Intra- and inter-population variation in the bladder grasshopper *Bullacris unicolor*

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Abstract

Although the processes that promote biodiversity and speciation remain poorly understood, ecological factors are thought to be one of the causal agents responsible for promoting variation. Bladder grasshoppers (Orthoptera; Pneumoroidea) are a group of endemic southern African insects that provide an ideal model system to study the role of ecology in speciation. All species rely on acoustic communication for mate location, with males producing an extremely loud advertisement call that is highly species specific. Any alteration to the male call would thus likely have implications for species integrity. In this study, I examined geographic variation as well as potential ecological drivers of biological diversity within *Bullacris unicolor*, a variable and wide-ranging species of bladder grasshopper. The main aims of the study were to characterise the extent of acoustic and morphological variation among individuals sampled from five geographic locations and to correlate this variation with environmental data and host plant use.
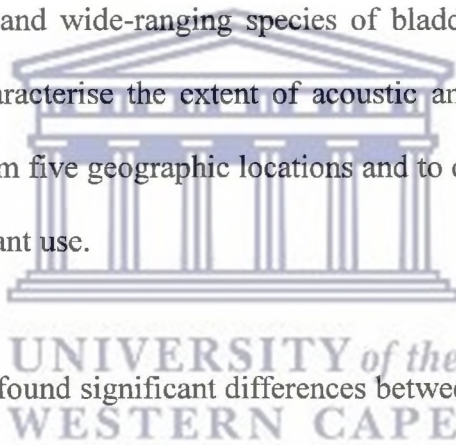
At the inter-population level, I found significant differences between populations with respect to both morphological and acoustic characters. Results of multivariate analyses showed significant differences in the body length, pronotum length and head width of males and in the pronotum length, abdomen width and head width of females. Similarly, the acoustic signals of males from the five populations differed significantly in both temporal and frequency components, with all but one variable (peak frequency of the introductory syllables) showing a significant difference. However, there was no correlation between morphological and acoustic variables among populations, and acoustic characters showed far greater divergence among populations than did morphology. In both males and females, the morphological variables that differed among populations were negatively correlated with mean annual temperature, indicating that grasshoppers are larger in areas with lower

temperatures. Also, some of the call characteristics of males were correlated with temperature, precipitation, altitude, and slope. Although grasshoppers were observed feeding on a range of host plant species, neither morphology nor signal characteristics were found to vary according to host plant.

At the intra-population level, multivariate analyses revealed that all acoustic characters differed significantly among individuals. Morphology may be influencing signal properties within a population to some extent as there were significant correlations between some of the call characters and both abdomen width and tibia length, with lengths of these two morphological variables being positively correlated with temporal components of the call and abdomen width being negatively correlated with frequency components. I also found a significant difference in the carrier frequency of male calls, as well as in some of the morphological characters of males and females, according to host plant. This indicates that host plant use has a greater effect on variation at the intra-population level than at the inter-population level.
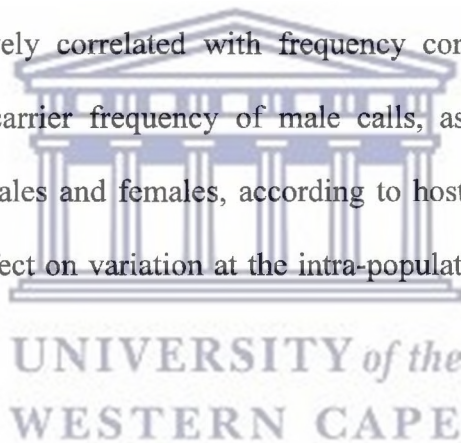
In conclusion, the results of my study reveal significant variation in the morphology and acoustic signals of *B. unicolor*, both within and among populations, and suggest that this variation is at least partially related to ecological factors.

1.   Introduction

1.1 Speciation

Explaining what has evolved in the past and starting to predict what might evolve in the future is the fascinating science of evolution. At this juncture, understanding speciation is one of the main goals in evolutionary biology (e.g.: Castellano & Giacoma 2000; Panhuis *et al.* 2001; Amezquita *et al.* 2009; Keller & Seehausen 2012; Mendelson & Shaw 2012). Divergence of genes in different gene pools is impacted by various processes, including the founder effect, sexual selection, ecological differences and random genetic divergence, although the crucial mechanisms needed for the evolution of reproductive isolation and speciation are still debated. Many of the most significant questions about speciation are difficult to address and answer (Santini *et al.* 2012); therefore, identifying the origin of reproductive isolating mechanisms in a divergent population is imperative in speciation research (Price 2007). It will help us to understand more about the processes and patterns of species origination.

During the development of a modern synthesis, many studies of speciation have focussed on genetic mechanisms (Guerrero *et al.* 2012; Martin & Mendelson 2012; Via 2012), such as how population divergence occurs via mutation and genetic drift (Coyne & Orr 2004; McNiven *et al.* 2011). However, there is very little information on the number of genes that are involved in the speciation process (Santini *et al.* 2012). Much progress has been made in the search for individual 'speciation genes' that cause reproductive isolation and this has created a shift in the study of speciation (Nosil & Feder 2012). Indeed, genetic studies shed tremendous light on evolutionary biology.

There are several non-genetic mechanisms that can also influence evolutionary change, such as learned behaviours. Learned mate preferences and learned display traits can contribute to sexual selection, evolution of reproductive isolation, population divergence and sexual conflict (Verzijden *et al.* 2012). However, many animals life span is too short to get an opportunity to acquire feedback for learning to occur (eg: most insects).

Recent research has also re-evaluated the role of geography in speciation, which has long been a major area of debate among speciation biologists. Mayr (1942, 1947, & 1963) proposed that speciation would be an eventual outcome when populations became geographically isolated from one another. Geographically separated populations diverge from each other both morphologically and genetically and this is termed as allopatric speciation. This model of speciation is widely recognised, where isolation is caused purely by genetic drift, adapting to an altered environment, sexual selection, or incompatible mutations. However, speciation also occurs in overlapping populations even in the absence of geographic barriers and this is termed sympatric speciation. This has been a much more contentious speciation model as there are no physical barriers preventing gene flow. In this model, divergence is driven primarily by fine-scale ecological differentiation and by sexual selection. A third scenario is parapatric speciation. In this model, the populations of a species are adjacent to each other with a small, continuous overlap between populations. Here the gene flow level between populations is lower than in sympatric speciation, but higher than in allopatric speciation. However, the phenomenon of parapatric speciation has received less attention compared to allopatric or sympatric speciation (reviewed by Safran & Nosil 2012). However, categorising speciation into allopatric, parapatric and sympatric does not provided a satisfactory framework (Marie Curie Speciation Network 2012), as the underlying mechanisms may be the same regardless of the extent of geographic isolation.

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1.2 Ecological speciation

By studying the factors that give rise to variation between populations, we can understand why there are so many species (Sobel *et al.* 2010). Gene flow barriers that evolve between populations as a result of divergent selection between different ecological environments are termed as ecological speciation (Schluter 2000; 2009; Maan & Seehausen 2011; Nosil 2012). This can happen under any spatial arrangement of populations (allopatric, sympatric, and parapatric). It has been proposed that ecologically based divergent natural selection is the ultimate source of reproductive isolation and ultimately speciation (Rundle & Nosil 2005; Rasanen & Hendry 2008; Wagner *et al.* 2012). Various empirical studies have shown evidence for ecologically based divergent selection (reviewed by Rundle & Nosil 2005). Populations adapting in different ecological environments diverge from each other in the way they look and behave. Gradually, gene flow between populations decreases as ecological differences increase. Finally they avoid mating with each other. This results in the formation of separate species (Safran & Nosil 2012). For example, variation in physical factors such as altitude, temperature, rainfall, sunlight and resource quality and availability may initiate diversity by causing a corresponding change in one or more of the morphological characteristics of an organism (Malhothra & Thorpe 1997). Reproductive isolation may also be promoted by the use of different host plants. Egan (2012) provided evidence that divergent host use promotes speciation among gall wasp populations. They found differences in body size and gall structure associated with divergent host use but no difference between populations using the same host plant. More specifically, small scale differences in ecological conditions or microhabitat within the same area may play a central role in the adaptive divergence of ecotypes within a population (Gavrilets & Vose 2007).

1.3 Environmental factors

Variation in the environment is broadly considered to be important for speciation (West-Eberhard 2003; Hua & Wiens 2013; Wiens *et al.* 2013), but it remains poorly understood exactly how it influences speciation. Particular environmental conditions may cause populations living in isolated areas to diverge from one another (Newton 2003) and direct variation in morphological and acoustic characters (Patten *et al.* 2004). Evidence that environmental factors play a role in acoustic and morphological divergence is well documented in anurans, birds, and insects. For example, environmental factors have been shown to influence morphological variation in frogs (Schäuble 2004), divergence of songs in birds (Ruegg *et al.* 2006), as well as intra- and inter-specific variation in sexual size dimorphism in insects (Laiolo *et al.* 2013). Thus, the role of environmental factors in speciation events has been identified as one of the most important factors in the generation of biological diversity (Coyne & Orr 2004). However, few studies have examined the relative role of environmental factors in intra- and inter-population variation in insects.
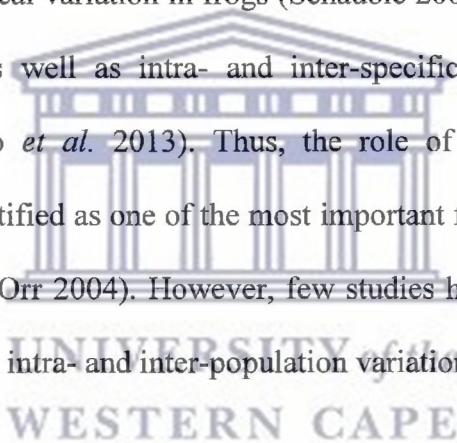
1.4 Acoustic divergence and reproductive isolation

The acoustic properties of signals are likely to serve as criteria for mate choice and thus play an important role in speciation (Boake 2002). At different social levels, animals have developed a remarkable variety of signals to communicate to conspecifics (Searcy & Nowicki 2005). Each signal might have some biologically significant information, i.e. signaller's identity (individual, sex, population, and species), size, physical condition, genetic fitness, or motivational state, such as its readiness to mate or fight (Gerhardt & Huber 2002). Therefore, advertisement calls of males are subject to selection in many species (Hoffman *et al.* 2006) and sexual signals of males' may exhibit minor inter-population difference (Zuk *et al.* 2001;

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Gerhardt & Huber 2002; Philips & Johnston 2008). Divergence in the acoustic characters of males among populations as a result of sexual selection may have important consequences for speciation (Prohl *et al.* 2007). Recent studies (Grace & Shaw 2012; Mandelson & Shaw 2005) have indicated that the evolution of sexual signalling is associated with diversification and accelerated rates of speciation. However, more studies are needed on the contribution of adaptive signal divergence to reproductive isolation (Rundle & Nosil 2005). The main advantage of studying acoustic communication is the potential for encoding biologically important information and preferences for signals which can bias the direction of future changes in communication systems (Gerhardt & Huber 2002).

Most previous studies have analysed female preferences for acoustic signals at the interspecific level (Gray & Cade 2000; Oh *et al.* 2012). A few studies (Foerschler & Kalko 2007; Koetz *et al.* 2007; Filatova *et al.* 2012) have obtained evidence to show that the signal structure and signalling behaviour vary within a species (geographical variation) and how this influences speciation, but do not consider the relative contributions of ecology to reproductive isolation at intra- and inter-population levels. Any alteration of the environment under which signalling occurs can critically influence both the nature and perception of signals. This divergence in mating signals and preferences resulting from ecological differences is referred to as sensory drive (Endler 1992, 1993).

The role of environmental factors in the speciation process remains relatively unexplored; therefore it is critical to identify the degree of environmental variation within and among populations in the early stages of divergence, because these studies provide the strongest support for the evolutionary effects of the environment on signalling behaviour and signals (Gerhardt & Huber 2002).

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1.5 Bladder grasshoppers (Orthoptera; Pneumoroidea)

Acoustic communication in insects presents ideal opportunities to biologists interested in evolutionary processes. Specifically, grasshoppers are good model species to address many interesting evolutionary questions that explore biodiversity (Song 2010). Bladder grasshoppers (Orthoptera; Pneumoroidea) are endemic southern African insects specialised for long range acoustic communication. The small family of pneumorids consists of 17 species in nine genera (Dirsh 1965), with each species living and feeding on either one or a small number of host plant species. Pneumorids are broadly distributed in the Succulent Karoo, Fynbos Biome, Savannah Biome and Forest Biome in coastal areas of South Africa (Rutherford 1997; Alexander & van Staaden 1989). The environmental conditions, such as annual rainfall, topography, humidity and plant diversity of these four habitats varies extensively (see Couldridge & van Staaden 2004), so that the development of phenotypic variation in these species is high. Gene flow in pneumorids is thought to be inhibited by two factors, namely low vagility and high host plant fidelity. These features create isolated populations and high potential for genetic drift (N. Donelson unpublished PhD thesis).

The distinctive feature of this family is the strongly inflated, balloon-like abdominal bladder of males that acts as an acoustic resonator and includes six pairs of abdominal hearing organs, unique among the animal world (van Staaden & Römer 1998). Acoustic communication systems are fairly well understood in pneumorids (van Staaden & Römer 1997, 1998; van Staaden et al. 2003; Couldridge & van Staaden 2004, 2006). Males generate a loud signal at night by rubbing their hind legs and abdomen together, creating a transmission distance of 1.5 - 1.9 km (van Staaden & Römer 1997). Females respond acoustically to male advertisement calls based on their detectability and attractiveness, thereby allowing flighted males to locate them (Couldridge & van Staaden 2004). While the male call varies greatly between species,

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the softer female signal is relatively simple in structure and is not species-specific. Alternate male morphs (sneaker males) are present at low numbers, and exploit the acoustic signalling system between primary males and females to their own benefit (Donelson & van Staaden 2005).

1.6 *Bullacris unicolor*

Figure 1 *Bullacris unicolor* male (A) and female (B).

Bullacris unicolor (Fig. 1) is a fairly wide ranging pneumorid, with a geographic distribution extending along the coastal areas of the western region of South Africa. *B. unicolor* is found

in the sparsely vegetated Succulent Karoo, noted for its steep climatic gradients and high species diversity (Linder & Hardy 2004), as well as in the Fynbos Biome, which is extremely rich in plant diversity. Very little research has previously been conducted on *B. unicolor*.

1.7 Aims of the study

The purpose of this study was to examine morphological and acoustic variation in the bladder grasshopper species *B. unicolor*, both within and between populations, and to correlate this variation with ecological factors, including local climatic conditions and host plant species, in order to evaluate their role in promoting divergence. This can be broken down into the following five specific aims: 1) characterise acoustic and morphological variation between populations that are sampled at several locations throughout the geographic range of the species, 2) correlate environmental data from each sampling location with measured physical and acoustic characters to evaluate the relative contribution of ecological factors to generating diversity, 3) determine whether or not the acoustic characters of the study species vary in relation to morphological characters, 4) determine whether or not the acoustic and morphological variation is associated with divergent host plant use, and 5) finally, determine the extent of intra-population variation in the acoustic characters of males, and also whether host plants and morphological characters correlate with the acoustic characters of individuals within a single population. Through the careful examination of the patterns of divergence within this ideal experimental system, the factors that contribute to speciation might be better understood.

2. Materials and Methods

2.1 Specimen acquisition and rearing

Bullacris unicolor was collected during the spring and early summer from five field sites located throughout the coastal region of the south-western Cape of South Africa – Springbok, Kamieskroon, Groenriversmond, Cederberg (near Citrusdal), and Melkbosstrand (Fig. 2). The vegetation biome of Springbok, Kamieskroon, and Groenriversmond is succulent Karoo with lower growing, sparser succulent shrubs and rare grasses, except in some sandy areas. Cederberg and Melbosstrand are part of the Fynbos biome, consisting of shrubs with small and hard leaves, and there are very few trees or grasses. All of these areas are characterized by winter rainfall, between 20 and 290 mm per year, and extreme summer aridity. Due to the seasonal occurrence of pneumorids, sampling was done to coincide with peak times of emergence (September to November) during 2008, 2010, 2012 and 2013. A total of 96 grasshoppers were individually captured by hand from their natural stands of host plants. As far as possible, adults or final instar nymphs were collected in order to avoid the potentially homogenising effects of controlled laboratory conditions (59 adults and 37 nymphs). Alternate males were excluded from the study due to their low numbers (n = 4). Alternate males were only found at Springbok.

Animals collected in the field were transported to the laboratory. Nymphs were raised to adulthood in the laboratory under identical rearing conditions. Adults and nymphs were housed in small groups in identical plastic storage bins with a wire mesh top to allow for airflow and light. To prevent fighting, adult males were kept separate from each other. Individuals were maintained under natural lighting conditions in a temperature controlled room maintained at 25 °C. Individuals were fed *ad libitum* either on their host plant species

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(*Didelta spinosa, Roepera morgsana,* and *Tripteris oppositifolia*) or, if this was unavailable, fed on a diet of organically grown lettuce. Grasshoppers were examined daily to replenish their water and food, and to determine their moulting.
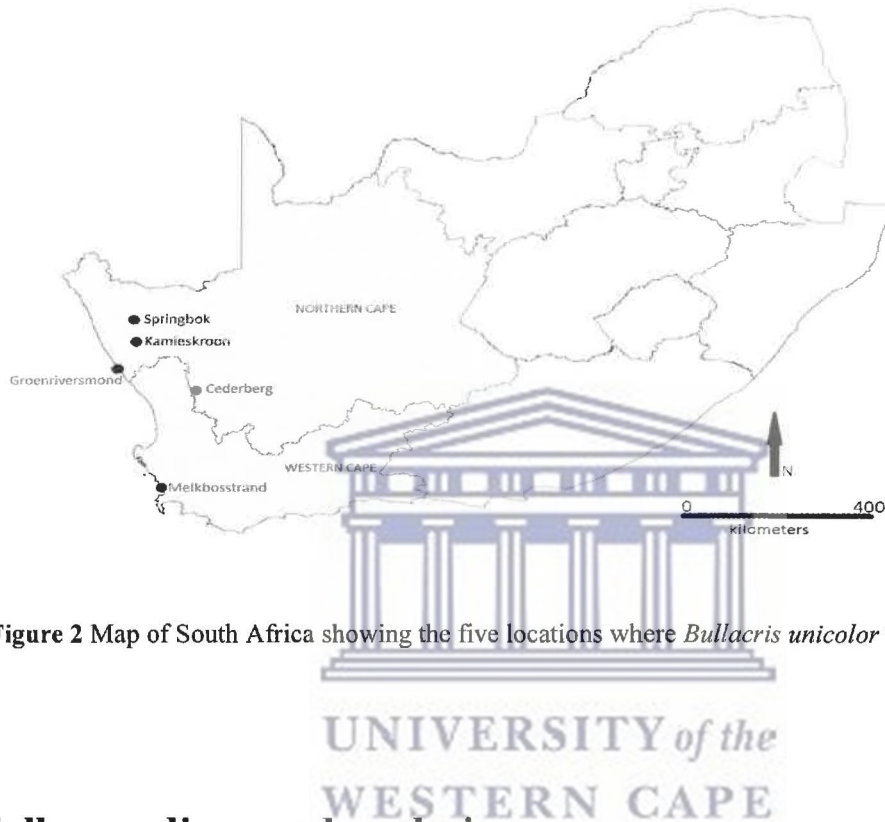
Figure 2 Map of South Africa showing the five locations where *Bullacris unicolor* was collected.

2.2 Call recordings and analysis

To record male advertisement calls, individual adult males were placed in a clear plastic container fitted with a mesh cover. The temperature in the room was maintained at approximately 20° C during recording and continuously monitored. Upon calling, males were recorded using a Marantz PMD-670 digital recorder and a Sennheiser K6/ME-66 microphone. The tip of the microphone was positioned at a distance of approximately 1 m in front of the calling male. The male's calls were spontaneous. Songs of 42 males were downloaded onto a computer and analysed using Raven Pro 1.3 software (Cornell Bioacoustics Research Programme).

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Bullacris unicolor produces a relatively short advertisement call (< 3 seconds) with only two short introductory syllables preceding the final long resonant syllable, and a carrier frequency of around 2 kHz (Fig. 3). The song recordings were characterized in terms of their temporal structure and frequency spectra. All the measurements were done after filtering background noise to remove frequencies below 500 Hz. For each call I measured seven characteristics: length of the introductory syllables, inter syllable pause, length of the final syllable, entire call length, peak frequency of the introductory and the final syllable, and the rise time (time taken to reach maximum amplitude) of the final syllable (Fig. 3). Data from ten calls per male were averaged and the mean values obtained were used in further statistical analyses.

2.3 Physical measurements

Following the death of grasshoppers, all material was preserved in 90% ethanol. Morphometric measurements of both male and female specimens were made to the nearest 0.01 mm using digital callipers. All the body parts were measured carefully without damaging the fragile specimens and the measurements were made on the right side of each specimen in order to standardise results.
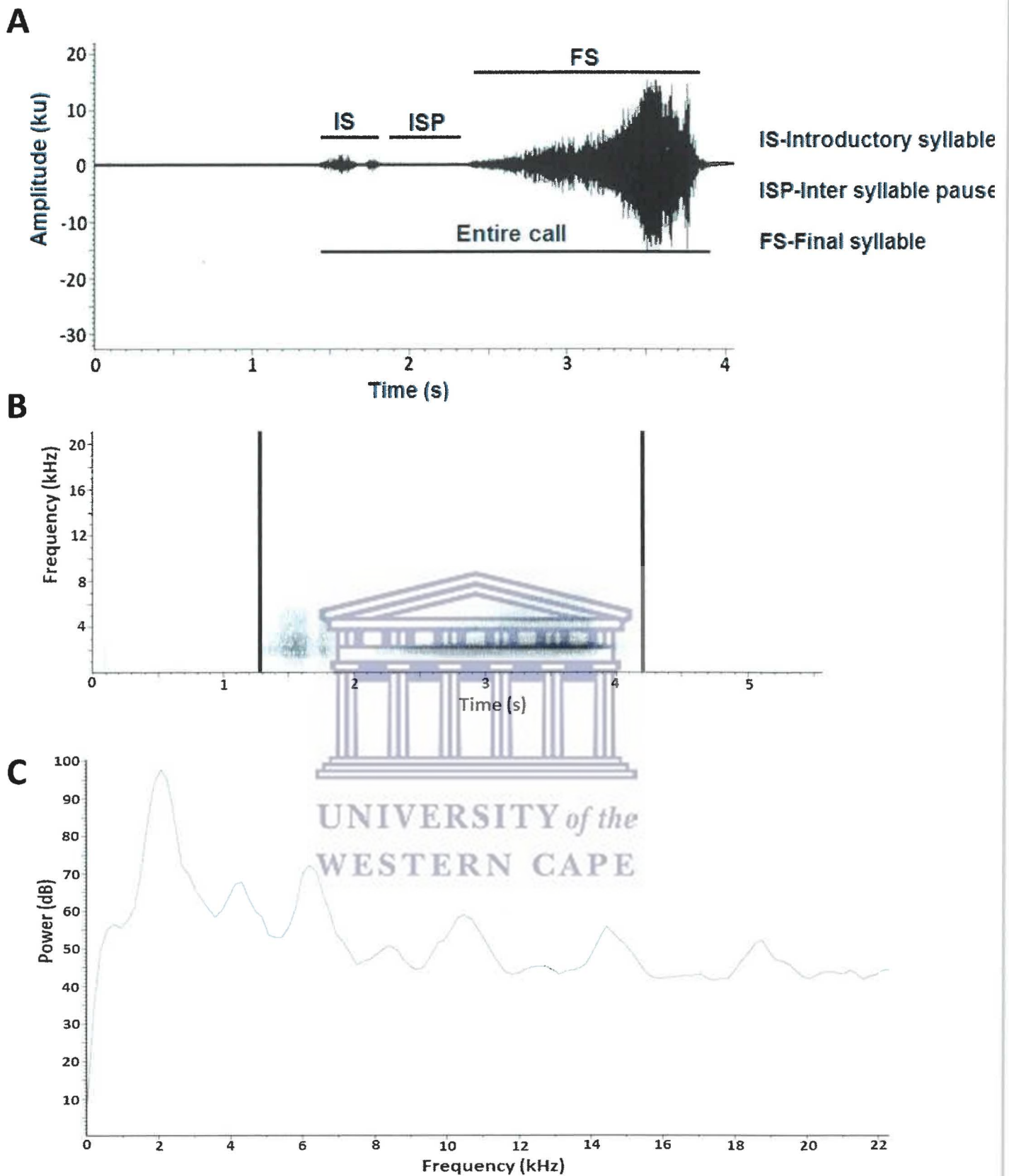
Figure 3 Oscillogram (A), sonogram (B), and power spectrum (C) exemplars of the male advertisement call of

B. unicolor. Space between two lines in figure B indicates the section taken for the power spectrum.

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Following Donelson & van Staaden (2005) a series of seven linear measurements (mm) were obtained from each male and female specimen, which included antennal length, body length, head width, abdomen width, femur length, tibial length, and pronotum length (Fig. 4). Antennal length (AL) was measured from the base of the antenna to its tip. Body length (BL) was measured from the most anterior point of the head to the end of the abdomen. Head width (HW) was measured immediately behind the compound eyes. Male abdomen width (AW) was measured from the point directly between the two stridulatory ridges and female abdomen width was measured from the point directly between the second and third abdominal segments. Hind femur length (FL) was measured from the point of articulation with the trochanter to the point of articulation with the tibia. Hind tibia length (TL) was measured from the point of articulation with the femur to the point of articulation with the tarsus. Pronotum length (PL) was measured linearly from the base of the ridge where it meets the head to its pointed end.

PL - Pronotum length

AL - Antennal length

HW - Head width

FL - Femur length

TL - Tibia length

AW - Abdomen width

BL - Body length
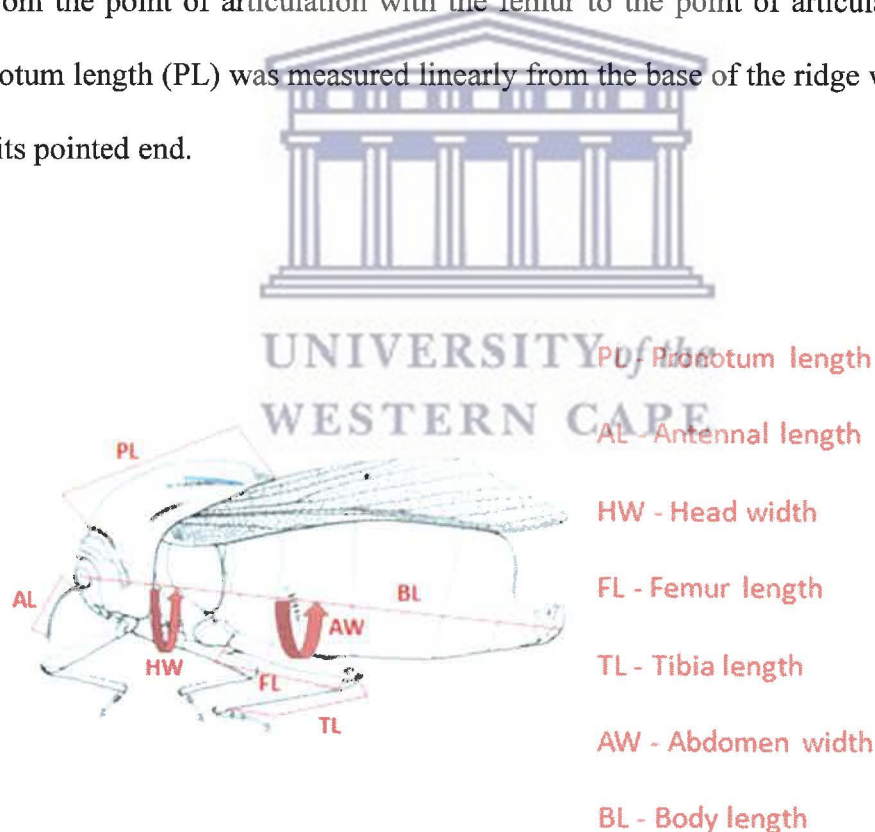
Figure 4 Diagram of male *Bullacris unicolor* showing the seven linear anatomical measurements taken in both males and females. For paired structures, right sides were used for each measurement. Line drawing taken from Dirsh (1965).

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2.4 Environmental variation

The individuals used in this study were obtained from the five field sites across the Western and Northern Cape provinces of South Africa. Latitude/longitude co-ordinates were marked in the field for each location and altitude, annual precipitation, and annual mean temperature for each of these locations (n = 5) were obtained from WorldClim – Global Climate Data (http://www.worldclim.org/bioclim). This is free online climate data for ecological modelling and GIS. The representations of bioclimatic variables are in annual trends (mean annual temperature and precipitation), seasonality (annual range in temperature and precipitation), and extreme or limiting environmental factors (temperature of the coldest and warmest month, and precipitation of wet and dry quarters).

The vegetation biome, moisture availability, slope, and terrain for each point location were collected from GIS shape file layers provided by the Department of Agriculture, Forestry and Fisheries (Republic of South Africa), and were extracted using DIVA-GIS. The vegetation biome was either Succulent Karoo or Fynbos Biome; designated according to the geographic position of each location. Moisture availability was measured as three categories (slight, moderate, and very severe). Slope was measured as one of four different ranges (<2%, 3 - 5%, 4 - 5%, 13 - 20%), and terrain was divided into four categories (open low hills, level plains, open high hills, open low mountains) (Table 1).

Table 1 Environmental characteristics of the five sampling locations.

Location	Latitude	Longitude	Altitude	Annual precipitation	Annual mean temp	Vegetation biome	Moisture availability	Slope (%)	Terrain
Cederberg	-32.6	19.01	192	327	18.27	Fynbos	Slight	3 to 5	Open low hills
Groenriversmond	-30.85	17.6	56	154	17.65	Succulent Karoo	Very severe	4 to 5	Level plains
Kamieskroon	-30.2	17.93	839	221	15.63	Succulent Karoo	Very severe	13 to 20	Open high hills
Melkbosstrand	-33.73	18.5	61	621	16.55	Fynbos	Moderate	<2	Level plains
Springbok	-29.66	17.88	954	182	17.34	Succulent Karoo	Very severe	13 to 20	Open low mountains

2.5 Host plants

To examine how host plant affects the morphology and acoustic characters, I recorded the host plant species on which each individual was found and used this data in further analysis. *Bullacris unicolor* was found on seven different host plants: (1) *Didelta spinosa*, (2) *Muraltia spinosa*, (3) *Roepera morgsana*, (4) *Salvia africana-lutea*, (5) *Tripteris oppositifolia*, (6) *Osteospermum moniliferum*, and (7) *Roepera* sp. Details of the number of individuals collected from each host plant among populations are shown in Table 2. Flying adult males were often collected at night and no host plant information is therefore available for these individuals (n = 25). Nymphs and adult females do not fly and were always found on a host plant.

Table 2 Number of *Bullacris unicolor* collected from each host plant species at each location.
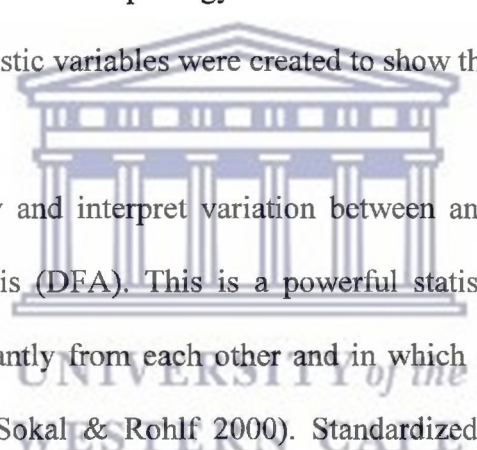
Host plant	Springbok	Groenriversmond	Kamieskroon	Cederberg	Melkbostrand
Didelta spinosa	22		9	12	
Muraltia spinosa					3
Roepera mogsana	13	2	1		
Salvia africana-lutea		2			
Tripteris oppositifolia		4			
Osteospermum moniliferum					2
Roepera sp.			1		

2.6 Statistical analysis

All statistical tests were performed with IBM SPSS version 22. I used several approaches to assess morphological and acoustic variation of individuals from different locations. I

summarized means and standard deviations of morphological characters of males and females, and of acoustic characters of males. To assess the variation in both morphological and acoustic characters between different populations I performed multivariate analysis of variance (MANOVA), and Canonical Discriminant Analysis.

In the first part of the analysis, I compared all variables using MANOVA, with morphological/acoustic variables as the dependent variables and location as the main fixed factor. The MANOVA compared the differences in body parts and call properties of grasshoppers from the different locations. Significant differences would indicate that these populations have distinctly different morphology and acoustic characters. Contingency tables for the morphological and acoustic variables were created to show the population variation.

In the second part, to identify and interpret variation between and within groups, I did a Discriminant Function Analysis (DFA). This is a powerful statistical technique to reveal whether groups differ significantly from each other and in which direction (in multivariate space) those differences lie (Sokal & Rohlf 2000). Standardized coefficients of the first discriminant functions directly estimate the degree of morphological and acoustic divergence: higher values show larger divergence of the variable. I compared morphology and acoustic characters of the study populations and classified them by means of canonical discriminant analysis (morphology/location of males and females, and acoustic/location of males), and created DFA canonical centroid plots to visually represents how the populations differed morphologically and acoustically from each other.

In the third part, to estimate the relationship between environmental variables and morphology/acoustic characters I used bivariate correlations. Partial correlation was used to

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determine the relationship between morphology and call characteristics. To examine the effects of host plants I included host plant as a grouping variable in the MANOVA to compare acoustic and morphological characters among populations.

Finally, I conducted a similar analysis to examine the variation of acoustic characters among individuals within a single population to test whether calls of individuals from one population could be reliably distinguished from each other. Males from Springbok were used in the intra-population analysis as this was the population with the greatest sample size. To test the correlation between morphology and call characters among individuals, I used bivariate correlation. Association between host plants and acoustic characters and morphology of males within this population was assessed by independent samples t-tests with two host plants (*Didelta spinosa* and *Roepera morgsana*) as defined groups.

3. Results

3.1 Inter-population variation

3.1.1 Morphological variation of males and females among populations

3.1.1.1 MANOVA results

Mean values and standard deviations for morphological characters of males and females are shown in Table 3 (A and B). Results of the MANOVA revealed that there was significant variation in some of the morphological characters of males and females among *Bullacris unicolor* populations. Males showed significant differences in head width, body length, and pronotum length ($F_{2.545}$, $p < 0.000$) (Table 4A; Fig. 5A). Females showed significant differences in head width, abdomen width, and pronotum length ($F_{2.232}$, $p < 0.003$) among populations (Table 4B; Fig. 5B). In addition, femur length of females was only marginally non-significant ($p = 0.08$).
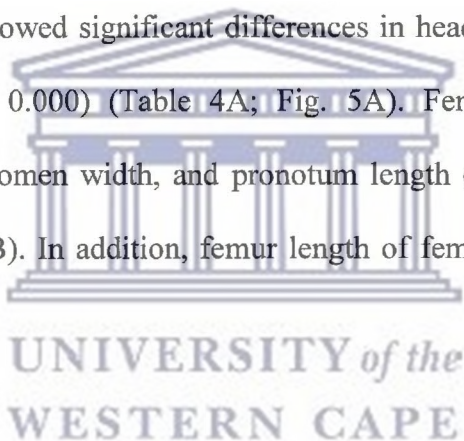
Table 3 Anatomical measurements of males (A) and females (B) of *Bullacris unicolor* from five locations, expressed as mean and standard deviation. Abbreviations are: antennae length (AL), head width (HW), body length (BL), abdomen width (AW), femur length (FL), tibia length (TL), and pronotum length (PL).

A

Variables	Mean ± SD (n=54)				
	Springbok	Kamieskroon	Groenriversmond	Cederberg	Melkbosstrand
AL	6.18 ± 1.03	6.00 ± 0.73	5.75 ± 0.95	6.15 ± 0.59	6.61 ± 0.77
HW	4.57 ± 0.25	4.47 ± 0.21	4.33 ± 0.16	4.22 ± 0.15	4.69 ± 0.43
BL	38.38 ± 1.79	39.61 ± 2.04	37.74 ± 1.75	35.05 ± 1.28	39.94 ± 2.57
AW	10.90 ± 0.77	11.49 ± 0.82	10.72 ± 0.71	11.20 ± 0.52	11.27 ± 0.66
FL	11.02 ± 0.48	10.88 ± 0.82	10.75 ± 0.61	10.35 ± 0.25	11.16 ± 0.62
TL	11.44 ± 0.73	11.24 ± 0.63	10.99 ± 0.34	11.05 ± 0.59	11.59 ± 0.69
PL	17.19 ± 0.85	17.33 ± 1.10	16.68 ± 0.60	15.91 ± 0.47	18.40 ± 0.55

B

Variables	Mean ± SD (n=31)				
	Springbok	Kamieskroon	Groenriversmond	Cederberg	Melkbosstrand
AL	5.67 ± 0.59	5.91 ± 0.58	5.17 ± 0.74	5.27 ± 0.47	5.74 ± 1.68
HW	5.51 ± 0.36	5.84 ± 0.51	4.92 ± 0.29	4.85 ± 0.19	5.80 ± 0.19
BL	38.61 ± 3.55	38.29 ± 2.02	35.43 ± 1.86	39.01 ± 4.68	39.15 ± 0.70
AW	8.70 ± 0.71	9.35 ± 0.31	7.41 ± 0.59	7.59 ± 1.43	8.67 ± 0.26
FL	10.45 ± 0.86	11.11 ± 0.12	9.96 ± 0.72	10.25 ± 0.27	11.41 ± 0.75
TL	11.23 ± 0.89	11.50 ± 0.26	10.69 ± 0.68	10.90 ± 0.39	11.21 ± 1.40
PL	21.8 ± 1.08	21.89 ± 1.92	20.20 ± 0.69	20.73 ± 0.99	24.50 ± 1.26

Table 4 MANOVA results of the variation in morphological characters of males (A) and females (B).

A

Dependent Variables	Sum of squares	Df	Mean square	F	p
AL	2.95	4	0.73	1.01	0.41
HW	0.97	4	0.24	3.76	0.00*
BL	112.99	4	28.24	7.39	0.00*
AW	4.47	4	1.12	1.96	0.11
FL	2.65	4	0.66	1.41	0.24
TL	1.99	4	0.49	1.2	0.32
PL	22.32	4	5.58	7.36	0.00*

*Significant, $p<0.05$.

B

Dependent Variables	Sum of squares	Df	Mean square	F	p
AL	1.97	4	0.49	1.07	0.38
HW	4.10	4	1.02	9.06	0.00*
BL	53.8	4	13.45	1.16	0.34
AW	13.54	4	3.38	4.62	0.00*
FL	4.81	4	1.20	2.33	0.08
TL	1.99	4	0.49	0.83	0.51
PL	34.25	4	8.56	7.04	0.00*

*Significant, $p<0.05$.

A
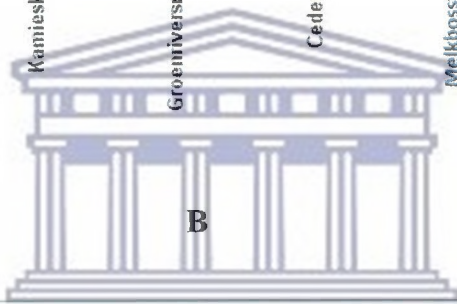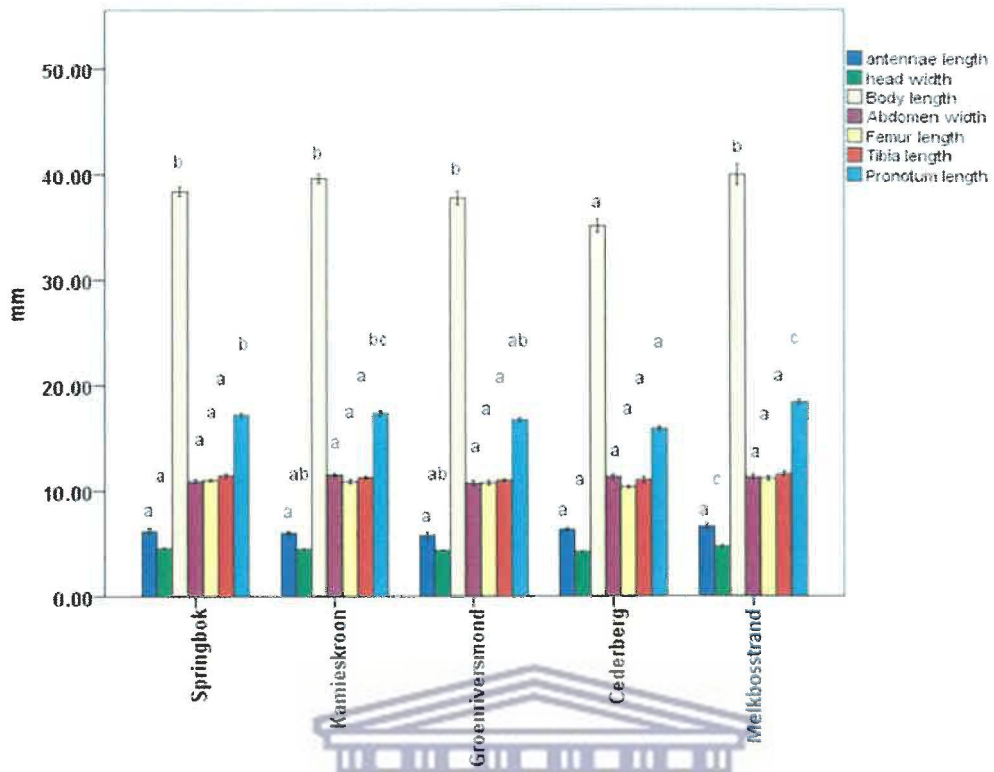
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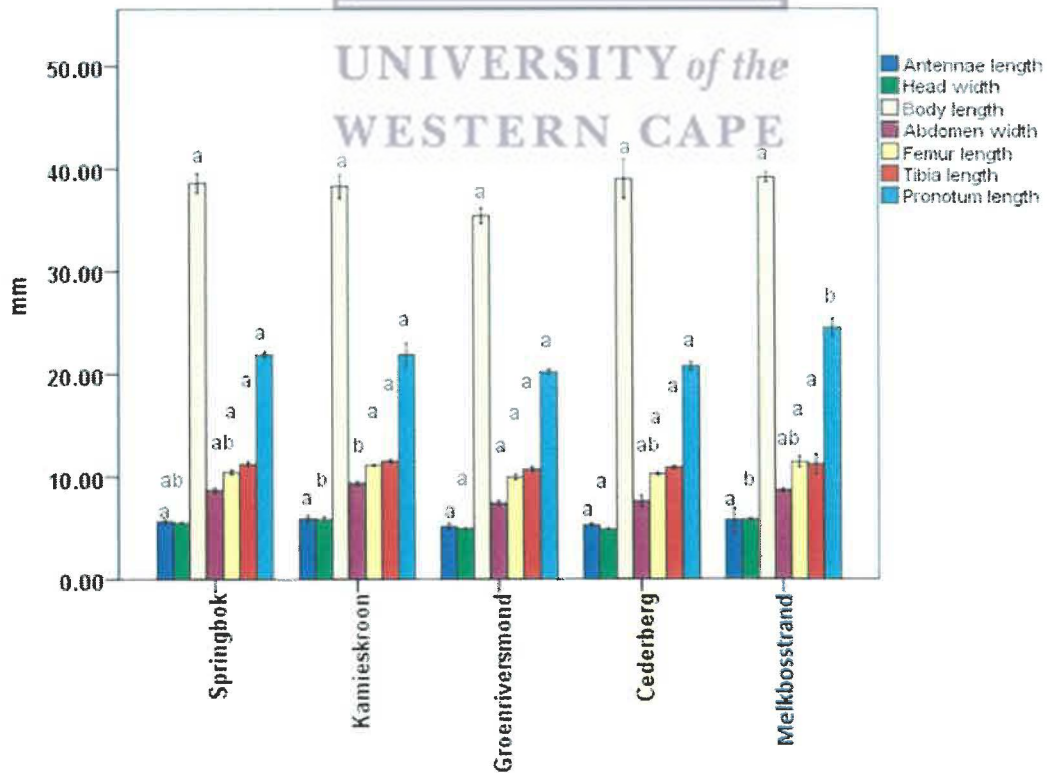
Figure 5 Variation in morphological characters of *Bullacris unicolor* among five allopatric populations (A) males and (B) females. Letters above error bars (a-c) indicate significance for each characteristic, with different letters indicating significant difference at the 5% level and the same letter indicating no significant difference.

3.1.1.2 DFA results

DFA was performed on 54 males and on 31 females (Table 5; Fig. 6). For males, DF1 explained 70.2% of total variance and DF2 explained 18.2%. Body length and abdomen width showed the highest standardized canonical discriminant function coefficient with DF1, whereas antennae and body length showed the highest correlation with DF2. For females, DF1 explained 66.7% and DF2 explained 22.6% of the total variance. Head width and tibia length show the highest correlation with DF1 and tibia and pronotum length show the highest correlation with DF2. Therefore, in males, high scores on the first DF depict grasshoppers with relatively large body length and small abdomen widths, whereas for females, high scores on the first DF depicts grasshoppers with a large head width and small tibia length.

Table 5 Standardized canonical discriminant function (DF) coefficients for seven morphological variables in male (n = 54) and female (n = 31) *B. unicolor* sampled from different populations.

Variables	Males		Females	
	DF1	DF2	DF1	DF2
Antennae length	0.106	0.745	0.034	0.261
Head width	0.318	0.407	0.633	0.901
Body length	0.825	-0.931	-0.455	-0.389
Abdomen width	-0.684	0.267	0.508	0.029
Femur length	0.32	-0.375	0.463	-0.523
Tibia length	-0.211	0.431	-0.605	0.961
Pronotum length	0.506	0.512	0.49	-1.178

https://etd.uwc.ac.za/
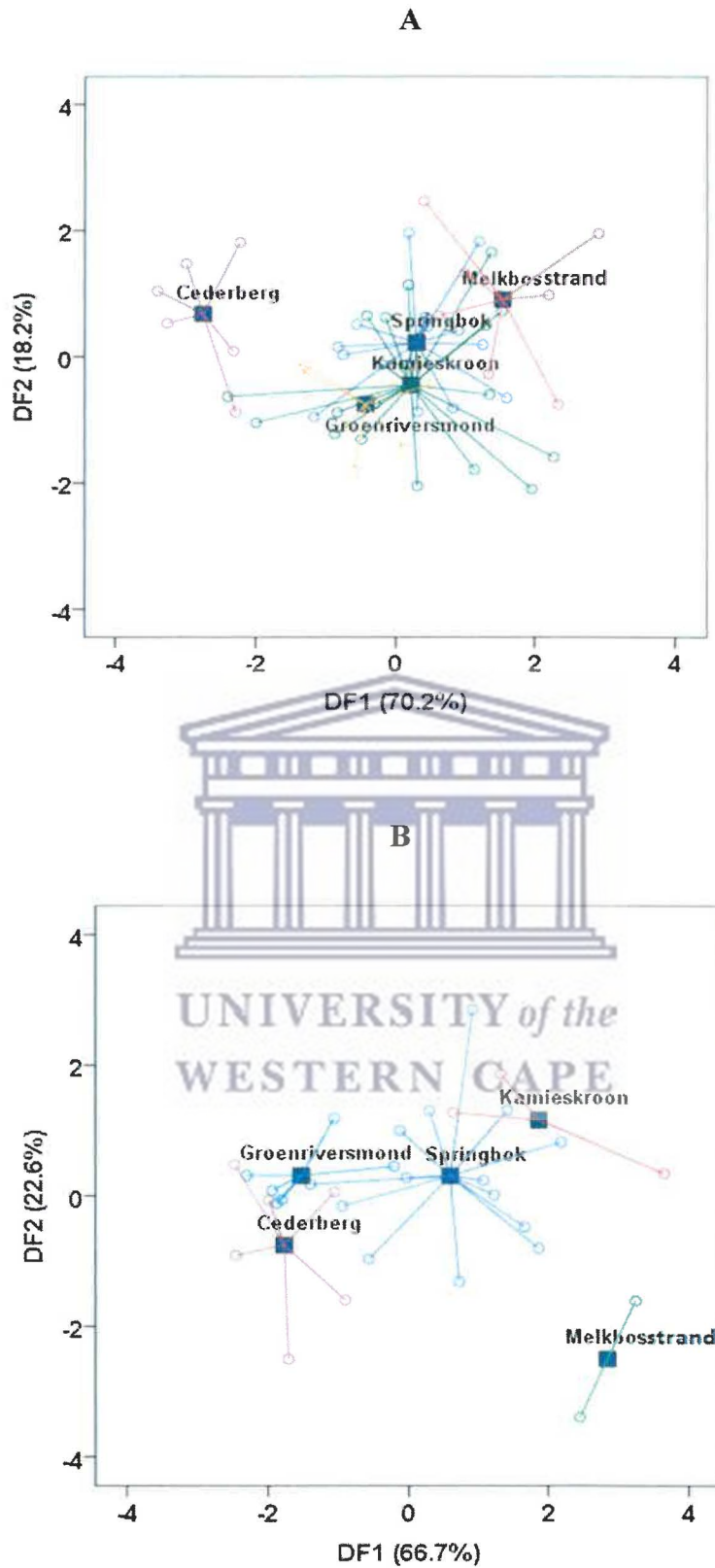
Figure 6 Canonical centroid plot of the discriminant function analysis for morphological data. Canonical discriminant plots DF1-DF2 depict individual scores on DF1 and DF2 for each sex: **A** males (n = 54), **B** females (n = 31). Coloured lines from the centroid represent the different individuals from each population.

3.1.1.3 Classification results of canonical discriminant analysis

Canonical discriminant analysis of male morphological comparison revealed that 55.6% of original group cases were classified correctly (Fig. 6A, Table 6A). Morphological measurements of males from Cederberg are separated with a 100% correct classification. Males from Melkbosstrand (71.4%) are separated with high percentage of correct classification, but show minor overlap with males from Kamieskroon. The separation of Springbok (46.7%), Groenriversmond (42.9%), and Kamieskroon (47.4%) was less obvious, with just under half these males being classified correctly, and all three populations showing slight overlap with each other.

Canonical discriminant analysis of female morphology revealed that 74.2% of original group cases were correctly classified (Fig. 6B, Table 6B). Females from Melkbosstrand and Kamieskroon were 100% correctly classified. Groenriversmond (83.3%) shows a high percentage of classification, although females of this location show minor overlap with Springbok. Females from Springbok (64.3%) and Cederberg (66.7%) show the least amount of correct classification, overlapping with Cederberg and Kamieskroon, and with Groenriversmond respectively.

Table 6 Classification results of canonical discriminant analysis showing real and predicted group membership of *Bullacris unicolor* from five study areas, derived from morphological characters of males (A) and females (B) (percentages in brackets).

A

Predicted group membership: 55.6% original grouped cases correctly classified				
Location	Springbok	Groenriversmond	Kamieskroon	Cederberg	Melkbosstrand
Springbok	7 (46.7)	3 (20.0)	3 (20.0)	0	2 (13.3)
Groenriversmond	2 (28.6)	3 (42.9)	2 (28.6)	0	0
Kamieskroon	3 (15.8)	1 (5.3)	9 (47.4)	2 (10.5)	4 (21.1)
Cederberg	0	0	0	6 (100.0)	0
Melkbosstrand	0	0	2 (28.6)	0	5 (71.4)

B

Predicted group membership : 74.2% of original grouped cases correctly classified				
Location	Springbok	Melkbosstrand	Groenriversmond	Cederberg	Kamieskroon
Springbok	9 (64.3)	0	0	2 (14.3)	3 (21.4)
Melkbosstrand	0	2 (100.0)	0	0	0
Groenriversmond	1 (16.7)	0	5 (83.3)	0	0
Cederberg	0	0	2 (33.3)	4 (66.7)	0
Kamieskroon	0	0	0	0	3 (100.0)

The MANOVA and canonical discriminant analysis of male and female morphological comparison revealed that variability within populations was relatively low and between populations was relatively high. However, the canonical centroid plots (Fig. 6A & 6B) show

some overlap of individuals from different populations. However, female morphology did not show as much overlap as male morphology.

3.1.2 Acoustic variation of males among populations

Visual inspection of calls among populations revealed the absence of an inter syllable pause from the individuals from Melkbosstrand, whereas individuals from the other populations show more similar song types (Fig. 7).
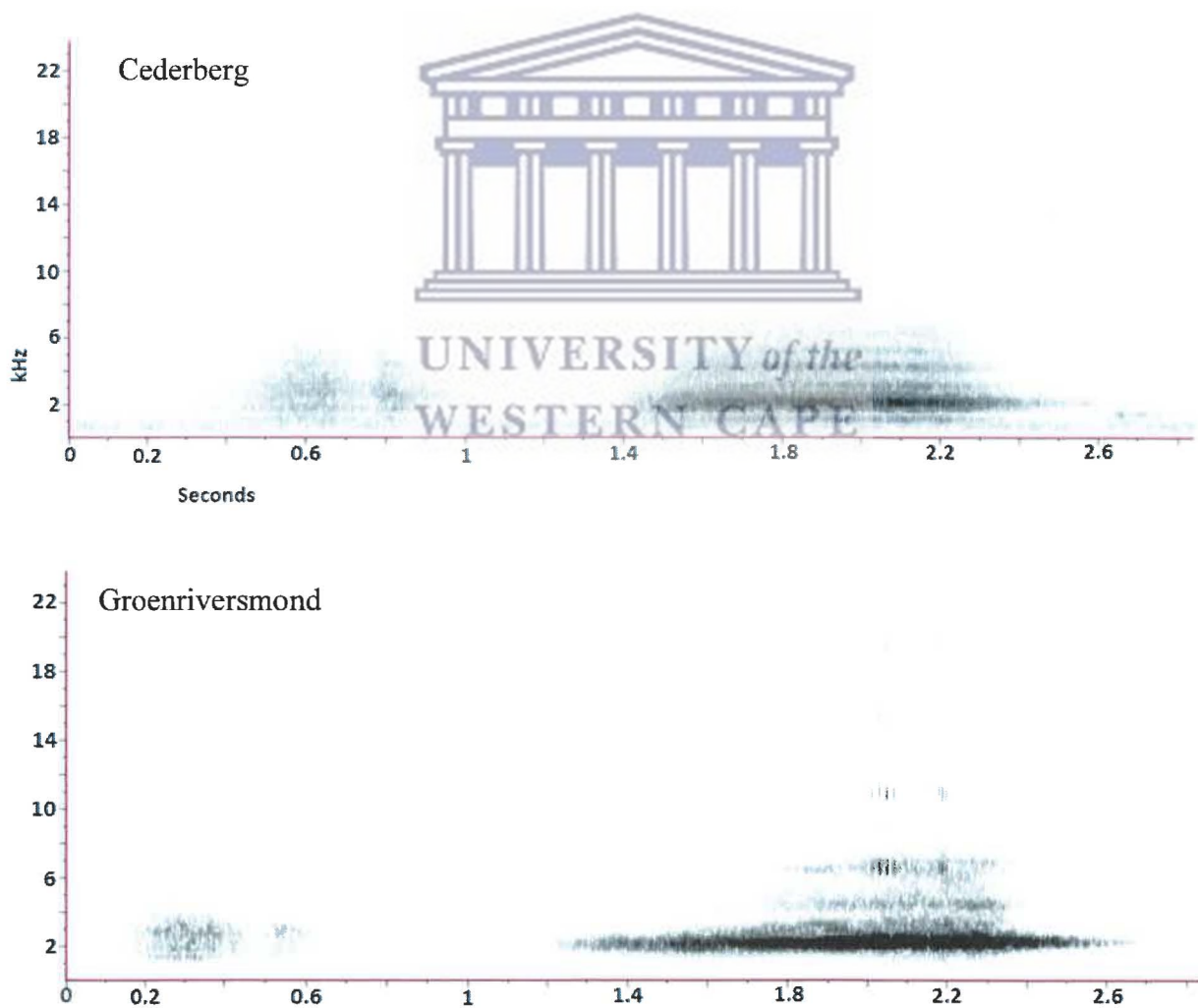
Figure 7 (continued on following page)
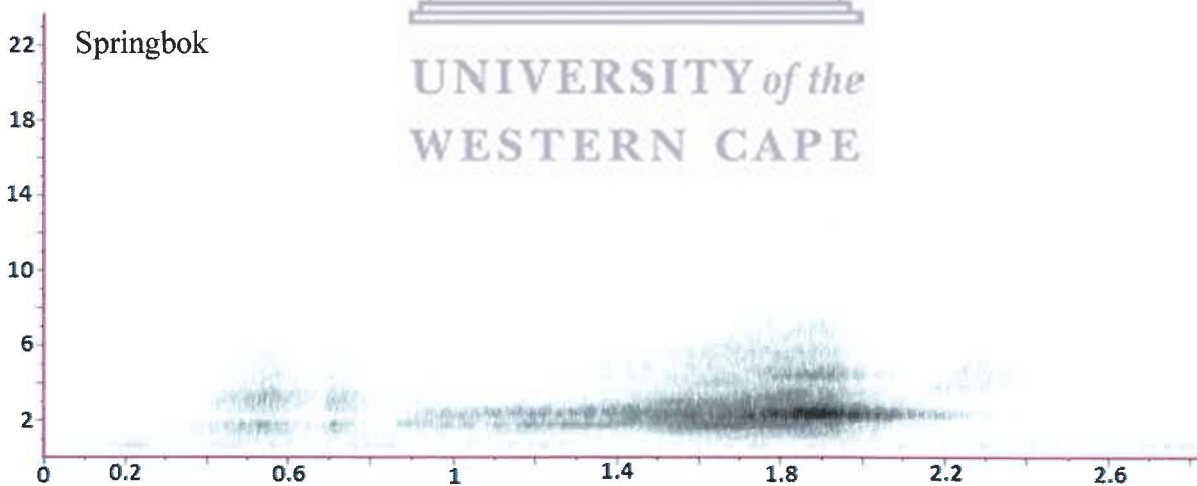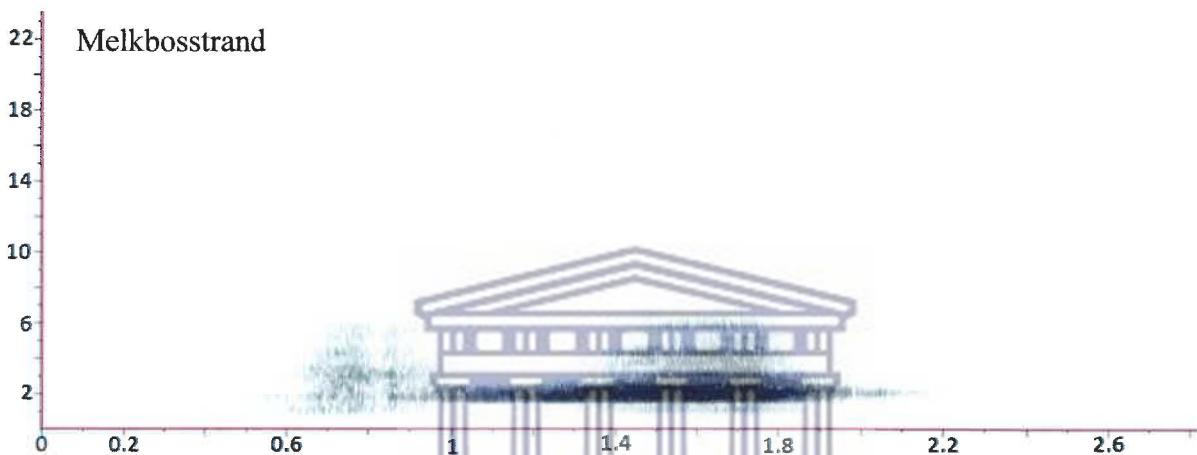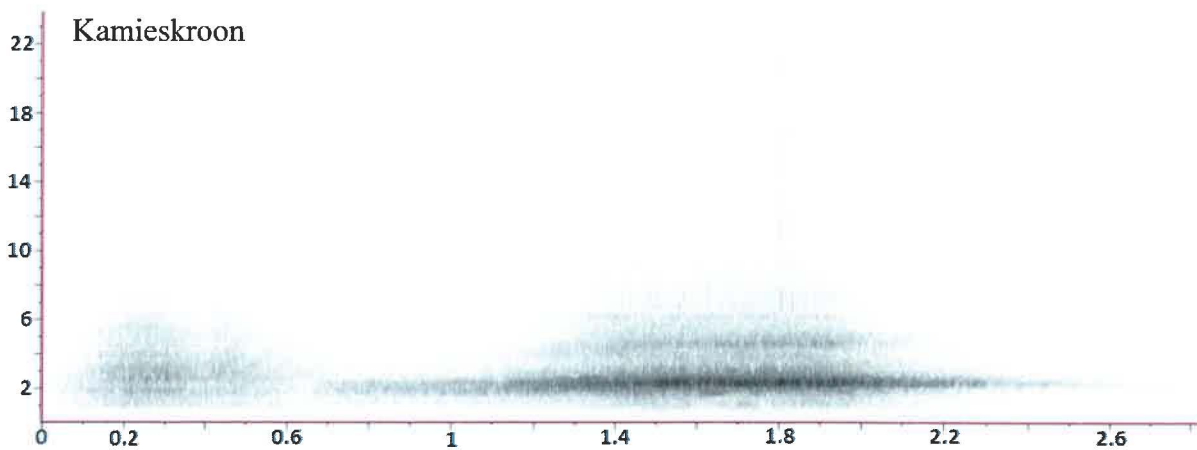
Figure 7 Sonogram exemplars of *Bullacris unicolor* male calls from the five populations.

3.1.2.1 MANOVA results

Mean values and standard deviations for acoustic characters of males are shown in Table 7. MANOVA results show that all acoustic characters of males from the five allopatric populations differed significantly ($F_{9.264}$, $p < 0.000$) (Fig. 8, Table 8), with the exception of peak frequency of the introductory syllables.

Table 7 Measurements of call characters of *Bullacris uncolor* males from five populations, expressed as mean and standard deviation.

Variables	Length of introductory syllable	Length of inter syllable pause	Length of final syllable	Length of entire call	Peak frequency of introductory syllable	Peak frequency of final syllable	Rise time of final syllable
			Mean ± SD				
Springbok (n=14)	0.439 ± 0.101	0.177 ± 0.107	1.586 ± 0.289	2.192 ± 0.432	2331.10 ± 573.51	2102.67 ± 121.03	1.166 ± 0.327
Groenriversmond (n=5)	0.583 ± 0.048	0.508 ± 0.076	1.699 ± 0.152	2.791 ± 0.214	2431.87 ± 222.01	2238.75 ± 109.81	1.243 ± 0.139
Kamieskroon (n=14)	0.518 ± 0.080	0.146 ± 0.068	1.431 ± 0.251	2.098 ± 0.319	2199.27 ± 349.87	2179.18 ± 122.58	0.831 ± 0.285
Cederberg (n=6)	0.407 ± 0.097	0.381 ± 0.083	0.881 ± 0.124	1.659 ± 0.271	1914.95 ± 324.02	2196.18 ± 75.23	0.430 ± 0.077
Melkbosstrand (n=3)	0.115 ± 0.199	0	1.501 ± 0.714	1.594 ± 0.742	2769.231	1690.70 ± 383.36	0.885 ± 0.234

Table 8 MANOVA table showing the variation in acoustic characters of males from five different locations.

Dependent variables	Sum of squares	df	Mean square	F	p
Length of introductory syllable	0.146	4	0.037	4.671	0.004*
Length of inter syllable pause	0.714	4	0.179	23.183	0.000*
Length of final syllable	2.515	4	0.629	10.563	0.000*
Length of entire call	3.759	4	0.94	7.626	0.000*
Peak frequency of introductory syllable	1236005.453	4	309001.363	1.641	0.186
Peak frequency of final syllable	134874.926	4	33718.732	2.553	0.056*
rise time of final syllable	2.937	4	0.734	10.034	0.000*

*Significant, $p<0.05$.

A
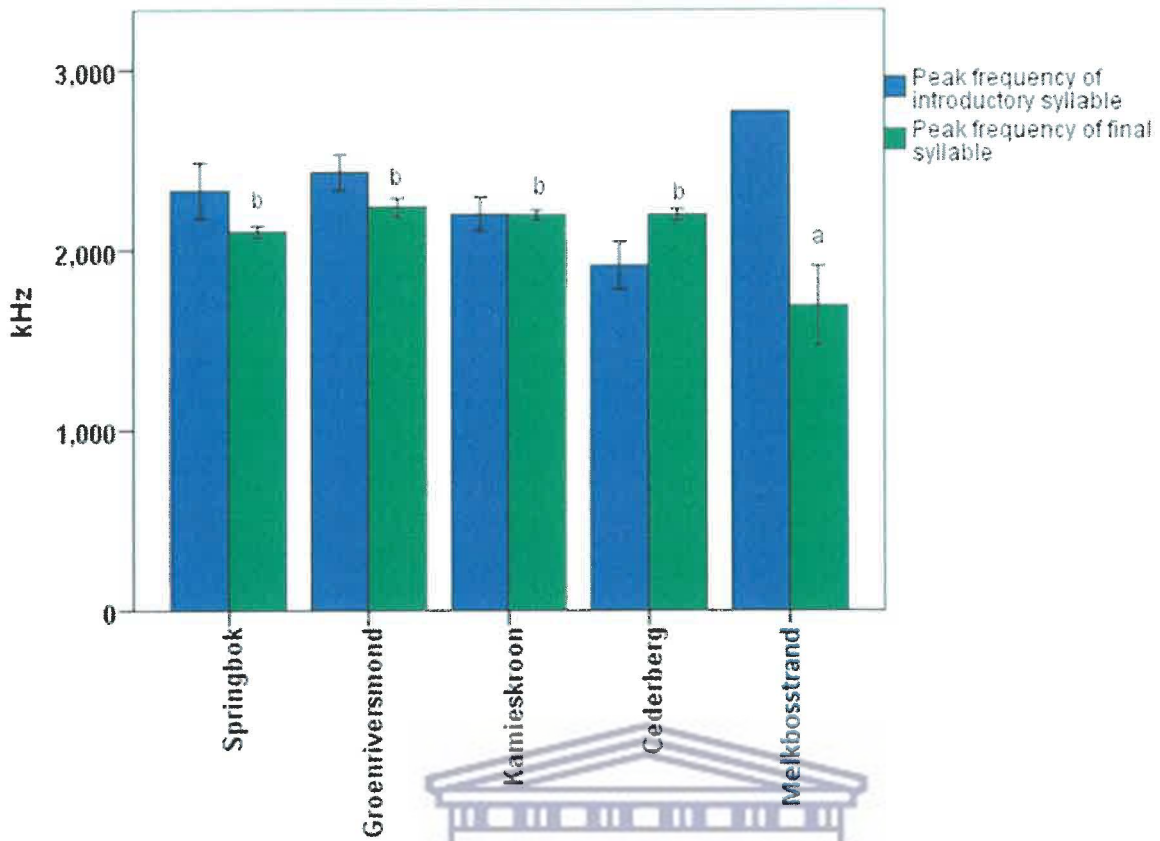
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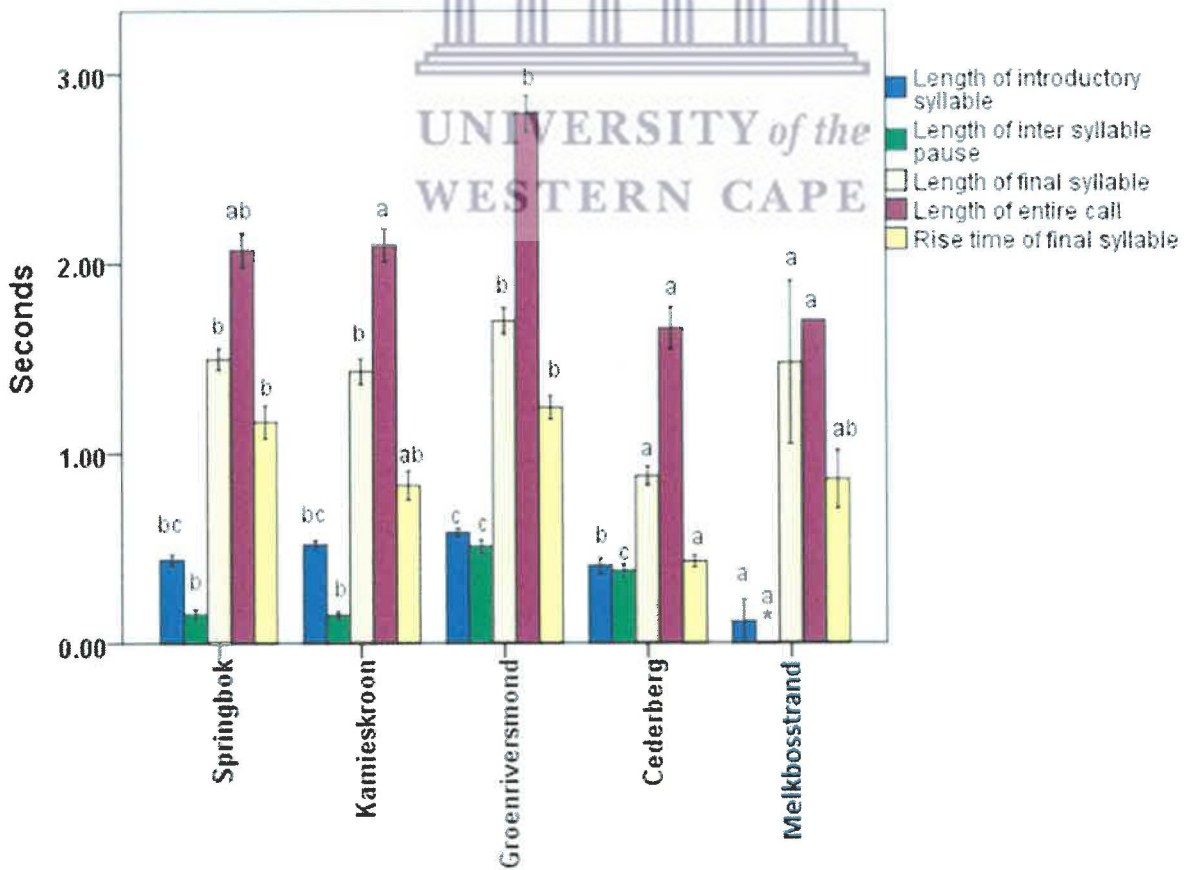
Figure 8 Variation in male advertisement call parameters among five populations of *B. unicolor*: (A) frequency components; (B) temporal components. Letters above error bars (a-c) indicate individual significance for each character, with different letters representing a significant difference at the 5% level and the same letter indicating no significant difference.

Note: * Inter syllable pause was absent from the calls of individuals from Melkbosstrand.

3.1.2.2 DFA results

DFA was performed on the acoustic characters of 42 males. DF1 explained 73.5% of the total variance and DF2 explained 14.4% (Fig. 9). Length of the entire call shows the highest correlation with DF1 and length of the final syllable shows the highest correlation with DF2 (Table 9), although length of the final syllable and the entire call length both contribute greatly to DF1 and DF2. Thus, high scores on the first two DFs depict grasshoppers with a long final syllable and entire call. The length of final syllable does not show much contribution to length of entire call.

Table 9 Standardized canonical discriminant function (DF) coefficients for seven acoustic variables in male (n = 42) *Bullacris unicolor* sampled from different populations.

Variables	DF1	DF2
Length of introductory syllable	1.652	-0.707
Length of inter syllable pause	3.728	-0.789
Length of final syllable	5.816	-4.749
Length of entire call	-7.954	4.532
Peak frequency of introductory syllable	0.47	1.077
Peak frequency of final syllable	0.135	-0.535
Rise time of final syllable	-1.465	2.074
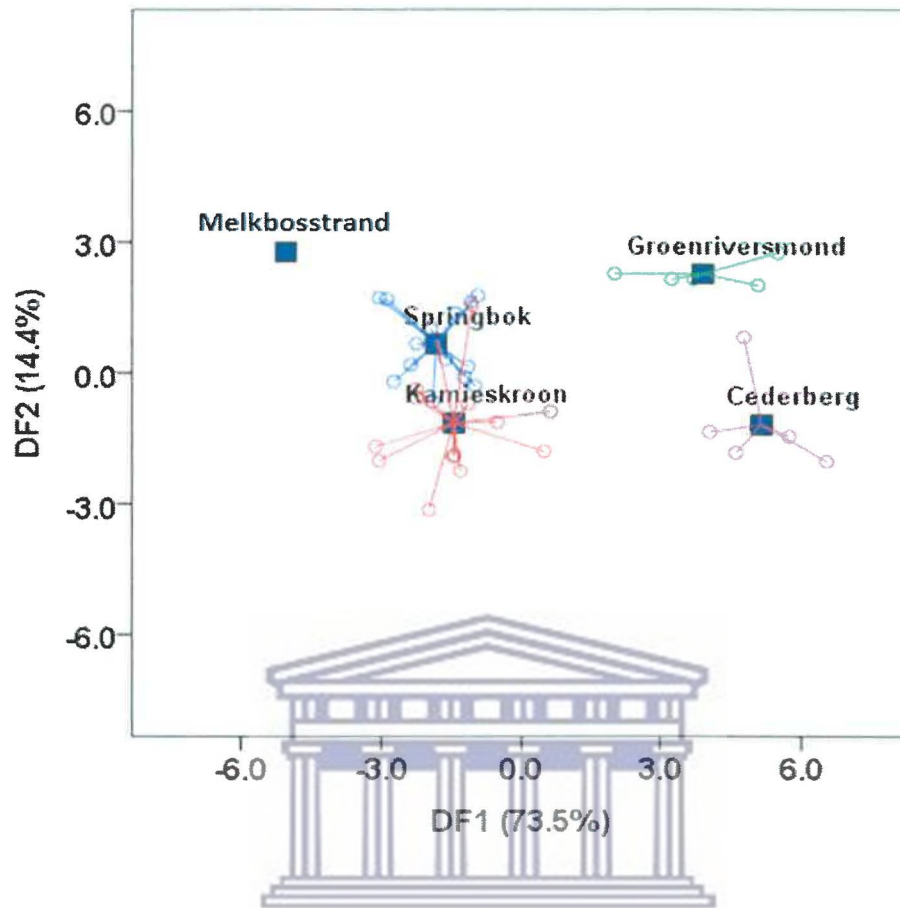
Figure 9 Canonical centroid plot of the discriminant function analysis showing the separation of male advertisement calls according to population. Lines from the centroid connect to individuals from each population.

3.1.2.3 Classification results of canonical discriminant analysis

By means of canonical discriminant analysis, 90.0% of original group cases were classified correctly (Fig. 9, Table 10). Correct classification was 100% for Melkbosstrand, Groenriversmond, and Cederberg. Calls of males from Springbok and Kamieskroon show a high percentage of correct classification (92.9% and 78.6% respectively), although they overlapped slightly with each other. One of 14 calls of males from Springbok (7.1%) was

incorrectly classified as Kamieskroon, and three calls of males from Kamieskroon (21.4%) were incorrectly classified as Springbok. Canonical discriminant analysis of acoustic characters clearly revealed that there was greater variation in male calls between populations than within populations.

Table 10 Classification results of canonical discriminant analysis showing real and predicted group membership of *Bullacris unicolor* from five study areas, derived from acoustic characters of males (percentages in brackets).

	Predicted group membership: 90.0% of original grouped cases correctly classified				
Location	Springbok	Groenriversmond	Kamieskroon	Cederberg	Melkbosstrand
Springbok	13 (92.9)	0	1 (17.1)	0	0
Groenriversmond	0	5 (100.0)	0	0	0
Kamieskroon	3 (21.4)	0	11 (78.6)	0	0
Cederberg	0	0	0	6 (100.0)	0
Melkbosstrand	0	0	0	0	1 (100.0)
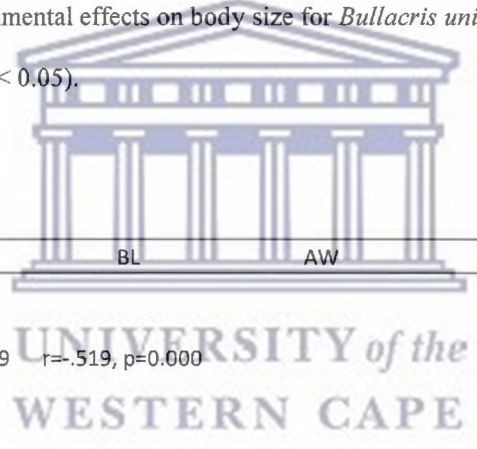
3.1.3 The effects of environmental variation on morphology

Correlation analysis revealed a relationship between environmental factors and morphology in this species. In males, bivariate correlation between environmental factors and morphological variation revealed that mean annual temperature and annual precipitation were both correlated with aspects of morphology (Table 11). I observed a significant negative correlation between body length and temperature. Relationships between pronotum length and head width with temperature were also negatively correlated, although these correlations

were weaker. Pronotum length was weakly positively correlated with precipitation. I found no correlation between any other environmental factors and morphological characters.

In females, bivariate correlation results revealed that mean annual temperature and, to a lesser extent, precipitation and slope, were significantly correlated with some of the morphological characters. Significant negative correlations were detected between temperature and head width, abdomen width, femur length and pronotum length of females (Table 11). As with males, pronotum length was positively correlated with precipitation. In addition, there were weak correlations with head and abdomen width with slope.

Table 11 Correlation table of environmental effects on body size for *Bullacris unicolor* males and females. Only significant correlations are shown ($p < 0.05$).

Variables	HW	BL	AW	FL	PL
Males					
Temperature	r=-.297, p=0.029	r=-.519, p=0.000			r=-.364, p=0.007
Precipitation					r=0.343, p=0.011
Females					
Temperature	r=-.645, p=0.000		r=-.511, p=0.003	r=-.408, p=0.023	r=-.418, p=0.019
Precipitation					r=0.419, p=0.019
Slope	r=0.362, p=0.046		r=0.372, p=0.040		

3.1.4 The effects of environmental variation on acoustic characters

Detailed analysis of the relationship between environmental factors and acoustic divergence revealed that environmental conditions may play an important role in acoustic differentiation. I found significant correlations between annual temperature, annual precipitation, altitude, and slope with some of the acoustic characters of males (Table 12). The result showed a

strong negative correlation between altitude and length of the inter-syllable pause, and positive correlations were found between temperature and inter-syllable pause, as well as length of the final syllable. The correlations between annual precipitation and length of the introductory syllables, length of the entire call, peak frequency of the final syllable, and rise time of the final syllable were all significantly negative. Slope was negatively correlated with length of the inter-syllable pause and positively correlated with length of the introductory syllables, length of the final syllable, rise time and peak frequency of the final syllable (Table 12). These correlations demonstrate that certain song features may decrease in length or frequency with an increase in altitude and annual precipitation. But, greater slope and temperature increases are related to an increase in the length of call components.

Table 12 Correlation table of environmental effects on acoustic characters for *Bullacris unicolor* males. Only significant correlations are shown ($p < 0.05$).

Variables	Length of introductory syllable	Length of inter syllable pause	Length of final syllable	Length of entire call	Peak frequency of final syllable	Rise time of final syllable
Temperature		r=0.485, p=0.004	r=0.566, p=0.000			
Precipitation	r=-.677, p=0.000			r=-.493, p=0.001	r=-.553, p=0.000	r=-.358, p=0.020
Altitude		r=-.691, p=0.000				
Slope	r=0.513, p=0.001	r=-.464, p=0.003	r=0.335, p=0.030		r=0.336, p=0.029	r=0.349, p=0.024

3.1.5 Relationship between morphological and acoustic characters

I tested whether call differences relate to body size using partial correlation tests. However, the analysis did not show any significant relationships between morphological and acoustic

characters. Therefore, dissimilarities in call characters between sampled localities were not related to differences in morphology.

3.1.6 Host plant effects

Partial correlation analysis did not show any significant relationship between morphological characters and host plants. In addition, no correlation was found between host plants and acoustic characters among populations in this study. Therefore, host plant was not a reliable predictor for the inter-population variation in morphology and acoustic characters observed in the study species.

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3.2 Intra-population variation

3.2.1 Acoustic variation of males within a population

3.2.1.1 MANOVA results

I examined the acoustic characters of 14 males (9 collected as adults and 5 collected as nymphs) from Springbok to look at the intra-population variation of acoustic characters. Mean values and standard deviations for acoustic characters are shown in Table 13. MANOVA results show that all acoustic characters of individuals from Springbok differed significantly among individuals ($F_{12.024}$, $p < 0.001$) (Table 14).
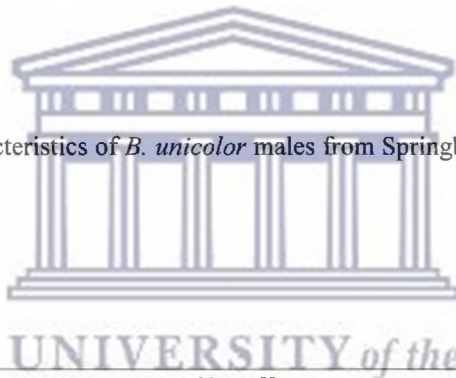
Table 13 Measurements of call characteristics of *B. unicolor* males from Springbok (n = 14), expressed as mean and standard deviation.

Males				Mean ± SD			
	Length of introductory syllable	Length of inter syllable pause	Length of final syllable	Length of entire call	Peak frequency of introductory syllable	Peak frequency of final syllable	Rise time of final syllable
1	0.45 ± 0.07	0.22 ± 0.06	2.00 ± 0.20	2.59 ± 0.12	2531.25 ± 511.03	2250.0 ± 0.00	1.74 ± 0.23
2	0.59 ± 0.12	0.42 ± 0.05	2.26 ± 0.08	3.28 ± 0.20	1854.16 ± 112.67	2062.50 ± 0.00	1.77 ± 0.12
3	0.40 ± 0.05	0.16 ± 0.06	1.46 ± 0.11	2.04 ± 0.11	2250.00 ± 559.01	2025.00 ± 79.05	1.01 ± 0.17
4	0.46 ± 0.02	0.15 ± 0.06	1.59 ± 0.20	2.21 ± 0.16	2381.25 ± 599.80	2062.50 ± 0.00	1.23 ± 0.30
5	0.46 ± 0.02	0.15 ± 0.06	1.59 ± 0.20	2.21 ± 0.16	2381.25 ± 599.80	2062.50 ± 0.00	1.23 ± 0.30
6	0.42 ± 0.01	0.24 ± 0.05	1.38 ± 0.04	2.04 ± 0.07	1050.0 ± 167.70	1875.0 ± 0.00	1.05 ± 0.01
7	0.38 ± 0.02	0.18 ± 0.04	1.63 ± 0.19	2.14 ± 0.10	2175.0 ± 539.09	2062.50 ± 0.00	1.27 ± 0.22
8	0.28 ± 0.02	0.04 ± 0.02	1.51 ± 0.06	1.84 ± 0.06	3125.0 ± 634.92	2062.50 ± 0.00	1.27 ± 0.22
9	0.30 ± 0.03	0.09 ± 0.02	1.51 ± 0.20	1.90 ± 0.20	2737.50 ± 674.30	2250.00 ± 0.00	1.12 ± 0.20
10	0.59 ± 0.10	0.16 ± 0.07	1.52 ± 0.13	2.29 ± 0.22	1762.50 ± 96.824	1912.50 ± 79.05	0.69 ± 0.15
11	0.57 ± 0.02	0.15 ± 0.03	1.25 ± 0.04	1.98 ± 0.04	2212.50 ± 147.90	2100.00 ± 79.05	0.69 ± 0.15
12	0.38 ± 0.03	0.08 ± 0.01	1.28 ± 0.07	1.76 ± 0.07	2850.00 ± 660.25	2231.25 ± 59.29	0.78 ± 0.13
13	0.36 ± 0.04	0.08 ± 0.03	1.33 ± 0.14	1.78 ± 0.14	2943.75 ± 177.87	2193.75 ± 90.57	0.92 ± 0.12
14	0.38 ± 0.00	0.09 ± 0.04	1.51 ± 0.05	1.98 ± 0.09	2906.25 ± 132.58	2250.00 ± 0.00	1.07 ± 0.04

Table 14 MANOVA table showing the variation of acoustic characters of 14 males within one population (Springbok).

Dependent variables	Sum of squares	df	Mean square	F	p
Length of introductory syllable	1.099	13	0.08	25.33	0.000*
Length of inter syllable pause	0.93	13	0.07	26.26	0.000*
Length of final syllable	8.63	13	0.66	29.39	0.000*
Length of entire call	17.86	13	1.37	63.54	0.000*
Peak frequency of introductory syllable	26717675.78	13	2055205.829	8.87	0.000*
Peak frequency of final syllable	1434082.031	13	110314.002	42.64	0.000*
Rise time of final syllable	11.191	13	0.861	20.6	0.000*

*Significant, $p < 0.05$

3.2.1.2 DFA results

DFA was performed on the acoustic characters of 14 males within one population. DF1 explained 52.6% of the total variance and DF2 explained 31.4% (Fig. 10; Table 15). Length of the entire call shows the highest correlation with DF1 and peak frequency of the final syllable shows the highest correlation with DF2. Therefore, DF1 and DF2 separate calls predominantly on the basis of length of entire call and peak frequency of final syllable. Thus, high scores on the first two DFs depict grasshoppers with long call and a high peak frequency of the final syllable. The percentage of variation accounted for by discriminant functions decreased rapidly, indicating the inter-correlation between variables, even though the canonical plot showed a better separation of individuals.
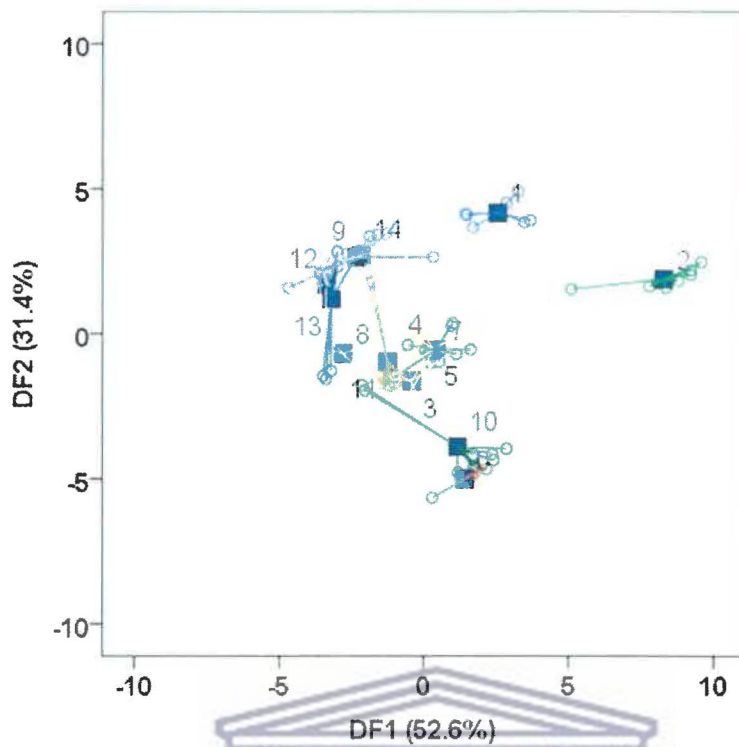
Figure 10 Canonical centroid plot of the discriminant function analysis showing the separation of male advertisement calls (n = 14) within one population (Springbok). Lines from the centroid represent the different calls measured from each individual.
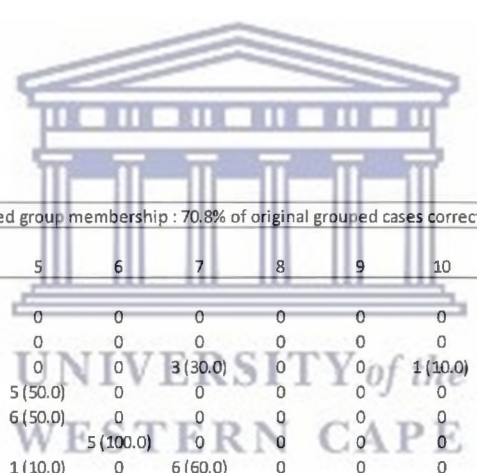
Table 15 Standardized canonical discriminant function (DF) coefficients for seven acoustic variables of individuals of *B. unicolor* sampled from Springbok.

Variables	DF1	DF2
Length of introductory syllable	-0.036	-0.128
Length of inter syllable pause	0.447	0.099
Length of final syllable	-0.001	0.085
Length of entire call	0.694	0.282
Peak frequency of introductory syllable	-0.305	0.298
Peak frequency of final syllable	-0.113	0.923
Rise time of final syllable	0.165	0.047

3.2.1.3 Classification results of canonical discriminant analysis

By means of canonical discriminant analysis, 70.8% of original group cases were classified correctly (Table 16). Some of the individuals show 100% of correct classification and some other males show a high percentage of correct classification (80%, 70%, and 60%). However, a number of males were classified incorrectly and overlapped with each other in their acoustic characters.

Table 16 Classification results of the canonical discriminant analysis showing real and predicted group membership of *B. unicolor* within one population, derived from the acoustic characters of males (percentages in brackets).

Males	Predicted group membership : 70.8% of original grouped cases correctly classified													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	8 (100)	0	0		0	0	0	0	0	0	0	0	0	0
2	0	9 (100)	0		0	0	0	0	0	0	0	0	0	0
3	0	0	5 (50.0)		0	0	3 (30.0)	0	0	1 (10.0)	1 (10.0)	0	0	0
4	0	0	1 (10.0)	4 (40.0)	5 (50.0)	0	0	0	0	0	0	0	0	0
5	0	0	1 (10.0)	5 (40.0)	6 (50.0)	0	0	0	0	0	0	0	0	0
6	0	0	0		5 (100.0)	0	0	0	0	0	0	0	0	0
7	0	0	2 (20.0)	1 (10.0)	1 (10.0)	0	6 (60.0)	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	6 (100.0)	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	8 (80.0)	0	0	1 (10.0)	1 (10.0)	0
10	0	0	2 (20.0)	0	0	0	0	0	0	8 (80.0)	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	10 (100.0)	0	0	0
12	0	0	0	0	0	0	0	1 (10.0)	1 (10.0)		0	7 (70.0)	1 (10.0)	0
13	0	0	0	0	0	0	0	2 (20.0)	1 (10.0)	0	0	3 (30.0)	2 (20.0)	2 (20.0)
14	0	0	0	0	0	0	0	0	0	0	0	0	0	2 (100.0)

3.2.2 Relationship between morphological and acoustic characters

An analysis of bivariate correlation was performed between morphological and acoustic characters of males from the Springbok population to look at the relationship between body sizes and call characters. Results show some of the call characters are correlated with

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morphological characters, although none of these were highly significant. I found a significant inverse relationship between abdomen width and the peak frequencies of introductory syllables and final syllable. However, lengths of the inter-syllable pause and of the entire call were positively correlated with abdomen width (Table 17). Furthermore, a positive relationship was found between tibia length and the rise time and length of the final syllable of male calls from this population.

Table 17 Correlation table showing the effects of morphological variation on acoustic characters for *B. unicolor* males within a population.

Variables	Length of inter syllable pause	Length of final syllable	Length of entire call	Peak frequency of introductory syllable	Peak frquency of final syllable	rise time of final syllable
AW	r=.600, p=0.023		r=.563, p=0.036	r=-.544, p=0.044	r=-.625, p=0.017	
TL		r=.548, p=0.042				r=.646, p=0.013
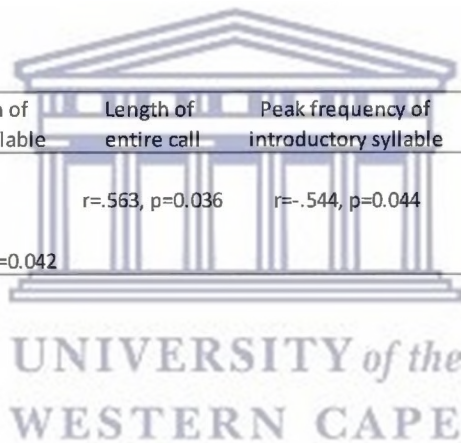
3.2.3 Host plant effects

Acoustic features varied between individuals and I therefore performed independent samples t-tests to determine whether the variation in acoustic characters of individuals was due to the effect of feeding on different host plants. *B. unicolor* at Springbok was collected from two host plants, namely *Roepera morgsana* and *Didelta spinosa*, for which individuals in this population show strong host plant specificity and each individual is confined to their food plants (see also Römer *et al.* 2014). Results show a significant difference for the peak frequency of final syllable (t = 4.360, p < 0.001). However, there was no significant difference for all other examined acoustic characters.

Similarly, some morphological characters of individuals also differed with host plants. I found a significant difference in pronotum length of males according to host plant (t = 3.416, p < 0.001). In females, head width (t = 3.051, p < 0.010), femur length (t = 3.941, p < 0.002), and tibial length (t = 3.385, p < 0.005) also differed significantly with host plant. In all cases, the analysis showed larger mean values for *Didelta spinosa* than for *Roepera morgsana*.

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4. Discussion

4.1 Inter-population variation

All the individuals of given species located in a specific area is called a population. In this study, grasshoppers from the five sampling areas were treated as separate populations. However, it is possible that there is some degree of gene flow between them. There was significant morphological and acoustic variation among populations of *B. unicolor*. However, when considering the specific differences of both sets of characters of each population, acoustic characters are much more strongly differentiated between populations than morphology. Some of the variation was significantly correlated with specific environmental factors for both morphological and acoustic traits, suggesting the role of environmental factors in divergence of morphology and acoustic properties in *Bullacris unicolor*. However, some morphological and acoustic characters were strongly differentiated among populations, but that difference was not strongly or consistently correlated with environmental differences, suggesting that other ecological factors such as difference in food abundance (Sofaer *et al.* 2013), predation (Okuyama 2008; Cothran *et al.* 2013), and interspecific competition (Lankau 2009; Vellend 2006) may also be contributing to diversification in the study species. Furthermore, population variation in *B. unicolor* might be partly due to the effects of stochastic (Fox & Kendall 2002; Vellend 2010) or genetic processes (Neal & Schall 2013) or variation could also be epigenetic in nature.

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4.1.1 Divergence in morphology

The fine scale analysis of morphological variation conducted in this present study revealed that *Bullacris unicolor* populations from different locations in the Western and Northern Cape are differentiated to some extent. Males of different populations were found to differ significantly in body length, pronotum length and head width, whereas females differed significantly in pronotum length, head width and abdomen width. The reason why females were not found to differ in body length may be due to the fact that the abdomen of females can be extended and contracted, becoming distended with eggs, unlike the abdomen of males which is much more rigid and fixed in size. Therefore, pronotum length may be a more reliable indicator of body size in females. This is supported by the observation that the body length and pronotum length measurements of males followed the same pattern of differentiation among populations, whereas female body length and pronotum length measurements showed little correlation with each other, and female pronotum lengths more closely mirrored the pattern of variation observed among male pronotum and body length measurements (Table 3). Although females were found to differ significantly in abdomen width, egg development can also cause some degree of variation in abdomen width, and so this result should be interpreted with caution.

A previous study by Donelson (2007) that investigated morphological differentiation within *B. unicolor* concluded that this species did not show distinct patterns of size differences across its geographic range, and that any body size variation was not consistently linked to environmental variation. These findings are in contrast to the present study, which indicates that geographically separated populations do differ significantly in their morphology and that temperature is strongly linked to this size variation. There are several possible reasons why

this study yielded different results to the study conducted by Donelson (2007), as the two studies differ in a number of ways. Firstly, the previous study was conducted on museum specimens and only considered male morphology. Secondly, the specimens examined were collected over a broader geographical area. It is therefore possible that more fine-scale differences in morphology were overlooked by this pattern of grouping. In contrast, my study examines morphology of both males and females and does so by focusing on selected locations, rather than grouping locations together, and thus defining populations more narrowly. It therefore may not be surprising that the results of these two studies differ. Thirdly, although the previous study did not focus on acoustic variation, my results indicate that populations actually differ more strongly in the acoustic properties of male calls than they do in morphology.
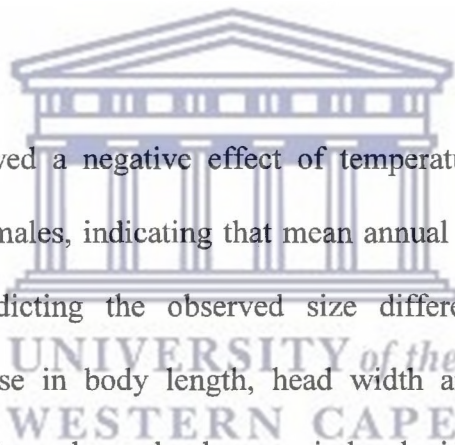
The results of my study showed a negative effect of temperature on the morphological variables of both males and females, indicating that mean annual temperature was the main environmental factor for predicting the observed size difference among *B. unicolor* populations. I found a decrease in body length, head width and pronotum length with increasing annual temperature for males and a decrease in head width, abdomen width, femur length and pronotum length with increasing annual temperature for females, revealing that the largest grasshoppers occurred in areas with the lowest temperatures, and vice versa. Shortly, this result supports Bergmann's rule, which states that there is a relationship between cold climate and larger body size. Interestingly, the effect of slope was positively correlated with two of the female body measurements (head width and abdomen width), although these were not strong correlations. Only one morphological variable (pronotum length) was correlated with precipitation, but the relationship held for both males and females, indicating that rainfall may have a slight positive effect on size. The above correlations between

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environmental factors and the morphology of males and females show that at least some variation in body size can be explained by differences in temperature, and possibly also precipitation and slope, although to a lesser extent.

I observed that the differences in average temperature between the various locations were relatively low, ranging from $15.7^{o}C$ to $18.2^{o}C$. However, even a small difference in temperature can affect development, survival, and reproduction in insects (Visser & Both 2005; Winkler *et al.* 2013). Numerous studies provide evidence for the effect of temperature on the daily cycle of insects and other ectotherms (Kingsolver *et al.* 2009; Paaijmans *et al.* 2010; Estay *et al.* 2011; Folguera *et al.* 2011; Treasure & Chown 2014). Paaijmans *et al* (2013) studied the effects of temperature for a range of terrestrial insects and revealed that temperature fluctuation reduces development under warm conditions and increases development under cool conditions. Also, variation in temperature has been shown to affect the behaviour and mating success of male insects (Dick *et al.* 2013).

This analysis reveals that the population with the largest body size was in Melkbosstrand, which had the second lowest annual temperature of $16.5^{o}C$ and the population with smallest overall body size was in Cederberg, which had the highest temperature of $18.2^{o}C$. Kamieskroon had the lowest temperature, and males from this location were second largest. Annual temperatures of Groenriversmond and Springbok varied between $16^{o}C$ and $17^{o}C$, and populations from these locations also differentiated according to this temperature range. Slight positive effects of altitude and slope with body size may be result from temperatures being influenced by variations in elevations (Enrique *et al.* 2013). However, overlapping of individuals from Springbok, with a higher elevation, and Melkbosstrand with a lower

elevation, revealed that changes in elevation were not predictable factors for the morphological difference in *B. unicolor*.

Under the scenario of habitat dependent divergence, the four ecological distributions of pneumorids are Forest Biome, Savannah Biome, Fynbos Biome and Succulent Karoo. *B. unicolor* is found in two of these biomes – Fynbos Biome and Succulent Karoo, where it feeds on a variety of host plant species. This species was collected from seven different host plants (see Table 2), but is documented to feed on additional host plants as well (MJ van Staaden, unpublished data). Another explanation of population divergence in this study could therefore be that the morphological differentiation is associated with these different host plants. According to Nosil & Rundle (2009) habitat isolation occurs when populations are separated in different environments. In particular, divergent host plant preference causes partial reproductive isolation in many herbivorous insect populations (reviewed in Nosil & Rundle 2009). Ecologists have also identified morphological differentiation associated with host plants. In contrast, my results showed no evidence for the association between host plants and morphology from the five allopatric populations that I examined. In this study, most of the individuals were collected from the host plants *Didelta spinosa* and *Roepera morgsana* (Table 2), and the sample size of individuals feeding on other host plants was very small in some cases. I therefore tested host associated divergence between individuals at the intra-population level only for one population where sample sizes were sufficient for this type of comparison (Springbok). At the intra-population level, many host plant species did not overlap between different populations, making it difficult to determine the extent to which they influenced body size. On the other hand, a relatively large number of individuals were found feeding on *Didelta spinosa* across three populations, and size differences between these populations were apparent, indicating that host plant species has less of an influence on

size than other factors. Nevertheless, I estimated the influence of host plants based on a relatively small size of samples across host plant species, and this may not accurately characterize the association of host plant with morphology among populations.

Furthermore, it is important to identify how changes in the climate with latitude and longitude affect plant phenology, plant zonation patterns, etc. For example, Watson & Pennings (2008) studied latitudinal variation in species composition of tettigoniid grasshoppers and found variation in feeding preferences in this species. Mainly, domination of host plants across latitude and longitude was due to climate differences. Thus, latitudinal variation in plant palatability contributes to species composition and body size. Also, plant resistance to herbivores (Pennings *et al.* 2001) and herbivorous pressure (Pennings & Silliman 2005) may contribute to latitudinal variation in body size. It would therefore be useful to have more data to examine the roles of host plants and their effects on morphology to gain a deeper understanding of divergence in this species.
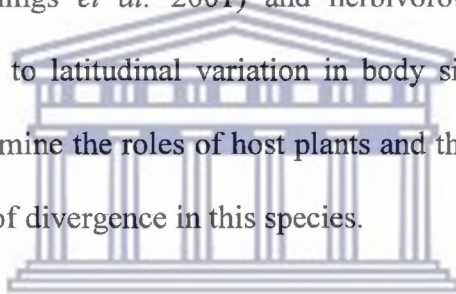
The comparison of morphology across the spatial geographic range of the species was relatively complex. Some morphological characters between populations that are close geographically show significant differences, whereas comparison of populations which are well separated in space show much less difference. For example, body size of male grasshoppers from Melkbosstrand and Kamieskroon showed slight overlap in discriminant function classification (Fig. 5; Table 5), and assigned almost 30% of grasshoppers from Melkbosstrand to Kamieskroon, despite the fact that these sampling locations are separated by a long distance (± 500 km). This may be because size differences did not follow a gradient from north to south, but rather that grasshoppers from areas with a similar temperature were more likely to be similar in size. According to Janzen (1967), temperature usually increases

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with latitude, but this trend was not observed in the current study. Within the geographic distribution of *B. unicolor*, temperature may be influenced by multiple interacting variables, rather than simply being a function of latitude. Consequently individuals were larger in the northern and southernmost parts of the distribution and smaller in the centre.

I found evidence for the role of environmental factors on the divergence of morphology in *B. unicolor*, although the divergence in morphology may not be predicted by differences in environmental factors alone (Schluter 2000; Ohmer *et al.* 2009). Populations were not entirely morphologically distinct, and all populations showed some degree of overlap with each other. This may indicate some degree of gene flow between allopatric populations. However, it is important to note that morphological variation may also proceed in the absence of geographical isolation between populations, such that ecologically mediated selection might still be an important driver of morphological variation (Bolnick *et al.* 2011; Shafer & Wolf 2013).
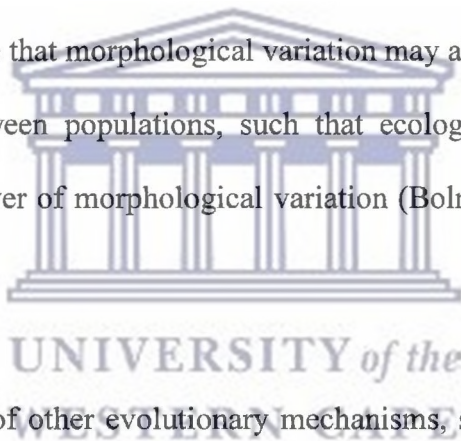
An investigation into the role of other evolutionary mechanisms, such as female preference (Sathyan & Couldridge 2013; Rosenblum 2006; Richards & Knowles 2007), genetic drift (Nosil & Feder 2013; Hollander *et al.* 2013) or both (Slatkin 1985; Conte & Schulter 2013), between populations will do much to clarify the pattern of morphological variation. In addition, studies of the correlation between the neutral differentiation in genes and phenotypic and environmental variation among populations are also needed to indicate ecological effects of trait variation within species (Bolnick *et al.* 2011; Saetre & Saether 2010). Therefore, future experiments that explore divergence in populations adapted to different environments by specific genes that cause reproductive isolation may be especially useful to provide general insights into speciation in *Bullacris unicolor*.

4.1.2 Divergence in acoustic characters

This study clearly shows the call of *B. unicolor* varies geographically, primarily in temporal, but also in frequency components. Correlations between acoustic and environmental factors revealed that environmental differences may influence the evolution of the call in *B. unicolor*. The highest variability in the calling songs of males was seen in the length of final syllable and in the entire call length. Rainfall and slope were the two environmental variables that were most consistently correlated with acoustic properties of the male call. In addition, temperature and altitude were also correlated with aspects of the male call, but consistent patterns regarding these two factors were less obvious. Populations were very distinct in their call properties, showing very clear patterns of clustering (Fig. 7). Indeed, it was only Springbok and Kamieskroon that showed very slight overlap with each other. Overlapping of calls of individuals from Springbok and Kamieskroon may be explained by the fact of migration (eg: Attisano *et al.* 2013; Bell *et al.* 2013) promoting similarity among these two geographically adjacent populations. Further research to investigate the isolating barriers among adjacent and distant population pairs will do much to shed light on the recent migration and the extent of isolation of *B. unicolor* populations.

Two important and interlinked drivers of acoustic divergence are different climatic conditions and different habitats. For example, climate influences acoustic divergence through selection processes (Coyne & Orr 2004). I found that environmental factors may influence the properties of acoustic calls in *B. unicolor*, but environmental factors also result in different habitat and vegetation types. Other forces that drive the divergence of songs include habitat dependent call transmission (Wiley & Richards 1992; Slabbekoorn *et al.* 2002). The five populations under examination here represented two different vegetation biomes – Fynbos Biome and Succulent Karoo. Cederberg and Melkbosstrand fall within the Fynbos Biome and

the other three populations are within the succulent Karoo. Calls of individuals within the same vegetation biome differed slightly between populations (Fig 7), but too few locations from each of these biomes were sampled for a robust comparison between the two biomes. In general, the acoustic signals of the Fynbos populations (Cederberg and Melkbosstrand) were shorter in entire length than the Succulent Karoo populations, but otherwise no discernible pattern of differences between the two biomes was observed. However, it is possible that changes in call characteristics may be influenced by the effects of different habitats. For example, Couldridge & van Staaden (2004) conducted a study investigating the influence of differences in habitat on the call transmission of bladder grasshoppers across different biomes. They identified habitat dependent signal degradation in bladder grasshoppers; suggesting that the Fynbos Biome was characterized by high inconsistency in signal degradation compared to the Succulent Karoo, Savannah Biome, and Forest Biomes. Detailed sound transmission studies will be needed to reveal the adaptive significance of habitat difference on call divergence in this species.
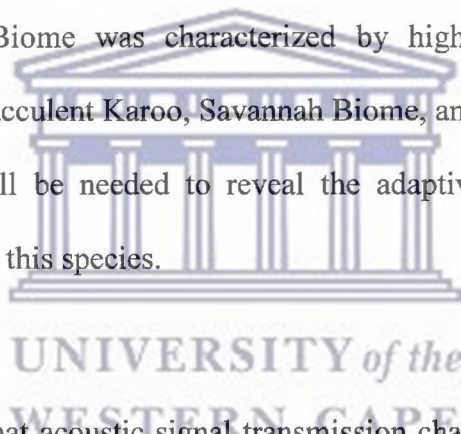
Previous studies have shown that acoustic signal transmission changes due to differences in temperature, rainfall, etc. (Ruegg *et al.* 2006). For example, variation in temperature affects the fine scale temporal properties of the calls of insects (Gerhardtz & Huber 2002; Snell-Rood 2012; Paaijmans *et al.* 2013). van Staaden & Römer (1997) demonstrated that increased signal propagation in pneumorids is a function of adaptations for increased output of sound as well as exploitation of ideal weather conditions in the form of nocturnal temperature inversions. Sound waves are refracted by temperature, as well as humidity and wind velocity, which affects the propagation of signals. While temperature was found to be the biggest predictor of morphological variation among populations of *B. unicolor*, it was not as strongly linked to acoustic variation. I found a positive effect of temperature with length of final

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syllable and inter-syllable pause only. Most of the other call properties were negatively correlated with precipitation or slope, which appeared to be stronger predictors of acoustic variation.

Male acoustic signals and the information they convey are often critical determinants of female mate choice. The influences of female choice on the variation in onset and offset cues are other possible explanations for acoustic divergence (Balakrishnan *et al.* 2001; von Helversen *et al.* 2004). Many studies on anurans (Nevo & Capranica 1985; Castellano *et al.* 1999) have confirmed an inverse relationship between body size and call frequency (Gingras *et al.* 2013). Thus, populations that differ in body size often vary in call spectral properties (Gasser *et al* 2009). Ronacher and Stange (2013) suggested that female grasshoppers could extract information about the size and the health of a potential mate from the songs he produces. In anurans and fish, pulse variables (pulse duration, pulse interval and pulse rate) are known to be important to mate choice (Smith *et al.* 2003; Parmentier *et al.* 2005) and these characters show significant differences among populations. Similarly, in insects, females generally tend to prefer intermediate values of carrier frequency and syllable rate, indicating that these may be used as species identity cues (Couldridge & van Staaden 2006). Ronacher & Stange (2013) tested female preference in the grasshopper *C. biguttulus*. They presented song models with varying syllable and pause duration and found female preference to pause durations with increasing syllable duration.

My work on *B. unicolor* indicates that there are significant differences in call characters, which could have implications for mate choice. However, I did not find any correlation between body size and call properties when comparing different populations. Even within a population, only two of the morphological variables showed any correlation with acoustic

characters. This result was unexpected, but may indicate that morphological and acoustic characters are evolving independently under different selection pressures. Given the nature of the sexual signalling system in *B. unicolor*, it seems likely that the male call is assessed by females and that difference in the advertisement call lead to differential male mating success. However, no mate choice studies have been conducted in this species and it is not known on which acoustic criteria females may base their mating preferences. A previous study of female preference in *Bullacris membracioides* (Couldridge & van Staaden 2006) revealed that females discriminate amongst males on the basis of their advertisement calls, so it is not unreasonable to assume that females of *B. unicolor* would also be choosy. At present, we cannot exclude that signal variation may also be the result of sexual selection. Further study of female preferences for these specific acoustic characters will provide insights as to whether the variation in calls is a result of divergent female preferences.
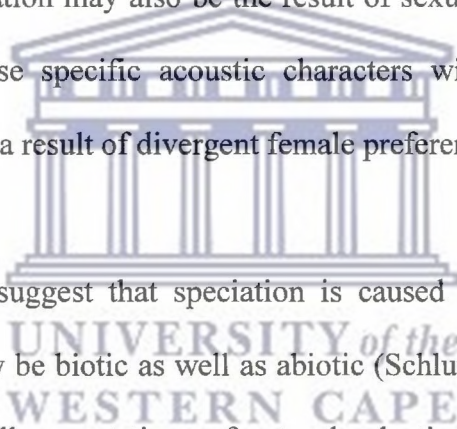
Ecological speciation models suggest that speciation is caused by divergent selection in different environments that may be biotic as well as abiotic (Schluter 2001). Communication systems are concordant with all expectations of natural selection and may also evolve to reduce predation pressure and interspecific competition (Endler 1992). Geographic variation in predation pressure also plays a role in signal divergence in many species (Doan & Arizabal 2002; Trillo *et al.* 2013). For example, locations with lower predation lead to more complex visual signals, and locations with higher predation lead to less conspicuous signals in fish (Endler 1983; Basolo & Wagner 2004). Future studies that focus on the variation in predation pressure across populations of the study species will indeed contribute to the knowledge of the evolution of mating signals (Trillo *et al.* 2013). Competition with heterospecifics may likewise influence signal evolution. *Bullacris unicolor* coexists with either one or two other species of bladder grasshopper at each of the five sampled locations. At both Springbok and

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Kamieskroon, it coexists with *Peringueyacris namaqua*; at Groenriversmond with *Bullacris obliqua* and at both the Cederberg and Melkbosstrand with *Physemacris variolosus* and *B. obliqua*. It is possible that the coexistence of similar species places selection pressure on the calls of *B. unicolor* as well as on the calls of these other species, but this has not yet been studied. However, the fact that different populations of *B. unicolor* may coexist with the same species yet are still distinct from each other indicates that interspecific competition among pneumorids cannot fully explain the pattern of call divergence in *B. unicolor*. Furthermore, it was only at Groenriviersmond that *B. unicolor* and *B. obliqua* showed a distinct temporal separation in their nocturnal signalling patterns, whereas at all other locations all coexisting species were observed to call simultaneously in the same area. This indicates that, for the most part, coexisting species of bladder grasshopper call at the same time and place and do not actively avoid each other.
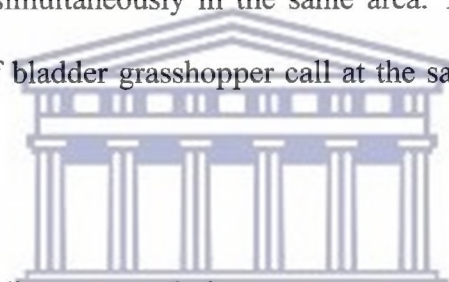
This study determined that adjacent populations were more similar than non-adjacent populations in terms of acoustic characters, with distant populations showing the highest divergence. In other words, there appears to be preferential gene flow between populations of a similar type of habitat. Interestingly, calls of males from Melkbosstrand completely lack pauses between the introductory and final syllables (see Fig. 6B). The calls are thus very short without this inter-syllable pause. Furthermore, males regularly omitted the introductory syllables altogether, producing only the final syllable. When the introductory syllables were produced they were very brief compared to other populations. This unusual adjustment of the call structure, together with the relatively low carrier frequency of the call, makes this population more acoustically distinctive than the other populations. This may be due to Melkbostrand being the most southerly and geographically isolated of the sampled locations, or because of stronger selective pressures at this location. Melkbostrand is fairly built-up

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compared to the other locations, and populations in urban environments are usually more fragmented and also have to contend with higher levels of noise pollution. Previous studies have shown that anthropogenic noise significantly impacts animal sound communication systems in grasshoppers and other animals (McMullen *et al.* 2014). It would be interesting to test to what extent anthropogenic influences are impacting sound communication in bladder grasshopper populations that exist in urban environments.

Genetic drift in isolated populations that results in random changes in call characteristics is another explanation for call divergence. The identification of individual genes conferring reproductive isolation potentially provides unique insight into ecological speciation (Rundle & Nosil 2005). Whether by drift or selection, the study of genetic differences between populations is the next logical step to see whether the divergence in calls among populations has a genetic basis (Presgraves *et al.* 2003; Coyne & Orr 2004; Oh *et al.* 2012). The calls of individuals from Springbok and Kamieskroon overlapped each other. Conversely, Groenriversmond, Cederberg, and Melkbosstrand were highly separated. It would be interesting to see the genetic relationships among these populations.

Altogether, I detected inter-population variation in most of the acoustic characters. Six of the seven variables show a significant difference between the five populations. I also show that differences in environmental factors may help to elucidate the patterns of acoustic variation in *B. unicolor*, while there is no evidence for body size and host plants to play a role in acoustic divergence. Whether females perceive the differences in acoustic cues and the impact of female preference on call divergence remains to be tested. However, my study findings lend further support for the divergence with environmental difference model of

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speciation by providing evidence of the effect of environmental factors on at least some of the acoustic properties.

4.2 Intra population variation

4.2.1 Divergence in acoustic characters

Acoustic characters of fourteen males from one population were studied to investigate intra-population variation. All acoustic characters varied significantly between individuals within this population. However, the canonical centroid plot showed a great deal of overlap of individual male calls (Fig. 8) with only four males showing 100% correct classification of songs. DFA assigned length of entire call to DF1 and peak frequency of final syllable to DF2, indicating these call characters are extremely diverse between individuals despite being from the same area.
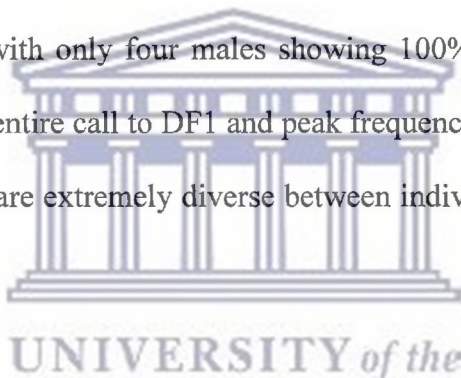
A comparison of acoustic divergence with host plants and morphology suggests local habitat and body size may influence the evolution of call divergence in this species. This hypothesis is supported by my two main findings: 1) the significant correlation between morphology and acoustic characters, and 2) the significant correlation of host plants with male calls and morphology of males and females. Abdominal width and tibia length both had a significant relationship with call characters. A larger width of the male's abdomen decreases with the peak frequency of calls, so larger males produce significantly lower frequency calls. Also, the length of the entire call and the intersyllable pause correlate positively with abdomen width. This result suggests that morphology is a predictor of acoustic differences in males within a population, but the analysis also revealed that many other body variables have no relationship

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with any of the acoustic characters of males. In summary, the analysis of relationships between body size and call characters indicates that the body size plays a partial role in the acoustic variation of individuals within a population.

Seven acoustic characters were found to be associated with morphology, although only abdomen width was consistently correlated with call properties (Table 17). It is possible that morphology directly influences the sound properties of male calls. For example, significant negative correlations between peak frequency of both introductory and final syllables with abdomen width might allow for the identification of larger males. A decrease in peak frequency with increasing abdomen width has not been reported previously in bladder grasshoppers, although many studies have provided evidence that an increase of body size causes a decrease in call dominant frequency in various species (Gerhardt & Huber 2002; Gingras *et al.* 2013). Bladder grasshoppers produce their signals by stridulation of their hind legs against air filled abdominal resonators (Römer *et al.* 2014). Increasing or decreasing the size of the abdomen could thus be expected to affect the frequency of the calls. This relationship between body size and acoustic characters is somewhat supported when comparing individuals within one population (Springbok), but does not hold when comparing males from different populations. This suggests that different selective pressures may be operating within versus between populations. Couldridge and van Staaden (2006) suggested that sexual selection plays an important role in the bladder grasshopper *Bullacris membracioides*. Consequently, the differences observed here could be also due to the effects of sexual selection. Certainly, there is sufficient variation among the calls of individual males on which sexual selection could operate. Clearly, multiple factors may thus contribute to the diversification of calls in *Bullacris unicolor*.

There are various scenarios for the divergence of acoustic signals and morphology within a population. In particular, shifts in host plants may favour acoustic signal as well as body size divergence. Not only do host plants differ in their nutritional value which can affect growth, but the structure of the plants also affects signal transmission properties, such that signalling or developing on different host plants can affect the frequency of calls (McNett & Cocroft 2008). The present study revealed a slight association of calls and host plants, with the mean value of the carrier frequency differing between males feeding on *Didelta spinosa* versus *Roepera morgsana*. When comparing host plants and morphology, I observed a significant difference only in the pronotum length of males between the two host plant species, while females differed in three of the measured morphological characteristics – head width, femur length and tibia length. Therefore, host plants may influence morphological variation within this population to some extent. Further study of the effects of the environment on host plants and how it affects call characters and morphology is necessary to clearly reveal whether divergence has been imposed by host plant use. Multiple measurements of host species may be important to understand this hypothesis. Furthermore, individual variation in morphology may also be strongly dependent on variable or unfavourable environmental conditions (see Tatsuta *et al.* 2001). For example, variation in temperature can influence morphological variation between individuals within a population.

The present study revealed significant differentiation of acoustic characters among individual males at a single location in both temporal and frequency components of the call. My result indicates that the call differences were dependent to some extent on body size and host plant use. However, it seems likely that sexual selection is also operating at the intraspecific level.

5. Conclusion

Here I incorporated an examination of morphological and acoustic characters together with environmental data to examine patterns of variation in *Bullacris unicolor*. I found a potential role of environmental variation in morphological and acoustic divergence between populations, and also that divergence in calls is partially associated with host plants and morphology within a population in this species. The present study of inter-population variation of morphological and acoustic characters of *B. unicolor* reveals significant variation according to specific environmental conditions. The presented data show that the five populations are highly separated in terms of the acoustic characters of males and also in male and female morphology, although to a lesser extent than acoustic separation. Results also indicate that abiotic environmental conditions, such as differences in temperature, precipitation, altitude and slope may be reliable predictors of inter-population divergence.

At the intra-population level, variation of acoustic characters suggests a potential role of body size and host plants in the initiation of call divergence in this species. However, evolution of acoustic signals can be shaped by several selective pressures, such as sexual selection, physiology, phylogeny, predation, parasitism, competition and the environment. Moreover, local environmental characteristics, such as vegetation structure, temperature and background noise effects acoustic signals. So, I predict that local environmental characteristics might play an important role for the intra-population divergence in acoustic characters in *Bullacris unicolor*.

There are many questions that remain regarding signal divergence and its role in speciation. Among the interesting questions are those pertaining to the degree of isolation between

populations. Requirements for tackling these questions are detailed genetic studies that examine genetic variation within and between populations. Moreover, female mate choice experiments that evaluate whether females discriminate amongst males both within and between populations would elucidate whether call differences serve as isolating mechanisms between populations and also whether females exhibit preferences for particular call properties. In addition, a more detailed analysis of the effects of habitat acoustics would indubitably benefit to understand fully the significance of geographic variation in habitat on the calls of this important group of insects.

The results of my study further support prior research which has suggested that environmental variation and local habitat variation are imperative to signal divergence. My data suggest that variation in the local environment, in combination with other ecological cues, likely contributed to the diversification of morphological and acoustic characters within and among populations of *B. unicolor*.

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