should be directly proportional to the variation within populations ($W$), i.e. $B \propto W$ (Ackermann and Cheverud 2002). Significant deviations from this null model imply other non-neutral forces acting on phenotype of species, possibly natural selection.

The process of deriving $B$ and $W$ is illustrated in Figure 2. First, $W$ (Fig. 2, grey box) was estimated through MANOVA with 12 phenotypic traits (Tables A1 and A2) as the dependent variables. Population (locality) and sex were specified as the independent variables. From the MANOVA, the residual variance/covariance (V/CV) matrix was extracted (Ackermann and Cheverud 2004). This matrix provides an estimate of the portion of variation that remains unexplained by inter-population and sexual differences (some parameters were sexually dimorphic). A set of PCs from the V/CV matrix using PCA was generated and eigenvalues of the PCs were extracted to represent the within-population variance $W$ (Fig. 2, grey box; Ackermann and Cheverud 2002; 2004).
Fig 2: Lande’s Model conceptual flow diagram showing the steps taken to derive the population variance \( B \) and the within population variance \( W \) which are used in the regression to determine whether \( B \propto W \). Solid arrow depict data set transferred as it is to the proceeding analyses, whereas dashed arrow depict processed data extracted from an analysis being passed onto another analysis procedure. The analysis starts from the top, either from right (using all populations) or later from the left (excluding a population at a time).

Next, \( B \) was estimated by multiplying the matrix of eigenvectors (obtained from the PCA on the V/CV matrix) by the matrix of the trait means for each locality (trait = columns; population = rows; see Fig. 2, green boxes; Ackermann and Cheverud 2002). The product of these two matrices yielded a new set of PCs derivatives. The variances associated with each new PC loading were calculated as the mean square of loadings within each PC. This variance value represents the between group variance \( B \) for each new PC (Fig. 2, green
boxes; Ackermann and Cheverud 2004). The natural logarithms of $B$ were regressed against those $W$ to assess whether the between locality variance could be fully explained by the within locality variance, i.e., whether the regression slope ($\beta$) was significantly different from 0. All slopes of traits that differed significantly from 0 were tested for consistency with drift by testing the alternative hypothesis that the slope was not significantly different from a gradient of one.

The $\beta$-test also predicts that the new PCs (calculated from eigenvectors and trait means, as described above) should remain uncorrelated if drift is present. The Pearson’s correlation test was therefore used as further confirmation of drift (Ackermann and Cheverud 2002; de Azevedo et al. 2015). If the PCs are correlated, there is a possibility of co-selection on the corresponding traits (de Azevedo et al. 2015). Therefore correlation analyses among the new PCs were run to further substantiate selection, if any.

**Lande’s Model: step-wise exclusion of components**

Some localities/some PCs were excluded and the model re-ran to reveal how each specific population or phenotype influenced the slope of the regression of $B$ on $W$, because drift or selection may be differentially exerted on populations occurring in different habitats or across different phenotypes. This rationale was also based on similar reasoning as in de Azevedo et al. (2015) when they excluded a PC at a time to assess whether different regions of human skulls differentially experienced drift/selection. The Lande’s model was therefore repeated excluding 1) a population at a time 2) a PC at a time and 3) a combination of populations and PCs (Fig. 2; blue boxes). When a single locality was removed, the whole analysis procedure was repeated from the MANOVA stage up to the regression. Excluding a PC at a time was done only at the regression stage, meaning that only this stage would be repeated without a particular PC. Therefore, all populations were used in the analysis up to the regression after which the regression was repeated several times excluding a PC at a time,
to check for changes in the relationship between $B$ and $W$ with and without each trait. This procedure was repeated when a population at a time was excluded i.e., to gauge the relationship of $B$ and $W$ with all PCs versus without some PCs, for each time that a particular population was excluded (see Fig. 2; blue boxes). A detailed account on the locality/PC exclusion stages is presented in the following sub-sections.

Using cluster diagrams generated during the assessment of geographic variation earlier in the analysis, populations occurring furthest from the others in the phenotypic space were excluded one at a time and the Lande’s model re-ran at each stage with all the remaining populations. The change in $B$ and $W$ regression results was assessed at each stage. The regressions of $B$ and $W$ excluding a PC at a time were repeated for the ‘all-population-analysis’ for both species. This was done to check how $B$ responded to $W$ in the absence of some phenotypic traits. Accordingly the change in the gradient of the regression between $B$ and $W$ at the exclusion of a specific PC compared with the gradient when all PCs were included in the regression was assessed. Some PCs were also removed from the analyses where we excluded a population at a time and the regression was re-run between $B$ and $W$. In this case only PCs carrying the lowest eigenvalues were excluded to simplify the analyses.

**Results**

The data of the morphology and resting frequency of 111 *R. simulator* and 141 *R. swinnyi* (Table A2) were analysed.

**Precision**

From the field measurements, 12 of the 14 variables were at least as precise as the chosen standard, forearm length (Tables A1 and A3). These were: foot length – FL, upper tooth-row length – TR, head width – HW, tail length – TL, aspect ratio – AR, forearm length – FA, wing area – WA, wing-loading – WL, wing-span – WS, head-height – HH, and head
length – HL. Only these measurements were used for all the subsequent analyses. The tibia length and nose-leaf width were excluded.

**Sexual dimorphism**

MANOVA results showed that both sex and localities were phenotypically different within both species (*R. simulator*: MANONA sex $F_{12, 73} = 3.74; p < 0.001$; Locality $F_{96, 502} = 7.21; p < 0.001$. *R. swinnyi* Sex: $F_{12, 100} = 5.34; p < 0.001$ and Locality $F_{84, 620} = 5.09; p < 0.001$). Only 4 of the 12 variables in the two species were sexually dimorphic as indicated by significant effects based on a significance level of $\alpha = 0.05$ (WS: ANOVA: $F_{1, 93} = 7.7; p < 0.001$, WA: ANOVA: $F_{1, 93} = 6.9; p < 0.05$, WL: ANOVA: $F_{1, 93} = 4.5; p < 0.05$ and RF: ANOVA: $F_{1, 93} = 11.7, p < 0.001$ for *R. simulator* and, WS: ANOVA: $F_{1, 119} = 6.7; P < 0.05$, WA: ANOVA: $F_{1, 119} = 5.7; p < 0.05$, and RF: ANOVA: $F_{1, 119} = 47.9; p < 0.001$ for *R. swinnyi*). Both species exhibited dimorphism for the same parameters except in the case of WL which was only dimorphic in *R. simulator*. For the Lande’s model, sex was incorporated as a categorical predictor together with study sites, and variation due to sex differences was therefore taken out of the within-population V/CV matrices used in the modelling. For the exploratory stages (DFA) we balanced the proportions of the sexes for populations which were dimorphic.

**Geographic variation**

Geographic variation in phenotype was indicated for both species (Figs. 3a and 3b). There was a fairly distinct separation of study sites in the 2D phenotypic space (using canonical roots 1 and 2 from the DFA), with LOB, GKC and MM the most distinct from the rest in *R. simulator* (Fig. 3a), whereas KP was the most distinct site from the rest in *R. swinnyi* (Fig. 3b). Total classification success reached 84.7% for *R. simulator* (Wilks' Lambda: 0.0038, $F_{108, 667} = 7.1333; p < 0.001$) and 80.4% for *R. swinnyi* (Wilks' Lambda: 0.0324, $F_{96, 838} = 5.8034; p < 0.001$). In *R. simulator*, canonical roots 1 and 2 accounted for
88% of the variation. Root 1 explained 76% of the variation and was predominantly made up of WS, WA, AR, and RF. Root 2 explained 12% of the variation and was also predominantly made up of WS, WA and AR. These three variables were all associated with flight and detection and suggest differences in manoeuvrability and orientation.

**Fig 3:** Multidimensional scaling (MDS) plots for A) *Rhinolophus simulator* and B) *Rhinolophus swinnyi* and cluster diagrams for C) *Rhinolophus simulator* and D) *Rhinolophus swinnyi* based on Mahalanobis $D^2$ distances showing inter-population variation in phenotype (based on body size, flight morphology and echolocation parameters). Locality abbreviations are the same as in Table 1.
Rhinolophus swinnyi’s canonical roots 1 and 2 from the DFA accounted for 74% of the variance. Root 1 explained 60%, predominantly made up of FA, HL, TL, WL and RF. Root 2 accounted for 14% and was predominantly made up of HL, WS, WA, WL and RF. These are variables associated with body size, flight and detection and suggest differences in size, manoeuvrability and orientation.

Cluster diagrams showed a hierarchy in the phenotypic linkage-distances as a measure of dissimilarity between sites. Following this hierarchy, the sites could be arranged in order of greatest dissimilarity to the rest as follows: R. simulator; MM, (GKC, LOB), MT, KL, SH, SUD, CC, DM, MC, and R. swinnyi; KP, KL, JET, BU, DM, (OD, PA), (MC, CC), (Figs. 3c and 3d, respectively).

According to the Mantel Test results, geographic variation in phenotype among localities of the two species was not related to the geographic distances among them (R. simulator: Monte-Carlo test, Observation: 0.362, Simulated p-value: 0.075 and R. swinnyi, Monte-Carlo test, Observation: -0.082, Simulated p-value: 0.534: Based on 10000 replicates).

**Lande’s Model Results**

Within locality variances could not explain between locality variances, as \( B \) was not directly proportional to \( W \) in most of the tests (Tables 1 and 2; Fig. 4). When all localities were included in the analyses (Lande’s Model) for each of the two species, the slopes \( \beta \) of the regression lines of \( B \) on \( W \) were not significantly different from zero and therefore did not show proportionality (Figs. 4a and 4b; Tables 1 and 2). This indicated that non-neutral processes, such as natural selection, were mainly responsible for the geographic variation. These results did not change qualitatively when removing one population at a time. Likewise, re-running the model using all populations but excluding a PC at a time did not show any signals of drift (Lande’s regressions; excluding some PCs). Instead, the relationship
became strongly inverse with the removal of PC10 in *R. swinnyi* (slope = -0.67; *p* < 0.05). Of major concern were PCs 9, 10 and 11 because they showed a huge disparity between *B and W* for very minor eigenvalues and strongly influenced the regression line in both species. Consequently, when these last PCs were removed from each of the stages where a single population at a time was removed two cases where drift was supported were found. This was when the DM population and PCs 10 and 11 were removed from the *R. simulator* data and when the OD population and PCs 9 and 11 were removed from the *R. swinnyi* data. Here positive slopes which were significantly different from zero for both species were found (*R. simulator*: β = 0.74; *p* < 0.05, *R. swinnyi*: Slope = 0.73; *p* < 0.05) and consistent with a slope of drift i.e., gradient β ≈ 1 (*R. simulator*: Slope = 0.74; *p* = 0.17; Table 1; Fig. 4c, *R. swinnyi*: Slope = 0.73; *p* = 0.16; Table 2; Fig 4d).
Table 1: Results of Lande’s model tests for Rhinolophus simulator

<table>
<thead>
<tr>
<th>Pop</th>
<th>PCs used</th>
<th>N</th>
<th>β</th>
<th>± std err</th>
<th>R²</th>
<th>P value, $h_0:$ b = 0</th>
<th>P value, $h_0:$ b = 1</th>
<th>Correlated PCs</th>
<th>Consistent with drift?</th>
</tr>
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<tr>
<td>All</td>
<td>All</td>
<td>9</td>
<td>-0.059</td>
<td>0.333</td>
<td>0.003</td>
<td>0.864</td>
<td>4-3; 6-4; 6-5; 7-4; 7-3; 10-2</td>
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<td>-MM</td>
<td>All</td>
<td>8</td>
<td>-0.274</td>
<td>0.321</td>
<td>0.075</td>
<td>0.415</td>
<td>7-1; 10-2; 6-5; 11-5; 11-6</td>
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<tr>
<td>-MT</td>
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<td>-0.456</td>
<td>0.297</td>
<td>0.148</td>
<td>0.307</td>
<td>7-1; 6-5</td>
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<tr>
<td>-KL</td>
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<td>-SH</td>
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<td>10-2; 4-3; 7-3; 7-4; 8-4; 6-5</td>
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</table>

NB: We only showed PCs which when removed changed the relationship between B and W (9, 10 and 11). Localities: PA = Pafuri, JET = Jiri Estate – Triangle, MM = Monaci Mine, OD = Odzi German Shafts, DM = Dambanzara, MC = Mabura, KP = Kapamukombe, KL = Kalenda, SUD = Sudwala. Pop = Populations (N – number of) populations included, starting with all populations (All), and excluding one at a time (e.g., -MM = meaning Population MM is excluded). The regression is run with either all PCs (PCs used; All) or excluding some PCs (Exclude e.g., 10 & 11). b: estimation of regression slope, along with the standard error (± std err) and p-value for the null hypothesis of b = 0 (P value, $h_0:$ b = 0). Regressions significantly different from 0 are tested against the null hypothesis of b = 1 shown by p-values (P value, $h_0:$ b = 1). $R^2$: adjusted R squared value for the regression test. PCs: Principal components presenting significant correlations with p < 0.001. The models in bold are consistent with the Null model of drift.
Table 2: Results of Lande’s model tests for *Rhinolophus swinnyi*

<table>
<thead>
<tr>
<th>Pop</th>
<th>N</th>
<th>PCs used</th>
<th>b</th>
<th>±std err</th>
<th>$R^2$</th>
<th>P-value, $h_o = 0$</th>
<th>P-value, $h_o = 1$</th>
<th>Correlated PCs</th>
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<td>All</td>
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<td>All</td>
<td>-0.080</td>
<td>0.332</td>
<td>0.006</td>
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<td>11-3; 7-5; 11-5; 9-6; 8-7; 11-7</td>
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<td>-KP</td>
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<td>0.177</td>
<td>0.328</td>
<td>0.031</td>
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<td>-JET</td>
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<td>6-3; 5-4; 11-7; 11-10</td>
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<td>0.000</td>
<td>0.981</td>
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<td>0.888</td>
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<td>7</td>
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<td>-0.444</td>
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<td>0.198</td>
<td>0.231</td>
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<tr>
<td>-OD</td>
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<td>-0.040</td>
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</table>

Abbreviations same as in table 1
**Figure 4:** Regression of $B$ (between-group) and $W$ (within-group variance) for A) *Rhinolophus simulator* and B) *Rhinolophus swinnyi*. Locality abbreviations are the same as in Table 1. For the top panels (A and B), dot sizes indicate the PC's influence on the regression line (calculated as the difference between the slope values with and without that particular PC point). The regression lines (thin black lines) for both species have a much smaller and slightly negative compared to a slope ($b = 1$) based on the null hypothesis of drift (thicker black lines in panels A and B). Bottom panels: C) *R. simulator* without the DM population and excluding PC 10 and 11. D) shows *R. swinnyi* without the OD population and excluding PC 9 and 11; in this case dot size does not carry any value.
PC loadings for *R. swinnyi* data without the OD populations (Table A4) showed that PC 9 and 11 were predominantly made up of manoeuvrability, echolocation behaviour and size i.e., PC 9: RF (0.61) and FA (0.22) and PC 11: HL (0.47) and WS (0.19). Similarly, for *R. simulator* data without the DM population (Table A4), PC 10 and 11 were predominantly made up of manoeuvrability, size and orientation behaviour i.e., PC 10: WL (0.44), TL (0.19) and HL (0.16) whereas PC 11: RF (0.85). These results from the Lande’s model, however, still did not solely support drift as other phenotypic features were still under selection. The graphs of the regressions between \(B\) and \(W\) showed that some PCs fell outside the 95% confidence interval. Furthermore, the correlation probabilities also indicated that some PCs were significantly correlated in both species: *R. swinnyi*; PC 8 – HH, TR, TL, FA, HW, WL, RF and PC 6 – HH, CM, TL; *R. simulator* had Two PC pairs correlated: PC 6 – HL, FA, HW, FL and PC 4 – AR, TR, WS; PC 6 and PC 5 – FA, HL, HH, FL. Thus there was evidence for co-selection between some PC pairs (Tables 1 and 2). However, the pairs correlated were highly variable across the different cases analysed (Tables 1 and 2).

Comparing the results for geographic variation (Figs. 3a and 3b) with those from the Lande’s Models indicate that the least distinct sites DM (*R. simulator*: Fig. 3a) and OD (*R. swinnyi*: Fig. 3b) greatly influenced the relationship between \(B\) and \(W\) in the Lande’s results. When these (together with PCs carrying low eigenvalues) were excluded from the modelling the relationship became consistent with drift. The geographic variation in phenotype was mainly through manoeuvrability and echolocation behaviour in *R. simulator* whereas in *R. swinnyi* it was mainly through echolocation behaviour and size variables. The Lande’s model confirmed that selection was acting mainly on RF in both species. Size and manoeuvrability variables where under selection but also showed signals of drift in the two species when DM and OD populations were excluded in combination with PCs containing RF. All \(R^2\) values were small, less than 0.6 showing that the relationship between \(B\) and \(W\) was weak (Tables 1
and 2). Additionally, no support for drift was supported by evidence of co-selection in the form of correlations between PC scores. If drift was responsible these PCs would not be correlated (Ackermann and Cheverud 2002). Thus geographic variation and the evidence of co-selection amongst different traits suggest that variation in these two species was predominantly the result of selection.

Discussion

Selection was the predominant process implicated in phenotypic divergence in *R. simulator* and *R. swinnyi* with only a minor role for drift in both species in accordance with our prediction 1). Signals of drift were only indicated in a negligible proportion of the tests. This result was supported by the absence of a correlation between geographic distance and phenotypic differences across populations for both species. Furthermore, there was evidence for co-selection among the different traits analysed supporting a negligible role for drift. Population divergence in both species was the result of habitat mediated selection mostly on resting frequency (both species), followed by selection on flight and manoeuvrability (more-so in *R. simulator* than in *R. swinnyi*) and selection on size (stronger in *R. swinnyi* than on *R. simulator*). In accordance with prediction 2), selection was differentially exerted across populations with DM and OD experiencing the strongest selection. Without these populations in the analysis, there was a signal for drift when the RF and Manoeuvrability PCs were removed. Contrary to prediction 3) there was no significant difference (Fig. 4) in the signal for selection between the two species.

Our results contrast with those from studies on monkeys and humans which report a predominance of drift (Ackermann and Cheverud 2002; Ackermann and Cheverud 2004; Smith 2011). However, evidence for significant deviations from neutrality were also found in
craniofacial variation of early and late Holocene Native American groups (de Azevedo et al. 2015). In common with our study, these studies also found that selection (and drift) were differentially expressed across different features of the phenotype and/or across different localities (de Azevedo et al. 2015). There are currently no examples of studies in which this approach was used on bats or other comparable small mammals. This study therefore represents the first to apply this approach to a small non-primate mammalian species. Our results corroborate the finding from previous genetic approaches on the horseshoe bat, *Rhinolophus ferrumequinum* (Sun et al. 2013) and two species of grasshopper, *Melanoplus sanguinipes* and *M. devastator* (Roff and Mousseau 2005), which also suggested a predominance of natural selection.

The bat phenotype is characterised by traits that have direct fitness benefits and it is therefore not surprising that selection rather than drift appears to be the predominant process in the evolution of the bat phenotype. Traits associated with flight, feeding and sensory systems have severe consequences on survival and reproduction both separately and in combination and several adaptive complexes have evolved in bats (Norberg and Rayner 1987). For example, there are strong correlations between body size and echolocation (Jones 1996), wing loading and echolocation (Norberg and Rayner 1987) and skull features associated with feeding and echolocation (Jacobs et al. 2014). The bat phenotype is also characterized by tight associations with environmental factors. There are correlations between habitat and each of wing loading (Kalcounis and Brigham 1995) and echolocation (Schnitzler and Kalko 2001) and between echolocation and climatic factors (Mutumi et al. 2016). Bat morphology also correlates with climate following eco-geographic rules, including Allen’s Rule (Solick and Barclay 2006) and Bergmann’s Rule (Hand and York 1990). In both these studies, phenotypic variation was the result of adaptations for reduced heat loss, e.g., *Myotis evotis* had larger ears and wings in mountain populations where it was cooler and
wetter than in the lower lying areas. The predominance of selection over drift we report here
was also found in at least two other studies that investigated the relative roles of these two
processes, Sun et al. (2013) and Odendaal et al. (2014). Both the studies concluded that
divergent ecological selection rather than drift was responsible for the variation in RF across
populations. Although the focus of both these studies as well as ours were different
rhinolophid bat species, there is no reason to suspect that similar results would not be
obtained for other bats or any other organism whose life history is dependent on a tight
association between phenotypic traits and physical laws. This would especially include those
animals that rely on flight, swimming or have specialized sensory and mating systems (e.g.,
birds, frogs, fish and insects).

It is possible that drift may have occurred in parts of the phenotype we did not
consider here (e.g., the skull). Clear signals of drift were detected in the basi-cranium,
temporal bone, and face of modern human populations, and in some features of the skull
within primates (Ackermann and Cheverud 2004, de Azevedo et al. 2015). Even though
morphological/phenotypic integration theories specify that a phenotype mostly evolves as
whole, other features may still evolve somewhat independently. For example in mandibles
and crania of Rhinolophus ferumequinum, two separate modules were identified (Jojic et al.
2015). There is need for a partitioned analysis to investigate different structures of the
phenotype separately. Models can be structured independently for the skull, flight apparatus,
and perhaps also echolocation call features. However, the results presented here still provide
a valuable over-view and a starting point for analysing micro-evolutionary signatures
responsible for phenotypic diversification.

The signal for selection was not the same across traits. Selection was greatest on RF
in both species highlighting the significant role that sensory drive plays in the diversification
of organisms (Mutumi et al. 2016). The RF of the echolocation calls of both species is
influenced by climate mediated selection (Mutumi et al. 2016). *Rhinolophus swinnyi* used lower frequency calls in cooler, humid areas than in hot dry areas whereas *R. simulator* showed spatial structuring by latitude (Mutumi et al. 2016). Even though other stochastic factors may be responsible for the divergence in the phenotype of these two species, results in this paper show that sensory based selection drives the divergence, and that echolocation behaviour plays a pivotal role.

Despite differences in call frequency, selection was not more pronounced on the RF of *R. swinnyi* than on *R. simulator* (Fig 4; Tables 1 and 2). *R. swinnyi* uses higher RF than *R. simulator* meaning its echolocation experiences increased atmospheric attenuation and it would be expected that the RF of *R. swinnyi* would be under more stringent selection. However, the difference in echolocation between *R. swinnyi* and *R. simulator* (24 kHz) translates to only 1.16 mm difference in wavelength (http://www.wavelengthcalculator.com) and may not be large enough to equate to significant differences in their sensory or foraging ecology e.g., differences in prey sizes or habitat (Jacobs et al. 2007). Future research should compare species that have a substantial difference in the frequencies of their RF at lower ranges of the frequency spectrum i.e. ≤ 80 kHz (Jacobs et al. 2007). Such comparisons would involve differences in wavelengths which may be ecologically significant.

In contrast to that on RF, selection on traits associated with manoeuvrability and size differed between the two species. Selection was more pronounced on manoeuvrability than body size in *R. simulator* but the reverse was true for *R. swinnyi*. An explanation for these differences requires more detailed analyses of their habitats and, more importantly, of how these two species use their habitats.

The strong signal for selection suggests that the populations may be isolated enough so that the counteracting effects of gene flow are relatively low compared to the selection
pressure these populations experience. It is therefore likely that the phenotypic divergence reported in this study is a result of adaptation to local habitats reinforced by limited gene flow (to allow adaptive differences to accumulate) among populations. The vicariance responsible for the reduced gene flow cannot be a result of isolation by distance because the mantel test results did not show a correlation between geographic distance and phenotypic distance. The absence of such a correlation suggests that there are other barriers to dispersal. Localities closest to each other (Fig. 1) were not necessarily the most similar, e.g., CC – MC for *R. simulator* and KP – DM for *R. swinnyi* (Fig. 3). The topography between these sites showed that each pair is separated by an extensive mountain range. For example, in CC – MC pair, MC is in a low valley (the Sanyati cotton belt, Zimbabwe) and CC is situated in the northern part of the watershed of Zimbabwe which has the highest elevation in the country. Similarly, DM is separated from KP by the Matusadonha mountain range, Zimbabwe.

Relative to the rest of the other localities, *R. swinnyi* from KP have the larger measurements for 50% of the parameters measured (FA, HH, HL, TL, WS, WL; Table A2) and have the second lowest echolocation frequency, 103.28 kHz. This may be related to the situation of KP in an ecotone of two ecoregions (Zambezian/Mopane and Southern Miombo woodlands). Ecotones characteristically present diverse selective forces which may act as ecological barriers to gene flow (Harris and Reed 2002). Similarly LOB sits in an ecotone of three vegetation biomes (Kalahari Acacia-Baikiaea woodlands, Kalahari xeric savanna and the Southern Africa bushveld). Such ecological barriers to gene flow between LOB and the other nearby localities may make them more divergent. LOB has the highest RF and GKC the second highest whereas, MM has the lowest FA, HH, HL, and the highest FL and AR making these three sites different from the other populations of *R. simulator*. These differences are partly explained by differences in climatic variables but competition for discrete frequency bands in a social context (Mutumi et al. 2016), or isolation by habitat/ecology (Wang 2013;
Wang et al. 2013) may contribute to these differences. However, a more detailed analysis of the environment (including a consideration of co-existing congeners) and the manner in which these bats use the environment need to be undertaken before these differences can be explained.

Conclusion

In organisms with phenotypes that are highly sensitive to selection owing to the combined use of sophisticated sensory and locomotor systems (e.g., insects, frogs, birds and bats), selection rather than drift is still likely to be the predominant process in the evolution of phenotypic variation and ultimately lineage divergence, perhaps even when population sizes are small. Drift is therefore only likely to exert an influence on traits that do not have a severe impact on fitness.
CHAPTER 3

Geographic variation in the skulls of the horseshoe bats, *Rhinolophus simulator* and *R. swinnyi*: determining the relative contributions of adaptation and drift using geometric morphometrics.

**Abstract**

The relative contributions of adaptation and drift to the morphological diversification of skulls and mandibles of *Rhinolophus simulator* and *R. swinnyi* were investigated using the Lande’s model. Geometric 3D landmark coordinates were placed on skulls and mandibles of both species sampled at various localities across their geographical ranges within southern Africa. Although shape variation was evident particularly in the nasal dome and cranium region of both species, changes in the nasal dome of *R. swinnyi* were more prominent than in *R. simulator*. In the mandibles the situation was reversed with more prominent shape changes across localities in the mandibles of *R. simulator* than in *R. swinnyi*. Additionally, modularity was evident in the skull of *R. simulator* but absent in the mandibles of both species as well as the skull of *R. swinnyi*. The skulls of the two species appeared to be under different selection pressures. Selection acted mainly on the nasal dome region of *R. swinnyi* whereas selection acted more on the cranium than on the nasal domes of *R. simulator*. The shape of skull of *R. swinnyi* has probably the functions of an acoustic horn that allows the generation of sufficiently intense echolocation calls to optimize the range of its echolocation for high frequency sounds. Dietary functions of the skull of *R. swinnyi*, which uses higher frequency sound, may have been compromised to enhance the function of the head as an acoustic horn.
This suggests the existence of evolutionary trade-offs between the dietary acquisition structures and the echolocative structures of the head. The mandible shapes in *R. simulator* showed larger variation across localities than for *R. swinnyi’s* across localities, which could be due to an adaptation to diet. This variation in shape was predominantly attributed to selective forces. The predominance of selection over drift in the phenotypic diversification of bats seems greater than in those of primates. This probably reflects the more stringent association between environment and the optimal functioning of phenotypic characters associated with echolocation and feeding in bats.

**Introduction**

Understanding the relative contributions of drift and adaptation to organismal diversification is fundamental to studies of evolutionary ecology. To avoid overestimation of selection, drift should always be explicitly accounted for (Betti *et al.* 2010). However, quantifying the relative roles of these processes to phenotypic diversification is challenging because distinguishing the two processes and identifying their impacts on diversity is difficult (Brandon and Carson 1996; Millstein 2002; 2008; Brandon 2005). Fortunately there has been some progress in this regard (Millstein 2008). Adaptation is deterministic and results in phenotypic patterns correlated to environmental/climatic clines (Millstein 2008). Drift is neutral and results from random processes affecting the genetic composition of populations (Millstein 2008). In many cases, drift is assumed when the evidence for selection is not found (Millstein 2008). By using mathematical effects of drift on patterns of phenotypic variation (Lande 1976; 1979), it has become possible to directly determine the relative importance of drift and selection to phenotypic variation.
In Chapter three the Lande’s model was applied on soft tissue morphology and echolocation parameters which might be confounded by seasonal fluctuations, for example, body weight, due to variations in resources across different seasons or to behavioural flexibility in the case of echolocation parameters, respectively. Sampling such traits at discrete points in time might lead to different results when using Lande’s model on sets of data sampled at different times. In contrast, hard tissue e.g., bony skeletons including skulls provide a more permanent record of the evolutionary processes that a species has endured over its history. Several studies have therefore suggested the use of skulls and geometric morphometrics for enquiries into the relative roles of drift and selection (Evin et al. 2008).

Skulls serve functions crucial to the fitness of organisms within different habitats and may likely diversify primarily through adaptation (Santana et al. 2012). The neurosensory system (brain), diet acquisition structures, olfactory system, speech and sound systems are integrated and housed in the skull implying that it is subject to a wide array of selective forces (Cheverud 1982; Peddersen 1998; Klingenberg 2008). For example, the evolution of strong bite force shown in Chamaeleotis lizards was correlated to tall heads, prominent temporal ridge, huge jaw adductors (Herrel and Holanova 2008). Similarly, this has been demonstrated in many other vertebrates (Cleuren et al. 1995; Freemen and Lemen 2008; Curtis et al. 2010; Davis et al. 2010). Additionally, elongated snouts have been shown as an adaptation to facilitate feeding through suction in fish (Westneat 2005). Besides dietary adaptations seen in the skull morphology, other behaviours relevant to fitness have shaped the evolution of skull shape. These are grooming (Rosenberger and Strasser 1985) fighting with conspecifics (Hyghe et al. 2005), building shelters (Zuri et al. 1999; Santana and Dumont 2011; Hansell and Overhill 2000) and sensing the environment (Oelshlager and Stern-Kai 1990; Kirk 2007). Even though the evolution of shape and size of the skulls is presumed to be predominantly adaptive, some structures within the skull may evolve neutrally (Ackermann
and Cheverud 2002) and weighing adaptation against a model of neutrality may have several applications in taxonomy and evolutionary ecology.

Geographic variation in skull shapes and sizes has therefore been identified in many animals including primates, lizards and bats (Ackermann and Cheverud 2002; 2004; Kaliontzopoulou 2007; Jacobs et al. 2014). In some cases, this has been used to infer cryptic species and in other cases for thorough demonstration of variation in taxa (Taylor et al. 2012; Colangelo et al. 2012), and to infer migration routes of mammals (De Azevedo et al. 2015). Ackermann and Cheverud (2002; 2004) applied the Lande’s model (Lande 1976; 1979) to variation in the shape and size of human and monkey skulls and found that drift played a role in geographic variation in the skulls of these animals. No studies have used Lande’s model to weigh the relative importance of adaptation and drift to the diversification of skulls of non-primate mammals.

Particularly interesting is the evolution of skull morphology in animals that use an advanced navigation system such as echolocation, a feature found only among bats, dolphins, whales and some rodents (Santana and Lofgren 2013). Prominent resonant chambers (the nasal dome) exist in the nasal region of horseshoe bat skulls, as an adaptation of the head to function as an acoustic horn (Pedersen 1998), besides the dietary, and other behavioural functions the mammalian skull performs. It has also been shown that flight in these mammals has influenced the position and orientation of structures of the skull particularly the foramen magnum and the posture of the head (Pedersen 1998). Flight and echolocation for navigation severely dictate the ecological functioning of horseshoe bats across their habitats, implying tight links between environmental conditions and the skull size and shape. Evolutionary studies on mammalian skulls have also shown the existence of at least two modules, the cranium and the muzzle region that can evolve more or less independently owing to the
different functions they serve (Jojic et al. 2015). Horseshoe bats have maintained the two
module structure despite the modification of the skull to serve as an acoustic horn (Jojic et al.
2015). Therefore, the evolution of the nasal dome which resonates sound has not led to an
additional module. Selection acts on the muzzle as a whole and the evolution of the muzzle
structures represents a compromise between the optimization of the dietary and acoustic
functions of the muzzle. Where modularity exists between the cranium and muzzle, the nasal
dome would adapt to environmental changes without affecting the rest of the cranium, this
increases the evolutionary potential of the species (Melo and Marroig 2015). Otherwise,
changes in the shape and size of the muzzles of bats may be influenced by evolutionary trade-
offs (Jacobs et al. 2014).

Even though studies weighing the relative importance of adaptation and drift in
phenotypic geographic variation are generally scarce and differ widely in the methods used,
they show that drift is an important force during phenotypic evolution. Most of these studies
have used genetic approaches with a few (reviewed earlier) using morphometric data and
mathematical models. To provide just a few examples, Armstrong and Cole (2007) compared
quantitative traits (Qst) against neutral genetic markers (Fst) in the natter-jack toad (Bufo
calamita) and showed some signals of drift in the divergence of traits in this toad. Similarly,
signals of drift were found in grasshoppers (Melanopus species; Roff and Mousseau 2005), in
Partula Land-snails (Partula taeniata; Goodacre 2001), in Rhinolophus darlangi (Jacobs et al.
2013) and in Rhinolophus Monoceros (Chen et al. 2009). There are also studies that have
used genetic approaches and found evidence for selection as the leading force, e.g., in
Rhinolophus capensis (Odendaal et al. 2014), in R. ferrumequinum (Sun et al. 2013), in the
common frog (Rana temporaria; Palo et al. 2003), and in singing mice of the Neotropical
regions (Scotinomys teguina and S. xerampelinus; Campbell et al. 2010). Even though it
seems horseshoe bats’ phenotypic divergence is predominantly adaptive, here there are two
cases where it is not so, purely. It is thus imperative and relatively more robust to employ models that tease drift and adaptation apart before adaptive explanations can be concluded.

Here the intra-specific variation of skull shape and size was quantified in two horseshoe bats and the relative importance of drift and adaptation to variation seen was weighed using geometric morphometric techniques and Lande’s Model. It was hypothesised that 1) because the flight-echolocation adaptive complex operates in bats, the two species, *R. simulator* and *R. swinnyi* should show strong signals of selection contrary to primates where such an adaptive complex does not exist. 2) More prominent changes in nasal dome would be likely in *R. swinnyi* than *R. simulator*. *R. swinnyi* uses higher frequency sound that is more affected by atmospheric attenuation and probably has to emit its calls at greater intensity to achieve the same operational range as *R. simulator*. 3) Modularity should be evident in the skulls and not in the mandible because the flight-echolocation adaptive complex makes echolocation structures central to the selection experienced by bats. Independence between the cranium and muzzle allows for relatively more flexible response to sensory driven selection. Additionally modularity should confirm directional selection because drift and stabilizing selection are inefficient at creating modularity (Melo and Marroig 2015). Therefore if the Lande’s model shows selection, then lack of modularity would further confirm diversification is due to stabilising selection (Melo and Marroig 2015).

**Materials and Methods**

Skulls were extracted from vouchers collected for both species from each locality sampled (see Chapter 2 and 4). However, these vouchers were only a small sample size and had to be supplemented with museum specimen which did not have echolocation call data (Appendix 1). Specimens were grouped according to the geographic location from where they were captured (Fig 1a and b; Appendix 1). These groupings comprised of the North Eastern South Africa (NE), Northern Zimbabwe (NZ), Democratic Republic of Congo
(DRC), South Eastern South Africa (SE), Southern Zimbabwe and Northern South Africa (SZ) all shown in (Figs 1a and b; Appendix 1).

Figure 1. Localities from where skulls and mandibles of A) *Rhinolophus simulator* and B) *R. swinnyi* originated. Groupings used: NZ = Northern Zimbabwe (black dots), SZ = Southern Zimbabwe and parts of northern South Africa and south of Botswana (orange dots), NE = North Eastern South Africa (green dots), SE = south eastern South Africa (white dots), and Democratic republic of Congo (grey dots).
3D Images of each skull were captured through X-ray tomography (micro-focus) at the South African Nuclear Energy Corporation (NECSA, Pretoria, South Africa; Hoffman and De Beer 2012) following the same procedures as in Jacobs et al. (2013). All images were loaded into the 3D imaging software, Avizo (version 8.0; Visualization Sciences Working Group, Merignac, France) as volume files. After creating iso-surfaces from the volume files in Avizo, files were saved in ‘Stanford ply’ format and opened in Meshlab (version 1.3.3, Visual Computing Lab of ISTI - CNR, Italy) for placing landmarks. Landmarks were chosen depending on their homology (common and repeatable points on all skulls for each species). One skull and one mandible were first tested by repeating the land-marking process 10 times to determine the precision at which landmarks could be placed. Among the tested landmarks the most precise were selected, i.e., 24 landmarks for the skull and 15 for the mandible (Appendix 2). Only the right half of the skull and the right mandible were used to control for possible asymmetry (Jacobs et al. 2014). Each landmark in the 3D space had three coordinates (x, y and z). These sets of three coordinates were used in MorphoJ (version 1.7.0_45; Klingenberg 2011) to analyse shape variation in skulls and mandibles of the two species across different localities.

Landmark co-ordinates were analysed as follows. Firstly, a Procrustes superimposition was done to remove variation contributed by orientation and scale on the coordinates and to standardise the landmarks (Adams et al. 2004). Outliers were checked and extreme cases were double-checked against the original volume files; where necessary the landmarks were re-inserted on some skull images. Procrustes coordinates were used to generate a covariance matrix on which a principal components analysis was performed to explore shape variation of skulls amongst different localities of each species. A Procrustes ANOVA (provided in MorphoJ software) was used statistically test the differences in skull shapes across localities and between sexes. To visualise the shape differences, a Canonical
Variate Analysis (CVA) was used. CVA effectively shows the directions in which *a priori* groups can be discriminated by using variances within each group (Zelditch *et al.* 2012). Changes in the shape of skulls and mandibles were displayed visually using the wireframe outlines in MorphoJ compared against the average skull shape along each Canonical Variate (CV) with the outlines at the extremes of each CV.

Modularity was also tested using *a priori* hypotheses according to Klingenberg (2009). Modularity is differential evolution of different complexes. These complexes or structures tend to evolve relatively autonomously (Cheverud 1996; Klingenberg 2005). Modularity can be caused by genetic, developmental, functional, and environmental processes (Klingenberg 2005). Two subsets of 7 (ascending ramus) and 8 (alveolar region) landmarks were used to divide the mandible and similarly on the cranium into two subsets of 10 (basicranium) and 8 (rostrum) landmarks as in Jojic *et al.* (2015). The strength of association between hypothesized modules and all alternative partitions were tested by the RV coefficient. The RV coefficient measures the association between two blocks, i.e., the two modules using covariance matrices of the landmark coordinates placed on the hypothesised modules (Robert and Escoufier 1976). It takes values –0 (completely uncorrelated data) – 1 (correlated). Adjacency graphs were used to display spatial contiguity of the alternative partitions. Modularity is supported if the RV coefficient between hypothesized modules appears in the lower range of RV distribution for alternative partitions. That is, if the distribution graph shows a significant number of partitions (i.e., if Partitions – *p* < 0.05) appearing before the point where the RV value appears (to the left of the RV).

**Lande’s Model**

The relative roles of drift and adaptation to the variation in skull and mandible landmarks was tested by applying the principles of Lande’s model (Lande 1976; 1979) in the form of the *β*-test (Ackermann and Cheverud 2002), which is described in detail in Chapter 2.
The $\beta$-test is based on the hypothesis of a log-linear relationship between the variation of phenotypic characteristics between populations ($B$) and within populations ($W$). If the gradient of the linear relationship between these two is not statistically different from one, variations in phenotypic traits can be attributed to neutral evolutionary processes (mutation and drift). Otherwise, non-neutral evolutionary processes, such as natural selection, can be assumed as the dominant drivers of diversification.

All coordinates were used to generate Euclidean distances ($D$) for each selected landmark using the following formula:

$$D_i = \sqrt{(x_{i,1} - x_{i,2})^2 + (y_{i,1} - y_{i,2})^2 + (z_{i,1} - z_{i,2})^2}$$

where $x$, $y$, and $z$ are the 3D landmark coordinates, the subscripts 1 and 2 denote successive positions, and $D_i$ is the Euclidean distance for landmark $i$.

Next, the resulting multivariate response matrix comprising $D_i$ was used to derive the within locality ($W$) and between locality ($B$) variances following the procedure outlined in Chapter 2. Briefly, the $D_i$ response matrix was fitted using MANOVA with localities and sex as the categorical predictors to generate a variance/covariance (V/CV) matrix for each species. A measure of the within-population variance $W$ was then obtained in the form of eigenvalues derived from PCA applied to the V/CV matrix. The between population variation $B$ was estimated through matrix multiplication of the PCA-derived eigenvectors and the $D_i$ means of each locality. Finally, regression t-tests were applied to test the hypothesis that there was no significant difference between the regression slope $\beta$ and one as a function of:

$$\ln(B) = \beta_0 + \beta \ln(W) + \epsilon$$

where $\beta_0$ is the intercept term and $\epsilon$ is the error.
Results

A total of 56 skulls and 50 mandibles of *R. simulator* and 19 skulls and 14 mandibles of *R. swinnyi* were analysed. Procrustes ANOVA tests did not find significant differences between sexes in both species (both in size and shape of skulls and mandibles), therefore sexes were pooled for all analyses. There was variation in the shape of skulls across different localities within each species but not in size (*R. simulator*: $F_{195;3445} = 2.22; p < 0.001$, *R. swinnyi*: $F_{130;1040} = 2.37; p < 0.001$). The shape of only *R. swinnyi* mandibles was different across localities ($F_{76;418} = 1.52; p < 0.01$), *R. simulator* mandibles were not different both in shape ($F_{114;1786} = 0.38; p = 0.15$) and size ($F_{3;47} = 0.17; p = 0.91$). The sizes of all the skulls and mandibles were not significantly different for both species across the sampled localities, *R. simulator* skulls: $F_{3;53} = 0.15; p = 0.93$, *R. simulator* mandibles: $F_{3;47} = 0.17; p = 0.91$, *R. swinnyi* skulls: $F_{2;16} = 2.57; p = 0.11$, *R. swinnyi* mandibles: $F_{2;11} = 1.68; p = 0.23$.

Additionally, skulls (and only in *R. simulator*) but not mandibles were consistent with the hypothesis of modularity in both species.

Skulls

For *R. simulator*, the first two canonical variates of the canonical variate analysis (CVA) of shape variation amongst the localities of *R. simulator* explained 90% of the variation (Fig. 2). The first canonical variate (CV1) was associated with changes in the palatine, zygomatic arch, cranium and cochlea structure (see appendix 2 for a description of these structures). The NZ locality fell at the positive end of CV1 and appeared to have a narrower zygomatic arc, narrower cochlea and shorter palatines relative to the average. Conversely, the SE locality fell at the negative end of CV1 and had a reduced zygomatic arc, a broader cochlea and longer palatines relative to the average. Two localities (NE and SZ) fell within the intermediate zone of the CV prescribed shape space. CV2 was mostly associated with the anterior medial swelling (Fig. 2). The SZ locality fell at the positive end of CV2 and
had an outline implying decreased volume of the nasal dome relative to the average. Two localities (NE and NZ) fell at the negative end of CV2 showing a somewhat larger anterior medial swelling, and one locality (SE) was somewhat intermediate. CV3 (Fig. 3) contributing only 10% of the variation predominantly showed changes in the zygomatic arc. NZ and SE were on the negative end of the CV showing a broader zygomatic arc relative to the average shape and SZ and NE showed narrower zygomatic arcs relative to the average shape (Fig. 3).

Figure 2: The first two canonical variates of the canonical variate analysis (CV1 and CV2) of skull shape variation amongst localities of *R. simulator*. Light blue outline represents the average shape; dark blue outline shows the variation of shape from the average. Abbreviations are the same as in Fig. 1.
Figure 3: The first and third canonical variates of the canonical variate analysis (CV1 and 3) of skull shape variation amongst localities of *R. simulator*. Light blue outline represents the average shape; Dark blue outline shows the variation of shape from the average. Abbreviations are the same as in Fig. 1.

For *R. swinnyi*, the first two canonical variates of CVA of shape variation amongst the localities of *R. simulator* explained 100% of the variation (Fig. 4). CV1 was associated with changes in the cranium and anterior medial swelling. The NZ locality fell at the positive end of CV1 and appeared to have a larger nasal dome and a broader and more elongated cranium than the average shape. Conversely, DR locality fell at the negative end of CV1 and had a smaller nasal dome, a narrower and shorter cranium relative to the average. One locality (NE) fell within the intermediate shape zone. CV2 was associated with the cochlea and cranium dimensions (Fig. 4). All localities seemed to group on the average shape space for CV2. CV3 could not be derived from the *R. swinnyi* data set because of the small sample size.
Figure 4: The first two canonical variates of the canonical variate analysis (CV1 and 2) of skull shape variation amongst localities of *R. swinnyi*. Light blue outline represents the average shape; Dark blue outline shows the variation of shape from the average. Abbreviations are the same as in Fig. 1.

**Mandibles**

For *R. simulator*, the first two canonical variates of the canonical variate analysis (CVA) of shape variation amongst the localities of *R. simulator* explained 95% of the variation (Fig. 5). CV1 was only associated with the thickness of the mandible, all the other dimensions seemed consistent with the average shape (Fig. 5). The NE locality fell at the positive end of CV1 and had an outline implying a thicker alveolar bone relative to the
average. SZ, NZ and SE fell at the negative end of CV1 and appeared to have a thinner mandible relative to the average (Fig. 5). CV2 was associated with changes in height of the ascending ramus and the thickness of the alveolar bone. The SE and NZ locality fell at the positive end of CV2 and appeared to have a taller ascending ramus and a thinner mandible relative to the average. Conversely, SZ locality fell at the negative end of CV2 and had a shorter ascending ramus and a thicker mandible relative to the average. NE fell within the intermediate shape zone (Fig. 5). CV3 (Fig. 6) did not show much variation in the mandible, all the localities grouped on the average shape space except the NZ locality which seemed to have a slightly thicker mandible (at its tip end region of the bone).
Figure 5: The first two canonical variates of the canonical variate analysis (CV1 and 2) of mandible shape variation amongst localities of *R. simulator*. Light blue outline represents the average shape; Dark blue outline shows the variation of shape from the average. Abbreviations are the same as in Fig. 1.
**Figure 6:** The first and third canonical variates of the canonical variate analysis (CV1 and 3) of mandible shape variation amongst localities of *R. simulator*. Light blue outline represents the average shape; Dark blue outline shows the variation of shape from the average. Abbreviations are the same as in Fig. 1.

For *R. swinnyi*, the first two canonical variates of the CVA of shape variation amongst the localities explained 100% of the variation (Fig. 7). CV1 was associated with changes in the total length of the mandible and the thickness of the alveolar bone. The NZ localities fell at the positive end of CV1 and appeared to have a longer mandible and a thicker alveolar bone than the average shape. Conversely, DR and NE localities fell at the negative end of CV1 and had a shorter mandible and thinner alveolar bone relative to the average. CV2 was associated with ascending ramus dimensions and position of the teeth (Fig. 7). The DR locality was at the positive end of CV2 and had an outline implying a longer ascending ramus...
and more posterior incisors relative to the average. NZ was at the negative end of CV2 suggesting a slightly shorter ascending ramus and less posterior incisors than the average shape (Fig. 7).

**Figure 7:** The first two canonical variates of the canonical variate analysis (CV1 and 2) of mandible shape variation amongst localities of *R. swinnyi*. Light blue outline represents the average shape; Dark blue outline shows the variation of shape from the average. Abbreviations are the same as in Fig. 1.
**Modularity**

*Rhinolophus simulator* skulls showed that the skull and the rostrum were evolving as separate modules (Fig. 8); RV coefficient = 0.140, and only 35 (0.4% partitions with less than or equal to the a-priori hypothesis). Contrarily the whole mandible (ascending ramus and alveolar bone) evolved as a complete module; RV coefficient = 0.931, and 29% partitions with less than or equal to the a-priory hypothesis (Fig. 9). Both skulls and mandibles of *R. swinnyi* skulls did not show strong modularity between the partitions analysed. For skulls (between rostrum and the skull; Fig. 10), 19% of the partitions were less than or equal to the *a-priori* hypothesis and the RV coefficient was high (0.421). For mandibles, (between ascending ramus and alveolar bone; Fig. 11), 8% of the partitions were less than the *a-priori* hypothesis and the RV coefficient was high (0.442).
Figure 8: Evaluation of the \textit{a priori} cranial hypotheses of modularity in \textit{Rhinolophus simulator}. The graph shows the partitions of the cranium into basicranium (red dots) and rostrum (blue dots) regions (also showing the adjacency graphs). RV coefficients, cranium: \(RV = 0.133\) and proportions (P) of partitions with RV lower than or equal to the \textit{a priori} hypothesis (cranium: \(P = 0.003\)) are indicated by arrows.
Figure 9: Evaluation of the *a priori* mandible hypotheses of modularity in *Rhinolophus simulator*. The graph shows the partitions of the mandible into ascending ramus (blue dots) and alveolar bone (red dots) regions (also showing the adjacency graphs). RV coefficients, mandible RV = 0.938, and proportions (P) of partitions with RV lower than or equal to the *a priori* hypothesis (P = 0.296) indicated by arrows.
**Figure 10:** Evaluation of the *a priori* cranial hypothesis of modularity in *Rhinolophus swinnyi*. The graph shows the partitions of the cranium into basicranium (red dots) and rostrum (blue dots) regions (also showing the adjacency graphs). RV coefficients (RV = 0.418) and proportions (P) of partitions with RV lower than or equal to the a priori hypothesis (P = 0.186) indicated by arrows.
Figure 11: Evaluation of the *a priori* mandible hypotheses of modularity in *Rhinolophus swinnyi*. The graph shows the partitions of the mandible into ascending ramus (blue dots) and alveolar bone (red dots) regions (also showing the adjacency graphs). RV coefficients (RV = 0.426) and proportions (P) of partitions with RV lower than or equal to the *a priori* hypothesis (P = 0.115) are indicated by arrows.

**Lande’s Model**

All regression slopes describing the relationship between Ln (W) and Ln (B) differed significantly from one (Table 1) and showed no consistency in the direction of slopes (Fig. 12). All the graphs were positive except the *R. swinnyi* mandibles which showed a negative trend (Fig. 12). This indicated that none of the tests were consistent with drift and that the shape and size of skulls and mandibles of *R. simulator* and *R. swinnyi* may have evolved predominantly through selection and possibly the strongest selection was on *R. swinnyi* mandibles. The selective force responsible for the variation in skull shapes and sizes appeared to be disruptive because there was less variation between localities than within for both
species. The limited sample size did not permit analyses by exclusion as in Chapter 2. This means that site specific signals of drift could not been detected by the analysis.

Table 1. Results from Lande’s model on the 3D coordinate landmarks of skulls and mandibles of *Rhinolophus simulator* and *R. swinnyi* from different localities within southern Africa.

<table>
<thead>
<tr>
<th></th>
<th>Slope</th>
<th>±SE</th>
<th>R²</th>
<th>$H_0 ; b = 0$</th>
<th>$H_0 ; b = 1$</th>
<th>Consistent with Drift</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. simulator</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skulls</td>
<td>0.180611</td>
<td>0.078109</td>
<td>0.210943</td>
<td>P &gt; 0.05</td>
<td>P &lt; 0.001</td>
<td>No</td>
</tr>
<tr>
<td>Mandibles</td>
<td>0.294812</td>
<td>0.035762</td>
<td>0.860686</td>
<td>P &gt; 0.05</td>
<td>P &lt; 0.001</td>
<td>No</td>
</tr>
<tr>
<td><em>R. swinnyi</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skulls</td>
<td>0.442914</td>
<td>0.130897</td>
<td>0.468286</td>
<td>P &gt; 0.05</td>
<td>P &lt; 0.001</td>
<td>No</td>
</tr>
<tr>
<td>Mandibles</td>
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<td>0.177081</td>
<td>0.059677</td>
<td>P &gt; 0.05</td>
<td>P &lt; 0.001</td>
<td>No</td>
</tr>
</tbody>
</table>
Figure 12: Lande’s model results showing the relationship of within population variances (LnW) and between population variances (LnB) of skull landmarks in *Rhinolophus simulator* and *R. swinnyi* captured from southern Africa.

**Discussion**

Geographic variation evident in the skulls and mandibles of both species and as hypothesised was predominantly through selection. Modularity was only supported in *R. simulator* skulls, separating the evolution of the cranium and muzzle. The ascending ramus and alveolar bone evolved as a complete module in both species. In *R. swinnyi*, both the skull and the mandible did not show modularity. Even though these two closely related species
showed contrasting results on modularity, the Lande’s model results suggest that this was mainly attributed to selection. We also found evidence consistent with our hypothesis that more prominent changes in shape were seen in the nasal dome in *R. swinnyi* than in *R. simulator*. These results combined (modularity and Lande’s model) therefore show that the selection responsible for the diversification of *R. simulator* is predominantly directional (in the skull) and stabilising in the mandibles, whereas in *R. swinnyi*, it is mainly directional selection for both the skull and the mandible.

To some extent, these results contrast with previous findings on the relative contributions of drift and adaptation based on Lande’s model which identified signals of drift in some instances (Marroig and Cheverud 2004; Weaver *et al.* 2007). It is particularly interesting to note that in Chapter 2 signals of drift were inferred, but were based on a broader range of phenotypic features including flight, size and echolocation parameters. Perhaps the fact that the skull incorporates several functions (e.g., feeding and echolocation) crucial to fitness causes it to be under severe selection pressure that could eliminate any drift that might occur. This poses the question why previous studies did not find similar results for monkeys and humans (Marroig and Cheverud 2004; Weaver *et al.* 2007). This may have been due to the flight-echo adaptive complex that exists in bats using flight and echolocation for survival. Additionally, the head becomes the hotspot of selective forces because it houses the structures used for echolocation. By comparison to other mammals, bats’ fitness features therefore seem to be more intricately associated with environmental conditions.

Even though the main selective forces on bat skulls identified in other studies are related to dietary and echolocation production functions (Jacobs *et al.* 2013), this study also found variations in the shapes of cochlea in both species. This suggests that selection is strongly operating on both sound production and perception functions in the two species. Variations in the cochlea can be related to variations in perceptions of sound. The cochlea of
each bat has to be fine-tuned to the reception of echoes from its calls which are specific to the habitat in which the bat occurs to enhance perception of returning echoes under prevailing environmental conditions. Relative to *R. simulator*, selection in *R. swinnyi* acted more strongly on echolocation features than dietary features. This difference could be attributed to the different frequencies the two bats use. *R. swinnyi* uses higher RF (110 kHz) which is more prone to atmospheric attenuation than *R. simulator*’s lower RF (80 kHz). Higher frequency sound achieves higher resolution and travels shorter distances and is more sensitive to changes in habitat and climatic conditions across localities. It therefore seems *R. swinnyi*’s sound apparatus is fine-tuned to each specific habitat without room for a somewhat universal shape across different habitats as it thus appears in *R. simulator*. This implies that *R. swinnyi* have specialised into optimum habitats mainly through echolocation mediated adaptation, supporting the predictions of the *Sensory Drive Hypothesis* investigated in chapter 4.

The results of this study suggest existence of evolutionary trade-offs between sensory and dietary functions of the skull (Jacobs *et al.* 2014). Even though we found the existence of the cranium and rostrum modules, in *R. simulator* skulls as in rodents, carnivores, shrews, primates and in the horseshoe bat *R. ferrumequinum* (Jojic *et al.* 2015; Klingenberg 2013), *R. swinnyi* did not support modularity. Modularity was also supported for *R. simulator* in Santana and Lofren (2013) where analyses were done across 22 species of rhinolophid bats across the globe, at the genus level. Even though this study did not include *R. swinnyi*, a bat of similar size and echolocation calls, *R. dentii* was included and it showed modularity. This suggests that doing analyses at the species level may allow one to uncover the absence of modularity that may have been diluted in the study done at the genus level. Particularly, in *R. swinnyi* the nasal dome showed predominant geographic variation. Even though the nasal dome also varied in *R. simulator* the variation was not as pronounced as it was in the dietary features (cranium, the palatine, zygomatic arcs) of this species. It appears that the function of
the skull as an acoustic horn may have facilitated selection for increased nasal dome volume in *R. swinnyi* (a species using a higher sound frequency). This may be owing to the demands imposed by atmospheric attenuation on high frequency sound. It could be that the evolution of cranium and nasal dome has to be correlated to allow the skull to support the relatively larger nasal domes and the generation of increased sound intensity. *Rhinolophus swinnyi* needs greater sound intensities than *R. simulator* to achieve the same detection range. If so, all bats echolocating at similar frequencies to *R. swinnyi* and that are of similar size should have similarly high intensities and should display no modularity. The fact that *R. denti* displayed modularity (Santana and Lofren 2013) may be due to the dilution effect of doing analyses of modularity at the genus level. The assumption of this is that there is correlation between the volume of the nasal dome and the intensity at which Rhinolophids emit their echolocation calls as shown in Horseshoe bats of southern Africa (Jacobs *et al.* 2014). Therefore, *R. simulator* may not need a large horn because it does not need to emit calls at relatively high intensities and this may allow modularity to evolve. However, this assumption requires confirmation once more is known about the intensities at which different species emit their calls.

In both species, the mandible evolved as one complete module (ascending ramus and alveolar bone) contrary to the mandibular modularity found in *R. ferrumequinum* (Jojic *et al.* 2015). The variations across localities did not show any particular difference between the two species except the variations on the position of the incisors that were seen in *R. swinnyi* but not in *R. simulator*. Incisors may be used for grooming (Rosenberger and Strasser 1985) and the reason why grooming may not be as important in *R. simulator* as it is in *R. swinnyi* as shown here needs further research. However, this is evidence that more selective forces than just diet and echolocation may facilitate the variation of skull shapes in the two species.
The effects of evolutionary forces on bats may proceed in finer timescales than any other mammal especially in the event of fluctuating/changing environmental conditions. Selection weighs strongly relative to drift in both species. Most of the selection could be linked to echolocation through the flight-echolocation and diet-echolocation adaptive complex. Selection and drift work as complementary processes operating on all populations at all times. Evolutionary studies should always consider and weigh the two as alternatives and interpret them with a context-specific approach. Strong selection coefficients will influence populations despite small population sizes characteristic of mammals (more so of larger mammals, like primates) while weaker ones will be hard to trace, resulting in variation consistent with drift. The study here also shows that even different structures of organisms can experience evolutionary forces variably.
CHAPTER 4

Sensory drive mediated by climatic gradients partially explains divergence in acoustic signals in two Horseshoe Bat species, *Rhinolophus simulator* and *Rhinolophus swinnyi*.

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Sensory Drive Mediated by Climatic Gradients Partially Explains Divergence in Acoustic Signals in Two Horseshoe Bat Species, *Rhinolophus swinnyi* and *Rhinolophus simulator*.

**Authors’ contributions:**

Gregory Mutumi and David Jacobs conceived and designed the study and carried out the fieldwork. Gregory Mutumi and Henning Winker conducted the ecological analyses with input from David Jacobs. All authors contributed to the writing of the manuscript and read and approved the final manuscript.

**Abstract**

Geographic variation can be an indicator of still poorly understood evolutionary processes such as adaptation and drift. Sensory systems used in communication play a key role in mate choice and species recognition. Habitat-mediated (i.e., adaptive) differences in communication signals may therefore lead to diversification. Geographic variation in echolocation calls of African horseshoe bats, *Rhinolophus simulator* and *R. swinnyi* in the context of two adaptive hypotheses: 1) James’ Rule and 2) the Sensory Drive Hypothesis was investigated. According to James’ Rule body-size should vary in response to relative
humidity and temperature so that divergence in call frequency may therefore be the result of climate-mediated variation in body size because of the correlation between body size and call frequency. The *Sensory Drive Hypothesis* proposes that lineage diversification may be driven by environmentally-mediated differences in echolocation call frequency especially if such calls are used in communication. It predicts that increases in atmospheric attenuation selects for calls of lower frequency. The morphology and resting call frequency (RF) of 111 *R. simulator* and 126 *R. swinnyi* individuals was measured across their distributional range to test the above hypotheses. Contrary to the prediction of *James’ Rule*, divergence in body size could not explain the variation in RF. Instead, acoustic divergence in RF was best predicted by latitude, geography and climate-induced differences in atmospheric attenuation, as predicted by the *Sensory Drive Hypothesis*. Although variation in RF was strongly influenced by temperature and humidity, other climatic variables (associated with latitude and altitude) as well as drift (as suggested by a positive correlation between call variation and geographic distance, especially in *R. simulator*) may also play an important role.

**Key Words**

Acoustic adaptation, echolocation, ecological selection, relative humidity, speciation, sexual selection, temperature, vicariant evolution

**Abbreviations**

RF = Resting Frequency of the echolocation call signal, FA = Forearm length, RH = Relative humidity, AnnTemp = Mean Annual Temperature, Alt = Altitude

**Introduction**

Variation in phenotypic characteristics across the distributional range of a species is common to all organisms. Such geographic variation in phenotype could involve morphological features as well as behavioural characteristics such as sensory modalities (e.g.,
echolocation), foraging habitat and prey preferences. Variation in such characters over the distributional range of a species can be the result of dispersal and adaptation to novel environments (Magurran 1998; Lomolino *et al.* 2006; Morrone 2009). However, it may also be the result of stochastic factors such as random genetic drift especially when populations are small such as during founder events when new populations are established (Barton and Charlesworth 1984).

Although drift and selection can both play roles in the evolutionary history of organisms, traits that are heritable and have strong impacts on fitness, are less likely to be impacted by drift, unless populations are small (Barton and Charlesworth 1984; Gübitz *et al.* 2005). Thus geographic variation in traits associated with sensory systems employed in communication is likely to be adaptive because they play a key role in mate choice (Puechmaille *et al.* 2014) and species recognition (Bastian and Jacobs 2015). Even though communication-related traits are sometimes great examples of cultural evolution that have no adaptive function apart from making an individual more attractive, echolocation signals have to be produced, transmitted and perceived under prevailing local environmental conditions while remaining relevant and easy to detect. The importance of the role that the environment, particularly climate, plays in such acoustic signal variation is increasingly being recognized (Wilczynski and Ryan 1999; Luo *et al.* 2013). This has resulted in the formulation of the *Sensory Drive Hypothesis* which proposes that lineage diversification may be driven by environmentally-mediated differences in communication signals (Endler 1992). This hypothesis thus predicts an adaptive, rather than stochastic, response in acoustic signals to environmental variables.

Acoustic signals with high information content, such as bird song and the echolocation calls of bats, are particularly sensitive to environmental conditions (Ey and Fischer 2009). Echolocation calls are used for prey detection and orientation (Barclay 1982;
Fenton 1995) which have an ecological context making echolocation ideal for the study of geographic variation influenced by environmental factors. Furthermore, evidence is emerging that bats also use echolocation for communication (Barclay 1982; Knörnschild et al. 2012; Fenton 2003; Kazial and Masters 2004) and echolocation may play a role in mate choice. If so, echolocation may be implicated in lineage diversification (Puechmaille et al. 2014).

Acoustic signals may diverge along climatic gradients as a result of variation in atmospheric attenuation of sound. Atmospheric attenuation, the decrease in the energy of a sound as a result of scattering and absorption by the atmosphere, is the result of a complex nonlinear interaction between humidity, temperature and the frequency of the sound (Lawrence and Simmons 1982; Bastian and Jacobs 2015). For example, wood warblers and bats of the American south-west used lower frequencies in more humid environments (absorption is high) to optimise sound propagation (Snell-Rood 2012). In bats, differences in humidity and temperature across the geographic range of a species may select for different echolocation frequencies so that atmospheric attenuation due to these climatic factors is minimized and the detection range of echolocation is optimized. Furthermore, because higher frequency sound is attenuated to a greater degree than lower frequency sound (Lawrence and Simmons 1982), variation in the frequency of echolocation as a result of attenuation is likely to be more pronounced in bat species using calls of high frequency. Previous field studies e.g., Guillén et al. (2000) and Jiang et al. (2010) have focused on how the frequencies of acoustic signals change in response to changes in humidity but have ignored the effects of call frequency and temperature on atmospheric attenuation. Here we considered all three components of atmospheric attenuation under the Sensory Drive Hypothesis by comparing the effects of temperature and humidity on the call frequencies of two species of bats with very different mean echolocation frequencies. The adaptive response predicted by the Sensory Drive Hypothesis should result in lower call frequencies in habitats with higher
atmospheric attenuation (lower temperature and higher humidity) and this effect should be more pronounced for calls of higher frequency.

There has been evidence for an inverse relationship between body size and echolocation frequency (Jacobs et al. 2007; Jones 1999) as well as between body size and humidity and this may confound the relationship between echolocation frequency and climatic factors. James’ Rule (James 1970) proposes that animals in hot humid environments generally have smaller body sizes than animals of the same species that occur in cooler, humid areas, and the largest animals are expected to occur in cool, dry areas. This would in turn lead to differences in other morphological parameters if allometry is maintained. James’ Rule thus predicts smaller body sizes in hotter, more humid environments which should result in higher call frequencies. This is opposite to the relationship predicted for these two variables by the Sensory Drive Hypothesis.

The objectives of the study were to test the validity of sensory drive as an explanation for divergence in acoustic signals using two horseshoe bat species of similar size but with different echolocation frequencies. This minimized the effects of size on echolocation variation, allowing testing the influence of atmospheric attenuation on calls of different frequencies. An assessment of 1) the level of geographic variation present in echolocation frequency in each species and 2) the contributions of environmental variables and body size to call frequency divergence as predicted under James’s Rule and the Sensory Drive Hypotheses were done.

Methods

Study animals

Two species of insectivorous horseshoe bats Rhinolophus simulator and R. swinnyi were used. These species use high duty cycle (signal duration is long compared to the silent
period) echolocation calls dominated by a constant frequency component (Fig. S1) at means of 80 kHz and 107 kHz, respectively (Schoeman and Jacobs 2008). Both inhabit heterogeneous habitats within a savannah biome and are widely distributed throughout the more mesic eastern half of southern and central Africa (Monadjem 2010); Fig. 1. The savannah biome is composed of several woodland types with unique vegetation and climate, commonly classified into ecoregions (Olson et al. 2001). *Rhinolophus simulator* has a similar distribution to that of *R. swinnyi* but extends further north into central Ethiopia through western Kenya and central Tanzania (Monadjem 2010).

**Figure 1:** Sampling sites within southern Africa from where *R. simulator* and *R. swinnyi* were caught. Abbreviations: CC = Chinhoyi, Zimbabwe; DM = Dambanzara, Zimbabwe; GKC = Gatkop Cave, South Africa; JET = Jiri Estate Triangle, Zimbabwe; KL = Kalenda, Zambia; KP = Kapatamukombe, Zimbabwe; LOB = Lobatse Estate, Botswana; MC = Mabura Cave, Zimbabwe; MM = Monaci Mine, Zimbabwe; MT = Matobo Hills, Zimbabwe; OD = Odzi German Shafts, Zimbabwe; PA = Pafuri, South Africa; SH = Shimabala, Zambia; and SUD = Sudwala, South Africa.
**Sampling**

Bats were caught from caves and disused mine-shafts at 14 locations (Fig. 1) across the distributional ranges of the focal species along a latitudinal gradient ranging from 16˚S to 32˚S (Fig. 1). Hand-nets and continuously monitored harp-traps and mist-nets were used at cave and mine exits and where possible, within caves and mines. After capture, bats were held individually in soft cotton bags. Sex and reproductive status were checked immediately following capture and bats in late pregnancy or early lactation were released. Reproductive status was determined by examination of the nipples and palpation of the abdomen of female bats (Racey 1974). Juveniles were distinguished from adults by the presence of cartilaginous epiphyseal plates in their finger bones detected by trans-illuminating the bat’s wings (Anthony 1988). Only non-pregnant/lactating adults were used in subsequent analyses.

Forearm length (FA) was measured to the nearest 0.1 mm using dial callipers and body mass (to the nearest 0.5 g) using a portable electronic balance. FA was chosen as a measure of body size instead of mass because FA is not prone to seasonal and diurnal fluctuations (Odendaal et al. 2014).

Echolocation calls were recorded (for approximately 45 seconds per bat) from hand-held individuals 30 cm from an Avisoft UltraSoundGate 416 (Avisoft Bioacoustics, Berlin, Germany), using a condenser ultrasound microphone (Avisoft-Bioacoustics CM16/CMPA). Calls were recorded onto an HP Compaq nx7010 notebook computer with RECORDER USGH Software from Avisoft. Hand-held calls allow the determination of the resting peak frequency (RF; frequency of maximal energy when at rest) in rhinolophid bats (Siemers et al. 2005) and eliminate variation in peak frequency as a result of horseshoe bats compensating for Doppler shifts during flight (Schnitzler 1987). Recordings were slowed down by 10x and analysed using BatSound Pro software (version 3.20, Pettersson Elektronik AB, Uppsala,
Sweden) using a sampling frequency of 500 kHz, a resolution of 16 bits mono and a threshold of 15.

The frequency of the dominant 2nd harmonic of high-quality calls (i.e., high signal-to-noise ratio) were measured from the power spectrum using a Hanning window and the duration of calls were measured from the oscillogram. The first 10 calls in each recorded sequence were not analysed because horseshoe bats tune into their RF after periods of silence (Siemers et al. 2005). The constant frequency component of the calls usually stabilizes (i.e., little to no variation in the frequency) by the 11th call. Ten calls were selected for analyses and an average RF calculated for each bat. The frequency of an actual call closest to the average RF of these ten calls was used in subsequent analyses for each bat.

Environmental Variables

ArcGIS Shape files were downloaded from BIOCLIM (http://www.worldclim.org/bioclim) and OEI (www.en.openei.org) websites and analysed in ArcGIS v.10 for the following environmental variables: relative humidity (RH), mean annual temperature, altitude, latitude, and longitude. Altitude was used as a proxy for air pressure because a significant relationship between atmospheric pressure, Altitude and RF has previously been reported (Gillam et al. 2009; Zuckerwar 2002) and air pressure data could not be obtained.

The shape files (at a resolution of 30 arc seconds) for Mean annual temperature were based on monthly temperature values averaged over 50 years (1950 – 2000). Relative Humidity was based on source data taken at 10 m above the surface of the earth by NASA Surface meteorology and Solar Energy (SSE Release 6.0, Data Set; Nov 2007); a shape file based on 22-year monthly and annual average data set (July 1983 - June 2005; http://eosweb.larc.nasa.gov/sse/). These data were regional averages, not point data.
Coordinates of each study site were taken using a Garmin-GPS unit (model Colorado 300, Garmin International Inc, Kansas).

**Detection Range**

To understand how climatic variables have shaped RF in our focal species, detection distances of ‘prey’ for each population according to the online-calculator method developed in Stilz and Schnitzler (2012) were calculated. The calculator estimates the detection range using the following variables: 1) atmospheric conditions: Relative Humidity, Mean annual temperature, atmospheric pressure in Pascal’s - Pa, 2) sound properties: RF in hertz – Hz and call intensity in decibels dB [SPL root-mean-square (rms)], 3) energy absorption constant of the target – C1, and 4) two-way geometric spreading constant – C2 between a bat and target. It also gives the degree of attenuation in decibels dB [SPL root-mean-square (rms)] over the estimated detection range calculated from the same input as above.

For prey (the target), the function point-reflector, which best explains the differences in detection ranges for insects was used (Stilz and Schnitzler 2012). The maximum range at which a bat detects an echo from a target depends on the size of the target; the smaller the target, the weaker the echo, and is dependent on the specific frequency used by the bat. Field measured RFs for the sound frequency inputs were used. Call intensity of the similar sized horseshoe bat *R. blassi*, 117 dB (SPL rms); calculated at a distance of 10 cm from the bats’ nose (Schuchmann and Siemers 2010) was used as the dynamic range (assuming a hearing threshold of 0 dB SPL) because the intensity of echolocation calls of *R. simulator* and *R. swinnyi* are currently unknown. Atmospheric pressure was kept at the normal atmospheric condition of 1.013 x 10^5 Pa (Sternheim and Kane 1986; Zuckerwar 2002). The online software has an inbuilt algorithm to calculate target strength (C1) and geometric spreading...
loss (C2) depending on one’s choice of the target reflecting the pulse. Accordingly, the average detection ranges of the two species at different localities were calculated.

**Statistical analyses**

To account for potential multi-collinearity among Relative Humidity, Mean annual temperature and Altitude (Luo *et al.* 2013), and the interactive effect that these variables have on atmospheric attenuation, principal component analysis (PCA) was used to generate uncorrelated variables in the form of principal component scores; PCs (Dormann *et al.* 2013). The PCA results suggested that Relative Humidity, Mean annual temperature and Altitude across study sites could best be summarized as AnnTemp-PC1 and RH-PC2 based on their dominant eigenvalues (Fig. 2), which combined accounted for 96% (AnnTemp-PC1 = 63% and RH-PC2 = 33%) of the variation. Altitude was included in the PCA and Lat and Long were kept as separate predictor variables because Altitude was likely to carry a strong climate component associated with less spatial dependency (the higher you go, the cooler it becomes) than the actual spatial coordinates. For subsequent analysis, the PCs derived from Relative Humidity, Mean annual temperature and Altitude were therefore assumed to represent a potential latent effect of environmental variation, whereas Latitude and Longitude were included as spatial predictor variables to account for possible larger scale spatial structuring of sampling sites as a function of distance.
Fig 2. Variation in environmental conditions (relative humidity; RH, mean annual temperature; AnnTemp and altitude; Alt) across sites from which *Rhinolophus simulator* and *R. swinnyi* were captured (based on principle component analysis). AnnTemp-PC1 and RH-PC2 accounted for 96% of the variation. Site abbreviations are the same as in Fig. 1.

To construct models for testing the *Sensory Drive Hypothesis*, linear mixed effects models (LMEs) were used to relate the response of RF to environmental (AnnTemp-PC1 and RH-PC2) and spatial (Latitude and Longitude) predictor variables, while accounting for the effects FA and Sex.

Initial inspection of residual distribution and quantile-quantile plots revealed that the residuals closely approximated a normal distribution. However, further evaluation of residuals versus fitted values and of correlograms (Bjørnstad and Falk 2001) provided strong evidence for spatial structuring of residuals which violates the assumption of independence in the data. To determine the most adequate error structure, models were tested based on the
above covariates with and without sampling site as a random effect and in association with
either none or one of the following three spatial autocorrelation functions: exponential,
spherical and Gaussian (where distances were specified by Latitude and Longitude). The
random effect for sampling site was considered to account for the nested sampling design as a
result of sampling several individuals from a single location. Spatial autocorrelation is a
common phenomenon in animal ecology (Cliff and Ord 1970) given that populations in close
proximity to each other are likely to be more similar than those far apart as stated by “the first
law of geography” (Tobler 1970). This effectively decreases the number of degrees of
freedom, which in turn increases the likelihood of Type I error (incorrect rejection of the null
hypothesis) if not accounted for by adequate residual correlation structure within the model
(Zuur et al. 2009).

In addition, conventional Mantel tests were employed to provide further insights into
the effect of geographic distances among sampling sites on RF. To do so, the Euclidean
distances were calculated from geographic coordinates and RF differences were calculated to
represent absolute differences in RF between paired sites. A simple pairwise Mantel test
followed on the two dissimilarity matrices whereby RFs and geographic distances across sites
were regressed to analyse the associations between RF differences and geographic distances
in R version 3.1 using package Ade4 (Dray and Dufuor 2007). All the tests used 10000
permutations based on Monte-Carlo simulation tests (Barnard 1963).

For both species, the most parsimonious model structure (the one with the lowest
Akaike’s Information Criterion; AICc – adjusted for small sample sizes) was found to be an
LME with a random effect for site but without spatial autocorrelation. Inspection of
correlograms confirmed that the inclusion of the random-effect sufficiently removed the
spatial structuring of residuals (Fig. S3) and standard model validation graphs for residuals
showed satisfactory residual dispersal against predicted values and normality (Fig. S2)
To determine the optimal combination of covariates, a forward-backward stepwise model selection (on the global model, i.e., with AnnTemp-PC1, RH-PC2, FA, Sex, Lat and Long as predictors) based on AICc was performed using the stepAIC function of the package MASS adjusted to cater for small sample sizes (Kormann et al. 2015) in R. Based on the retained covariates, the ‘best’ model was summarised statistically with an analysis of variance (Crawley 2012) to determine which variables contributed significantly to the variation in the RF response. Only variables that explained a significant proportion of the variation in RF ($p < 0.05$) were included in the final models. This tested the predictions of the Sensory Drive Hypothesis against alternatives, i.e., whether environmental/climatic factors (relative humidity, mean annual temperature and altitude) or body size (James’ Rule), or other factors (sex, and spatial structuring) best explained the variation in resting frequency across sites.

To illustrate the nature of the relationship between covariates and RF divergence across populations, individual effects were predicted by fixing all covariates other than the effect of interest to standardized values (i.e., means across observations and female for sex). Uncoupling the individual environmental effects Mean annual temperature, Relative humidity and Altitude required a two-step approach. First, sets of “standardized” PCs for each variable Relative humidity, Mean annual temperature and Altitude were generated by fixing two of the variables to their respective means whilst allowing the other to vary [e.g., the designation PC (Alt) meant that Mean annual temperature and Relative humidity were fixed whilst Altitude was varied]. Then, the ‘standardized’ PCs were aligned with the other fixed covariates to predict the environmental effect of interest based on the ‘best’ LME.
Results

**Geographic variation in resting frequency**

We analysed the RF of 111 *R. simulator* and 126 *R. swinnyi* across 10 and 8 sites, respectively (S1 Tables 1a and b). *R. simulator* had an average RF of 80.32 ± 2.20 kHz and an average duration of 22.92 ± 9.39 ms. *R. swinnyi* had an average RF of 103.77 ± 1.70 kHz and an average duration of 28.07 ± 12.45 ms (Tables S1 a and b). Mean RF for *R. simulator* had a range across populations of approximately 7 kHz and that for *R. swinnyi* was 4 kHz.

**Geographic variation in detection range**

The inferred average detection range of echolocation signal across populations was longer for *R. simulator* (7.86 ± 0.30 m) than for *R. swinnyi* (6.13 ± 0.25 m). The range of these values was somewhat similar in magnitude (1 m) across populations from 7.5 – 8.5 m and 5.7 – 6.5 m, respectively. *R. simulator* experienced lower attenuation (average 3 dB and range 2 – 3 dB across sites) than *R. swinnyi* (average 4 dB and range 3 – 4 dB across sites). Thus there was an increase of ~ 1 dB/m in attenuation across a difference of 24 kHz (80 kHz and 104 kHz for *R. simulator* and *R. swinnyi*, respectively) between the echolocation frequencies of the two species.

**Effects of environmental variables on RF**

For both species, environmental variation (Relative humidity, Mean annual temperature and Altitude; comprising AnnTemp-PC1 and RH-PC2) as well as latitude and gender explained the variation in RF. In contrast, body size (FA) did not. Initial model selection using stepAICc dropped FA for *R. simulator* and dropped Long and the interaction ‘AnnTemp-PC1: RH-PC2’ for *R. swinnyi* (Table 1). ANOVA on the variables maintained in the ‘best’ model for each species yielded the interaction AnnTemp-PC1: RH-PC2, Lat and
Sex as significant predictor variables for \textit{R. simulator}; whereas for \textit{R. swinnyi}, AnnTemp-PC1, RH-PC2, Lat and Sex were significant predictor variables (Table 1). Only the significant variables were used further for predictive modelling.

**Table 1.** The ‘best’ model from forward-backward stepwise model selection on the global model of environmental variables, body size and sex against resting frequency for each of the two species, \textit{Rhinolophus simulator} and \textit{R. swinnyi}. Statistics are only presented for variables maintained in the best model.

<table>
<thead>
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<th></th>
<th><strong>Rhinolophus simulator</strong></th>
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<th><strong>Rhinolophus swinnyi</strong></th>
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<td>Number of Groups: 10</td>
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</table>

Abbreviations: AnnTemp-PC1 and RH-PC2 = Principle component factor 1 and 2 derived from relative humidity, mean annual temperature and altitude; Lat = Latitude; Long = Longitude; FA = Forearm length; RF = Resting frequency in kHz; numDF = numerator degrees of freedom; denDF= denominator degrees of freedom.
Fig 3. Predictive modelling plots showing how resting frequency (RF) in *Rhinolophus simulator* responded to environmental variation and sex. Abbreviations: e.g., PC (Altitude) represents a principle component generated by fixing mean-annual-temperature and relative humidity (rel.humidity) to their across-site-means while altitude was allowed to vary. The shaded areas and error bars represent 95% confidence limits. The final best model (only variables significant after ANOVA on the best model shown) was used for the modelling.
**Fig 4.** Predictive modelling plots showing how resting frequency (RF) in *Rhinolophus swinnyi* responded to environmental variation and sex. Abbreviations: e.g., PC (Altitude) represents a principle component generated by fixing mean-annual-temperature and relative humidity (rel.humidity) to their across-site-means whilst altitude is allowed to vary. The shaded areas and error bars represent 95% confidence limits. The final best model (with only variables shown significant after ANOVA on the best model) was used for the modelling.
The effects of each of the climatic variables (Relative humidity, Mean annual temperature and Altitude) were isolated by holding (controlling) the others constant at the across-site mean. The climatic variables exhibited predominantly linear relationships with RF across the different habitats, with the exceptions of non-linear relationships in Mean annual temperature and Altitude for R. simulator (Figs 3 and 4). This uncoupled effect appeared to be generally stronger in R. swinnyi than in R. simulator (Figs 3 and 4). These predictive modelling results may also indicate that each climatic variable was associated with variation in RF in the context of the other two. Importantly, our results could not attribute divergence in RF to climatic variables alone, as gender and region (North – South spatial structuring; Lat) were also associated with variation in RF (Table 1; Figs 3 and 4). RF increased significantly with a southward increase in distance for both species (Figs 3 and 4); whereas longitude did not explain significant variation in RF (Table 1).

The Mantel test showed variation in RF was positively associated with geographic distances in R. simulator: differences in resting frequency were larger among pairs of sampled sites that were further from each other than those that were nearer (Monte-Carlo test Observation: 0.647, 10,000 replicates; \( p < 0.01 \)). The association between RF differences and geographic distances was not significant for R. swinnyi (MC test Observation: 0.550; 10,000 replicates, \( p = 0.059 \)).

Sexual dimorphism was evident in both species but stronger in R. swinnyi than R. simulator (overall across all populations; Figs 3 & 4). At most sites with both sexes, males used higher RFs although these were not always statistically significant for both species. Females never used higher frequencies than males except at MT in R. simulator and at MC in R. swinnyi (Figs 3 & 4; Tables S1A and B). R. simulator males used higher RFs than females at three sites i.e. at CC, GKC and MC and in R. swinnyi males used higher RFs at two sites i.e., at KP and OD (Tables S1A and B).
Discussion

Body size (FA) was not correlated with RF in either *R. swinnyi* or *R. simulator* suggesting RF variation was not the result of climate-mediated variation in body size as proposed by James' Rule. In accordance with the Sensory Drive Hypothesis, RF in *R. swinnyi* was predominantly climate driven. However, this was not the case with *R. simulator*. Instead, RF in this species was predominantly latitude driven. Both species showed significant spatial structuring by latitude, and sexual dimorphism (which was stronger in *R. swinnyi*). The effect of latitude suggests that other climatic and environmental variables not considered here may also exert an influence.

The extent to which each species responded to climatic variables was dependent on its specific echolocation frequency. These observed changes were larger in *R. swinnyi* than *R. simulator* even though both species are exposed to the same climate (where they co-occurred) or to similar Relative humidity and Mean annual temperature (47 – 61% Relative humidity; 17.7 – 21.5°C Mean annual temperature and 51– 61% Relative humidity; 18.5 – 23.8°C Mean annual temperature for *R. simulator* and *R. swinnyi*, respectively; S1 Tables 1a and b). This was probably because atmospheric attenuation is more pronounced at the higher call frequencies used by *R. swinnyi*. Thus, lowering call frequency in response to higher Relative humidity is probably crucial for *R. swinnyi* to maintain comparable detection distances (mean = 6.13 ± 0.25 m; range = 5.7 – 6.5 m) across its distributional range.

The reason for the influence of latitude on RF (besides its influence exerted through Mean annual temperature, Relative humidity and Altitude) in both species is difficult to ascertain at this stage. A possible explanation for the latitudinal cline in RF could be the effect of isolation by distance and vicariance because the effect of distance between populations was stronger for *R. simulator*, before controlling for spatial autocorrelation (Fig. S3). Surprisingly, very few studies (Jiang *et al.* 2010) have found such correlations between
geographic co-ordinates and RF; reviewed in Jiang et al. (2015). However, this shows that atmospheric attenuation is not the only factor underlying the variation seen in RF, for instance, a change of Relative humidity from 50 to 62% (Fig. 4) at 20°C and 104 kHz and 101325 Pa changes atmospheric attenuation from 3.5 dB/m to 3.9 dB/m, i.e., an increase of about 0.4 dB/m (in R. swinnyi). Lowering RF from 104 to 101 kHz causes in turn a reduction of atmospheric attenuation of 0.1 dB/m (at 50%) or of 0.1 dB/m at 62% Relative humidity. Similarly, in R. simulator, a change of Relative humidity from 47 to 61% (Fig. 3) at 20°C and 82 kHz and 101325 Pa changes atmospheric attenuation from 2.6 dB/m to 2.9 dB/m, i.e. an increase of about 0.3 dB/m. Lowering RF from 82 to 79 kHz causes in turn a reduction of atmospheric attenuation of 0.1 dB/m (at 47%) or of 0.1 dB/m at 61% Relative humidity. The proposed reduction in RF caused by relative humidity can thus only compensate to about one quarter the increased atmospheric attenuation caused by the increased Relative humidity, making it likely that atmospheric attenuation is not the only factor underlying the divergence seen in RF for both species.

The strong north-to-south structuring in the RFs therefore raised a possibility of the existence of a latent variable in latitude, besides the tested variables in the PCs (Relative humidity, Altitude and Mean annual temperature). Altitude, for example, was found to be a significant influence on call frequency in Gillam et al (2009) and altitude is a composite of atmospheric pressure, temperature, relative humidity, and wind speed (with pressure having likely the least impact). Unfortunately, reliable data to directly test the influence of atmospheric pressure and wind speed (mean annual averages over 20 – 50 years) on RF comparable to our other climatic variables (Relative humidity and Mean annual temperature) could not be obtained.

The results at hand on the influence of climatic factors on geographic variation in call frequency generally support those of other studies but with some notable differences. Studies
on *Hipposideros ruber* (Guillén *et al.* 2000) and *Rhinolophus pusillus* (Jiang *et al.* 2010) in the tropics of Africa and Asia, respectively, found support for an association between mean annual rainfall and RF, where mean annual rainfall was used as a proxy for Relative humidity. However, Odendaal *et al.* (2014) found no association between Relative humidity and RF in *R. capensis*, although the species occurred in biomes ranging from desert to forest but across which, surprisingly, Relative humidity did not vary significantly. However, there appeared to be a correlation between RF and mean annual precipitation (David Jacobs, personal observation). These discrepancies between studies and between species within studies (reported here) suggest that the atmospheric attenuation experienced by bats is the result of a complex interaction between local temperature, humidity, atmospheric pressure and rainfall, as well as the frequency of the acoustic signal. All of these should be considered in attempts to understand how climatic factors drive acoustic variation.

The small range in RFs across populations of *R. swinnyi* (4 kHz) and *R. simulator* (7 kHz) raises the possibility that variation may not be ecologically relevant. The maximum difference in detection range across the frequencies used by *R. swinnyi* and *R. simulator* was 1.0 m and 0.8 m, respectively. Differences in detection ranges of 1m are likely to make considerable differences in the detection of prey and the avoidance of obstacles at increased flight speeds. Studies on exactly how these bats vary echolocation and flight speeds are sorely needed. The advent of multiple microphone arrays will facilitate this.

It is possible that part of the variation in RF could be driven by the presence of other horseshoe bat species in multi-species assemblages e.g., partitioning of frequency bands so that each species in bat assemblages has its own private band allowing more effective intraspecific communication (Acoustic Communication Hypothesis; Duellman and Pyles 1983; Heller and Helversen 1989; Jacobs *et al.* 2007). If so, it might explain the absence of a correlation between body size and RF. At Lobatse (Botswana) where *R. simulator* is syntopic
(occurring in the same cave) with only one other species of rhinolophid, *R. clivosus* (92 kHz; David Jacobs unpublished data) it calls at 85 kHz (S1 Table 1a). However, where *R. simulator* is syntopic with several species of rhinolophids including *R. blasii* which echolocates at 86 kHz (Schoeman and Jacobs 2008) it calls at 80 kHz (S1 Table 1a) which ensures that its call frequency does not overlap with that of *R. blasii*. Confirmation of this would require more detailed analyses comparing echolocation call frequencies of both *R. simulator* and *R. swinnyi* and several other rhinolophid species with overlapping geographic distributions in situations of syntopy and allopatry. It is also note-worthy that variations in RF may be driven by sexual selection in which female choice and male-male competition (Andersson 1994; Podos and Warren 2007) may drive the divergence in RF (Puechmaille *et al*. 2014; Jiang *et al*. 2015).

Males generally called at higher frequencies than females, although the differences were not always significant (Tables S1 a and b). The data analysed in this study do not allow testing the potential explanations for sexual dimorphism or why it varies across localities. Future analyses could focus on call parameters other than frequency (e.g., slope and minimum frequency of the frequency modulated component of the call, duration and interpulse interval) to provide more detail on sexual differences in calls. This should provide further insight into the potential communicative function of echolocation and how environmental/climatic conditions may influence information exchange between different sexes and how such processes may contribute to geographic variation.

The isolation by distance patterns obtained from the Mantel test indicated that there is differential gene flow between populations, at least in *R. simulator*, and that environmental factors may not be solely responsible for the variation in RF amongst populations. If populations are sufficiently small then stochastic factors such as genetic drift in combination with reduced gene flow may exert an influence (Hutchinson and Templeton 1999). However,
average geographic distances were in fact similar for both species but slightly lower for *R. simulator* than for *R. swinnyi* (mean mahalanobis distance of coordinates = 5.09 and 5.26, respectively) and suggest that other barriers besides distance may reduce gene flow between populations, at least in *R. swinnyi*.

The contribution of atmospheric conditions to variation in acoustic signals may not be restricted to animals using high frequency acoustic signals. There is also some support for atmospheric attenuation contributing to geographic variation in low frequency bird song (Snell-Rood 2012) suggesting that lineage diversification may be driven by habitat-mediated differences in communication signals in a variety of terrestrial (and perhaps also marine) taxa that rely on acoustic signals for orientation, food and mate acquisition. Sensory drive may have a greater effect on the generation of biodiversity than is currently appreciated. However, evidence that habitat driven variation in acoustic signals lead directly to lineage diversification is sorely needed. Climate driven changes in acoustic signals, as shown in this study, may have implications for the understanding not only of lineage diversification, but also of how organisms may respond to climate change over space and time.
The primary purpose of this thesis was to quantify the causes of phenotypic geographic variation in two horseshoe bats of southern Africa, *Rhinolophus simulator* and *R. swinnyi*. Several hypotheses were tested and a range of statistical procedures were used to elucidate the underlying evolutionary processes responsible for the geographic variation observed within the two species across their distributional ranges in southern Africa. Chapter two proceeded to weigh the relative contributions of adaptation and drift in soft tissue morphological parameters and echolocation calls using phenotypic covariance matrices in the Lande’s model. Because of the limitations of traditional morphometrics and soft tissue measures (Rolf et al. 2004), hard-tissue (skulls and mandibles) and geometric morphometrics were used as further confirmation of the relative contributions of adaptation and drift in Chapter three. To reconstruct selection, the predictions of the *Sensory Drive Hypothesis* in explaining the variation in echolocation calls across different sites of varying climatic conditions were explored in Chapter four.

Phenotypic diversification is multifaceted and can be facilitated by both deterministic and stochastic processes. As such, there have been efforts to investigate the relative contributions of selection and drift in phenotypic divergence in many studies (e.g., Lande 1976; Ackermann and Cheverud 2002; 2004; Weaver et al. 2007; Smith 2011; de Azevedo et al. 2015). Methods that are used to do so are predominantly genetics based (e.g., Bilgin et al. 2009). However, it is equally important to understand diversification using phenotypic features because these directly interact with the environment accruing fitness benefits.
Additionally, in most cases, where morphological data is available from museum specimens, genetic samples may not be available. This study used mathematical and statistical models which depend on phenotypic variation patterns to weigh the relative contribution of drift and selection. Particularly, the current study aimed not only to address adaptive explanations but rather to weigh these against a null model of drift. However, limitations on sample sizes were encountered and this compromised the number of populations that could be analysed, especially with regards to the skull data. Additionally, skulls loaned from museums could not be matched directly with echolocation data. So with this data set, only skull size and shape variations could be analysed without relating the variation to respective echolocation calls (e.g., Jacobs et al. 2014).

The Lande’s based Chapters (two and three) supported the evidence of selection shown in the climate data analyses in Chapter four. Chapter four asssed how two adaptive hypotheses: 1) James’ Rule and 2) the Sensory Drive Hypothesis could explain the geographic variation of echolocation calls of African horseshoe bats, *Rhinolophus simulator* and *R. swinnyi*. There was evidence to support the view that geographic variation in both species was predominantly caused by natural selection. The main difference among the three main Chapters was that skulls showed almost non-existent signals of drift, whereas linear phenotypic measurements from the rest of the body including flight parameters and echolocation showed traces of drift. Selection appeared to be strongest on the head shape relative to other morphological traits in both species. This may be related to the head’s function as an acoustical horn as well as to house structures used to perceive the returning echoes (Pedersen 1998). Direct selection on echolocation signal function was likely taking place here, because signals of drift could only be traced when echolocation-PCs were removed in both species. The results from Chapter four also showed that variations in sound frequency were not indirect effects of variations in body size as the effect of body size did not
explain the variation in RF. Instead, as predicted by the Sensory Drive Hypothesis, divergence in RF was best explained by latitude, geography and climate-induced differences in atmospheric attenuation. The major role played by environmental conditions on RF divergence warrants that additional climatic variables (associated with latitude and altitude) may also contribute to the variation. This leaves drift with an overall very minor role in the diversification of the two horseshoe bats considered here. Therefore the positive correlation between RF and geographic distance, especially in *R. simulator* shown in Chapter four may have been more to do with isolation by habitat as in (Wang 2013; Wang et al. 2013). Chapters 2 and 3 did not find further support for isolation by distance using all the phenotypic characters as a whole.

The presence of signals of drift identified for some phenotypic characters, were counter balanced by signals for selection on resting frequency (both species), manoeuvrability (*R. simulator* only) and size (*R. swinnyi* only). Additionally, there was correlated selection (co-selection) amongst phenotypic characters confirming that drift was not substantial. Overall, selective forces were mostly disruptive with less variation shown between than within populations. The phenotypic traits under selection differed between the two species, and between different localities, suggesting that differential ecological selection had occurred. Because phenotypic divergence was also not correlated to geographic distance, the most plausible explanation of the divergences in phenotypes observed in this study is predominantly adaptation to local habitats with reduced gene flow among them as a result of vicariance and/or isolation by habitat.

The shapes and sizes of the skulls of *R. simulator* and *R. swinnyi* (Chapter three) showed more prominent changes in the nasal dome in *R. swinnyi* than in *R. simulator*. Also more prominent shape changes across localities were identified in the mandibles of *R. simulator* than in *R. swinnyi*. All these variations in shape were attributed to selective forces
and these seemed to differ between the two species especially with respect to modularity. Two modules existed only in *R. simulator* skulls (between the cranium and rostrum). Of particular importance is that the skulls responded to selection in different ways between the two species. The nasal dome region of *R. swinnyi* showed more variation across localities than the cranium and the opposite was true for *R. simulator*. The contribution of selection on the two species differs, with more precedence given on rostrum in *R. swinnyi* than in *R. simulator*. Even though the two species are almost similar in size and occupy similar biomes they differ markedly in RF. Selection, because of its greater effect on the nasal dome in *R. swinnyi*, probably acts more on echolocation in *R. swinnyi* than in *R. simulator*. This finding is also supported by the results in Chapter 4 which showed stronger correlations between RF and climatic variables as well as latitude in *R. swinnyi* than in *R. simulator*. *Rhinolophus swinnyi* uses a higher RF which has to be propagated with a higher intensity (Brinkløv et al. 2009) to achieve a detection range comparable to *R. simulator* which calls at a lower RF (80 kHz). To resonate the sound to a higher intensity, the nasal dome has to be bigger and more robust. This might explain why there was more variation in the nasal dome of *R. swinnyi* than *R. simulator* and why *R. swinnyi* did not show modularity. Probably the rest of the cranium has to be correlated to the rostrum to support a relatively more robust nasal dome which meets the demands imposed by high RF production. It seems plausible that selection acts directly on RF at high RF characteristic of *R. swinnyi*, and morphology somewhat delays in response. Whereas in *R. simulator*, selection is likely to act first on diet, which then determines dimensions of structures used for echolocation. Chapter two also provides evidence that RF plays a fundamental role in the diversification of *R. simulator* and *R. swinnyi*, given that selection was found to act more strongly on echolocation than other phenotypic characters.
The predominance of selection as an explanation for phenotypic divergence in bats
seems more prevalent than in monkeys and humans (Ackermann and Cheverud 2002; 2004)
where a similar procedure was used to weigh the relative contributions of selection and drift.
This likely places bats, particularly *R. simulator* and *R. swinnyi*, as well as other animals
depending on echolocation and the adaptive complexes that form as a result, as highly
sensitive to environmental changes and needing more stringent conservation measures,
particularly with respect to human-induced climate change.

**Conclusions**

Our results show that selection pressures and the phenotypic responses to such
pressures can differ even amongst closely related species. Each species should therefore be
considered collectively with other congenerics but also separately. Selection can act on
acoustic signals directly and can be influenced by the nature of the acoustic signals
themselves (in this case different frequencies) – acoustic signals, even those used mainly for
orientation, could play a role in lineage diversification. Against a null model of drift,
phenotypic diversification in *R. simulator* and *R. swinnyi* was predominantly adaptive and
there was very little influence of drift. The evidence presented here therefore provides more
insight into the *Drift versus Selection* debate (Brandon and Carson 1996; Millstein 2002;
2008; Brandon 2005), i.e., it shows that selection can be the main driving force behind
divergence to the almost complete exclusion of drift.

The selection versus drift debate needs more empirical studies using more robust
approaches like the ones explored in this thesis. There is need to analyse as many different
taxa as possible, and to incorporate analyses both at genus and species level. It would be
interesting to also use genetic approaches as a further confirmation of the findings presented
here.
This thesis used the name *Rhinolophus swinnyi* as it is currently applied (Monadjem *et al.* 2010). However, recent genetic analysis suggests that the populations sampled in the current study differs from the nominate form of *R. swinnyi* and appears to be more closely allied with *R. simulator*. Thus further taxonomic revision of the *capensis* clade is required (Dool *et al.* 2016). The specimens used in Dool *et al.* (2016) and referred to by them as *R cf. simulator* came from the same populations sampled for this thesis.
REFERENCES


Funk, W., Cannatella, D. and Ryan, M. 2009. Genetic divergence is more tightly related to call variation than landscape features in the Amazonian frogs *Physalaemus petersi* and *P. freibergi*. *Journal of Evolutionary Biology* 22, 1839-1853.


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Lin, G., Ci, H., Zhang, T. and Su, J. 2008. Conformity to Bergmann’s rule in the plateau Pika 
(Ochotona curzoniae hodgson, 1857) on the qinghai-tibetan plateau. Acta Zoologica 
Academiae Scientiarum Hungaricae 54, 411–418.


Thorpe, R. S. and Malhotra, A. 1996. Molecular and morphological evolution within small islands. *Philosophical Transactions: Biological Sciences* 351(1341), 815-822


## APPENDICES

### Chapter 2

Table A1: Phenotypic parameters measured from live bats in the field, *Rhinolophus simulator* and *R. swinnyi*.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF</td>
<td>Resting Frequency</td>
<td>Peak frequency of the constant frequency component of the call measured in kilo hertz (kHz) from the power spectrum.</td>
</tr>
<tr>
<td>FA</td>
<td>Forearm Length</td>
<td>Forearm length measured in millimetres.</td>
</tr>
<tr>
<td>TR</td>
<td>Upper tooth-row length</td>
<td>Upper tooth-row length (measured in millimetres) from the end of the last molar to the front-end of the first molar.</td>
</tr>
<tr>
<td>HH</td>
<td>Head Height</td>
<td>Head-height measured (measured in millimetres) from beneath the jaw just in front of the auditory bulla to the highest point of the head.</td>
</tr>
<tr>
<td>HW</td>
<td>Head width</td>
<td>Maximum width of the head measured in millimetres across the head just behind the two ears.</td>
</tr>
<tr>
<td>HL</td>
<td>Head Length</td>
<td>Condyllobasal length (measured in millimetres) from the tip of the nose tip to the skull lambda.</td>
</tr>
<tr>
<td>FL</td>
<td>Foot length</td>
<td>Foot length measured (measured in millimetres) to the point of where the nail emerges.</td>
</tr>
<tr>
<td>TL</td>
<td>Tail length</td>
<td>Tip of tail to anus length measured in millimetres.</td>
</tr>
<tr>
<td>WS</td>
<td>Wingspan</td>
<td>Wing span length measured in millimetres between the tips of the outstretched wings including the body.</td>
</tr>
<tr>
<td>WA</td>
<td>Wing Area</td>
<td>Wing area measured in square meters as the combined area of the two wings, the tail membrane and the portion of the body between the wings.</td>
</tr>
<tr>
<td>AR</td>
<td>Aspect Ratio</td>
<td>Calculated as the square of the wingspan in meters divided by the wing area in square meters.</td>
</tr>
<tr>
<td>WL</td>
<td>Wing loading</td>
<td>Calculated as the weight divided by the wing area and is measured in Newtons per square meter (N.m$^{-2}$).</td>
</tr>
</tbody>
</table>
Table A2: Means and standard deviations (±SD) of phenotypic traits measured from live bats *Rhinolophus simulator* and *R. swinnyi* in the field.

<table>
<thead>
<tr>
<th>Study Site</th>
<th>N</th>
<th>RF</th>
<th>±SD</th>
<th>FA</th>
<th>±SD</th>
<th>TR</th>
<th>±SD</th>
<th>HH</th>
<th>±SD</th>
<th>HW</th>
<th>±SD</th>
<th>HL</th>
<th>±SD</th>
<th>FL</th>
<th>±SD</th>
<th>TL</th>
<th>±SD</th>
<th>WS</th>
<th>±SD</th>
<th>WA</th>
<th>±SD</th>
<th>AR</th>
<th>±SD</th>
<th>WL</th>
<th>±SD</th>
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</thead>
<tbody>
<tr>
<td>CC</td>
<td>8</td>
<td>80.1</td>
<td>0.59</td>
<td>44.2</td>
<td>0.97</td>
<td>68.5</td>
<td>0.52</td>
<td>6.70</td>
<td>0.24</td>
<td>7.09</td>
<td>0.52</td>
<td>19.8</td>
<td>0.25</td>
<td>1.10</td>
<td>0.91</td>
<td>7.33</td>
<td>0.11</td>
<td>20.6</td>
<td>0.25</td>
<td>0.011</td>
<td>0.011</td>
<td>0.009</td>
<td>5.60</td>
<td>0.37</td>
<td>4.97</td>
</tr>
<tr>
<td>DM</td>
<td>22</td>
<td>79.1</td>
<td>0.94</td>
<td>44.4</td>
<td>1.21</td>
<td>6.75</td>
<td>0.39</td>
<td>7.00</td>
<td>0.39</td>
<td>9.04</td>
<td>0.60</td>
<td>19.5</td>
<td>1.17</td>
<td>7.55</td>
<td>0.81</td>
<td>23.1</td>
<td>0.26</td>
<td>0.011</td>
<td>0.013</td>
<td>0.001</td>
<td>4.94</td>
<td>0.35</td>
<td>5.02</td>
<td>0.42</td>
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<tr>
<td>GKC</td>
<td>10</td>
<td>83.3</td>
<td>0.67</td>
<td>45.5</td>
<td>1.16</td>
<td></td>
<td></td>
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<tr>
<td>KL</td>
<td>6</td>
<td>77.8</td>
<td>0.54</td>
<td>45.1</td>
<td>0.97</td>
<td>6.96</td>
<td>0.52</td>
<td>6.70</td>
<td>0.24</td>
<td>9.50</td>
<td>0.52</td>
<td>18.9</td>
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<td>7.97</td>
<td>0.74</td>
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<td>1.71</td>
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<td>0.013</td>
<td>0.001</td>
<td>4.91</td>
<td>0.36</td>
<td>6.77</td>
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<tr>
<td>LOB</td>
<td>18</td>
<td>84.6</td>
<td>0.64</td>
<td>45.0</td>
<td>1.04</td>
<td>7.13</td>
<td>0.57</td>
<td>6.98</td>
<td>0.49</td>
<td>8.88</td>
<td>0.62</td>
<td>17.8</td>
<td>0.88</td>
<td>8.25</td>
<td>0.84</td>
<td>24.3</td>
<td>2.00</td>
<td>0.011</td>
<td>0.013</td>
<td>0.001</td>
<td>5.74</td>
<td>0.47</td>
<td>5.53</td>
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<tr>
<td>MC</td>
<td>8</td>
<td>79.5</td>
<td>0.77</td>
<td>45.2</td>
<td>1.27</td>
<td>6.51</td>
<td>0.45</td>
<td>7.30</td>
<td>0.31</td>
<td>8.96</td>
<td>0.29</td>
<td>20.1</td>
<td>0.37</td>
<td>7.41</td>
<td>0.76</td>
<td>20.7</td>
<td>2.00</td>
<td>0.021</td>
<td>0.013</td>
<td>0.001</td>
<td>5.15</td>
<td>0.49</td>
<td>4.99</td>
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<td>MM</td>
<td>4</td>
<td>81.2</td>
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**Rhinolophus simulator**

**Rhinolophus swinnyi**

Abbreviations for phenotypic parameters are given in Table 1. Study site abbreviations: PA = Pafuri, JET = Jiri Estate – Triangle, MM = Monaci Mine, OD = Odzi German. Shafts, DM = Dambanzara, MC = Mabura, KP = Kapamukombe, KL = Kalenda, SUD = Sudwala. RF was measured in kilohertz (kHz), FA, TR, HH, HW, HL, FL and TL in millimetres (mm), WS in meters, Wing Area in square meters, Aspect Ratio as wingspan squared divided by wing area, WL was expressed in Newtons per square meter.
Table A3: Precision; repeated voucher measurements showing deviation statistics and the number of times population paired differences were less than the measurement error for each parameter.

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Firstly the CV = coefficient of variation of the repeated measurements (from the voucher) should not exceed FA’s. If it does, it is excluded further assessments. Std.dev = standard deviation, #Sites = number of study sites/populations, Pairs = number of paired differences of population means compared, <Std.err = number of population pairs with differences less than the standard error of measurement (from the voucher’s repeated measurements - precision) and this is expressed as a percentage of the total number of pairs compared (% out). If the number of comparisons less than the standard error of precision exceed those of the chosen standard (Forearm - FA) then the parameter is not used in the modelling. Forearm was chosen because it was the easiest to measure under harsh field conditions.

More abbreviations: FA= forearm length in millimeters (mm), TR = Upper toothrow length in millimeters (mm), HH = Head height in millimeters (mm), HW = Head width in millimeters (mm), HL = Head length in millimeters (mm), NLW = Noseleaf width in millimeters (mm), TB = Tibia length in millimeters (mm), FL = Foot length in millimeters (mm), WL = Wingspan length in meters, WA = wing area in square meters, AR = Aspect Ratio.
Table A4: Standardised PC factor loadings after the V/CV matrix (without the DM population for *Rhinolophus simulator* and without OD population for *R. swinnyi* is analysed using PCA.

| PC  | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| FA  | 0.014 | 0.110 | 0.000 | 0.000 | 0.327 | 0.112 | 0.239 | 0.169 | 0.008 | 0.019 | 0.001 |
| TR  | 0.000 | 0.166 | 0.310 | 0.212 | 0.061 | 0.011 | 0.153 | 0.073 | 0.010 | 0.003 | 0.000 |
| HH  | 0.185 | 0.079 | 0.018 | 0.067 | 0.128 | 0.045 | 0.087 | 0.369 | 0.011 | 0.010 | 0.000 |
| HW  | 0.134 | 0.113 | 0.256 | 0.016 | 0.074 | 0.105 | 0.001 | 0.211 | 0.059 | 0.028 | 0.001 |
| HL  | 0.003 | 0.040 | 0.082 | 0.007 | 0.156 | 0.434 | 0.000 | 0.097 | 0.004 | 0.159 | 0.016 |
| FL  | 0.065 | 0.004 | 0.141 | 0.032 | 0.118 | 0.099 | 0.457 | 0.052 | 0.009 | 0.013 | 0.008 |
| TI  | 0.044 | 0.016 | 0.021 | 0.070 | 0.004 | 0.021 | 0.018 | 0.004 | 0.602 | 0.190 | 0.009 |
| WS  | 0.089 | 0.253 | 0.004 | 0.133 | 0.019 | 0.032 | 0.000 | 0.000 | 0.048 | 0.012 | 0.045 |
| WA  | 0.326 | 0.088 | 0.061 | 0.067 | 0.000 | 0.096 | 0.008 | 0.001 | 0.026 | 0.062 | 0.013 |
| AR  | 0.024 | 0.096 | 0.097 | 0.387 | 0.024 | 0.013 | 0.006 | 0.001 | 0.008 | 0.015 | 0.038 |
| WL  | 0.114 | 0.033 | 0.009 | 0.009 | 0.086 | 0.031 | 0.031 | 0.010 | 0.213 | 0.441 | 0.019 |
| RF  | 0.002 | 0.000 | 0.000 | 0.000 | 0.003 | 0.000 | 0.000 | 0.013 | 0.001 | 0.047 | 0.849 |

The PCA loadings in bold weigh at least 0.10 (1/12 variables – rounded off to the nearest first decimal – 0.1)
Chapter 3

Appendix 1: Skulls and mandibles of *Rhinolophus simulator* and *R. swinnyi* collected from museums and from recent field trips in the Animal Evolution and Systematics Lab, University of Cape Town (AES UCT).

Abbreviations: *Rsi* = *Rhinolophus simulator*, *Rsw* = *Rhinolophus swinnyi*, F = female, M = male, Lat = latitude, Long = longitude, AES UCT = Animal Evolution and Systematics Lab at University of Cape Town; these skulls were extracted from voucher specimen captured during field work (2003 – 2013).

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Appendix 2: 3D Landmark coordinates used for the skulls and mandibles of *Rhinolophus simulator* and *R. swinnyi*.

Cranium
Mandible

Descriptions of the Landmarks shown in the diagrams

Craniun

1. Most anterior point at the base of the canine
2. Most anterior point of the anterior medial swelling on the midline between the left and right anterior medial swellings.
3. The widest point of the anterior medial swelling.
4. Most dorsal point of the anterior medial swelling
5. Most posterior point of the anterior medial swelling on the midline of the skull.
6. Most anterior point of the sagittal crest on the midline of the skull.
7. Most dorsal point of the sagittal crest.
8. Most posterior and lowest point of the sagittal crest above the parietal depression.
9. Most posterior point of the skull at the sagittal and lambdoid crests.
10. Most lateral point of the occipital condyle.
11. Most Anterior point of the foramen magnum.
12. Most ventral point of the auditory bulla.
13. Most posterior point of the external auditory meatus at the junction with the paraoccipital process.
14. Widest point of the cranium where the zygomatic arch originates from the squamosal.
15. Widest point of the zygomatic arch.
16. Most posterior point of the foramen ovale.
17. End of tooth row at the base of the third molar.
18. Suture between the palatines at the midline.
19. Suture between the premaxilla and maxilla at the midline.
22. Between 1st Molar and 1 Premolar at the base.
23. Between 1st and 2nd premolar at the base.
24. Between canine and first premolar at the base.

*Mandible*

1. Tip of the coronoid process.
2. Tip of the condylar process at external edge
3. Tip of the condylar process at internal edge
4. Tip of the angular process midway along the width
5. Point of extreme curvature at the incisura praemasseterica
6. Point of extreme curvature on the lower edge of mandibular corpus
7. Most anterior point of the lower edge of mandible corpus at the point where the two mandibles join.

8. Midpoint of the aboral edge of the mental foramen

9. Most anterior point of the mandible corpus

10. Midpoint at the base, between Incisor 1 and Canine 1

11. Midpoint at the base, between C1 and PM1

12. Midpoint at the base, between PM3 and M1

13. Midpoint at the base, between M1 and M2

14. Midpoint at the base, between M2 and M3

15. At the end of Molar 3 (M3).
Chapter 4

Fig. S1: Typical echolocation calls for a) *Rhinolophus swinnyi* and b) *Rhinolophus simulator*.

Fig. S2 (below): Residual distribution and model validation graphs for *Rhinolophus simulator* (top panel) and *Rhinolophus swinnyi* (bottom panel). Within each panel; from the top we show the linear-mixed-effects model as a stand-alone; below this we show the best model, i.e., after all spatial autocorrelation structures with and without study sites as a random effect have been tested. In this case, both species showing the best model structure as linear mixed effects with study sites as random effects.
Fig. S3: Spline correlograms of the residuals (with 95% confidence intervals) from a linear mixed effects model with study sites as random effects, including all predictor variables. *Rhinolophus simulator* and *Rhinolophus swinnyi* (bottom). The correlation is measured in Moran’s I spatial auto-correlation index.
Table S1:

a). Phenotypic parameters and environmental variables (Mean ± SD) for *R. simulator* from different sites within southern Africa.

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Total N 111

Abbreviations: AnnPrec = Mean Annual Precipitation (mm), AnnTemp = Mean Annual Temperature (°C), RH = Relative Humidity (%), Lat = Latitude, Long = Longitude, N = sample size, M = Males, F = Females, SD = standard deviation, RF = resting frequency of the echolocation call (kHz), Dur = Call Duration (ms), FA = Forearm length (cm), Mass = Mass (g). Locality codes, CC = Chinhoyi Cave, DM = Dambanzara, Zimbabwe; GKC = Gatkop Cave, South Africa, KL = Kalenda, Zambia; LOB = Lobatse Estate, Botswana; MC = Mabura Cave, Zimbabwe; MM = Monaci Mine, Zimbabwe; MT = Matobo, Zimbabwe; SH = Shimabala, Zambia; SUD = Sudwala Cave, South Africa.
b). Phenotypic parameters and environmental variables (Mean ± SD) for *R. swinnyi* from different sites within southern Africa.

<table>
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<tr>
<th>Site/Sex</th>
<th>N</th>
<th>FA   ±SD</th>
<th>RF   ±SD</th>
<th>Dur(ms) ±SD</th>
<th>Lat</th>
<th>Long</th>
<th>Alt(m)</th>
<th>RH(%)</th>
<th>AnnTemp</th>
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<td>106.55 ±0.91</td>
<td>24.94 ±11.08</td>
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</tbody>
</table>

Total N 126

Abbreviations: JET = Jiri Estate – Triangle, Zimbabwe; KP = Kapatamukombe, Zimbabwe; OD = Odzi German Shafts, Zimbabwe; PA = Pafuri, South Africa. All other abbreviations are given in table 1 b.