Evolutionary implications of variation in the calling song of the cricket *Gryllus bimaculatus* De Geer (Orthoptera: Gryllidae)

By

Marna Ferreira

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University of Pretoria

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Supervisor: Prof. J. Willem H. Ferguson
Department: Centre for Environmental Studies, University of Pretoria, Pretoria, 0002, South Africa

Abstract

The acoustic communication system of the field cricket, *Gryllus bimaculatus*, comprises male signals that elicit phonotaxis by females. Although calling song traits have been implicated in mate recognition as well as sexual selection, the degree to which these two categories of call traits differ as well as the sources of variation that affect them have not been investigated. Variation in signaling traits implicated in mate recognition or sexual selection can bring about speciation. *Gryllus bimaculatus* occurs in Africa, Asia and Europe, and its communication system is easily measured, making it an ideal study organism to investigate the evolutionary implications of variation in signaling traits.

Calling song data was used to investigate how call traits change with male ageing in order to determine if call traits can be used by females to judge male age. In general, older males produced shorter syllables and slower chirp rates. It has been predicted that females can use call traits to judge male size. However, this study found only weak correlations of male size with call traits and therefore suggests that females probably cannot use the calling song as an indicator of male size. Environmental effects could act as noise on the cricket's signaling system and, in turn, influence the reliability with which a male signal reflects his quality. Within-male variation in call traits was mostly caused by large degrees of between-chirp variance. There were significant between-season differences in call

and morphometric measurements, with large degrees of seasonal stability across years. Geographical variation in call traits and morphometrics of eight South African and two European populations as well as six captive-reared F_1 populations was also investigated. I found larger degrees of between-population differences within regions than between continents. An isolation-by-distance effect could not explain inter-continental and regional variation in call and morphological traits. To determine to what degree environmental effects contribute to geographical variation in these traits, variances of wild populations were compared with variances of their captive reared F_1 offspring. This study found that a significant part of the geographical variation is due to an environmental component.

Intraspecific patterns of genetic variation were investigated in seven South African and two European populations, by sequencing part of the mitochondrial cytochrome *b* gene. There was a small degree of within-population genetic variation in Europe and a large degree of within-population genetic variation in South Africa. Thirty one haplotypes were identified from 161 individuals, one of which occurred in all nine populations. While some gene flow did occur among South African and European populations, large amounts of gene flow occurred within South Africa. Nested clade analysis predicted that isolation by distance in combination with restricted gene flow explained gene flow patterns within South Africa. The genetic diversity of *G. bimaculatus* is probably maintained by a large gene pool, and inter-continental and regional gene flow is possibly maintained by means of land, sea or air transport.

Keywords: *Gryllus bimaculatus*, Orthoptera, Gryllidae, South Africa, Europe, Wild-caught populations, Captive-reared F_1 populations, Calling song variation, Morphometrics, Mitochondrial DNA, Cytochrome *b*, Phylogeography

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CHAPTER 1

General introduction

The effects of sexual selection and mate recognition on signaling traits

The process of intersexual selection includes female preference whereby she prefers to mate with some males above others. Various sexual selection models have been proposed to explain female preference. Some indirect selection models include the 'good genes' hypothesis where female preference involves traits that enhance the lifetime fitness of offspring (Endler & Houde, 1995; see review in Møller & Alatalo, 1999), the 'Hamilton-Zuk' hypothesis where female preference involves traits whose expressions are health-dependent and reflect resistance to genetic diseases (Hamilton & Zuk, 1982), e.g. pathogen resistance in house crickets, *Acheta domesticus* (Ryder & Siva-Jothy, 2000), or the 'chase-away' model described by Holland & Rice (1998). In this model antagonistic coevolution occurs between the sexes whereby females had a preexisting sensory bias for a specific trait, but they now prefer not to mate with males that display this trait since they might induce females to mate in a suboptimal manner, leaving males to evolve even more elaborate display traits (Holland & Rice, 1998). On the other hand, females might prefer traits that are correlated with some aspect of male quality from which they can benefit directly, e.g. female decorated crickets, *Gryllodes sigillatus*, would obtain greater nutritional awards when mating with larger males (Sakaluk *et al*., 1992). It has been predicted that traits involved in sexual selection should show directional selection and have large degrees of between-male variation (Etges, 2002; Klappert & Reinhold, 2003). Sexual selection also has between-population diversifying effects on mating signals (Panhuis *et al*., 2001).

Sexual section implies that a female prefers to mate with a particular male, either by having prior preferences or by preferring to mate with a male that is more active and persistent in competing for her attention (O' Donald, 1983). Mate recognition, on the other hand, is regarded by Paterson (1985) as '…a specific response by one partner to a specific signal from the other', ensuring that an animal mates with an appropriate partner. The function of mate recognition is

therefore, to ensure mating between conspecific individuals. Paterson's Specific-Mate Recognition System (SMRS) includes signals and responses that lead to fertilization and Paterson (1985) predicted that mate recognition traits that form part of the fertilization system is subject to stabilizing selection throughout a species range while this species occupies its normal habitat. Butlin (1995) suggested that, if there are between-population differences in mate recognition traits, then it is probably due to environmental effects. Furthermore, one would expect mate recognition signals to have small degrees of between-individual variation (Etges, 2002). Indeed, Ryan *et al*. (1996) and Ferreira & Ferguson (2002) showed that call traits important for mate recognition in the túngara frog, *Physalaemus pustulosus*, and the field cricket, *Gryllus bimaculatus*, respectively had lower degrees of variation compared with other call traits. Henderson & Lambert (1982) found considerable stability in the mate recognition system among worldwide populations of the fruit fly, *Drosophila melanogaster*.

Some authors suggested that sexual selection and mate recognition are independent processes (e.g. Paterson, 1993) while others believe the contrary is true (Pfennig, 1998). Several studies have shown that different traits are involved in mate recognition and sexual selection (European green toad, *Bufo viridis*: Castellano & Giacoma, 1998; Hawaiian picture-winged fly, *D*. *heteroneura*: Boake *et al*., 1997; Túngara frog, *P. pustulosus*: Ryan *et al*., 1996). Ryan & Rand (1993) suggested that mate recognition and mate choice through sexual selection are separate processes, but with recognition having effects on mate choice and *vice versa*. On the other hand, Backwell & Jennions (1993) argued that mate choice involves selection on all aspects of communication systems, including mate recognition, and that mate recognition systems can therefore not be distinct from mate choice systems.

The role of signaling traits in speciation

Paterson (1985) suggested that if a small population becomes isolated in a new and distinct environment, then mate recognition traits of the fertilization system could be subjected to directional selection. However, once the SMRS has improved its effectiveness in the new habitat, the mate recognition traits will again be under stabilizing selection. If this new fertilization system is different from the parental one, then speciation has occurred.

Several sexual selection models aim to explain geographic divergence in sexually selected traits and female preferences (see review in Andersson, 1994). Fisher's runaway process predicts diversity in courtship signals through genetic correlation between female preferences and male traits (Fisher, 1958). Lande (1981) predicted through modeling on polygenic traits that the male-female signaling system may be unstable, depending on the genetic covariance between the male signal and the female preference. This will lead to rapid between-population differentiation in the signaling system, as was shown in female preferences of guppies, *Poecilia reticulata* (Brooks & Endler, 2001). Iwasa & Pomiankowski (1995) and Pomiankowski & Iwasa (1998) predicted through mathematical modelling that Fisher's runaway process is unstable and naturally leads to continual change in sexual traits.

When the same trait or genetically correlated traits are used for both species recognition and sexual selection (i.e. mate quality recognition), conflict could arise between species recognition and sexual selection, resulting in inappropriate mate choices (i.e. preferences for heterospecifics or lower-quality males; Pfennig, 1998). It has been shown in laboratory studies that females could respond to calls of heterospecific males (Ryan & Rand, 1993; Backwell & Jennions, 1993) or that they could prefer heterospecifics above conspecifics in the absence of a mate recognition cue (Hankison & Morris, 2002). Female túngara frogs, *P. pustulosus*, had a preference for heterospecific male song when part of a heterospecific male's song was added to the conspecific call (Ryan & Rand, 1993). Although female Neotropical frogs, *Hyla ebraccata*, preferred conspecific male calls, they did respond to heterospecific male calls when no alternative was available (Backwell & Jennions, 1993). Female pygmy swordtails, *Xiphophorus pygmaeus*, use multiple cues for mate recognition and sexual selection and Hankison & Morris (2002) found that females do prefer heterospecific males when subjected to certain choices in laboratory tests. An understanding of how mate recognition and sexual selection processes affect signaling systems should therefore enable scientists to predict what the evolutionary implications of variation in signaling systems could be, and if a species might be susceptible to speciation.

Rationale for this thesis

Understanding the sources and magnitude of variation in the communication systems of animals, enables one to have a better understanding of the

mechanisms of mate recognition and sexual selection within a species, as well as their potential interaction. I will investigate several sources of variance that potentially contribute to variation in the signaling traits of the field cricket, *G. bimaculatus*. This includes variation within an individual, between individuals, between populations, between continents and between seasons. I will also investigate whether different call traits are involved in sexual selection and mate recognition.

The male signals of *G. bimaculatus* are stereotype and easily quantified, which makes it an ideal species for studying variation in signaling traits. In addition, these crickets occur almost worldwide (Harrison & Bogdanowicz, 1995; Ragge, 1972), giving one the opportunity to study large-scale geographical variation in signaling traits. It is also relatively easy to sample these crickets in the field, since calling males can be tracked easily. It is also easy to breed and rear them in captive conditions, which makes it easy to study variation in the signaling traits of captive-reared F_1 offspring.

The acoustic communication system and call production in G. bimaculatus

The acoustic communication system of *G. bimaculatus* comprises calling songs, courtship songs and aggressive songs (Alexander, 1962). Males produce calling songs to attract females. The calling song consists of chirps and each chirp comprises three to six syllables (Desutter-Grandcolas & Robillard, 2003). Syllables are produced by wing closing movements, when the plectrum of the one wing traverses the file of the other wing. Wing opening movements result in quiet inter-syllable intervals (Bennet-Clark, 1989). Several calling song characteristics have been implicated in mate recognition (Bennet-Clark, 1989) and sexual selection (Simmons, 1988; Simmons & Zuk, 1992).

Mate recognition and sexual selection in the calling song of G. bimaculatus

Two calling song characteristics, namely calling song frequency and syllable period, are important for mate recognition in *G. bimaculatus* (Bennet-Clark, 1989; Schildberger *et al*., 1989) while chirp duration, chirp rate and syllable rate have been identified as sexually selected call traits (Simmons, 1988). Within this framework of mate recognition, I addressed the following question: Do between-individual, between-population and between-seasonal factors have a smaller

effect on variation in mate recognition traits than on other call and morphological traits in *G. bimaculatus*?

It is not clear to what extent the calling song of *G. bimaculatus* is used in sexual selection. It has been suggested that females use the calling song to determine male age and male size. Simmons & Zuk (1992) predicted that females either decide post-copulatory whether they intend to mate with a certain male or they use a cue in the calling song to determine a male's age. Older males had a significantly higher daily mating rate than younger males, even though gregarine infection did not influence the mating success of older *G. bimaculatus* males (Simmons & Zuk, 1992). It is known that *G. bimaculatus* females prefer large males and they accept more sperm from large males compared with small males (Bateman *et al*., 2001; Simmons, 1986a, b). It has been shown in several cricket species that females could use call traits to assess male size (Brown *et al*., 1996; Gray, 1997; Simmons, 1988) and Simmons (1988) suggested that call traits provide information relating to male size. However, there are energetic costs involved in calling song production and Prestwich (1994) showed that the energetic cost of calling increases linearly with calling rate in several cricket species. Within the framework of sexual selection, the following questions were addressed: (a) Do the calling song characteristics change with male ageing? (b) Do larger males spend more hours per day calling than smaller males? (c) Do males with shorter life spans spend more hours per day calling than males with longer life spans? (d) Do larger males have longer life spans than smaller males? (e) Is there a correlation between male size and calling song characteristics? (f) What is the contribution of the within-individual variance to the total population-level variation in call traits? (g) Does rearing temperature affects male and female body sizes at adult eclosion?

Phylogeography of G. bimaculatus

One of the aims in population genetics is to determine the amount of genetic variation that exists in natural populations and to ideally explain this variation in terms of its origin, maintenance and evolutionary importance (Hartl, 1987). The mitochondrial (mt) DNA genome is maternally inherited, haploid and non-recombining (Hartl, 1987) which make it useful for studying intraspecific patterns of genetic differentiation among populations with the use of gene trees (i.e. phylogeography). Phylogeographical analyses could provide information about the

present and historical processes that gave rise to the present geographical distribution of *G. bimaculatus* (Templeton *et al*., 1995; Templeton, 1998). Although the phylogeny of *Gryllus* is well understood, little is known about the intraspecific patterns of gene flow and population structure of *G*. *bimaculatus*. By sequencing part of the cytochrome *b* mtDNA gene, I addressed the following questions: (a) What is the degree of gene flow that occur among South African and European populations (i.e. inter-continental) as well as within South Africa and Europe (i.e. regional)? (b) Which biogeographical processes provide the best explanation for the current genetic structure of seven South African populations of *G. bimaculatus*?

Geographical variation in the calling song and morphometrics of wild-caught and captive-reared F_1 populations of G. bimaculatus

"Patterns of geographic variation within species give some indication of both the potential for future evolution and the past history of selection and constraints" (Ryan *et al*., 1996). It is important to have an estimate of between-population variability in the calling song of *G. bimaculatus*, since the calling song is regarded as one of the most important premating barriers in crickets (Otte, 1989). Isolation-by-distance effects explained geographical variation in the mating call of the green treefrog, *Hyla cinerea* (Asquith *et al*., 1988) and the advertisement call of the túngara frog, *P*. *pustulosus* (Ryan *et al*., 1996). However, it is not clear what caused geographical variation in the advertisement call of the frog, *Leptodactylus fuscus* (Heyer & Reid, 2003) or the calling song of the bushcricket, *Ephippiger ephippiger* (Ritchie, 1992). Genetic and environmental variation, as well as an interaction between them, contributes to the total phenotypic variation of a trait (Falconer & Mackay, 1996). Mousseau & Howard (1998) compared wild-caught with laboratory-reared populations of two species of ground crickets, *Allonemobius fasciatus* and *A. socius*, as well as a hybrid population and they found a significant effect of the environment on call traits. My aims incorporated the following questions: (a) Does an isolation-by-distance effect explain the geographical variation in the call and morphological traits of *G. bimaculatus* males from South Africa and Europe? (b) Does geographical variation in the call and morphological traits resemble gene flow patterns? (c) When comparing geographical variation in call and morphological traits in wild-caught populations

from South Africa and Europe with captive-reared F_1 populations, can part of this variation be due to environmental effects?

Knowledge of the degree of variation that exists in sexually selected traits compared with mate recognition traits, as well as having an estimate of environmental effects on the communication system, could therefore help us to understand whether these classes of signals are distinct in crickets and how sexual selection and mate recognition act on the signaling system of *G. bimaculatus*. This study aspires to make a contribution towards such a better understanding.

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CHAPTER 2

Interactive effects of age and body size on calling song traits of male field crickets, *Gryllus bimaculatus* De Geer (Orthoptera: Gryllidae)

Abstract

Some authors suggested that female crickets prefer to mate with older males, based on their call traits. This study aims to determine whether calling song characteristics of the field cricket *Gryllus bimaculatus* change with age and whether the calling song can be used by females to discriminate between songs of young and old males. I make use of sound recordings of fifteen captive males, recorded throughout their entire lives, as well as records of all calling activity during their entire lives. Older males have slower chirp rates with shorter syllables and longer inter-syllable intervals, reflecting a general slowing down in most temporal call traits. There was a strong interaction between size and age of a male with old, large males calling at slower chirp rates than young, small males. Large males also called for significantly longer periods than small males. Highest calling activity occurred at 22 days after adult eclosion. Males with shorter life spans did not compensate for lost reproductive opportunities by calling faster or for longer periods than males with longer life spans. Although most males showed rigid circadian rhythms in calling throughout their life spans, there was a large degree of between-male variation in circadian rhythm. Females can, therefore, potentially select old males on the basis of call traits. In contrast, two call traits crucial for mate recognition (calling song frequency and syllable period) remained constant throughout the realistic outdoor cricket life span of 50 days, suggesting different modes of selection on this type of signaling trait.

Keywords: *Gryllus bimaculatus*, Calling song, Body size, Male age

Introduction

Male field crickets, *Gryllus bimaculatus*, produce calling songs to attract receptive females (Alexander, 1962). The calling song consists of discrete chirps, each of which comprises a number of syllables. Each syllable is produced by a complete wing-closing movement, where the teeth of the plectrum from one wing traverses the file of the other wing. Inter-syllable intervals are due to wing-opening movements (Bennet-Clark, 1989). Simmons (1988a) suggested that female crickets use male calling song to assess male quality, and showed that females preferred calling songs of large males. Following the 'good genes' model of sexual selection, males should be chosen on the basis of genetic quality, of which longevity is an important measure (Hansen & Price, 1995). Kokko (1998) showed through modelling that a strong correlation between genetic quality and survival should be expected. Long-lived males may have a smaller chance of possessing deleterious mutations (Manning, 1985). Females could use age-dependent male ornaments as an indicator of male fitness with older males having a higher fitness than younger males (Manning, 1985). In the willow warbler, *Phylloscopus trochilus*, and the European starling, *Sturnus vulgaris*, females use song repertoire size as an indicator of male age which, could in turn, convey information about the male's condition, competitive experience, reproductive experience and genetic quality (Gil *et al*., 2001; Mountjoy & Lemon, 1995). On the other hand, the outcome of a simulation model by Beck & Powell (2000) suggested that the 'good-genes' model of sexual selection could not explain female preference for older males in species where males only provide sperm. Accordingly, female preference for young to intermediate mates is more likely, because male genetic quality could decrease with age, based on the assumption that many mutations have deleterious effects. Also, males of lower genetic quality show a decrease in reproductive success as they age (Alatalo *et al*., 1986; Beck & Powell, 2000; Price & Hansen, 1998).

The effects of male age in crickets have been studied by a number of workers. Some authors have suggested mechanisms explaining the higher mating success of older males, but in some cases it is unclear why females would prefer older males as there may be negative consequences, for example, older *G. bimaculatus* males produce fewer progeny (Simmons, 1988b). Although older *G. bimaculatus* males have a significantly lower daily mating rate than younger males (Simmons, 1988b), they have a higher mating success than younger males

(Simmons & Zuk, 1992). In *G. veletis* and *G. pennsylvanicus* males found paired with females in the wild were older and less infected with gregarine parasites (Zuk, 1988) than were solitary calling males (Zuk, 1987). While gregarine infection had no influence on the mating success of older *G. bimaculatus* males, younger infected males did not mate and young uninfected males only had a small chance of mating success (Simmons & Zuk, 1992). In *G. integer*, young males call less actively than older ones (Bertram, 2000) since the calling song attracts parasitoid flies, and the risk of being parasitized outweighs the cost of mating at a young age. However, another explanation for this could be that younger males call less to avoid aggressive interactions with older males (Dixon & Cade, 1986).

There are two possible mechanisms explaining why female *G. bimaculatus* mate with older males: (a) they use a cue in the calling song of the male to determine his age or (b) females only show a post-copulation preference by accepting larger sperm quantities from preferred males (Simmons & Zuk, 1992). The present study investigates the first of these mechanisms by determining age effects on the calling behaviour of male *G. bimaculatus*.

The aims of this study are:

1. To determine whether calling song traits in the field cricket, *G*. *bimaculatus*, change with age; a requirement for females using calling songs to detect male age.
2. To determine how much of the population-level variation in calling song traits can be explained by male age and the interaction between age and male size.
3. To determine the amount of time males invest in producing calling songs, in order to determine whether males with shorter life spans call more intensively each day in order to compensate for reduced mating opportunities.

Materials and methods

Cricket rearing

Fifteen penultimate *G. bimaculatus* males were randomly chosen from a laboratory-reared colony in a climate room (27°C ± 2°C) with 12h:12h Light:Dark (L:D) regime. Each male was placed in a 2-litre container in the climate room with egg carton for shelter, food (Pronutro® and fish flakes) and water *ad libitum*. During this period they were exposed to the calling songs of all the males in the

colony. At nine days post adult eclosion, each male was transferred to a sound-damped recording chamber.

Calling song recordings

Sound-damped recording chambers (31 cm x 35 cm x 26 cm; acoustic isolation 50.13 ± 1.73 dB (mean ± SE) between neighbouring chambers) were maintained at 25°C ± 1°C using an electric heating element. Each chamber was insulated with glass fibre mat (thickness 5 cm) and equipped with a Behringer XM200S dynamic microphone (50 Hz – 10 kHz ± 3 dB; Behringer Spezielle Studiotechnik GmbH, Germany). Each male was placed in a gauze container (11 cm x 11 cm x 12 cm) with a cardboard floor and a small piece of egg carton for shelter. Fresh food (Pronutro®) and water were provided every second day. A 12h:12h L:D light regime was implemented using an 8000 mCd light-emitting diode in the roof of each gauze container. When a male called, the sound was recorded automatically on the hard disk of a computer, utilizing a Maya 4 sound card (Audiotrak, Korea). Each male was monitored for its life span, i.e. life span is measured from 10 days post adult eclosion until death. Three sound recordings of 30 seconds duration each were made each night during which a particular male called, with between-recording intervals of at least 5 minutes. The equipment also kept a continuous record of all minutes during which a male called for longer than 40 seconds and the number of minutes spent calling per hour was monitored continuously throughout each male's life span.

Calling song characteristics and morphometric measurements

Canary V1.2.4 (Cornell Laboratory of Ornithology, Ithaca, New York) was used on an Apple Macintosh computer for spectrographic analysis of the calls. The power spectrum (Figure 2.1a) and oscillogram (Figure 2.1b) of each recording were used as a basis for measuring the call traits (Table 2.1) of three consecutive chirps every third night. After its death, each cricket was measured and weighed. A Video Blaster FS200 video software kit (Creative Laboratories, Singapore; resolution = 16 microns) was used for the morphological measurements (Table 2.1). Thorax area was calculated as thorax width x thorax length (mm^2) and was used as an indicator of body size. Mass was assumed to represent wet mass. After the body measurements were taken, the wings of each male were removed and the harp size of the right wing was measured by calculating the right angled

triangular area formed by points H1, H2 and H3 as noted in Ferreira & Ferguson (2002). Repeatability ± standard error was calculated to estimate the reliability of the measurement procedure, following Becker (1984). The call traits and body measurements (Table 2.1; excluding tibia-R, tibia-L and mass) were measured twice for 30 individuals. Repeat measurements were blind with respect to previous measurements and separated by more than 24 hours.

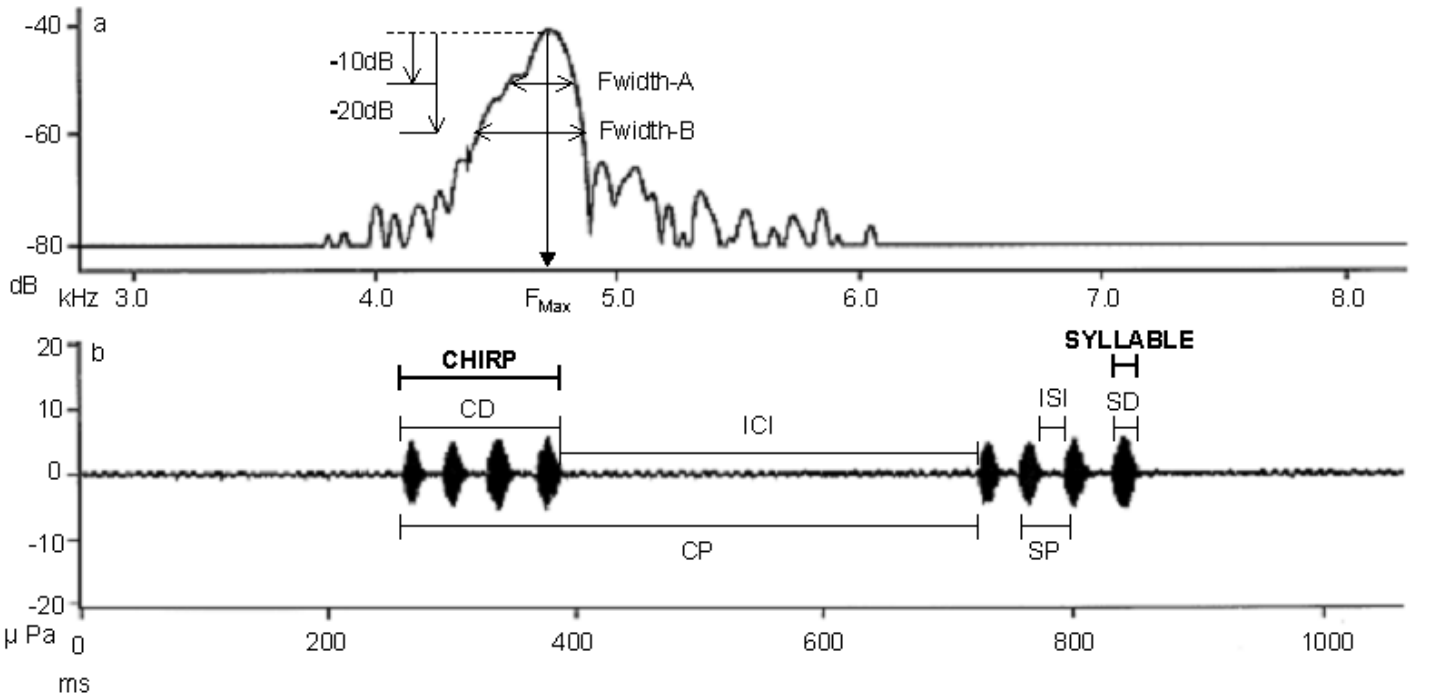


Figure 2.1. Graphical illustration of part of a male field cricket's, *G. bimaculatus*, calling song. A power spectrum (a) and an oscillogram (b) were used to measure the calling song characteristics (see Table 2.1).

Table 2.1. Description of the calling song characteristics, body measurements and wing measurement for the 15 *G. bimaculatus* males. The explained variance components for each of the call traits are shown, resulting from analysis of variance on a 6-day interval data set up to the adult age of 69 days. Error variance and unknown factors contributed to the remaining variation in the data set

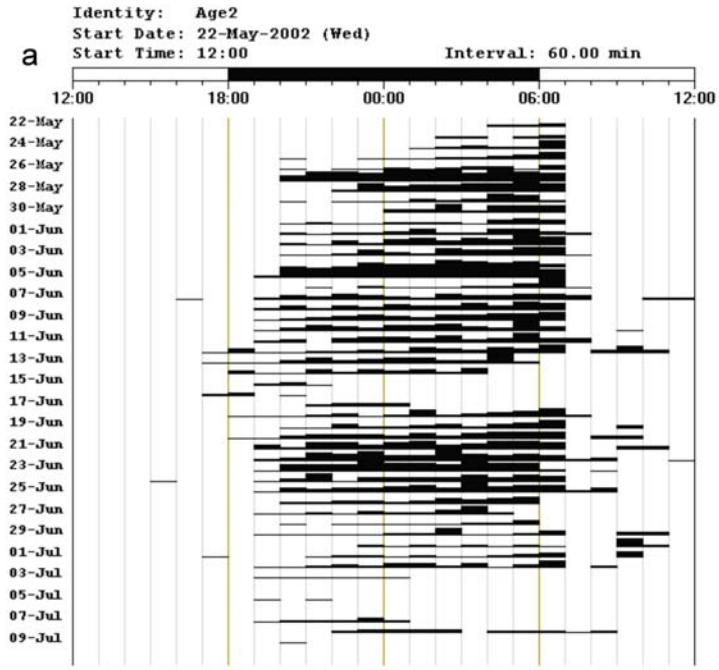
		Variance components	
		Between individuals	Within individual (age effect)
Variable	Definition		
Call traits			
F_{max} (kHz)	Emphasized frequency for each syllable (Fig. 2.1a)	0.16	0.12
Fwidth-A (kHz)	Frequency range at 10 dB below the amplitude of F_{max} (Fig. 2.1a)	0.52	0.20
Fwidth-B (kHz)	Frequency range at 20 dB below the amplitude of F_{max} (Fig. 2.1a)	0.51	0.16
ICI (s)	Interval between chirps (Fig. 2.1b)	0.11	0.20
CD (s)	Duration of one chirp (Fig. 2.1b)	0.37	0.19
CP (s)	Duration from the beginning of a chirp to the beginning of the next chirp (Fig. 2.1b)	0.17	0.26
SD (s)	Duration of one syllable (Fig. 2.1b)	0.57	0.13
ISI (s)	Interval between syllables (Fig. 2.1b)	0.27	0.39
SP (s)	Duration from the beginning of a syllable to the beginning of the next syllable (Fig. 2.1b)	0.52	0.06
S_C	Number of syllables per chirp	0.21	0.23
Body measurements			
Thorax width (mm)	Width of the thorax		
Thorax length (mm)	Length of the thorax		
Head width (mm)	Width of the head		
Head length (mm)	Length of the head		
Femur-R (mm)	Length of the right hind femur		
Femur-L (mm)	Length of the left hind femur		
Tibia-R (mm)	Length of the right hind tibia		
Tibia-L (mm)	Length of the left hind tibia		
Mass (g)	Wet mass of the cricket immediately after death		
Wing measurement			
Harp (mm^2)	Total area of the harp		

Statistical analyses

Circadian patterns of calling

Actiview^{TM} V1.2 (Mini Mitter Co., Oregon) was used to draw an actogram of each male's calling activity. All 15 actograms were inspected to determine a circadian calling pattern during the life span of each male. Males were classified accordingly into five circadian categories based on the following circadian patterns: (1) males calling mostly during the early morning hours, from midnight – 6am (Figure 2.2a), (2) males mostly calling during the early evening from 6pm – midnight (Figure 2.2b), (3) males whose calls were spread evenly throughout the night from 6pm – 6am (Figure 2.2c), (4) males that showed no pattern whatsoever throughout their life span and (5) males that changed their circadian patterns and shifted from early evening calling to early morning calling. Statistica V5.5 (StatSoft, Inc. (1999), Tulsa, USA) was used to perform a univariate analysis of variance (ANOVA) to compare the means of the call traits, thorax area and lifespan between the circadian calling pattern categories. This was done to determine firstly whether males with different circadian calling patterns also differ in their calling song traits, secondly whether small males have different circadian calling patterns compared with large males and lastly whether males with different circadian calling patterns have different life spans.

a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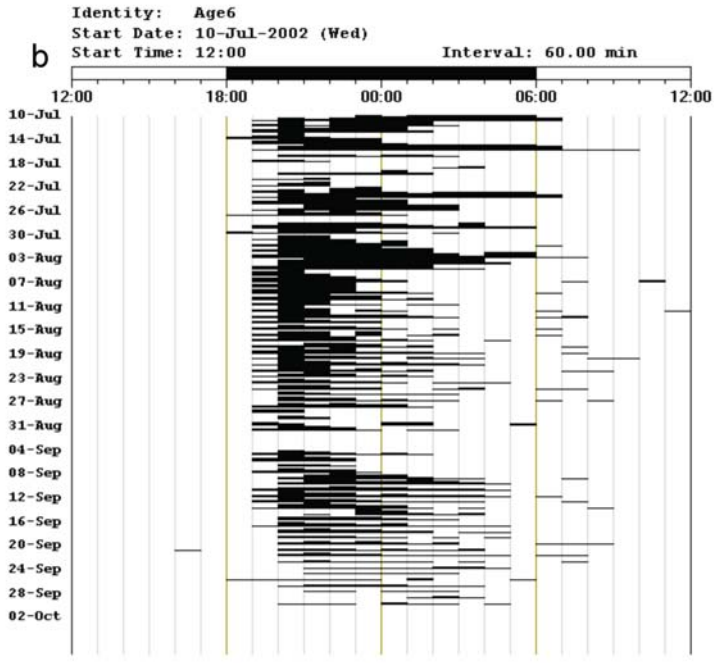
b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07-Aug
11-Aug
15-Aug
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02-Oct

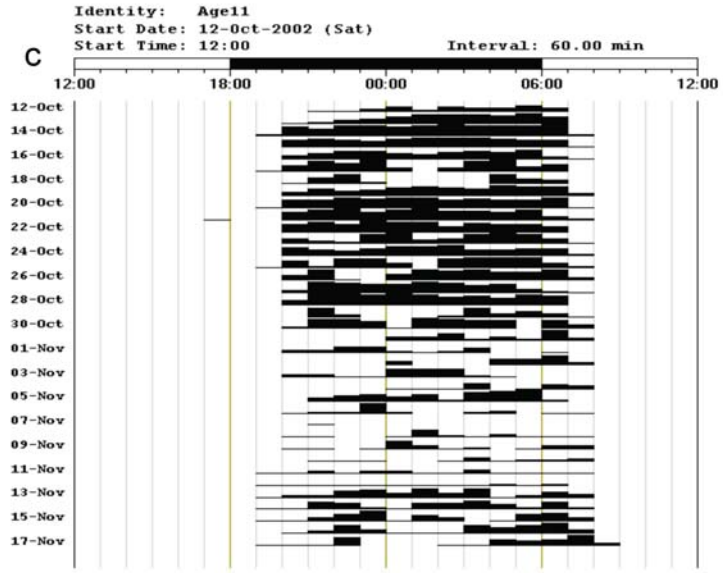

Figure 2.2. Three actograms showing between-male and within-male variation in the circadian calling activity of *G. bimaculatus*: (a) early-morning calling (category 1), (b) early-evening calling (category 2) and (c) calling throughout the night (category 3).

Age effects on calling activity

Because of a large degree of between-male variation in calling activity per day, raw data were transformed to relative calling activity (RCA). RCA reflected the time that each male spent calling per day as a fraction of the highest calling activity logged for that individual, i.e. (number of seconds called per day) / (largest number of seconds called per day during its lifetime). Mean RCA was calculated at 2-day intervals for each of the 15 males.

Age and body size effects on calling song characteristics

The call traits of each male were analysed at 3-day intervals, using Statistica. Male calls were studied over two spans of ageing after adult eclosion. A forward stepwise multiple regression analysis was performed on call traits for the periods 10 – 49 days and 10 – 140 days post adult eclosion to determine whether temporal changes in the call traits (Table 2.1) are correlated with male age and/or male body size. Following the results of the multiple regression analysis on the 10 – 140 days data set, a non-linear multiple regression was performed on the call traits that were significantly influenced by both male age and body size, using NLREG V5.4 (Sherrod, 2002). In addition, Pearson product moment correlations (Statistica) were performed for each male, to determine any significant correlation between male age (for the period 10 – 49 days) and call traits. A Bonferroni adjustment was applied to detect significant correlations (Rice, 1989).

The call traits of each male were grouped into 6-day age classes spanning the period nine to 69 days (because some males did not call ten days after adult eclosion but did call nine days after adult eclosion, the call recordings for the ninth day was used for these analyses). SAS V8.02 (Proc Varcomp; SAS Institute Inc., Cary, NC, USA) was used to determine the within-individual and between-individual variance components for the call traits of the males using restricted maximum-likelihood estimation (REML).

Relationship between life span, body size and calling activity

Pearson product moment correlation analysis was performed, using Statistica, to identify any significant correlation of the morphometric measurements (Table 2.1) with life span. This gave an indication whether small males had a longer or shorter life span than large males. Statistica was also used to calculate Pearson product moment correlation between the mean number of seconds called per day and life

span. This gave an indication whether males with a short life span called for longer or shorter durations than males with a long life span. To determine the effect of male size and life span on calling activity, a forward stepwise multiple regression analysis was performed, using Statistica, with mean number of seconds called per day as the dependent variable and body size and life span as independent variables.

Effect of male size and male age on calling duration and calling intensity

To relate *calling intensity* (the mean number of minutes called during a particular hour) and *calling duration* (the mean number of hours called per day, which included only those hours with a minimum calling duration of 100 seconds) with male size and male age, forward stepwise multiple regression analyses were performed (Statistica) using body size and male age as the independent variables and calling duration and calling intensity as the dependent variable, respectively.

Relationship between calling duration, calling intensity and chirp rate

Statistica was used to perform forward stepwise multiple regression analysis to relate the effects of calling duration and calling intensity on the *chirp rate* (number of chirps per second) of a particular male.

Results

Repeatability of measurements of call traits and body measurements

There were significant differences between the 30 individuals for all the call traits ($21846.6 > F_{29, 57} > 64.54$, $P < 0.001$) and body measurements ($115.75 > F_{29, 57} > 7.31$, $P < 0.001$). The call traits and body measurements were highly repeatable (call traits: $1.0 \pm 0.0 > r > 0.985 \pm 0.006$; body measurements: $0.984 \pm 0.006 > r > 0.759 \pm 0.079$).

Circadian patterns of calling

There was a large degree of between-male variation in calling activity per day (Figure 2.2). Males started calling within two hours after the Light:Dark transition at 6pm and terminated calling within one hour prior until three hours after the Dark:Light transition at 6am. The majority of the males called either early in the evening (category 2, $n = 4$; Figure 2.2b) or throughout the whole night (category 3, $n = 4$; Figure 2.2c). The remainder of the males called either early in the morning

(category 1, n = 2; Figure 2.2a) or showed irregular patterns of calling behaviour (category 4, n = 3; category 5, n = 2).

Due to the small sample sizes of categories four and five, they were combined for the ANOVA. Univariate ANOVA revealed no significant differences between the four circadian groups for the call traits, thorax area and life span. The circadian patterns of calling behaviour of small males did not differ significantly from large males (thorax area: $F_{3, 14} = 0.07$, $P > 0.05$, n = 15). In addition, older males did not have different circadian calling patterns compared with younger males (lifespan: $F_{3, 14} = 0.71$, $P > 0.05$, n = 15). There were also no significant differences between the circadian groups for the temporal ($3.09 > F_{3, 14} > 0.32$, $P > 0.07$, n = 15) or spectral call traits ($0.43 > F_{3, 14} > 0.1$, $P > 0.76$, n = 15).

Age effects on calling activity

There was a large degree of within-male variation for the time spent calling per day (Figure 2.2). Males showed two peaks for calling activity throughout their life spans (Figure 2.3). The first peak was at 22 days (RCA = 0.551 ± 0.063 (mean ± SE); n = 15) and the second peak was at 72 days (RCA = 0.463 ± 0.111 (mean ± SE); n = 4). When the mean number of seconds called per day was plotted against age (i.e. using untransformed data, not RCA), the same trend in calling activity was observed.

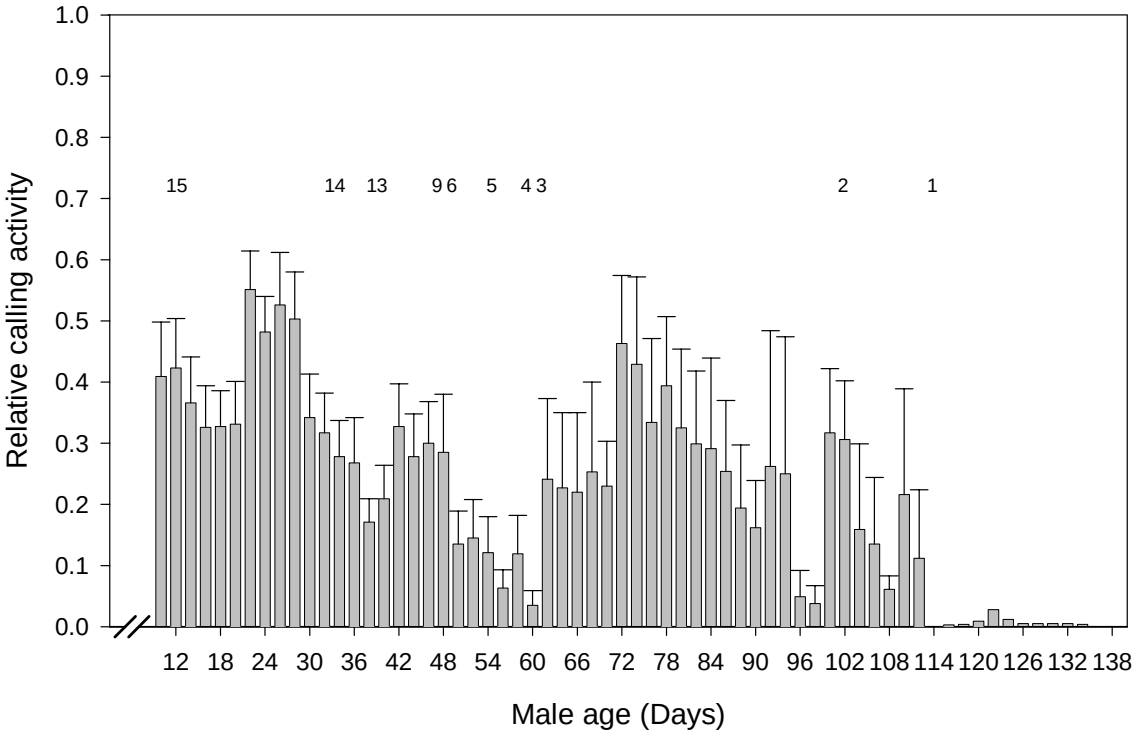

Figure 2.3. The relative calling activity (RCA) reflecting the time duration that *G. bimaculatus* males spent calling per day. Horizontal bars represent mean values calculated for all the males at 2-day intervals, while Y-error bars indicate standard error. Values above the Y-error bars represent the sample size of males that reached that particular age.

Age and body size effects on calling song characteristics

(i) *Ten – 49 days post adult eclosion*

Forward stepwise multiple regression analyses found only a significant correlation of body size (thorax area) with two chirp traits (CD and CP) and bandwidth. After controlling for body size, all the temporal call traits except syllable period (Figure 2.4) and the spectral call traits correlated significantly with male age. The sharp increase in the values of the standard errors of the call traits after 50 days (Figure 2.5) suggested that the temporal changes taking place during the first 50 days after adult eclosion are more informative than temporal changes at an older age.

Chirp call traits: While older males produced significantly longer inter-chirp intervals there was no significant correlation of thorax area with inter-chirp interval ($F_{2, 136} = 5.70$, $P < 0.01$, $n = 139$; male age partial correlation: r = 0.23, $P < 0.01$; thorax area partial correlation: r = 0.14, $P > 0.05$). Both older and larger males produced longer chirp durations compared with younger and smaller males ($F_{2, 136} = 10.41$, $P < 0.001$, $n = 139$; male age partial correlation: r = 0.28, $P < 0.001$; thorax area partial correlation: r = 0.23, $P < 0.01$). This increase in chirp duration resulted in the significantly longer chirp periods produced by these males ($F_{2, 136} = 13.10$, $P < 0.001$, $n = 139$; male age partial correlation: r = 0.33, $P < 0.001$; thorax area partial correlation: r = 0.22, $P < 0.01$). Older males produced significantly more syllables per chirp than younger males, while there was no significant correlation of number of syllables per chirp with thorax area ($F_{2, 136} = 9.89$, $P < 0.001$, $n = 139$; male age partial correlation: r = 0.32, $P < 0.001$; thorax area partial correlation: r = 0.16, $P > 0.05$).

Syllable call traits: There was no significant correlation of syllable period with either male age or thorax area ($F_{1, 137} = 3.71$, $P > 0.05$, $n = 139$; male age partial correlation: r = 0.16, $P > 0.05$; thorax area partial correlation: r = 0.22, $P > 0.05$). While syllable duration decreased significantly with male aging, the correlation of syllable duration with thorax area was not significant ($F_{1, 137} = 12.86$, $P < 0.001$, $n = 139$; male age partial correlation: r = -0.29, $P < 0.001$; thorax area partial correlation: r = -0.05, $P > 0.05$). Inter-syllable interval increased significantly with male ageing, while thorax area was not significantly correlated with this temporal call trait ($F_{1, 137} = 12.86$, $P < 0.001$, $n = 139$; male age partial correlation: r = 0.43, $P < 0.001$; thorax area partial correlation: r = 0.05, $P > 0.05$)

Spectral call traits: Calling song frequency did not correlate significantly with either male age or thorax area ($F_{2, 136} = 0.01$, $P > 0.05$, $n = 139$; male age partial

correlation: r = -0.01, $P > 0.05$; thorax area partial correlation: r = 0.004, $P > 0.05$). Larger males produced significantly wider bandwidths than smaller males, while male age did not correlate significantly with bandwidth (Fwidth-A: $F_{1, 137} = 8.40$, $P < 0.01$, $n = 139$; male age partial correlation: r = -0.02, $P > 0.05$; male size partial correlation: r = 0.24, $P < 0.01$; Fwidth-B: $F_{1, 137} = 16.64$, $P < 0.001$, $n = 139$; male age partial correlation: r = -0.06, $P > 0.05$; male size partial correlation: r = 0.33, $P < 0.001$).

Pearson product moment correlations performed separately for each male revealed similar results to the multiple regression analyses performed on the combined data set. These results are reported in three sections below:

a) *Chirp call traits:* Twelve of the males showed positive trends towards either producing more syllables per chirp, longer chirp durations and longer chirp periods with age while 13 produced longer inter-chirp intervals with age. However, results for only seven of the 15 individuals were significant at $P < 0.05$. Three males produced significantly more syllables per chirp ($r > 0.59$, $P < 0.05$) as well as significantly longer chirps ($r > 0.59$, $P < 0.05$). On the contrary, two males produced significantly shorter chirps ($r = -0.65$, $P < 0.05$) as they aged. One male produced significantly longer inter-chirp intervals ($r = 0.75$, $P < 0.01$). Chirp period increased significantly with male age in four males ($r > 0.61$, $P < 0.05$). After a Bonferroni adjustment the negative correlations were not significant while one male produced significantly more syllables per chirp ($r = 0.87$, $P < 0.01$), two males produced longer chirp durations ($r > 0.66$, $P < 0.01$), one male produced longer inter-chirp intervals ($r = 0.75$, $P < 0.01$) and two males produced longer chirp periods ($r > 0.73$, $P < 0.01$) with age.

b) *Syllable call traits:* Similar trends were observed among individuals for each of the syllable traits with male ageing. Fourteen males produced shorter syllable durations as well as longer inter-syllable intervals with male age while ten males produced longer syllable periods as they aged. Nine individuals had significant correlations between male age and syllable traits. Seven males had significantly shorter syllable durations ($r < -0.56$, $P < 0.05$) of which five also had significantly longer inter-syllable intervals ($r > 0.70$, $P < 0.05$) as they aged. Two more males had significant correlations between male age and syllable period ($r > 0.68$, $P < 0.05$), of which one had longer inter-syllable intervals as he aged ($r = 0.63$, $P < 0.05$). After a Bonferroni adjustment, there was a significant correlation between male age and syllable period for two males ($r > 0.68$, $P < 0.05$). Two more males

produced significantly shorter syllable durations ($r < -0.75$, $P < 0.01$). One of these as well as a third male produced significantly longer inter-syllable intervals ($r > 0.81$, $P < 0.01$, $n = 2$) as they aged.

c) *Spectral call traits:* Individual correlations between the spectral call traits and male age revealed no clear trends. Some males produced higher calling song frequencies and broader bandwidths as they aged, while others produced lower calling song frequencies and narrower bandwidths with age. These trends were still observed after a Bonferroni adjustment.

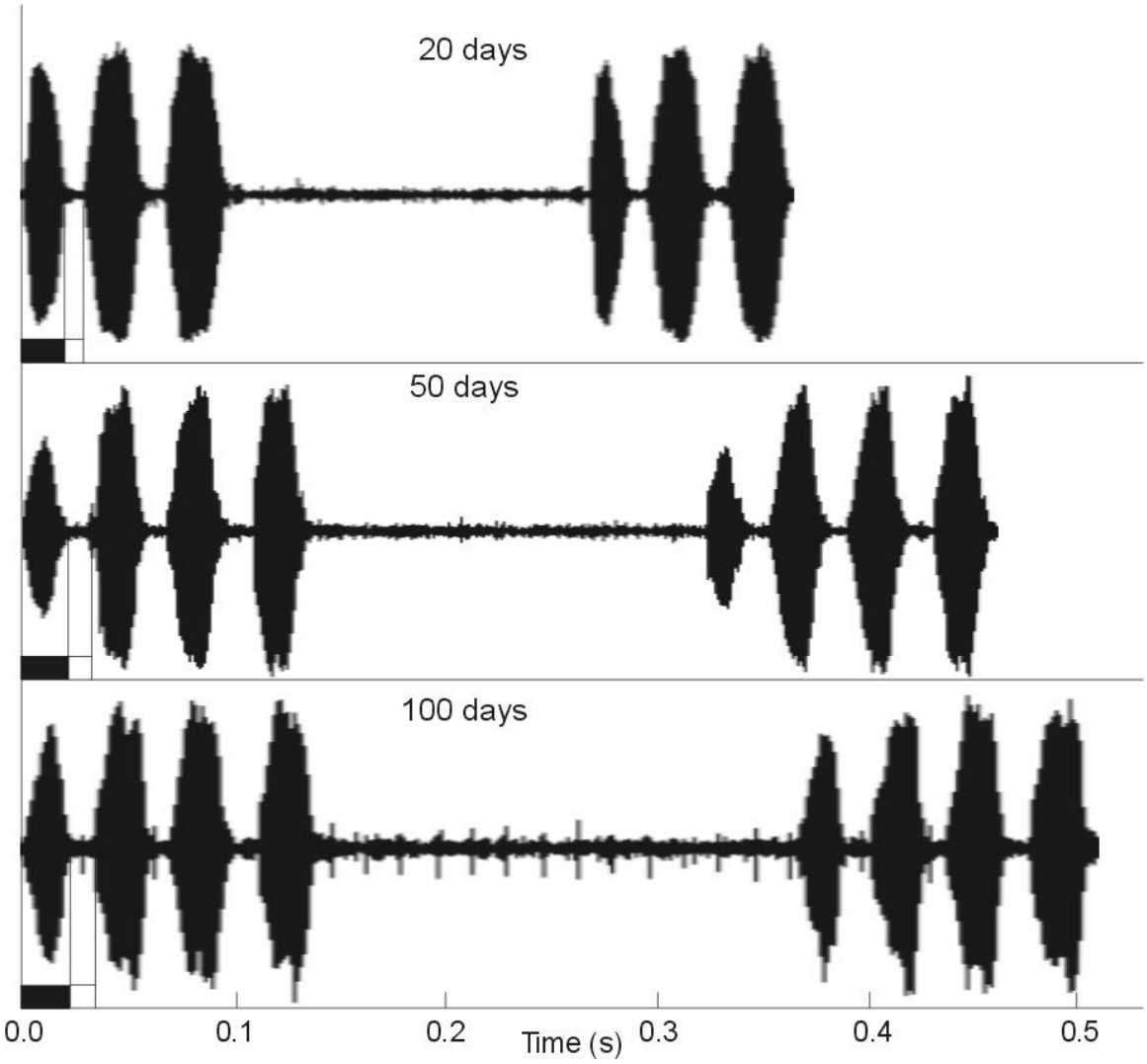

Figure 2.4. Graphical representation of the temporal changes in the calling song of adult male A1 at different ages (days after adult eclosion). Syllable duration (indicated by black bars) and inter-syllable interval (indicated by white bars) are shown for the first syllable at each time period.

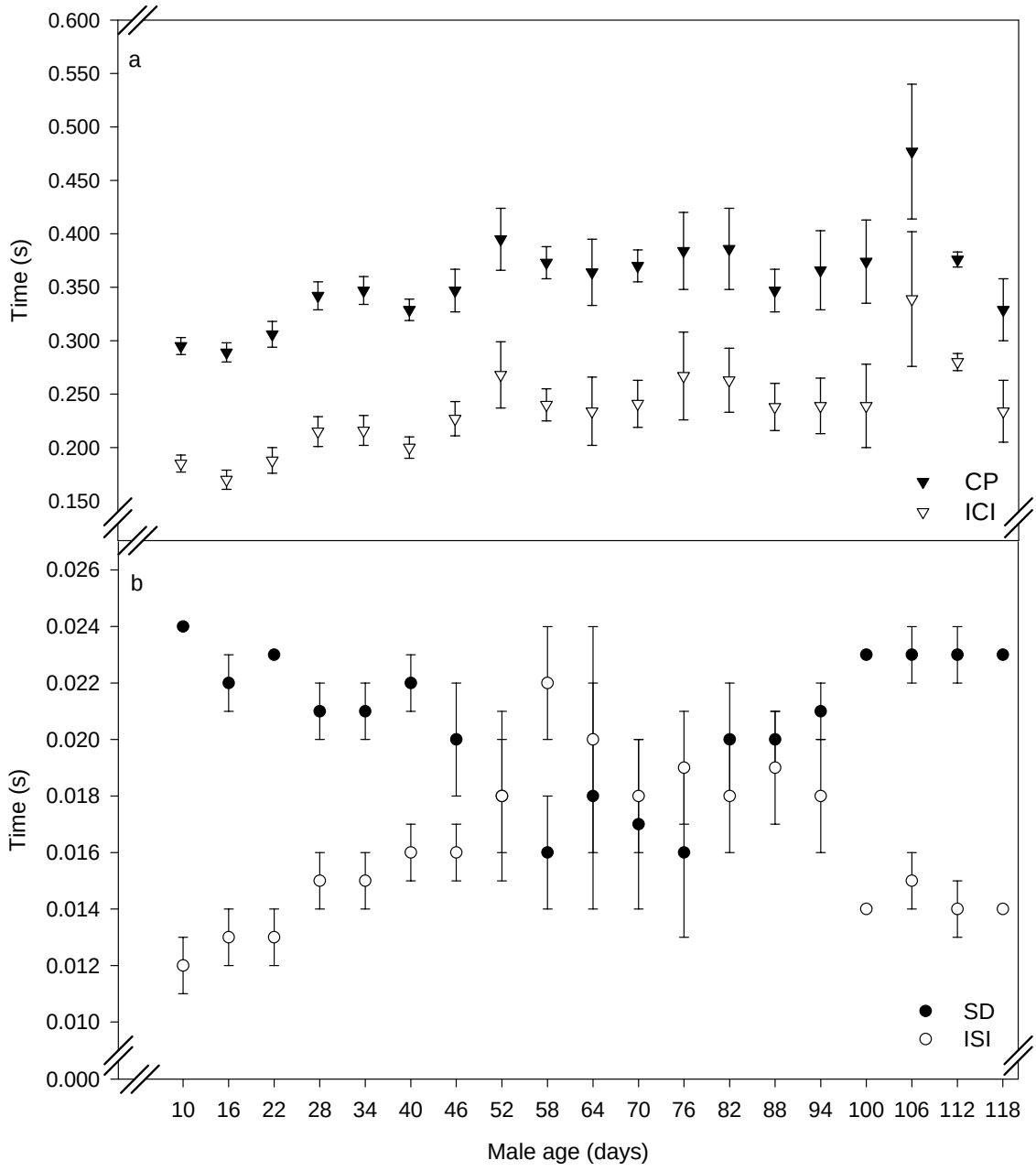

Figure 2.5. The relationship between male age and (a) two chirp and (b) two syllable traits of the calling song of male *G. bimaculatus*. Bullets indicate mean values, while the Y-error bars represent standard error.

(ii) *Ten – 140 days post adult eclosion*

The results of the statistical analyses over this range in life span should be interpreted with caution, since only three males had a life span of more than 62 days. Body size (thorax area) was significantly correlated with all the temporal and spectral call traits, except with syllable duration and calling song frequency. After controlling for body size, age had a significant correlation with two spectral call traits and all of the temporal call traits except number of syllables per chirp and chirp duration.

Chirp call traits: Although male age did not correlate significantly with chirp duration, there was a significantly correlation of thorax area with chirp duration ($F_{2, 201} = 8.14$, $P < 0.001$, $n = 204$; male age partial correlation: r = 0.07, $P > 0.05$; thorax area partial correlation: r = 0.26, $P < 0.001$). The same trend was also observed for syllables per chirp: there was no significant correlation of male age with syllables per chirp, while larger males produced significantly more syllables per chirp compared with smaller males ($F_{1, 202} = 8.80$, $P < 0.01$, $n = 204$; male age partial correlation: r = 0.06, $P > 0.05$; thorax area partial correlation: r = 0.20, $P < 0.01$). Older and larger males produced significantly longer inter-chirp intervals compared with younger and smaller males respectively ($F_{2, 201} = 28.10$, $P < 0.001$, $n = 204$; male age partial correlation: r = 0.42, $P < 0.001$; thorax area partial correlation: r = 0.22, $P < 0.01$). This gave rise to the significantly longer chirp periods produced by these males ($F_{2, 201} = 37.33$, $P < 0.001$, $n = 204$; male age partial correlation: r = 0.44, $P < 0.001$; thorax area partial correlation: r = 0.31, $P < 0.001$).

Syllable call traits: Older males produced significantly shorter syllable durations while thorax area did not correlate significantly with syllable duration ($F_{2, 201} = 9.88$, $P < 0.001$, $n = 204$; male age partial correlation: r = -0.27, $P < 0.001$; thorax area partial correlation: r = -0.13, $P > 0.05$). Older and larger males produced significantly longer inter-syllable intervals than younger and smaller males respectively ($F_{2, 201} = 25.66$, $P < 0.001$, $n = 204$; male age partial correlation: r = 0.41, $P < 0.001$; thorax area partial correlation: r = 0.20, $P < 0.01$). This lead to the significantly longer syllable periods produced by them ($F_{2, 201} = 5.96$, $P < 0.01$, $n = 204$; male age partial correlation: r = 0.18, $P < 0.01$; thorax area partial correlation: r = 0.14, $P < 0.05$).

Spectral call traits: Older males produced significantly lower calling song frequencies than younger males, while thorax area did not correlate significantly

with this spectral trait ($F_{2, 201}$ = 6.91, P < 0.01, n = 204; male age partial correlation: r = -0.23, P < 0.001; thorax area partial correlation: r = 0.13, P > 0.05). While Fwidth-A only correlated significantly with thorax area and not male age, Fwidth-B on the other hand, correlated significantly with male age and thorax area respectively (Fwidth-A: $F_{2, 201}$ = 19.88, P < 0.001, n = 204; male age partial correlation: r = -0.12, P > 0.05; thorax area partial correlation: r = 0.40, P < 0.001; Fwidth-B: $F_{2, 201}$ = 23.65, P < 0.001, n = 204; male age partial correlation: r = -0.15, P < 0.05; thorax area partial correlation: r = 0.43, P < 0.001).

(iii) *Age and body size effects on calling song characteristics: Ten – 140 days post adult eclosion*

Nonlinear regression analysis predicted that an older, large male should call at a slower chirp and syllable rate than a younger, small male (CP: adjusted nonlinear R^2 = 29.42%, Figure 2.6; SP: adjusted nonlinear R^2 = 4.88%) and the rate at which chirp period and syllable period changes with male ageing is faster during the first 49 days of an adult male's life span, than after 49 days (Figure 2.6). These effects were mainly caused by the longer inter-chirp intervals and inter-syllable intervals that were produced by larger and older males (ICI: adjusted nonlinear R^2 = 21.75%; ISI: adjusted nonlinear R^2 = 25.55%). Bandwidth also changed at a faster rate in younger males, compared with older males, with older males producing narrower bandwidths than younger males. On the other hand, larger males produced wider bandwidths (Fwidth-A: adjusted nonlinear R^2 = 14.30%; Fwidth-B: adjusted nonlinear R^2 = 17.50%).

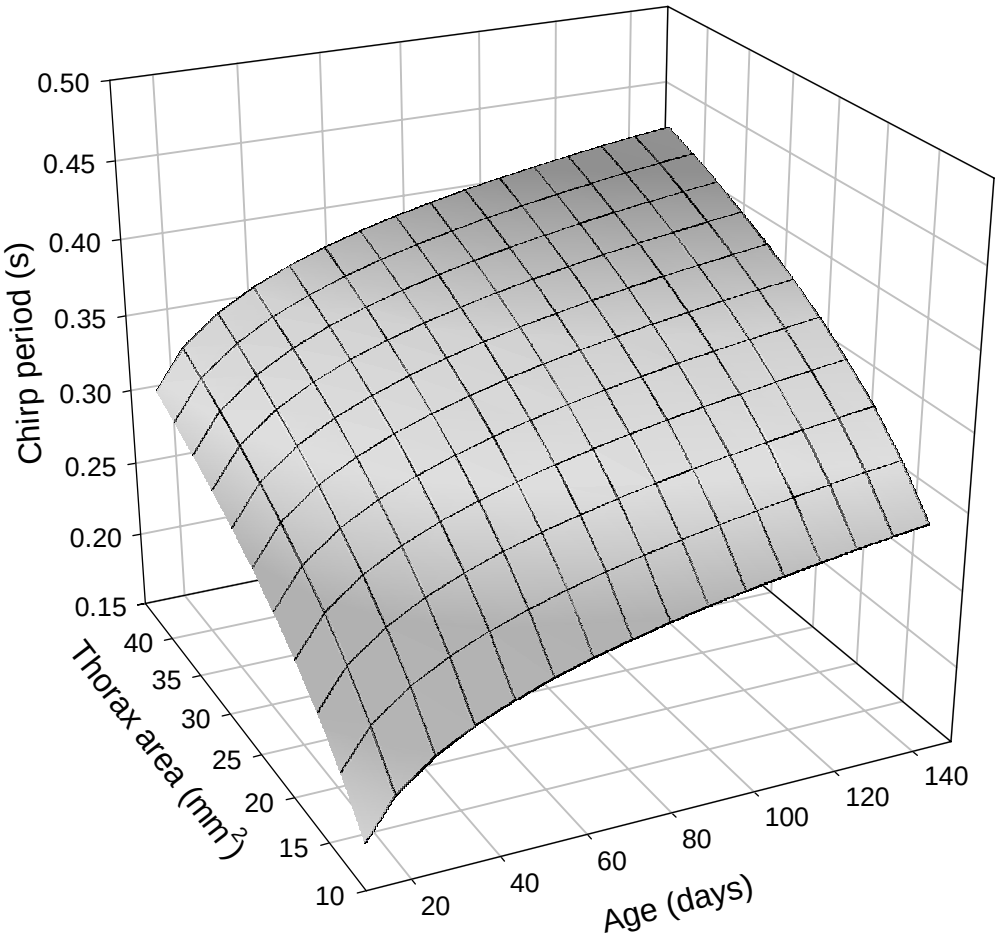

Figure 2.6. Predicted values for chirp period (adjusted nonlinear R^2 = 29.42%) of *G. bimaculatus* males as a function of body size (thorax area) and male age, based on nonlinear regression analysis (see results).

Between-individual variation in the calling song characteristics

Following REML analysis for nine – 69 days of adult age, the between-individual variance component contributed largely to the variation in bandwidth, chirp duration, syllable period and syllable duration (Table 2.1), while the within-individual variance component contributed largely to the variation in inter-syllable interval (Table 2.1). The variation in chirp period, inter-chirp interval, number of syllables per chirp and calling song frequency were mainly caused by the error variance component and unknown factors (Table 2.1).

Relationship between life span, body size and calling activity

There was no significant correlation between life span and any of the ten morphometric measurements (Table 2.1), i.e. life span was not size dependent (-0.142 < r < 0.323, $P > 0.05$, $n = 15$). There was also no detectable correlation between the mean number of seconds called per day and life span (r = -0.08, $P > 0.05$, $n = 15$; Figure 2.7a). After taking the effect of male size into account, forward stepwise multiple regression revealed that the partial correlation between the mean number of seconds called per day and life span was not significant (r = -0.24, > 0.05, $n = 15$). However, there was a significant partial correlation between the mean number of seconds called per day and thorax area (r = 0.52, $P < 0.05$, $n = 15$; Figure 2.7b), suggesting that while larger males spent more time calling per day than smaller males, life span had little influence on the daily calling activity of males.

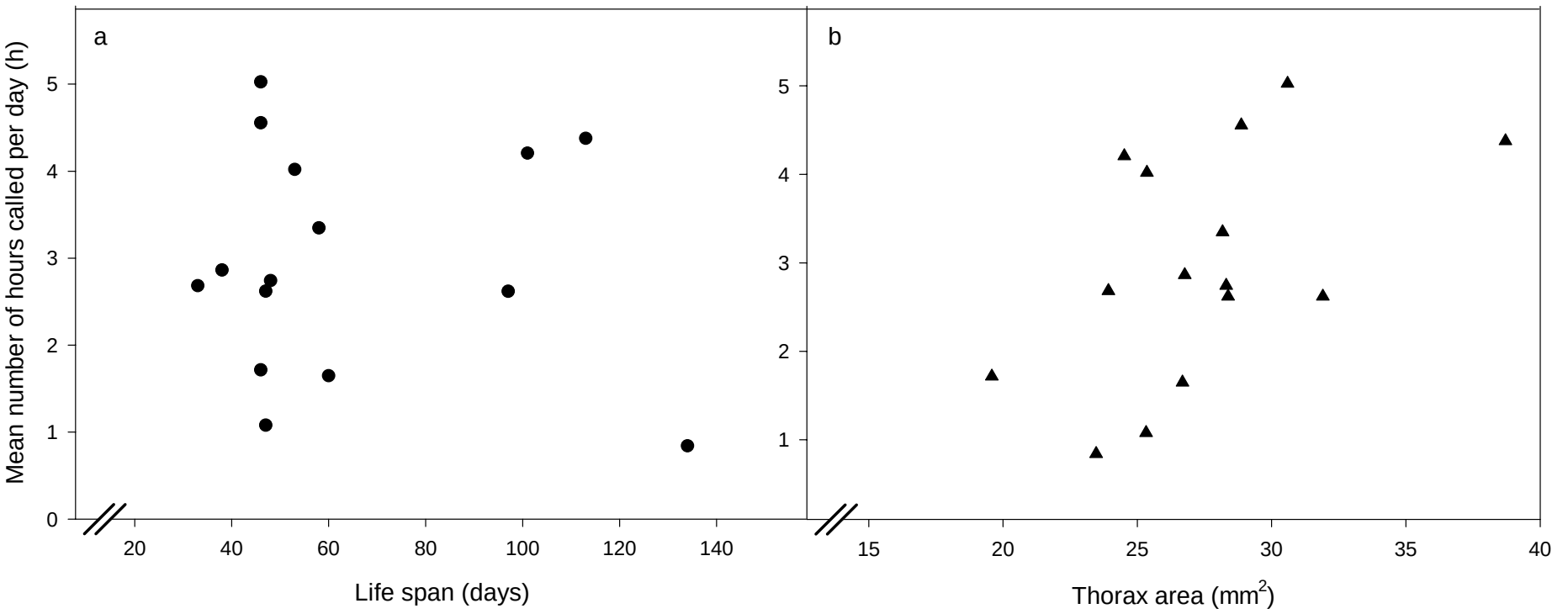

Figure 2.7. Mean number of hours (seconds were converted to hours for easier interpretation) that each *G. bimaculatus* male spent calling per day as a function of (a) life span and (b) male body size.

Effect of male size and male age on calling duration and calling intensity

Larger males spent significantly more hours per day calling (calling duration: partial correlation: r = 0.18, $P < 0.001$, $n = 655$) and more minutes per hour calling (calling intensity: partial correlation: r = 0.29, $P < 0.001$, $n = 654$), than smaller males. However, regardless of the effect of male size, calling intensity and calling duration decreased significantly with male age (calling intensity: partial correlation: r = -0.26, $P < 0.001$, $n = 654$; calling duration: partial correlation: r = -0.25, $P < 0.001$, $n = 655$).

Relationship between calling duration, calling intensity and chirp rate

Males that called at a faster chirp rate spent fewer minutes per hour calling than males with slower chirp rates (calling intensity: partial correlation: r = -0.19, $P < 0.01$, $n = 198$). However, this effect was not evident at a time scale longer than an hour (calling duration: partial correlation: r = -0.04, $P > 0.05$, $n = 197$).

Discussion

Effect of male age on the calling song characteristics

Two trends were observed in the temporal call traits within a realistic life span of 50 days for wild *G. bimaculatus* males (see below). Similar results were obtained irrespective of whether analyses were performed on a combined data set or separately on individual males. Firstly, at the chirp level, older males produced longer chirps and increased the intervals between chirps, resulting in longer chirp periods (Figure 2.5a). Secondly, at the syllable level, older males produced shorter syllables and longer intervals between syllables (Figure 2.5b). This suggests that a female could use syllable duration and chirp period to determine a male's age. The shorter syllables could be due to physical wear of the file, which resulted in a shorter contact period between the plectrum and the file. In the bushcricket, *Ephippiger ephippiger*, older males produced shorter syllables and had fewer pegs on the file, which form part of the stridulatory organs (Ritchie *et al*., 1995). Up to 49 days of age there was no significant effect of male age on the two calling song characteristics important for mate recognition in this species, namely syllable period and calling song frequency (Huber & Thorson, 1985; Schildberger *et al*., 1989). Calling song frequency either increased or decreased with male age (up to 49 days of age), suggesting that there are no directional changes that take place in calling song frequency with male ageing. Syllable period changed very little up to 49 days of age

because, as syllable duration decreased, inter-syllable interval increased with male age (Figure 2.5b). Although two males had significantly longer syllable periods up to 49 days as they aged, a clear trend was only observed in one of them, while the other one had a variable syllable period. This supports the findings of Ferreira (2006, Chapter 3) that there is a large degree of within-individual stability in calling song frequency and syllable period (Table 2.1). The large within-individual variance component for inter-syllable interval largely reflects male ageing (Table 2.1). The inter-syllable interval is produced by the wing opening movement (Bennet-Clark, 1989) and ageing could cause older males to take longer in the stridulatory movements of their wings, compared with younger males. However, a physiological study is required to determine whether this is indeed the case. In contrast to my results, existing data describing the effect of age on cricket calls of several species do not show strong trends. Souroukis *et al*. (1992) found no significant correlation between syllable rate, percentage of missed syllables per trill and male age for *G. integer*. Supporting this, Martin *et al*. (2000) found no significant effect of male age on any of the call traits measured, for G. integer, except for a slight effect on duty cycle that could be explained by the experimental design or the fact that the result is not strongly significant. In *G. rubens*, syllable rate did not change significantly with age when measured at two days after adult eclosion and again after 25 days (Walker, 2000). The F_1 and F_2 male offspring of wild caught *G. integer* females showed no significant correlation between number of syllables per trill and male age (Gray & Cade, 1999). Ciceran *et al*. (1994) found no significant correlation of male age with syllable rate, number of syllables per chirp, inter-chirp duration or chirp duration in *G. pennsylvanicus*. No significant correlation of amplitude, number of syllables per chirp, frequency and inter-chirp interval with male age was found in the house cricket, *Acheta domesticus* (Gray, 1997). Brown *et al*. (1996) found no significant correlation of male age with frequency or syllable period in the black-horned tree cricket, *Oecanthus nigricornis*, but syllable duration decreased significantly with male ageing.

Relative calling activity throughout a male's life span

Two periods of intensive calling throughout the males' life spans were observed, firstly at around 22 days and secondly at around 72 days (Figure 2.3). The first peak at 22 days coincided with the highest median calling effort reported at 20 days after adult eclosion for *Teleogryllus commodus* reared on a high protein diet (Hunt *et al*.,

2004). The second peak at 72 days is probably of no biological significance for three reasons. Firstly, the error in this estimate may be substantial due to the small sample size (*n* = 3) of animals at 72 days of age and older. Secondly, a single male contributed to most of the calling activity after 70 days of age and thirdly, it is highly unlikely that males become this old under natural conditions. Wild *G. bimaculatus* males caught by Simmons & Zuk (1992) had a mean (± SE) adult age of 12.6 ± 0.40 days (*n* = 97). Captive males in the present study had a mean (± SE) life span of 64.00 ± 7.98 days (*n* = 15), compared with Simmons's (1988b) study on lifetime mating success where captive *G. bimaculatus* males had a mean (± SE) life span of 37.46 ± 2.48 days (*n* = 10).

Relationships between male size, life span and calling activity

This study showed no significant correlation of male size with life span for *G. bimaculatus*, supporting Simmons's (1988b) results. No significant correlation was found between body mass and male life span for *G. pennsylvanicus* (Ciceran *et al*., 1994). In *T. commodus* male adult life span was affected by a combination of the protein quality of the rearing diet and reproductive effort, in the form of calling. Males reared on a high protein diet were heavier at adult eclosion but they invested more energy in calling during early adulthood and therefore died sooner (Hunt *et al*., 2004). This suggests that larger males do not necessarily live longer than smaller males. In addition, this study found no evidence that males with longer life spans have different circadian patterns of calling behaviour than males with shorter life spans. During my study, male size had a significant effect on calling activity, calling duration and calling intensity but not on the circadian pattern of calling behaviour. Larger males spent longer time periods per day calling than smaller males (Figure 2.7b), but this was not confined to early age as reported by Hunt *et al.* (2004). Large males could therefore increase their chances of mating through the longer durations of calling activity as shown by Hunt *et al.* (2004), where female *T. commodus* showed strong positive selection on calling effort in playback experiments in the wild. This study found no evidence that small males have different circadian patterns of calling behaviour compared with large males. This suggests that small and large males compete for the same females at a given time, since a small male does not avoid this competition by calling at a different time than a large male.

Interaction between body size and male age with respect to calling song characteristics

Several studies have shown that *G. bimaculatus* females prefer large males (Bateman *et al*., 2001; Simmons, 1986a, b) and Simmons (1988a) suggested that temporal traits could provide information relating to male size. Simmons & Zuk (1992) found that females preferred older males and suggested that females use male calling song as an indicator of male age. However, preference for older males could be an artifact of selection for large males or *vice versa*. Non-linear regression predicted that older, larger males produced slower chirp periods than younger, smaller males (Figure 2.6). It would therefore be necessary to control for male age or male size when female preference studies involving either one of these traits are conducted.

Effect of life span and male age on calling activity

Hunt *et al*. (2004) found that the life span of males on a high protein diet (fish pellets, containing 45% protein) decreased with an increase in mean nightly calling effort. Conversely, my study investigated the effect of life span on calling activity and found no correlation between life span and the mean number of seconds that a male spent calling per day, suggesting that males of all life spans probably invest the same amount of time in calling, and that life span has little influence on the amount of time that a male spent calling (Figure 2.7a). Males in my study were fed Pronutro® containing 16% protein, reflecting a high protein diet. On the other hand, male age was a significant determinant of the time that a male spent calling per day: young males spent more hours per day and more minutes per hour calling than older males, potentially increasing their chances of mating success.

The role of energetic constraints on calling intensity and circadian pattern of calling

This study showed that chirp rate did influence calling intensity (number of minutes called per hour). Males with faster chirp rates spent fewer minutes per hour calling than males with slower chirp rates. The energetic cost of calling at a faster chirp rate could limit the amount of time that males spent calling per day, since the energetic cost of calling increases linearly with calling rate (syllables per second) in several

cricket species (Prestwich, 1994). However, at a longer time scale, chirp rate did not influence calling duration (number of hours called per day) probably because the energetic constraints on calling are more important at the scale of short time intervals. Energetic and feeding constraints may impose an upper limit to the number of hours a cricket can call each night. Indeed, most of the males did not call for the whole duration of the night, but for substantially shorter periods. The mean start time ranged from 7pm to 8pm, while the mean stop time ranged from 5am to 8am (Figure 2.2). Although there were large between-male differences in the circadian patterns, most males showed consistency throughout their life spans regarding their individual circadian patterns. In addition, this study found no significant differences between the categories of circadian patterns of calling behaviour for any of the call traits, suggesting that time of day, in a controlled environment, does not affect calling song characteristics.

Conclusion

In conclusion, female field crickets could potentially distinguish older males from younger ones based on the shorter syllable durations and slower chirp rates produced by older males. These findings provide a mechanism for the hypothesis of Simmons & Zuk (1992) that female *G. bimaculatus* choose older males by using cues in the male calling song. In contrast, two of the calling song characteristics important for mate recognition in *G. bimaculatus* (Huber & Thorson, 1985; Schildberger *et al*., 1989) changed very little during the realistic free-living life span (50 days) of a male cricket. These are calling song frequency (nominally 4.8 kHz) and syllable period (nominally 38 milliseconds). The shortening of the syllable duration during the first 50 days is compensated for by a corresponding lengthening of inter-syllable interval, resulting in a constant syllable period. These differences in degree of constancy between call traits of male crickets probably reflect different selective forces that operate on separate parts of the cricket communication system. In-depth studies are required for a comprehensive understanding of age effects within the context of sexual selection in crickets.

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CHAPTER 3

High levels of environmentally-induced variation in the calling song of the field cricket, *Gryllus bimaculatus* De Geer (Orthoptera: Gryllidae)

Abstract

The calling song of the field cricket is involved in both sexual selection and species recognition. Several environmental parameters could influence the calling song traits and, in turn, the reliability with which a male signal reflects its quality. This study investigates variation in the calling songs of two wild cricket populations in South Africa, recorded under standardised conditions. Depending on the particular call trait measured, up to 62% of the total within-population variation was explained by within-individual variation in call traits, suggesting considerable flexibility in the communication system of these crickets. There were differences in the partitioning of variation in the two populations. However, the call traits suggested to be involved in species recognition had a smaller degree of within-individual variance. In addition, male size and call traits differed between seasons: males were smaller, produced higher calling song frequencies and called at a faster syllable rate during summer, compared with winter. An experiment was performed to test whether these seasonal differences are attributable to temperature-related body size differences. Males raised at 27°C were significantly heavier than those raised at 22°C. We conclude that other environmental factors such as food availability and brood size result in seasonal differences in body size. In addition, this study found only weak correlations between male body size and call traits and suggests that females probably cannot use male calling song as an indicator of male body size. Call traits at a particular site were largely consistent between years.

Keywords: *Gryllus bimaculatus*, Calling song, Within-population variation, Within-individual variation, Seasonal variation

Introduction

Male field crickets, *Gryllus bimaculatus*, produce calling songs that attract females (Alexander, 1962). Several calling song characteristics are suggested to reflect male phenotypic attributes and to be sexually selected (number of syllables per chirp: *Acheta domesticus*: Gray, 1997; syllable rate, chirp rate, chirp duration: *G. bimaculatus*: Simmons, 1988; calling song frequency: *G. campestris*: Simmons & Ritchie, 1996; chirp duration: *Teleogryllus oceanicus*: Simmons *et al*., 2001). As with most quantitative traits, phenotypic variation in the calling song of crickets comprises both genetic and environmental components (Falconer & Mackay, 1996). Since additive genetic variance determines the predictable genetic properties of individual males (Lynch & Walsh, 1998) it forms the basis on which selection, e.g. sexual selection, can act. After strong sexual selection one would expect environmental variation to constitute the bulk of the remaining phenotypic variation. However, in certain studies this was not the case and the maintenance of additive variation in sexually selected characteristics has been explained as the result of changing selective pressures on these communication systems (Iwasa & Pomiankowski, 1995; Pomiankowski & Møller, 1995). On the other hand, substantial remaining environmental variation could be thought of as a noise factor that obscures efficient communication during courtship and that slows down the rate at which selection removes additive genetic variation. This could decrease the selection pressure on sexually selected characteristics as envisaged by the above authors. An aspect of cricket communication that has received virtually no attention is the degree of stability in the calls of an individual male. This would determine the reliability with which a female could judge the call and, consequently, the quality of a male before mating (e.g. Simmons, 1988; Simmons & Ritchie, 1996). An understanding of the degree of environmentally-induced variation is therefore important for understanding sexual selection in crickets since it complements estimates of heritability on call traits (Gray & Cade, 1999). Direct measurements of environmental variation could be derived from multiple measurements on the same individuals (the ‘special environmental component’; Falconer & Mackay, 1996). Furthermore, variation in communication characteristics over short time periods (e.g. between seasons of the same year) within the same population is also likely to comprise mostly an environmental component since the genetic composition of a population is expected to remain nearly constant over such short periods. Environmentally-induced variation in the

cricket communication system could also be brought about by a correlation between signaling characteristics and environmentally affected morphological characteristics such as body size. Variation in cricket song could result from three causes: (a) variation in motivation for calling, (b) immediate environmental effects on the physical and physiological processes controlling calling behaviour and (c) developmental effects brought about by the environment.

Body size in many invertebrates is significantly affected by rearing environment (Sibly & Atkinson, 1994). Environmentally induced developmental variation can also cause variation in adult behaviour and the interaction between developmental environment and adult environment can cause variation in call traits of crickets (Grace & Shaw, 2004). For instance, calling temperature, rearing temperature and the interaction between them had a significant effect on call traits in the striped ground cricket, *Allonemobius fasciatus* (Olvido & Mousseau, 1995). Conversely, Grace & Shaw (2004) found that the effect of the rearing environment on the calling song in the Hawaiian cricket, *Laupala cerasina*, was not permanent. The quality of the rearing environment, i.e. food availability and food quality, could influence a male's communicatory system. *Gryllus campestris* males reared on a poor diet produced a high carrier frequency due to disproportionate reduction in the harp size (Scheuber *et al*., 2003). However, this effect is not universal, as none of the courtship song characteristics measured for *G. texensis* and *G. lineaticeps* were affected by food treatment in nymphs (Gray & Eckhardt, 2001) and in adults (Wagner & Reiser, 2000).

Two studies on female preferences in *G. bimaculatus* showed that females not only preferred large males, but that they also received more sperm from them (Bateman *et al*., 2001; Simmons, 1986). Several studies have demonstrated a relationship between male body size and calling song characteristics (Brown *et al*., 1996; Gray, 1997; Simmons, 1988). Small-scale local differences in the environment could affect male body size and could, in turn, influence the call traits. *Gryllus campestris* females preferred low calling song frequencies (Simmons & Ritchie, 1996) that, in turn, resulted in the selection of larger males (Scheuber *et al*., 2003). Large male black-horned tree crickets, *Oecanthus nigricornis*, produced calls with a lower frequency that could potentially be used by females as a pre-copulatory mechanism to choose larger males (Brown *et al*., 1996). Gray (1997) found that large male house crickets, *A. domesticus*, produced more syllables per chirp and that females preferred the calling song of these

males. Since *G. bimaculatus* females respond to male calls through phonotaxis, it could be to a female's advantage to judge the size of a calling male from its calling song. Chirp rate and calling rate could potentially be used in certain cricket species as reliable indicators of the current condition of males. *Gryllus campestris* males that received more food had a significant increase in body mass and called for significant longer periods at a higher chirp rate than males that did not (Holzer *et al*., 2003; Scheuber *et al*., 2003). Although *G. lineaticeps* males did not show a significant increase in body mass when fed high nutrient food, they did call for significant longer periods than males that received low nutrient food (Wagner & Hoback, 1999). Conversely, Gray & Eckhardt (2001) found that, although *G. texensis* males fed on a high quality diet gained significantly more weight than males on a low quality diet, none of their courtship song characteristics were affected by residual body mass. The investment of energy appears to be species-specific where some taxa invest energy into body reserves as well as calling rate with others investing it into either.

The aims of this study are:

1. To quantify the within-individual variation in male calling song in order to assess the degree to which (within a framework of sexual selection) a male's signal, at a particular moment, is a reliable indicator of longer term signaling characteristics.
2. To determine whether the signaling characteristics within a population remain stable with time over seasons of the same year and over different years in order to test the hypothesis that the communication system has a large degree of temporal consistency at the same geographical site.
3. To determine whether sexually selected signaling characteristics differ in the above-mentioned respects from signaling characteristics not subject to sexual selection.
4. To determine whether, within a population, male size is correlated with call traits in order to test the hypothesis that the calling song can potentially be used by females to judge the size of a male.
5. To determine whether the rearing temperature affects body size in male and female crickets at adult eclosion. This tests the hypothesis that seasonal differences in body size are attributable to temperature differences.

Materials and methods

Within-individual and between-individual variation in calling songs of wild-caught males

Sampling localities

Wild male field crickets, *G. bimaculatus*, were captured at Pretoria (25°41'S, 28°13'E) and Makhado (23°05'S, 29°59'E) in South Africa, during 2001 – 2003. During 2000 – 2002, the annual rainfall was higher at Makhado than Pretoria (Makhado: mean = 1285 mm, range 414 – 2407 mm; Pretoria: mean = 765 mm, range = 426 – 1268 mm). In addition to being moister, Makhado, a montane site, had less extreme temperature fluctuations than Pretoria (Figure 3.1). Each population was analysed separately.

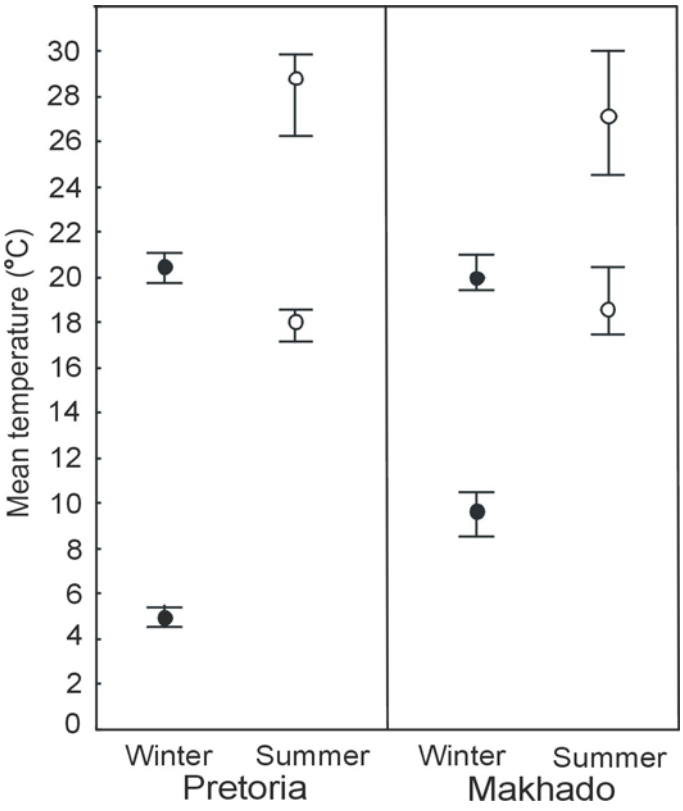

Figure 3.1. Mean minimum and mean maximum temperatures (with ranges) for winter and summer for Pretoria and Makhado during 1999 – 2003.

Calling song recordings

Males were placed in sound damped recording chambers where the calling song of each male was recorded. Sound-damped recording chambers (31 cm x 35 cm x 26 cm; acoustic isolation 50.13 ± 1.73 dB (mean ± SE) between neighbouring chambers) were maintained at 25°C ± 1°C using an electronically controlled heater. Each chamber was sound damped with a glass fibre mat (thickness 5 cm) and equipped with a Beringer XM200S dynamic microphone (50 Hz – 10 kHz ± 3 dB; Behringer Spezielle Studiotechnik GmbH, Germany). A Sony TC-D5M cassette recorder was used to record the calling songs. Each male was placed in a gauze container (11 cm x 11 cm x 12 cm) with a cardboard floor and a small piece of egg carton for shelter. High protein cereal (Pronutro®) and water were provided *ad libitum*. A 12h:12h Light:Dark (L:D) light regime was implemented using an 8000 mCd light-emitting diode above each gauze container. Three sound recordings of 30 seconds duration each were made per night for at least two nights (i.e. a total of six recordings) with between-recording intervals of at least five minutes.

Calling song characteristics

Canary V1.2.4 (Cornell Laboratory of Ornithology, Ithaca, New York) was used for spectrographic analysis of the calls. A power spectrum was generated to measure calling song frequency with maximum amplitude (F_{max}), bandwidth at 10 db below maximum amplitude (Fwidth-A) and bandwidth at 20 db below maximum amplitude (Fwidth-B) for each recording. An oscillogram was used to measure number of syllables per chirp (S_C), syllable period (SP), syllable duration (SD), inter-syllable interval (ISI), chirp period (CP), chirp duration (CD) and inter-chirp interval (ICI) for each recording. For formal definitions of these characteristics see Ferreira (2006, Chapter 2). To measure within-recording variation in call traits, six chirps were measured for the first recording of the first night with a 10 second interval between the first three and the last three chirps. Longer-term variation was measured by measuring two different recordings from the same night and between-night variation was measured using at least two recordings from each of two different nights. Three consecutive chirps from each of these recordings were measured. Each chirp comprises three to six syllables (Desutter-Grandcolas & Robillard, 2003).

Statistical analyses

For each population, SAS V8.02 (Proc Varcomp; SAS Institute Inc., Cary, NC, USA) was used to determine the within-individual and between-individual variance components for the call traits using restricted maximum-likelihood estimation (REML). Within-individual variation was broken down into four levels: (a) between nights within an individual, (b) between recordings within a night, (c) among repeats (six chirps were measured with a 10 second interval between the first three and last three chirps) within a recording and (d) among chirps within a repeat. The magnitude of the variance components of each of these sources of variation as well as the magnitude of their interactions were calculated.

Several of the analyses below reflect four subsets of data: (a) morphometric data, (b) chirp call traits (ICI, CD, CP and S_C), (c) syllable call traits (ISI, SD and SP) and (d) spectral call traits (F_{max}, Fwidth-A and Fwidth-B).

Seasonal and annual variation in calling songs and body size of wild-caught crickets

Data set

Calling song recordings of wild male crickets from Pretoria and Makhado were classified into a season, based on capturing dates, as follows: summer: 1 December – 28 February; autumn: 1 March – 31 May; winter: 1 June – 31 August; spring: 1 September – 30 November. Since cricket population densities fluctuate greatly in space and time, an even representation across all seasons was not possible. Data from Pretoria were available for six seasons: autumn 2001, spring 2001, summer 2002, autumn 2002, winter 2002 and autumn 2003. Data from Makhado were available for three seasons: winter 2001, autumn 2002 and summer 2003. Since the data from Makhado represent three different seasons from three different years, care needs to be taken with respect to the temporal interpretation of these analyses (see discussion). Between-year comparisons utilised the autumn data collected from Pretoria males during 2001 – 2003.

Calling song characteristics and morphometric measurements

Male calling songs were recorded and measured as described above. After killing a male with ethyl acetate, its thorax width, thorax length and the length of the right hind femur (Fem-R) were measured using the VideoBlaster FS200 video kit (Creative Laboratories, Singapore; resolution = 16 microns). Thorax area was calculated as: thorax width x thorax length (mm^2). The wings of each male were then removed and the harp size of each right wing was measured, following Ferreira (2006, Chapter 2).

Statistical analyses

For each separate population, SAS (Proc Varcomp) was used to determine the between-season variance component for the call traits of the wild-caught males. Statistica V5.5 (StatSoft, Inc. (1999), Tulsa, USA) was used to perform fixed effects analysis of variance (ANOVA) to compare the means of the call traits as well as the means of the morphometric measurements between seasons and between years. Tukey HSD *post-hoc* analysis was performed to determine which seasons or which years (autumn, Pretoria) differed significantly from one another. A Bonferroni adjustment (Rice, 1989) was applied to the Tukey HSD *post-hoc* data of both populations. Using Statistica, a principal components analysis (PCA; varimax rotation on normalised data) was performed to determine which call traits and morphometric measurements contributed the most to the total variation of the data set. Thorax width and thorax length were excluded from the PCA, due to the inclusion of thorax area.

Effect of rearing temperature on body size of laboratory-reared crickets

Setup of breeding colonies and morphometric measurements

Breeding colonies from three geographical sites from South Africa (wild-caught crickets from Queenstown (31°52'S, 26°52'E) and Wolmaransstad (27°23'S, 26°00'E) as well as a laboratory colony from Pretoria) were established in a climate room at 27°C ± 2°C with a 12h:12h L:D light regime. Egg-laying trays from each of these colonies were collected and placed separately in labelled 2-litre plastic containers. When the eggs hatched, 75 first instar nymphs were randomly chosen from each of the three colonies and divided into batches of 25 nymphs in three 9-litre plastic containers. One container per colony was placed in incubating chambers set at constant temperatures of 15°C, 22°C and 27°C. High protein

food (Pronutro® and fish flakes) and water were provided *ad libitum* and one egg carton was placed in each of the 9-litre containers to provide shelter. Crickets were killed with ethyl acetate after adult eclosion, sexed and weighed to the nearest milligram on a Mettler Toledo AG135 scale. The Creative Labs FS200 video kit was used to measure thorax width, thorax length, head width, head length, Fem-R, right hind tibia (Tib-R), left hind femur (Fem-L), left hind tibia (Tib-L) and harp size of the right male wing. Thorax area was calculated as: thorax width x thorax length (mm^2).

Statistical analyses

Due to the high mortality rate of nymphs in the temperature treatments at 15°C and 22°C, the offspring from all three different localities were combined for each of the temperature treatments and could not be analysed separately. Differences between the morphometric measurements of the temperature treatments were analysed using a two-way fixed effects ANOVA with treatment and sex as independent variables, using Statistica.

Body size as a factor explaining within-population variation in call structure of wild-caught crickets

Calling song characteristics and morphometric measurements

Call traits (described above) of Pretoria males were obtained for four seasons (summer, autumn, winter and spring). For each male, morphometric measurements comprised thorax length, thorax width, thorax area, head length, head width, Fem-R, Tib-R, Fem-L, Tib-L and harp size.

Statistical analyses

Using SAS (Proc GLM), an analysis of covariance (ANCOVA) on the data for each of the four seasons was performed on each of the call traits, using thorax area as a covariate, to determine the effect of size on the calling song during the four seasons. Following the ANCOVA, summer data differed significantly from the other seasons and were excluded from the following analyses (see results below). Linear least-square regression analysis was performed, using SAS (Proc Reg), on the morphometric measurements and call traits to determine any significant relationship between body size and calling song. Variables with significant relationships were subjected to forward stepwise multiple regression analysis,

using Statistica, to determine which morphometric measurements contributed mostly to the variation found in the call traits. Pearson product moment correlations between morphometric measurements were calculated using Statistica.

Results

Between-individual variation in calling songs of wild-caught males

For both Makhado and Pretoria, the between-individual variance component was larger than the within-individual variance component in the spectral traits (Table 3.1). This effect was pronounced in Pretoria comprising a larger sample size. However, the chirp traits mostly had a larger within-individual variance component than between-individual variance component, reflecting the relatively large variation in chirp traits at the individual level (Table 3.1). Syllable traits for Pretoria males had a somewhat larger between-individual than within-individual variance component, while the converse was found for the Makhado males (Table 3.1).

Table 3.1. Proportions (%) of the between-season, between-individual and within-individual effects contributing to the explainable variance in the calling song characteristics of wild *G. bimaculatus* from Makhado (*n* = 57) and Pretoria (*n* = 115)

	Makhado			Pretoria		
Call traits	Between season	Between individual	Within individual	Between season	Between individual	Within individual
S_C	11.84	54.52	33.63	0.00	48.14	51.86
ICI	0.00	35.32	64.68	1.35	34.97	63.69
CP	0.00	37.16	62.84	2.84	40.27	56.89
CD	28.76	41.29	29.95	7.12	49.29	43.59
ISI	24.15	20.31	55.54	12.05	66.13	21.82
SD	13.06	43.03	43.91	4.4	52.74	42.87
SP	42.86	19.14	38.01	32.38	54.4	13.22
F_{max}	15.05	68.12	16.83	30.5	54.74	14.76
Fwidth-A	13.01	56.83	30.17	16.91	47.71	35.38
Fwidth-B	19.25	45.09	35.66	15.39	47.2	37.41

Within-individual variation in calling songs of wild-caught males

The between-night variance component and the between-chirp variance component were mainly responsible for the variation found within male calls from Makhado (Figure 3.2a) and Pretoria (Figure 3.2b). The call traits of both populations showed a large degree of stability between recordings as well as between repeats within the same recording. Syllable period and calling song frequency showed a large degree of between-night stability in the Pretoria population, with a large degree of between-chirp variation (Figure 3.3). The large between-chirp variation in syllable period nullified between-individual variation (Figure 3.2). In contrast, a large degree of between-chirp stability was observed for Makhado males. This constituted an important difference in the distribution of sources of variation between the two study sites.

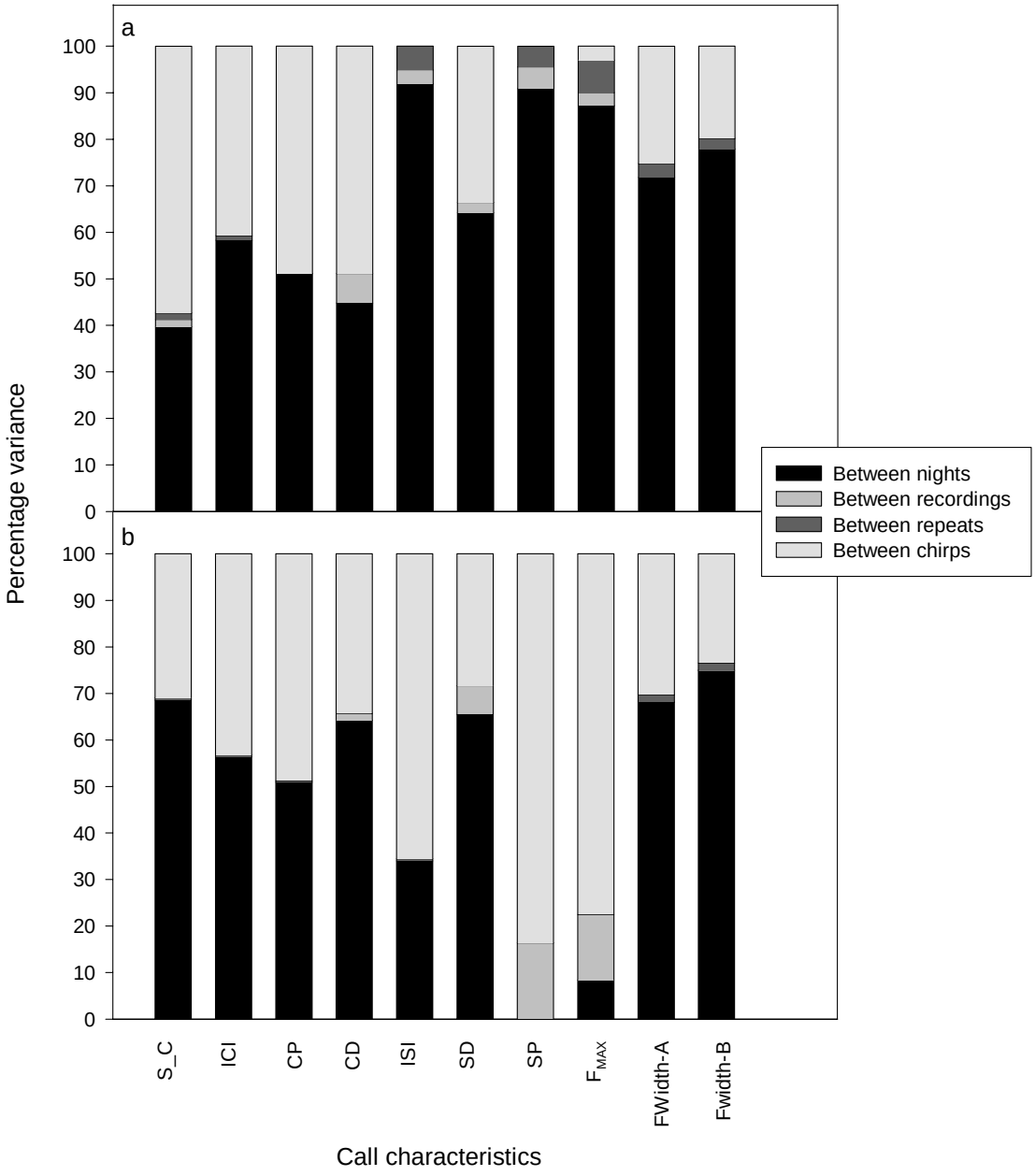

Figure 3.2. Results of a random-effects ANOVA, indicating sources of explainable variance contributing to within-individual variation in the calling song of the field cricket for (a) Makhado males (*n* = 49) and (b) Pretoria males (*n* = 102).

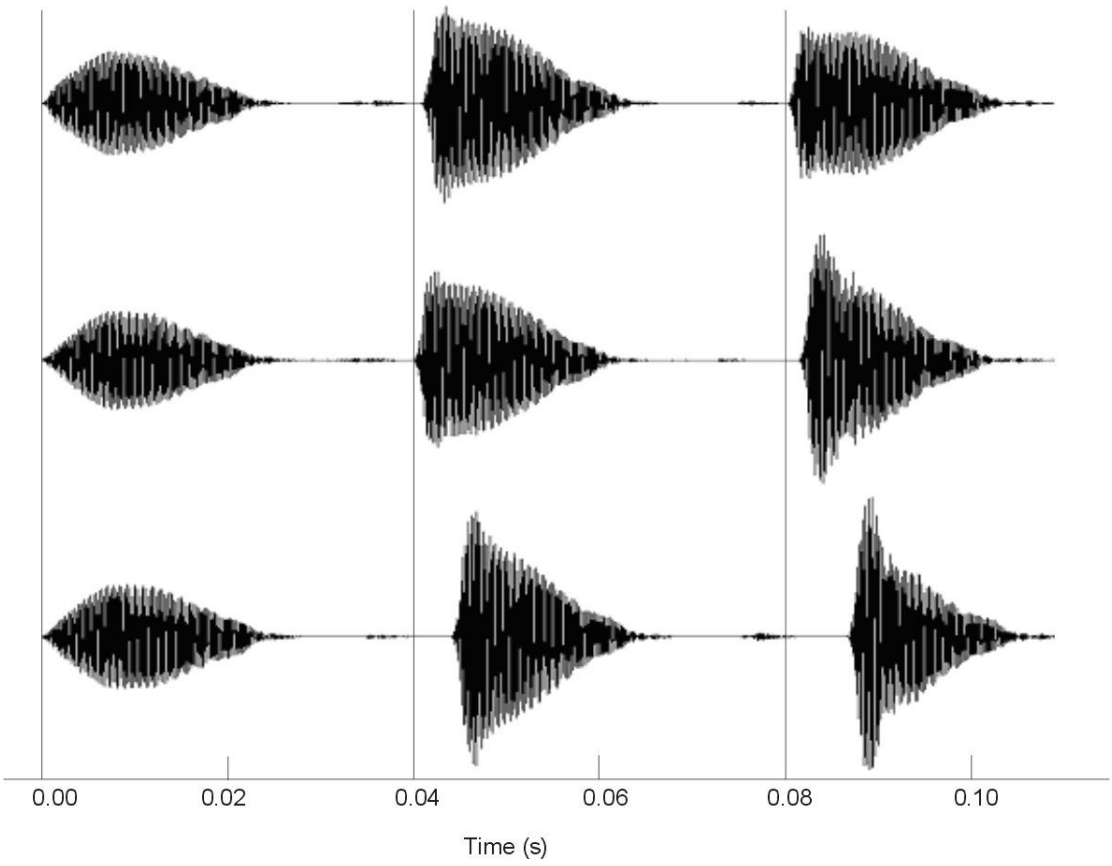

Figure 3.3. Oscillograms of three consecutive calling song chirps of male WPT 13, from Pretoria, indicating the between-chirp variation of syllable period.

Between-year comparisons of body size and call traits of wild-caught males

For the Pretoria data collected during autumn over three years, there was no clear systematic trend of between-year differences in body size (Table 3.2). Following ANOVA, after a Bonferroni adjustment, multiple comparisons revealed that male thorax length was significantly longer in 2001 compared with 2002, whereas Fem-R was significantly shorter in 2001 compared with 2003. There was no significant difference in harp size or any other body size measurement between years. Only two chirp-level measures showed significant between-year differences: males emitted slower calls during 2003 by producing significantly longer inter-chirp intervals and chirp periods in 2003 compared with 2002 (Table 3.2).

Table 3.2. Between-year analysis of the calling song characteristics and morphometric measurements of wild-caught Pretoria males (fixed effects ANOVA) as well as mean (±SE) values. A Tukey HSD *post-hoc* analysis, after a Bonferroni adjustment, indicates significant between-year differences. '*' indicates significance values for $P < 0.05$, while 'NS' indicates non-significant values for $P > 0.05$. '*n*' represents the number of crickets measured for each year

	Mean (SE)			ANOVA	Tukey HSD test		
Variable	2001	2002	2003	*F* statistic	2001-2002	2001-2003	2002-2003
Call characteristics							
n	8	20	15	$F_{2, 40}$			
S_C	3.681 (0.159)	3.467 (0.097)	3.526 (0.116)	0.671	NS	NS	NS
CD (s)	0.128 (0.006)	0.118 (0.004)	0.119 (0.004)	0.886	NS	NS	NS
ICI (s)	0.193 (0.021)	0.179 (0.009)	0.22 (0.011)	3.855*	NS	NS	*
CP (s)	0.321 (0.018)	0.297 (0.01)	0.339 (0.012)	3.606*	NS	NS	*
SD (s)	0.027 (0.001)	0.025 (0.0)	0.025 (0.001)	1.837	NS	NS	NS
SP (s)	0.038 (0.001)	0.038 (0.001)	0.037 (0.001)	0.337	NS	NS	NS
ISI (s)	0.011 (0.001)	0.013 (0.0)	0.012 (0.001)	0.907	NS	NS	NS
F_{max} (kHz)	4.774 (0.028)	4.897 (0.033)	4.927 (0.057)	2.235	NS	NS	NS
Fwidth-A (kHz)	0.254 (0.02)	0.307 (0.024)	0.254 (0.021)	1.828	NS	NS	NS
Fwidth-B (kHz)	0.455 (0.033)	0.517 (0.031)	0.439 (0.032)	1.813	NS	NS	NS
Body measurements (mm)							
n	8	20	15	$F_{2, 40}$			
Thorax length	4.767 (0.242)	4.175 (0.099)	4.446 (0.098)	4.669*	*	NS	NS
Thorax width	6.841 (0.347)	7.508 (0.204)	7.588 (0.191)	2.155	NS	NS	NS
Thorax area (mm^2)	32.263 (1.556)	31.669 (1.438)	33.878 (1.45)	0.625	NS	NS	NS
n	7	20	12	$F_{2, 36}$			
Fem-R	10.975 (0.278)	11.721 (0.19)	12.028 (0.28)	3.273*	NS	*	NS
Wing measurement (mm^2)							
n	8	17	15	$F_{2, 37}$			
Harp	16.735 (0.532)	16.594 (0.428)	16.776 (0.468)	0.047	NS	NS	NS

Between-season comparisons of body size and call traits of wild-caught males

Different trends in body size were observed between populations, reflected by results from fixed effects ANOVA. While Pretoria males were significantly smaller in summer (Table 3.3a), Makhado males were significantly smaller in autumn (Table 3.3b) compared with other seasons. Fixed effects ANOVA revealed significant between-season differences in the call traits of both populations. Several spectral and syllable related characteristics of the Pretoria males during summer differed significantly from those of other seasons (Table 3.3a). For Makhado, two syllable traits and one chirp trait differed significantly between the seasons (Table 3.3b). For both populations, the between-season variance component was less than the between-individual and within-individual variance components, affecting both spectral and chirp traits (Table 3.1). However, the between-season variance component contributed largely to the variation in the syllable traits of both populations.

Principal components analyses (PCA) showed that, for both populations, five principal components (eigenvalues > 1) explained more than 85% of the total variation (Table 3.4). The variables contributing most to the existing variation were similar for both the Makhado and Pretoria populations (Table 3.4). The large degree of variation in body size, encountered in the ANOVA's, were clearly reflected in the PCA, since body size contributed significantly to principal components 1 & 2 in both the Pretoria and Makhado populations, representing > 20% of the total variation. For Makhado, the chirp traits contributed largely to principal components 1 & 3, while for Pretoria they largely contributed to principal components 2 & 4, representing some 30% of the total variation for both populations. Syllable traits only contributed to the fifth principal component of both populations that comprised less than 16% of the total variation (Table 3.4). Calling song frequency did not contribute in a measurable way to the total variation. Although seasonal trends were detected in the PCA's there was no distinct seasonal clustering of the R-rotated data.

Table 3.3. Mean values (±SE; *n* if not the same as *n* in the first row) for the call, body and wing characteristic(s) of the respective seasons for male field crickets, *G*. *bimaculatus*, from (a) Pretoria and (b) Makhado. The *F* statistic and its significance for a fixed effects ANOVA, as well as the results for a Tukey HSD *post-hoc* comparison test, after a Bonferroni adjustment, are presented. Significance values are indicated with asterisks: $P < 0.05$*, $P < 0.01$**, $P < 0.001$***, NS = Not significant ($P > 0.05$). '*n*' represents the number of crickets measured for each season. '–' indicates seasons not sampled for Makhado

	Variable	Mean (SE; *n* if not the same as *n* in the first row)				ANOVA	Tukey HSD test					
		Summer (S)	Autumn (A)	Winter (W)	Spring (P)	*F* statistic	S-A	S-W	S-P	A-W	A-P	W-P
a	*n*	22	43	11	26	$F_{3, 98}$						
	S_C	3.557 (0.098)	3.527 (0.067)	3.75 (0.117)	3.497 (0.087)	0.929	NS	NS	NS	NS	NS	NS
	CD (s)	0.111 (0.004)	0.12 (0.003)	0.135 (0.004)	0.123 (0.004)	4.661**	NS	NS	NS	NS	NS	NS
	ICI (s)	0.178 (0.007)	0.196 (0.007)	0.164 (0.011)	0.205 (0.008)	3.458*	NS	NS	NS	NS	NS	NS
	CP (s)	0.289 (0.01)	0.316 (0.008)	0.299 (0.011)	0.328 (0.009)	3.143*	NS	NS	NS	NS	NS	NS
	SD (s)	0.024 (0.0)	0.025 (0.0)	0.025 (0.001)	0.026 (0.001)	4.243**	NS	NS	NS	NS	NS	NS
	SP (s)	0.034 (0.001)	0.037 (0.0)	0.040 (0.001)	0.038 (0.0)	23.403***	***	***	***	NS	NS	NS
	ISI (s)	0.01 (0.0)	0.012 (0.0)	0.014 (0.001)	0.012 (0.0)	5.7**	NS	NS	NS	NS	NS	NS
	F_{max} (kHz)	5.378 (0.084)	4.884 (0.027)	4.78 (0.054)	4.883 (0.029)	27.893***	***	***	***	NS	NS	NS
	Fwidth-A (kHz)	0.414 (0.029)	0.278 (0.014)	0.298 (0.027)	0.26 (0.016)	11.208***	***	NS	***	NS	NS	NS
	Fwidth-B (kHz)	0.646 (0.035)	0.478 (0.02)	0.52 (0.04)	0.457 (0.024)	9.325***	***	NS	***	NS	NS	NS
	Thorax length (mm)	3.648 (0.173)	4.379 (0.079)	4.064 (0.098)	4.551 (0.092)	11.794***	***	NS	***	NS	NS	NS
	Thorax width (mm)	7.09 (0.176)	7.412 (0.136)	7.525 (0.124)	7.628 (0.123)	1.999	NS	NS	NS	NS	NS	NS
	Thorax area (mm^2)	25.539 (1.015)	32.55 (0.882)	30.661 (1.136)	34.728 (0.918)	14.052***	***	*	***	NS	NS	NS
	Femur-R (mm)	9.256 (0.203; 21)	11.681 (0.148; 39)	11.716 (0.148; 10)	11.834 (0.143; 22)	48.083***; $F_{3, 88}$	***	***	***	NS	NS	NS
	Harp (mm^2)	14.611 (0.264)	16.691 (0.268; 40)	16.614 (0.35)	17.045 (0.272)	12.981***; $F_{3, 95}$	***	NS	***	NS	NS	NS
b	*n*	18	21	10	-	$F_{2, 46}$						
	S_C	3.741 (0.099)	3.417 (0.093)	3.7 (0.105)	-	3.48*	NS	NS	-	NS	-	-
	CD (s)	0.124 (0.004)	0.108 (0.004)	0.132 (0.003)	-	11.39***	NS	NS	-	***	-	-
	ICI (s)	0.185 (0.008)	0.187 (0.011)	0.172 (0.016)	-	0.42	NS	NS	-	NS	-	-
	CP (s)	0.309 (0.008)	0.295 (0.012)	0.303 (0.016)	-	0.467	NS	NS	-	NS	-	-
	SD (s)	0.025 (0.0)	0.024 (0.0)	0.025 (0.001)	-	2.03	NS	NS	-	NS	-	-
	SP (s)	0.036 (0.001)	0.034 (0.0)	0.04 (0.001)	-	17.891***	NS	***	-	***	-	-
	ISI (s)	0.011 (0.001)	0.011 (0.001)	0.015 (0.001)	-	9.331***	NS	NS	-	***	-	-
	F_{max} (kHz)	5.079 (0.033)	5.077 (0.043)	4.908 (0.061)	-	3.644*	NS	NS	-	NS	-	-
	Fwidth-A (kHz)	0.315 (0.022)	0.399 (0.034)	0.262 (0.031)	-	4.618*	NS	NS	-	NS	-	-
	Fwidth-B (kHz)	0.491 (0.032)	0.607 (0.038)	0.425 (0.043)	-	5.688**	NS	NS	-	NS	-	-
	Thorax length (mm)	4.527 (0.111; 19)	4.193 (0.079)	4.778 (0.125)	-	7.386**; $F_{2, 47}$	NS	NS	-	NS	-	-
	Thorax width (mm)	7.859 (0.174; 19)	7.36 (0.083)	7.898 (0.139)	-	5.052*; $F_{2, 47}$	NS	NS	-	NS	-	-
	Thorax area (mm^2)	35.88 (1.627; 19)	30.947 (0.838)	37.86 (1.548)	-	6.801**; $F_{2, 47}$	*	NS	-	**	-	-

Table 3.3b (continued)

Fem-R (mm)	12.45 (0.241; 19)	11.789 (0.214; 20)	12.147 (0.21)	-	2.384	NS	NS	-	NS	-	-
Harp (mm^2)	17.016 (0.447; 19)	15.849 (0.222)	17.674 (0.525; 9)	-	5.382**	NS	NS	-	NS	-	-

Table 3.4. Principal component analyses, with varimax normalised rotation, of seasonal variation in call traits and morphometric measurements of wild male crickets from Makhado (M) and Pretoria (P). Loadings indicated by 'M' and 'P' are > 0.700 for the respective geographical site. Calling song frequency did not contribute in a measurable way to the variation in either of the data sets

Variable	PC1	PC2	PC3	PC4	PC5
S_C		P	M		
CD		P	M		
ICI	M			P	
CP	M			P	
SD					P
SP					M
ISI					M, P
Fwidth-A			P	M	
Fwidth-B			P	M	
Thorax area	P	M			
Fem-R	P	M			
Harp	P	M			
Total variance explained % (M)	16.0	22.7	15.7	17.4	19.9
Total variance explained % (P)	24.90	16.40	18.10	16.70	12.00

Body size as a factor explaining variation in the call traits of wild-caught males

The ANCOVA revealed a significant seasonal effect ($P < 0.05$, $n = 102$) on all the call traits except for number of syllables per chirp. The only significant effect of body size was on calling song frequency ($F_{3, 100} = 126.65$, $P < 0.001$, $n = 102$). The ANOVA revealed that Pretoria males were smaller during summer than during the other seasons (Table 3.3a), while none of the remaining three seasons differed significantly with respect to any of the variables analysed below. For this reason the summer data were omitted from the correlation and regression analyses.

Although there was a highly significant correlation ($P < 0.01$ in all cases, $n = 56$) between harp size and several body size measurements (head length: r = 0.347, head width: r = 0.606, thorax width: r = 0.671, thorax length: r = 0.457, thorax area: r = 0.76, Fem-R: r = 0.575, Tib-R: r = 0.5, Fem-L: r = 0.501 and Tib-L: r = 0.398), the relationships between body size and call traits were much more tenuous. Both calling song frequency and inter-syllable interval showed a significant inverse relationship with harp size (F_{max}: $F_{1, 75} = 10.028$, $P = 0.002$, $n = 77$; ISI: $F_{1, 75} = 5.007$, $P = 0.028$, $n = 77$). Harp size explained 10.6% of the variation in calling song frequency and 5% of the variation in inter-syllable interval. There was a significant inverse correlation between thorax area and calling song frequency ($F_{1, 78} = 7.504$, $P = 0.008$, $n = 80$), with thorax area explaining 8% of the variation in calling song frequency (Figure 3.4). Forward stepwise regression analysis revealed that harp size contributed more to the variation in calling song frequency (partial correlation = -0.343, $P = 0.002$, $n = 77$) than did thorax width or thorax area.

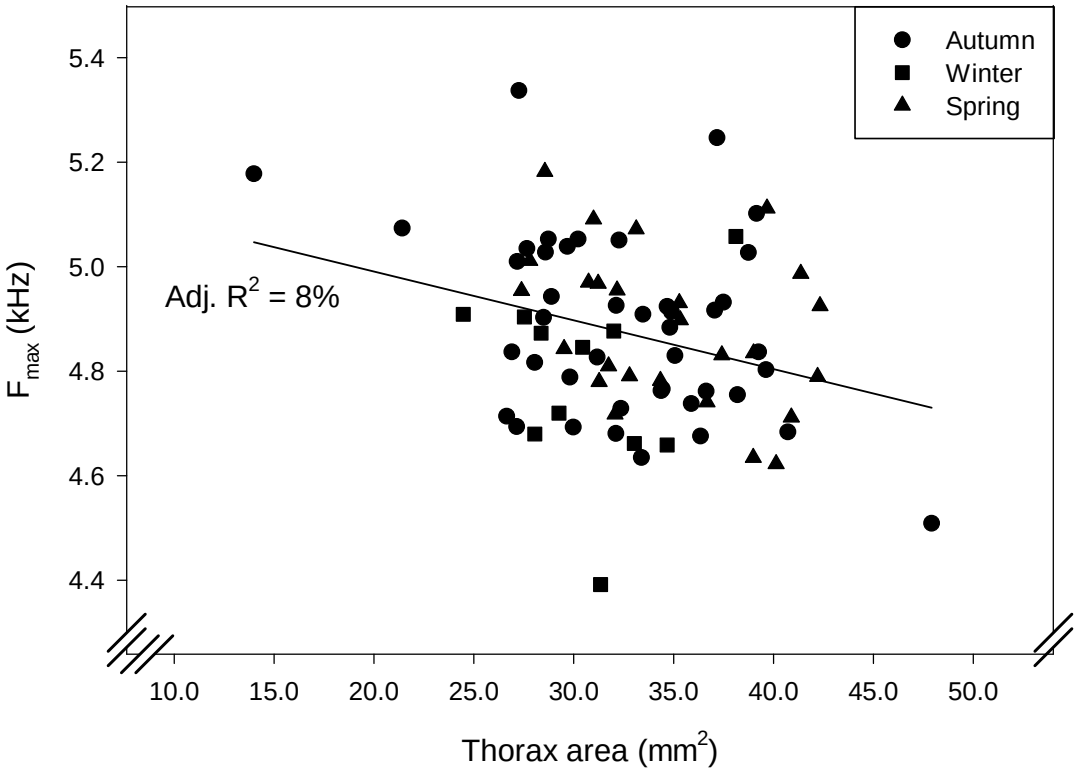

Figure 3.4. Scatter diagram indicating the relationship between calling song frequency (F_{max}) and thorax area for the Pretoria males for three different seasons of the year.

Effect of rearing temperature on body size of laboratory-reared crickets

There was a mortality of 100% at the 15°C treatment before the second instar was reached, therefore no useful data were obtained for this treatment. At 22°C the survival rate was 13%, with four males and six females reaching adulthood across the three breeding lines. At 27°C the survival rate was much higher at 47%, with 16 males and 19 females that reached adulthood across the three breeding lines.

Body size of crickets raised at 27°C was larger than for those raised at 22°C (significant effects for leg measurements and body mass; Table 3.5). Two-way fixed effects ANOVA revealed a strong treatment effect with respect to age at adult eclosion as well as a sex-specific treatment effect in body size (Table 3.5). Except for head length and right tibia at 22°C, females were always larger than males across both treatments. This effect was larger at 27°C, although only one of the size measurements showed a significant interaction effect, indicating a significantly stronger between-sex difference at the higher temperature. Females at 22°C were younger at adult eclosion than males at the same treatment (Table 3.5). Male harp size did not differ significantly between 22°C and 27°C (Table 3.5).

Table 3.5. Mean values (±SE) for the body and wing characteristic(s) of the males and females at adult eclosion for rearing environments at 22°C and 27°C. The *F* statistic and its significance for a two-way fixed effects ANOVA for the treatments (temperature (temp) and sex) and the interaction between them (temp-sex) are presented. Significance values are indicated as follows with asterisks: $P < 0.05$*, $P < 0.01$**, $P < 0.001$***. '*n*' represents the number of crickets measured

	Mean (SE)				Two-Way ANOVA		
	22°C		27°C		Temp	Sex	Temp-sex
Variable	Males	Females	Males	Females	*F* statistic	*F* statistic	*F* statistic
Body measurements (mm)							
n	4	6	16	19	$F_{1, 41}$	$F_{1, 41}$	$F_{1, 41}$
Thorax length	3.672 (0.114)	4.036 (0.138)	3.699 (0.131)	4.407 (0.075)	1.805	13.132***	1.349
Thorax width	6.25 (0.048)	6.38 (0.174)	6.433 (0.229)	7.088 (0.105)	3.552	2.749	1.237
Thorax area (mm^2)	22.954 (0.807)	25.856 (1.508)	24.215 (1.671)	31.362 (0.963)	3.321	7.322**	1.307
Head length	3.941 (0.157)	3.471 (0.09)	3.92 (0.146)	3.884 (0.105)	1.269	2.108	1.553
Head width	5.175 (0.062)	5.517 (0.237)	5.523 (0.196)	5.825 (0.079)	2.534	2.431	0.009
Mass (g)	0.5 (0.04)	0.613 (0.05)	0.592 (0.053)	0.852 (0.052)	5.046*	6.404*	0.994
n	4	6	16	18	$F_{1, 40}$	$F_{1, 40}$	$F_{1, 40}$
Fem-R	9.427 (0.163)	9.941 (0.277)	10.169 (0.268)	11.336 (0.162)	12.262**	7.589**	1.14
n	4	4	12	18	$F_{1, 34}$	$F_{1, 34}$	$F_{1, 34}$
Fem-L	9.441 (0.342)	9.564 (0.323)	10.225 (0.351)	11.359 (0.136)	14.425***	3.418	2.214
n	4	6	14	16	$F_{1, 36}$	$F_{1, 36}$	$F_{1, 36}$
Tibia-R	7.24 (0.172)	6.873 (0.163)	7.43 (0.225)	8.37 (0.188)	9.89**	1.139	5.941*
n	4	5	11	17	$F_{1, 33}$	$F_{1, 33}$	$F_{1, 33}$
Tibia-L	7.083 (0.243)	7.256 (0.141)	7.724 (0.78)	8.54 (0.143)	15.936***	4.249*	1.776
Wing measurement (mm^2)							
n	4		14		$F_{1, 16}$		
Harp	10.255 (0.573)		11.714 (0.635)		1.37		
Age at adult eclosion (days)							
n	4	6	16	19	$F_{1, 41}$	$F_{1, 41}$	$F_{1, 41}$
Age	191.5 (10.137)	167.667 (9.912)	73.438 (3.673)	79.842 (2.366)	350.173***	2.509	7.553**

Discussion

For both populations, PCA indicated that the frequency and syllable call traits contributed little to the total variation in the normalised data set (Table 3.4). Conversely, the chirp call traits and morphological characteristics contributed largely to the observed variation. This is consistent with the conclusion of Ferreira & Ferguson (2002) that several call traits involved in mate recognition have relatively little variance and may be subject to stabilizing selection.

Between-individual differences

Taking into account that between-individual variance in the calling song of both populations results mostly from both general environmental and additive genetic causes, the between-individual differences in this study are suggestive of the maximum heritability of a trait (Falconer & Mackay, 1996). This implies that call traits with large between-individual variation and small within-individual variation (Table 3.1) could potentially be subject to sexual selection.

One of the obvious between-individual differences was body size. This gives rise to the question of how this factor contributed to between-individual differences in call traits. This is especially important since some workers inferred that female crickets can judge the size of a male through his calling song (Gray, 1997; Simmons, 1988). This study showed that large males have large harp sizes and produced shorter inter-syllable intervals and lower calling song frequencies (Figure 3.4). However, the proportion of variation in calling song frequency and inter-syllable interval explained by male size is extremely low. This study found no significant effect of body size on any chirp trait, opposing the findings of Ferreira (2006, Chapter 2). The low correlation between body size and calling song frequency could probably be explained by the indirect effect of body size on harp size that, in turn, correlates with calling song frequency. The utility of calling song frequency for indicating male size is thus limited and body size is not a major determinant of between-individual differences in calling song characteristics. This study could not repeat the conclusions of Simmons (1988) or Simmons & Zuk (1992) that syllable rate and chirp duration correlate with male size. These relationships are highly variable, evident from Brown *et al*. (1996) who found a significant inverse correlation in *O. nigricornis* between calling song frequency and body size, Gray (1997) who found a positive correlation between male size and

number of syllables per chirp in *A. domesticus* and Wagner & Hoback (1999) who did not find any correlations in *G. lineaticeps.*

Within-individual differences

Within-male variation, measured here as temporal variation in calling song characteristics, results from variation in either the environment or of the individual insect (Falconer & Mackay, 1996). Environmental variance in signals acts as noise within a system of selection on the communication system. The high levels of within-individual variation in the chirp traits (Table 3.1; Figure 3.3) limits the heritability and increases the error in intersexual communication using these traits within a framework of sexual selection.

For both populations, within-individual variation largely comprised between-night and between-chirp differences in the calling song, with less variation between repeats within a recording and between recordings. However, the main sources of variation differed between populations. For Pretoria, syllable period and calling song frequency varied less between nights than between chirps within a recording (Figure 3.2b). Conversely, for Makhado, the between-night component of variation contributed largely to within-individual variation (Figure 3.2a). Overall, calling song frequency and syllable period, important for species recognition (Huber & Thorson, 1985; Schildberger *et al*., 1989), had a small degree of within-individual variance (Table 3.1).

Seasonal differences

There were significant between-season differences in male size and call traits (Table 3.3a). Male size and harp size were the smallest in the summer samples. For Pretoria, the between-season variance component explained a significant proportion of the variation in the syllable and spectral traits (Table 3.1). Calling song frequency was significantly higher during summer than during winter, consistent with the results of Van Wyk & Ferguson (1995), who found that males produced a lower calling song frequency during winter than autumn. In addition, syllable period during the present study was significantly shorter in summer, leading to rapid syllable rates. This could be explained by the temperature effect on the syllable and chirp characteristics. Doherty (1985) found that *G. bimaculatus* males produced shorter syllable and chirp periods at warmer temperatures. Males of three *Oecanthus* species produced faster chirp rates at warmer

temperatures than at cooler temperatures (Toms, 1992) and *Laupala cerasina* males produced significantly faster syllable rates at warmer temperatures than at cooler temperatures (Grace & Shaw, 2004).

Since cricket abundance varies temporally, reflecting environmental conditions such as rainfall and food availability, wild crickets could not be obtained for all consecutive seasons over the time period of this study. Therefore, some of the seasonal variation might be explained by medium-term temporal variation rather than fixed seasonal variation. This medium-term temporal variation could be caused by environmental conditions that the cricket experiences, such as food availability. Notwithstanding the distinction between fixed seasonal effects or less regular medium term effects, the temporal variation in calling song did not last from one year to the next.

If one extrapolates from the Pretoria crickets (which did not differ between years), the temporal calling song differences for Makhado (Table 3.3b) were probably due to seasonal differences. Both syllable and chirp traits of Makhado males differed significantly between seasons, with no significant between-season differences in body size (Table 3.3b). Significantly longer inter-syllable intervals during the winter sample caused longer syllable periods during winter. Overall, males from Makhado called at a slower chirp rate during the winter sample. This is consistent with the seasonal results from Pretoria. However, firm temporal interpretation of the data from Makhado is obscured by the irregular temporal structure of the samples.

Although the call characteristics important for species-specific communication differed between seasons, they contributed less to the variation than the chirp traits and the body measurements, evident from the PCA (Table 3.4). Seasonal variation is probably environmentally induced, since the genetic composition of a population remains more or less the same over short time spans. The between-season differences in the calling song were probably related to the local environment. Indeed, Pretoria males were the smallest during summer (Table 3.3a). If the environment (e.g. cricket density, food availability) affects body size, it may have an indirect effect on calling song characteristics. Since male size affects harp size which, in turn, correlates with calling song frequency, it is possible that the higher calling song frequency produced in summer was a consequence of the smaller body size in summer (Table 3.3a). However, it is unlikely that body size is singly important as it was only weakly correlated with the

call characteristics (Figure 3.4). For both populations syllable period was slower during winter than summer. This is probably explained by the colder temperatures experienced by these crickets during winter compared with summer. Indeed, *L. cerasina* males recorded at a constant temperature produced faster syllable rates when kept at warmer temperatures than at colder temperatures (Grace & Shaw, 2004). What remains is to have a better understanding of the relationship between body size and temperature to explain the relationship between body size and the calling song.

Body size effects due to temperature differences

Sibly & Atkinson (1994) predicted that ectotherms should develop faster and have a smaller body size at warmer temperatures under certain conditions. This study found that males and females developed faster at the warmer rearing temperature (Table 3.5). While females at the higher temperature had a larger body size, there was a smaller effect of rearing temperature on male body size. For males and females, development time from egg to adult was similar at each rearing temperature (Table 3.5). This suggests that between-season differences in male size are probably not temperature related but are rather affected by factors such as food availability, population density or other environmental factors. These results indicate that the seasonal differences in calling song characteristics of *G. bimaculatus* are not explained by a simple temperature-dependent effect of body size.

Annual differences

The Pretoria population was temporally stable in body size and call traits for autumn across three years. Only two chirp-level call traits differed between years (Table 3.2), whereas the syllable and spectral traits, important for communication, showed consistency over consecutive years. Harp size, an important correlate of calling song frequency, did not differ between years. Seasonal effects were more marked than between-year effects, indicating that across years the seasonal influence on the calling song probably remains the same.

Conclusion

Short-term variation in the sexually selected calling song characteristics possibly compromises the communication system. However, this effect could be negated,

firstly, due to the parallel coupling of female preferences to the male signaling system at different temperatures (Doherty, 1985; Grace & Shaw, 2004) and secondly, due to wide preference ranges of females (L. Verburgt, personal communication). The absence of large differences in male size between two rearing temperatures suggests that rearing temperature is not an important determinant of male size. Due to the weak correlations between male size and the calling song characteristics and due to the large degree of between-night variation in the call traits within males, females probably cannot use the male calling song as a reliable indicator of male size-based quality.

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CHAPTER 4

Inter-continental phylogeography and regional gene flow in the field cricket, *Gryllus bimaculatus* De Geer (Orthoptera: Gryllidae)

Abstract

I sequenced part of the mitochondrial cytochrome *b* gene to determine intraspecific patterns of genetic variation in *Gryllus bimaculatus*, within and among wild crickets of seven South African and two European populations. For South Africa, there was a large degree of within-population genetic differentiation and 30 haplotypes were identified from 133 individuals. Two ancestral haplotypes occurred in all of the South African populations. A large amount of gene flow occurred between all the South African populations, and two possible migration routes were established: (a) between the northeastern regions of the country and (b) between the southeastern regions of the country. NCA predicted that isolation by distance compounded by substantial localised gene flow was the consequence of geographical differentiation in South Africa. For South Africa and Europe, there was a large degree of between-population differentiation, with some gene flow occurring between one South African population (Queenstown) and both the European populations. Only two haplotypes were identified from 28 individuals in the European populations, of which one was a unique haplotype occurring only in Spain, and the other occurred throughout South Africa. The within-population genetic differentiation in Europe was very low, possibly reflecting the influence of the severe seasonal temperature changes on population numbers. NCA could not be performed on the African-European data set, due to the large non-sampled areas. This study concludes that gene flow in *G. bimaculatus* is not restricted to one continent, or even regions, and that inter-continental gene flow as well as regional gene flow occurs in this cricket, either by means of air, land or sea transport.

Keywords: *Gryllus bimaculatus*, Inter-continental phylogeography, Gene flow, Mitochondrial DNA, Cytochrome *b*

Introduction

Gryllus bimaculatus has a global distribution, spanning Africa, Asia and southern Europe (Harrison & Bogdanowicz, 1995; Ragge, 1972). It is an irruptive insect with large numbers appearing locally over a short time span, with low local population densities between these peaks. Moreover, *G. bimaculatus* outbreaks are unpredictable in time as well as in space, probably because of the specific habitat requirements for egg and larval development. Oviposition sites have to be warm and moist (personal observation) and immatures cannot survive cold conditions (Ferreira, 2006a, Chapter 3). Southern Europe has stronger seasonal trends and relatively severe winters imposing strong limitations on cricket population numbers, compared to the mild winters and low seasonality of Southern Africa (Martyn, 1992; Schulze, 1994). Although some parts of Asia experience dramatic changes in climatic conditions between seasons, the temperate areas preferred by *G. bimaculatus*, have a relatively low seasonality (Martyn, 1992). The ecological conditions and climatic conditions on each continent modulate the population numbers of crickets. For instance, changes in weather systems probably lead to mass migrations of *G. bimaculatus* from the West African coast towards the sea in 1969 (Ragge, 1972). During March and April 2005, a heat wave probably caused population explosions of *G. bimaculatus* in the Western Cape Province of South Africa (Breytenbach, 2005).

Genetic analyses are very valuable in understanding the spatial dynamics of this species since it can shed light on the degree of isolation between local populations, as well as among populations on different continents. Phylogeographical analyses have the potential of yielding information about present as well as historical processes that may have given rise to the present geographical distribution and patterns of irruption. By measuring intraspecific patterns of genetic variation in mitochondrial (mt) DNA it is possible to make statements on the effective sizes of populations as well as the amount of gene flow between them. Phylogeographical analyses also allow one to separate historical events from recurrent forces to explain phylogeographical patterns (Templeton *et al*., 1995; Templeton, 1998).

Intraspecific phylogeographical patterns have been studied within the context of hybridization between three closely related North American *Gryllus* species (*G. firmus, G. pennsylvanicus* and *G. ovisopis*: Broughton & Harrison, 2003; Willet *et al*., 1997). Broughton & Harrison (2003) found little geographical

structure and no correlation between genetic distance and geographical proximity for the *Ef1α*, *Cam*, and *Cyt-c* introns of the nuclear genes in *G. firmus* and *G. pennsylvanicus*. However, they argue that ancestral polymorphism have persisted through the divergence of *G. firmus*, *G. pennsylvanicus* & *G. ovisopis* and that this event rather than gene flow explains the observed deep coalescence of the nuclear loci. On the other hand, the COI-COII mtDNA gene revealed substantial intraspecific genetic structure in *G. firmus* and *G. pennsylvanicus* with both species having a north-south split (Willet *et al*., 1997). These authors suggested that, although contemporary gene flow accounted for intraspecific patterns of allele frequency variation, the distinctness of mtDNA haplotypes between northern and southern clades argued against the gene flow hypothesis, and that the biogeograhical history of this north-south divergence event remains a puzzle.

Phylogeographical analyses can give fundamental insights to understand geographical variation in morphological as well as behavioural traits of crickets. For instance, clinal variation was observed in the advertisement call of the túngara frog, *Physalaemus pustulosus,* and Ryan *et al*. (1996) proposed that call similarities between populations located close to each other were probably due to gene flow or might have originated through a common ancestor. Ferreira (2006b, Chapter 5) and Ferreira & Ferguson (2002) found significant geographical variation in the calling song as well as morphological traits of *G. bimaculatus*. In order to fully understand the underlying mechanisms contributing to this geographical variation, it is therefore necessary to quantify the degree of contemporary intraspecific gene flow as well as patterns of historical geographical differentiation for several populations of *G. bimaculatus*.

The aims of this study are:

1. To determine geographical variation in cytochrome *b* mtDNA sequences in *G. bimaculatus* from South Africa and Europe.
2. To estimate gene flow and migration rates between different geographical areas in southern Africa and Europe.
3. To infer historical biogeographical processes from the observed genetic variation.

Materials and methods

Sampling localities and genomic DNA extraction

Male crickets, *G. bimaculatus*, were sampled from 2000 to 2003 at seven localities in South Africa (Figure 4.1a; Dullstroom: 25°5'S, 30°00'E; Dundee: 28°15'S, 30°23'E; Hotazel: 27°14'S, 22°57'E; Makhado: 23°05'S, 29°59'E; Paarl: 33°14'S, 18°56'E; Pretoria 25°41'S, 28°13'E; Queenstown: 31°52'S, 26°52'E). One hundred and thirty three individuals from South Africa were sequenced. In addition, 28 crickets from two regions in Europe (Figure 4.1b; Italy and Spain) were sequenced. Crickets were stored at – 70°C after collection until DNA isolation. DNA was extracted from tarsi using 5% Chelex 100® (Walsh *et al*., 1991) and stored at – 20°C. The Italian sample comprised four males from Genova (44°24'N, 8°54'E), two from Modigliana (44°8'N, 11°46'E) and one each from Peccioli (43°32'N, 10°43'E) and Russi (44°22'N, 12°1'E). Due to the few animals from each locality in Italy, they were treated as a single Italian sample. Twenty crickets from Spain (Sevilla: 37°24'N, 6°59'W) were sequenced.

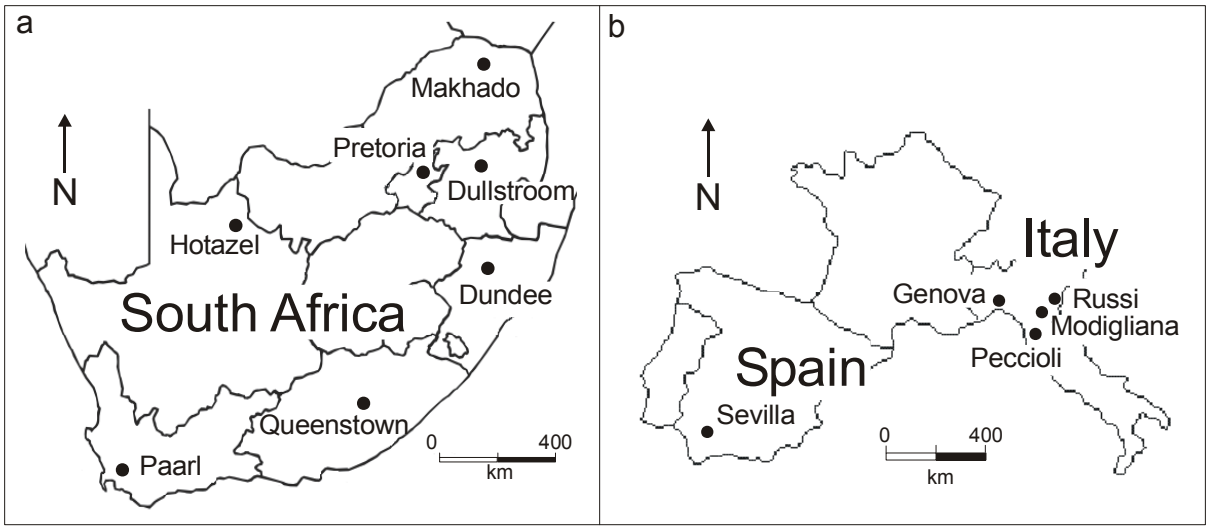

Figure 4.1. Map of sampling locations of *G. bimaculatus* in (a) South Africa and (b) Europe.

PCR amplification and sequencing of the cytochrome b region

A forward primer, labeled 197F (5'GTTGGACGTGGAATATATTA3') and complimentary reverse primer, labeled 925R (5'GCACCGATTCAAGTTAATAA3') were designed and used to amplify a 728bp fragment of the cytochrome *b* gene. Polymerase chain reaction (PCR) was performed using a GeneAmp system 2400 thermal cycler (Perkin Elmer Applied Biosystems, Norwalk, Connecticut) using a total reaction volume of 50μl. Concentrations and volumes of the reagents for each amplification reaction were as follows: 1μl of DNA template, 2.5μl of each primer (10pmol/μl), 4μl of dNTPs (2.5mM), 5μl of 10 X buffer, 1μl of *Taq* DNA polymerase (1U/μl; Biotools, Madrid, Spain) and 36.5μl of ddH_20. Thermal cycling parameters were as follows: 20 seconds preheat at 96°C, 1 cycle of initial denaturation at 95°C for 20 seconds, annealing at 50°C for 25 seconds, extension at 72°C for 60 seconds and 39 cycles of denaturation at 95°C for 15 seconds, annealing at 49.5°C for 15 seconds, extension at 72°C for 50 seconds and 1 cycle of final extension at 72°C for 60 seconds and held at 5°C. PCR negative controls containing no DNA template were included in each set of amplification reactions. After checking PCR products on a 1.5% agarose gel, stained with ethidium bromide, they were purified using the High Pure PCR Product Purification kit (Roche Diagnostics Corporation, Indianapolis, USA) following the manufacturer's specifications.

All PCR products were sequenced in both directions. Purified DNA was cycle-sequenced in 10μl reactions using 2μl of the Big Dye Terminator cycle Sequencing Ready Reaction kit (fourfold diluted; Perkin Elmer), 1μl of 5 X sequencing buffer, 1μl of primer (3.2 pmol/μl), 2 – 6μl of DNA template, 6 – 2μl of ddH_2O in a GeneAmp thermal cycler. The amount of DNA template and ddH_2O used were determined by the PCR product yield. Conditions for thermal cycling were as follows: preheat for 0.0 seconds at 96°C, followed by 25 cycles of denaturation at 96°C for 10 seconds, annealing at 54°C for 5 seconds and extension at 60°C for 4 min and held at 4°C. Sequencing was performed on ABI Prism 377 & 3100 DNA Sequencers (Perkin Elmer). Sequence chromatograms were visualized using Chromas V1.43 (Mc Carthy, 1996) and aligned and edited in Dapsa V4.9 (Harley, 2000).

Eliminating the analysis of nuclear copies of the cytochrome b gene

Nuclear copies of mtDNA (mtDNA pseudogenes) have been found in several orthopteran species namely *Schistocerca gregaria* (Zhang & Hewitt, 1996), ten grasshopper species from the families Acrididae, Podisminae, Calliptaminae, Cyrtacanthacridinae and Gomphocerinae (Bensasson *et al*., 2000), therefore all cytochrome *b* sequences in this study were checked for characteristics consistent with mtDNA pseudogenes (Arctander, 1995; Bensasson *et al*., 2001). In addition, the chromatograms of these sequences were checked for double peaks at each base pair. Finally, pure mtDNA of five individuals (two from Pretoria, one from a laboratory-reared colony and two from Sevilla) was extracted using Cesium Chloride (CsCl; Lansman *et al*., 1981). The two hind femurs and front wings were removed from each of the five individuals and stored at – 20°C, after which the remainder of the cricket was homogenized. For each individual the cytochrome *b* gene of the purified mtDNA was amplified and sequenced as described above. Chromatograms were visualized using Chromas, aligned and edited using Dapsa. These sequences were then aligned and compared to those obtained from the genomic DNA extractions (Chelex extraction of the two Sevilla males or Roche kit extraction of the two laboratory-reared males and one Pretoria male, following manufacturer's specifications).

In order to verify that it was free of nuclear DNA, the extracted pure mtDNA of each male was amplified using nuclear ribosomal primers (18_sF and 5.8_sR), yielding a 962 region of the ITS gene (Ji *et al*., 2003). It was assumed that a CsCl extraction of mtDNA contained no nuclear DNA if there was no PCR product using the ribosomal primers. Application of these primers to amplify genomic extractions (Roche kit) from these crickets yielded clear PCR products. Polymerase chain reaction was performed using a GeneAmp thermal cycler with a total reaction volume of 50μl. Concentrations and volumes of the reagents for each amplification reaction were as follows: 1μl of DNA template, 2.5μl of each primer (10pmol/μl), 4μl of dNTPs (2.5mM), 5μl of 10 X buffer, 1μl of *Taq* DNA polymerase (1U/μl; Biotools) and 36.5μl of ddH_20. Thermal cycling parameters were as follows: 4 min preheat at 94°C, 35 cycles of denaturation at 95°C for 20 seconds, annealing at 50°C for 40 seconds, extension at 72°C for 90 seconds and 1 cycle of final extension at 72°C for 20 seconds and held at 4°C. One positive control (genomic DNA template using ITS primers) and one negative control (no

DNA template using ITS primers) were included with each set of amplification reactions. PCR products were checked on a 1.5% agarose gel, stained with ethidium bromide.

Gene flow and genetic differentiation within and between populations

All the analyses were performed on two separate data sets, the first comprising both the South African and European populations (African-European data set) and the second comprising only South African populations (South African data set).

Modeltest V3.06 (Posada & Crandall, 1998) suggested that the most appropriate approach for estimating within-population genetic diversity for both data sets was the Tamura-Nei (TrN) model (Tamura & Nei, 1993). The estimated transition/transversion ratio was 11.048 for the African-European data set and 10.501 for the South African data set. Haplotype diversity (*h*) and nucleotide diversity (π) were calculated for each population using Arlequin V2.000 (Schneider *et al*., 2000).

A X^2 contingency test was performed to determine significant geographical associations using 1000 randomized permutations (Roff & Bentzen, 1989). In addition to this, a Mantel test (Mantel, 1967) was performed to test the null hypothesis of no association between the genetic distances obtained in Arlequin and the geographical distances between populations. One thousand randomized permutations were performed using Mantel V2.0 (Liedloff, 1999).

An analysis of molecular variance (AMOVA; Excoffier *et al*., 1992), using Arlequin, was performed to evaluate the degree of within-population and among population genetic differentiation for both data sets using pairwise F_{ST} statistics. This allowed estimation of the number of migrants between populations per generation. In addition, Migrate V1.7.6.1 (Beerli, 1997 – 2002) was used to calculate maximum likelihood estimates for migration rates among populations. This approach uses an expansion of coalescent theory that includes migration. The analysis on the African-European data set was inconclusive, and it was therefore decided to perform this analysis only on the South African data set. Migration was assumed to be symmetrical. One hundred short chains with 500 sampled genealogies each and three long chains with 5000 sampled genealogies each were run. One of every 20 reconstructed genealogies was sampled, therefore the total number of reconstructed genealogies was 10^4 for each short

chain and 10^5 for each long chain. Heating was set to adaptive, with temperature settings of 1.0, 1.2, 1.5 and 3.0.

Phylogeography and nested clade analysis (NCA)

TCS V1.13 (Clement *et al*., 2000) was used to estimate a single mutation haplotype genealogy (probability > 0.95) from DNA sequences for both data sets as described by Templeton *et al*. (1992). Identical sequences were collapsed to single haplotypes and haplotype frequencies were calculated for estimating haplotype outgroup probabilities, which correlate with haplotype age (Donnelly & Tavaré, 1986; Castelloe & Templeton, 1994). The haplotype network was converted into a nested series of clades using the nesting rules in Templeton *et al*. (1987) and Templeton & Sing (1993). After construction of the cladogram a NCA was performed, using the inference key of Templeton (2004) and Geodis V2.0 (Posada *et al*., 2000). This analysis attempted to discriminate between phylogeographical associations due to recurrent forces (e.g. gene flow, genetic drift and mating systems) vs. historical events (e.g. fragmentation or range expansion) at the population level (Templeton, 1998). The analysis performed on the African-European data set was inconclusive, mainly due to the large non-sampled area between South Africa and Europe. The European data were therefore excluded from this analysis. One thousand random permutations were performed to obtain statistical inferences at the 5% level.

Results

MtDNA haplotype variation

A 575 bp region of the targeted 728 bp region of the cytochrome *b* gene was used in the analyses (Table 4.1). The 161 crickets from South Africa and Europe yielded 31 haplotypes (Tables 4.2 & 4.3) defined by 35 polymorphic sites (Tables 4.1 & 4.2). Six haplotypes were found at two or more localities (h1, h2, h4, h6, h7 and h28; Table 4.3). The most frequently observed haplotype (h2, *n* = 71) was found in all the South African and European populations. The second most frequently observed haplotype (h1, *n* = 49) was found only in South Africa. Only two haplotypes were found in Europe (h2 and h31; Table 4.3). The first haplotype, namely h2, was the only haplotype found in Italy and was one of two Spanish haplotypes. The second haplotype, namely h31, was found in one individual in Spain.

Table 4.1. The 575 bp cytochrome *b* region for Pretoria male, G4. This sequence also represents haplotype 1. The 35 variable base pair positions reported on in Table 4.2 are indicated with asterisks and printed in bold

```
  *
TCATAGCTGCAGCTTTTATAGGATATGTATTACCATGAGGACAAATATCA
                              *   *    *
TTTTGAGGAGCTACTGTAATTACTAATCTTCTATCAGCAATTCCTTATTT
    *                                        *
AGGGACTGATTTAGTTCAATGAGTATGAGGAGGATTTGCAGTTGATAATG
      *                 *                    *
CCACACTAACTCGATTTTTTACATTCCATTTCATAATCCCATTTATCGTT
    *                    *          *
GCAGCATTCGTAATAATTCACTTACTTTTCCTTCACCAAACAGGATCTAA
    *                             *     *      *
CAACCCAATAGGAATTAATAGAAATCTAGATAAAATCCCATTCCATCCAT
     *               *   *                  *    *
ATTTTACTTTTAAGGACATCATAGGATTTTTAATCATACTAATATCATTA
  *
ACCATTTTATCACTTACAAATCCTTATTTATTAGGGGATCCAGATAATTT
           *                        *  *                    *
CACTCCTGCTAATCCTTTAGTTACCCCAGTTTCATATTCAACCAGAATGAT
                *       *    *    *          *
ATTTTCTTTTTGCTTTACGCCATTCTACGATCAATTCCTAACAAATTAGGA
                *                          *
GGAGTAATAGCTCTAATTGCATCTATTGCCATTCTATTTTTATTACCCTT
       *      *
TATATCCAATAATAAATTCCGAAGA
```

Table 4.2. Variable positions in the 575 bp of cytochrome *b* region defining the 31 haplotypes for *G. bimaculatus* sequenced from South Africa, Italy and Spain. h1 is the corresponding haplotype of Pretoria male G4 in Table 4.1

Haplotype	Nucleotide position
h1	c t t c g g c t c g c c c c t c t c c c c c t g t a t c c g t c c c t
h2	. . . . . t c
h3	. . . t . . . . . t c . . . . .
h4	. . . . . c . . . . .
h5	t . . . . . c . . t . . . . .
h6	 c
h7	. . . . . c . . . t . . . . . c . . . . .
h8	. . . . . t . . . . . a . . . . . t c
h9	. . . . . t . . . . .
h10	. . . . . c . . . t . . . . . c . . . . . t .
h11	. . . . c . . . . .
h12	. . . . . t . .
h13	. . . . . t . . . . .
h14	. . . . . c . . . t . t . . . . . c . . . . .
h15	. . . . . t . . . . .
h16	. . . . . t . . . . . t . . . . . t c
h17	. . c . . . . .
h18	. . . . . a . . . . . t . . . . . t c
h19	. . . . . t . . . . . t c
h20	. . . . . t . . . . .
h21	. . . . . t . . . . .
h22	. . . . . t . . . . . t . . . t c
h23	. . . . . c g . . . . c . . . .
h24	. . . . . t . . . . . t . . . . . t c
h25	. c . . . . . t . . . . . t c
h26	. . . . . c . t . . . . .
h27	a . . . . .
h28	. . . . . t c
h29	. . . . . t . t . . . . . c . . . . .
h30	. . . . . t . . . . . t . . c
h31	. . . . . a . . . . . t c

Table 4.3. The number of individuals sequenced (*n*) and the number of individuals representing each haplotype (h) are given below for each locality. The haplotype diversity (*h*) and nucleotide diversity (π) of the cytochrome *b* region of *G. bimaculatus* are also given for each locality

		Cytochrome *b* haplotype (h)																															
Locality	*n*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	*h*	π (SD)
Dullstroom	20	8	6	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0.720	0.058 (0.04)
Dundee	14	6	4	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0.714	0.059 (0.039)
Hotazel	20	9	4	0	0	1	0	0	1	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0.748	0.077 (0.048)
Makhado	20	5	7	0	0	0	1	2	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0.790	0.07 (0.044)
Paarl	20	8	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	2	0	0	0	0.755	0.056 (0.037)
Pretoria	20	10	4	0	1	0	1	2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0.690	0.059 (0.038)
Queenstown	19	3	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0.430	0.041 (0.029)
Italy	8	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.000	0.000 (0.000)
Sevilla	20	0	19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.095	0.004 (0.007)

Testing for mtDNA pseudogenes

Firstly, all the cytochrome *b* sequences translated successfully into proteins and no stop codons were encountered. Secondly, although isolated incidences of double peaks were encountered in some of the sequences, they did not occur consistently at the same positions of both forward and reverse sequences and it is suspected that these peaks were artifacts of the quality of the sequencing reaction itself. Thirdly, when the sequences obtained through CsCl extractions were compared with those from genomic DNA extractions, not a single inconsistency was found between the two sources of DNA. Fourthly, a large degree of sequence variation was not observed, comparable to those found in the nuclear copies of mitochondrial genes in grasshoppers (Zhang & Hewitt, 1996). The ITS primers yielded a good PCR product when using genomic DNA extractions. On the other hand, the CsCl extractions did not allow amplification of the ITS gene in any of the five individuals tested, suggesting that the purified mtDNA extraction was free of nuclear DNA.

Within-population genetic differentiation

Haplotype diversity (h) and nucleotide diversity (π) differed largely among populations (Table 4.3), with the lowest haplotype and nucleotide diversity values observed in the European populations. Nucleotide diversity in the total data set was 6.09%. For South Africa, the Hotazel population had the highest nucleotide diversity and Queenstown the lowest nucleotide diversity, indicating a large degree of within-population differentiation in the Hotazel population and a small degree of within-population differentiation in the Queenstown population. The low nucleotide diversity in the Queenstown population is also reflected in its haplotype diversity. Six of the seven South African populations had haplotype diversities larger than 0.69, indicative of a high degree of overall within-population genetic diversity. The low nucleotide diversity of the European populations was also reflected in the haplotype diversities and indicated a small degree of within-population genetic differentiation. The current effective maternal population sizes (estimated using Migrate; Table 4.4) reflected the degree of within-population differentiation (based on nucleotide diversity; Table 4.3) in some of the South African populations. While the Queenstown population had the smallest effective population size and the lowest nucleotide diversity, the Dullstroom population had

the largest effective population size and its nucleotide diversity was almost similar to three other populations.

Table 4.4. The estimated migration rates per generation between South African populations of *G. bimaculatus* derived from a maximum likelihood estimation (Beerli, 1997-2002). Migration was assumed to be symmetrical. '*n*' indicates the number of individuals sequenced at each locality

		Effective population size	Migration rate (4Nm)					
Population	*n*	(θ = 4Ne mu)	Dullstroom	Dundee	Hotazel	Makhado	Paarl	Pretoria
Dullstroom	20	0.01141						
Dundee	14	0.00587	37.79690					
Hotazel	20	0.00141	38.43680	0.02190				
Makhado	20	0.00037	61.97450	16.54800	0.01250			
Paarl	20	0.00088	86.76480	0.00630	22.29770	0.21390		
Pretoria	20	0.00170	58.82090	0.00017	0.02180	0.17910	58.21410	
Queenstown	19	0.00010	10.35310	0.01490	2.10070	0.31780	0.77200	0.00002

Between-population genetic differentiation

Although there was a larger degree of between-population genetic differentiation in the African-European data set than in the South African data set, the overall patterns of gene flow between the South African populations were the same for both analyses. For the African-European data set, AMOVA indicated that 80.51% of the genetic variation occurred within populations and 19.49% among populations, revealing significant between-population genetic differentiation (F_{ST} = 0.195, P < 0.001). For the South African data set, AMOVA also revealed significant between-population genetic differentiation (F_{ST} = 0.049, P < 0.05) and indicated that 5% of the genetic variation occurred among populations and 95% of the variation occurred within populations. As expected, the estimated number of migrants per generation was higher between the South African populations than between the South African and European populations (Table 4.5). The results of the Roff-Bentzen test supported that of the AMOVA. There was significant geographical differentiation in the African-European data set (observed X^2 = 271.5, P < 0.05, n = 161) as well as within the South African data set (observed X^2 = 190.19, P < 0.05, n = 133). This was probably due to the small number of haplotypes in Europe compared with South Africa in the African-European data set, and the large number of haplotypes found in single South African populations in the South African data set (Table 4.3). Three possible migration routes were suggested within South Africa, based on the migration estimates of Migrate (Maximum log likelihood = 3.585; Table 4.4). It was assumed that a migration route existed among populations if the migration rates among these populations were higher compared with other populations. Firstly, large degrees of gene flow occurred among the populations in the northeastern region of South Africa (Dullstroom, Dundee, Makhado and Pretoria). Secondly, migration took place among the Hotazel, Paarl and Queenstown populations that are situated in the southern and western regions of the country. Lastly, a smaller degree of migration occurred between the southern and northeastern regions of the country.

Table 4.5. Population pairwise F_{ST} statistics (Tamura-Nei distance parameter) for the South African and European populations of *G. bimaculatus*, derived from AMOVA (lower diagonal area of the table). Asterisks indicate significant F_{ST} statistics ($P < 0.05$ *, $P < 0.01$ ** and $P < 0.001$ ***), while negative values indicate a larger degree of within-population variation than between-population genetic differences. The upper diagonal area of the table shows the estimated number of migrants per generation (2Nm). 'Inf' indicates an infinite number of migrants per generation among populations

	Population pairwise F_{ST}'s and number of migrants per generation								
Population	Dundee	Dullstroom	Hotazel	Italy	Makhado	Paarl	Pretoria	Queenstown	Sevilla
Dundee		749.090	Inf	0.515	22.134	Inf	48.022	1.039	0.332
Dullstroom	0.001		Inf	0.629	Inf	Inf	89.959	1.585	0.444
Hotazel	-0.015	-0.016		0.703	51.417	Inf	Inf	1.278	0.494
Italy	0.493***	0.443***	0.416***		1.218	0.444	0.559	3.421	Inf
Makhado	0.022	-0.038	0.010	0.291***		Inf	52.020	3.157	0.842
Paarl	-0.092	-0.054	-0.181	0.53***	-0.156		Inf	1.406	0.315
Pretoria	0.010	0.006	-0.025	0.472***	0.010	-0.054		0.998	0.395
Queenstown	0.325***	0.24***	0.281**	0.128	0.137	0.262*	0.333***		2.171
Sevilla	0.601***	0.53***	0.503***	0.000	0.373***	0.613***	0.559***	0.187*	

Isolation by distance

No significant isolation-by-distance effect was observed among the South African populations (Mantel test: g = 0.306, r = 0.096, $P > 0.05$, $n = 133$), suggesting that, although there was significant between-population genetic differentiation, simple isolation by geographic distance was not the major determinant of these genetic differences. A significant isolation-by-distance effect was observed for the African-European data set (Mantel test: g = 3.607, r = 0.776, $P < 0.05$, $n = 161$), suggesting that, on a broader scale, isolation by geographic distance was a determinant of between-continent genetic differentiation.

Phylogeography and nested clade analysis

Haplotype networks constructed for both spatial data sets showed large similarities for the South African populations. Since inclusion of the European data did not result in robust conclusions for the African-European data set, only the results of the South African data set will be discussed.

The haplotype network included a loop, and two alternative cladograms were constructed that comprised two 2-step clades (two-step cladogram; Figure 4.2a) and two 3-step clades (three-step cladogram; Figure 4.2b) respectively, depending on where the loop was broken.

Two-step cladogram: The cladogram consisted of 30 haplotypes, nine one-step clades and two 2-step clades. Clades 2-1 & 2-2 included haplotypes from all the South African populations. Clade 2-1 mostly comprised haplotypes from the southern region of South Africa (Hotazel, Paarl and Queenstown), while clade 2-2 mostly comprised haplotypes from the northeastern region (Dullstroom, Dundee, Makhado and Pretoria).

Three-step cladogram: This cladogram differed from the 2-step cladogram by having three 2-step clades and two 3-step clades. Although clade 1-3 was now included in clade 3-1, this had a minor influence on the spatial representation of the populations. Most of the populations from the southern region of South Africa were nested as clade 3-1 and the populations from the northeastern region nested as clade 3-2.

The nested analysis showed significant differences for within-clade (*Dc*) and nested clade (*Dn*) distances (Appendix 1) for both cladograms.

Two-step cladogram: Significant geographical associations were only observed at the highest level (X^2 = 16.23, P = 0.01). Historic events rather than recurrent forces were the most likely explanation for the patterns in the clades. Restricted gene flow with isolation by distance was the most likely explanation for the pattern in clade 1-1. Clade 1-1 included individuals from all the South African populations (the furthest distance between two populations being 1770 km and closest distance being 220 km; Figure 4.1a), suggesting that, from a spatial distribution point of view, an isolation-by-distance effect be the best explanation for the pattern of gene flow. Within clade 1-1, the interior haplotype (assumed to be the oldest haplotype) represented all of the South African populations. The majority of the Queenstown individuals (n = 14) were represented by this interior haplotype. The tip haplotypes represented all of the South African populations, except the Dundee population, suggesting that although there were old haplotypes present in the populations, derived haplotypes evolved throughout most of the geographical distribution (Figure 4.2a). Restricted gene flow with isolation by distance was the most likely explanation for the pattern in clade 2-2. Clade 2-2 also comprised individuals from all of the South African populations and the same reasoning as for clade 1-1 would probably account for the pattern of gene flow in this clade, with the exception that the tip clades in clade 2-2 represented all of the South African populations. Clade 1-9 also had significant genetic structure but the sampling design was inadequate to discriminate between isolation by distance versus long distance dispersal. The geographical sampling was inadequate to explain the pattern in clade 1-2.

Three-step cladogram: Significant geographical associations were observed at the total cladogram level (X^2 = 12.21, P < 0.05). Clades 1-1, 1-2 & 1-9 comprised the same haplotypes as in the two-step cladogram, therefore yielding the same inferences. Inferences for clades 2-2 & 3-1 were inconclusive. Restricted gene flow with isolation by distance was the most likely explanation for the pattern in clade 2-3. The oldest interior clade (1-9) and the younger tip clades comprised haplotypes from all the South African populations. However, with the exception of clade 1-8, the geographical representation of the tip clades were restricted to certain regions of the country (clades 1-4 & 1-5 represented the northeastern region) indicating restricted gene flow in certain areas of the country.

Both cladograms indicated that, due to restricted gene flow between the South African populations, gene flow mainly occurred within two regions of the

country namely the northeastern (represented by clade 1-9) and southeastern (represented by clade 1-1) regions.

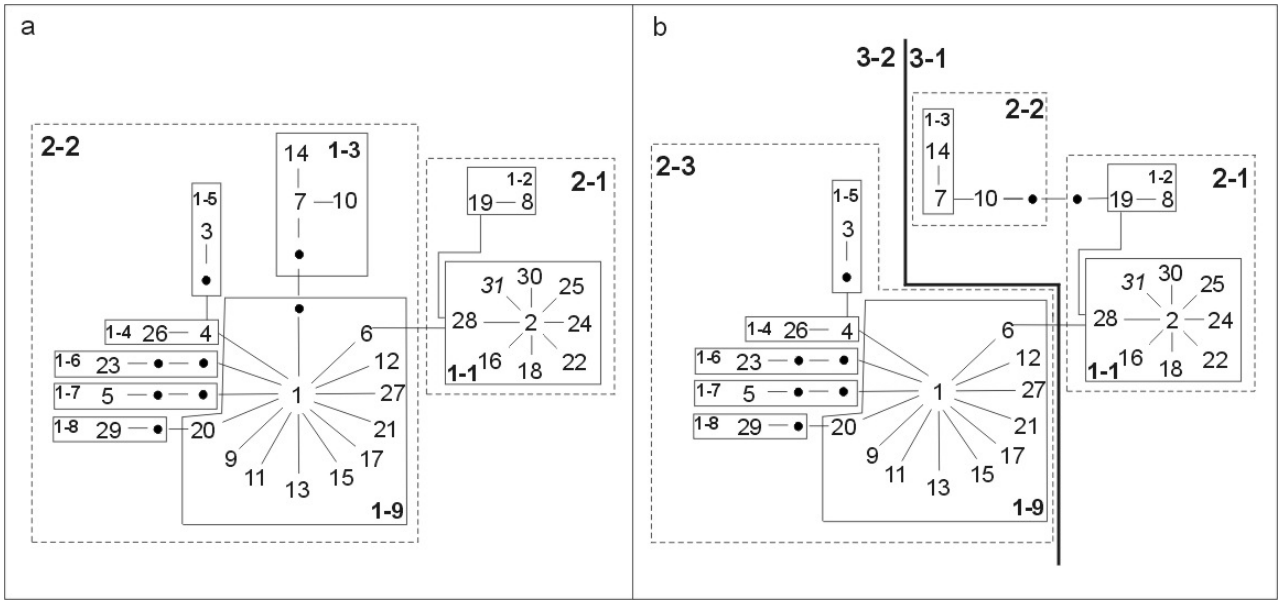

Figure 4.2. The cladogram and associated nested design for cytochrome *b* haplotypes from seven South African populations of *G*. *bimaculatus* based on the statistical parsimony of Templeton *et al*. (1987) and Templeton & Sing (1993). Haplotypes from Italy and Spain were excluded form the NCA due to large non-sampled areas between South Africa and Europe. The unique haplotype (h31) from the Spanish population is indicated in italics on the network (clade 1-1) and is only intended for explanatory purposes. Separate analyses performed to accommodate an internal loop in the haplotype network resulted in (a) a 2-step cladogram and (b) a 3-step cladogram. Haplotype numbers do not contain the prefix ‘h’ and are separated by one mutational step. Hypothetical haplotypes are indicated by a black dot. Numbers in bold indicate clade numbers and thin line boxes enclose one-step clades, dotted line boxes enclose two-step clades and the thick line indicates three-step clades for the nested clade analysis.

Discussion

Nuclear copies of the mtDNA have been identified in several orthopteran taxa, resulting in highly variable DNA sequences of particular genes (Bensasson *et al*., 2000; Zhang & Hewitt, 1996). Purified mtDNA sequences of five individuals matched their genomic DNA sequences, indicating that the results in this study reflected variation at the mitochondrial cytochrome *b* locus of *G. bimaculatus*, and that no nuclear products were amplified with the cytochrome *b* primers used in this study.

Genetic differentiation within South Africa and Europe

The degree of within-population genetic variation was much higher in the South African populations than in the European populations (Table 4.5). In Europe, severe regional seasonal changes in climate probably caused irruptive cricket populations in the warmer summer months with only a small portion of the population surviving through the colder winter months, consequently, reducing genetic variation (Table 4.5). For Italy and Sevilla, the coldest winter month had a 24-hour mean temperature of 1.3°C at Bologna (period 1808 – 1990), 8.7°C at Genova (period 1981 – 1990) and 10.7°C at Sevilla (period 1951 – 1990), while the hottest summer month had a 24-hour mean temperature of 25°C at Bologna (period 1808 – 1990), 24.6°C at Genova (period 1981 – 1990) and 26.7°C at Sevilla (period 1951 – 1990) (www.worldclimate.com). In addition, one would expect crickets in Europe to have fewer generations per year than crickets in South Africa, due to the less favourable climatic conditions in Europe (www.worldclimate.com). This could in turn contribute to the reduced genetic variation in the European populations compared to the larger degree of genetic variation in the South African populations (Table 4.5). On the other hand, South African populations experience less severe regional seasonal and annual climate changes (Schulze, 2001) that probably ensure a high survival rate through the winter months, resulting in higher haplotype and nucleotide diversities compared to the European populations. The coldest winter month had a 24-hour mean temperature of 12.4°C at Paarl (period 1994 – 2002), 11.2°C at Pretoria (period 1960 – 1991) and 10.5°C at Queenstown (period 1940 – 1991), while the hottest summer month had a 24-hour mean temperature of 25.4°C at Paarl (period 1994 – 2002), 22°C at both Pretoria (period 1960 – 1991) and Queenstown (period

1940 – 1991) (www.worldclimate.com). Although regional climate conditions potentially influenced the population dynamics, other factors such as food availability and rainfall probably also influenced population numbers, and therefore genetic diversity in this cricket.

Gene flow and migration rates among the South African and European populations

For the South-African data set, a large amount of gene flow occurred among populations, with the exception of Queenstown where only a small amount of gene flow occurred between Queenstown and the other South African populations (Table 4.5). Both the maximum likelihood estimation and the NCA revealed two similar general migration routes within South Africa (Table 4.4; Appendix 1). Firstly, migration occurred among the populations situated in the northeastern regions (Appendix 1) and among the populations situated in the southeastern regions (Appendix 1), corresponding largely to different climatic regions (Preston-Whyte & Tyson, 1988; Schulze, 1994). While the northeastern region of South Africa is categorised as a summer rainfall area with a high annual rainfall, the southern and western regions are categorised as a winter rainfall area with drier conditions (Preston-Whyte & Tyson, 1988; Schulze, 1994). Secondly, migration occurred along a North-South gradient among the northeastern and southeastern regions. One of the major inland road transport routes in South Africa follows a southwest to northeast direction (Brett & Mountain, 1997) and could incidentally be a major cause of the intraspecific gene flow within South Africa. Indeed, it is known that *G. bimaculatus* were accidentally transported from the Western Cape province to the Gauteng province in South Africa by road, covering a distance of more than 1000km (Spinman, 2005). For the African-European data set, there was no between-population genetic differentiation among the Italian and Spanish samples due to the large estimated number of migrants that migrate between these countries (Table 4.5). Although this number of migrants is based on the AMOVA's F_{ST} estimates that have been criticized by Whitlock & McCauley (1999), migration rates could not be obtained with Migrate since this study only included two populations from Europe and there are large unsampled areas between South Africa and Europe. In addition, more extensive sampling is required for a better understanding of cricket movements in Europe. As expected, the South African populations were significantly different from the European populations, except for

the Queenstown population (Table 4.5). Transport routes connecting South Africa and Europe through air and sea could increase the chances of inter-continental gene flow in *G. bimaculatus*, thereby maintaining the genetic diversity in this species. Although it is rare for these insects to fly long distances, swarms of *G. bimaculatus* were reported to land on ships passing the West coast of Africa on route from the Canary Islands to South Africa, the furthest ship being 933 km from land (Ragge, 1972).

Demographic processes explaining the genetic differentiation within southern Africa

The significant between-population genetic differentiation in South Africa was reflected by the large number of haplotypes from single localities (26 of 31 haplotypes). This is similar to the results of Willett *et al*. (1997) who found that, for the COI-COII mtDNA gene in *G. firmus* and *G. pennsylvanicus*, all except one of the mtDNA haplotypes were restricted to single localities. The Mantel test (correlating genetic distances with linear geographic distances) indicated that a simple model of isolation by distance was not suitable for explaining the geographical differentiation within South Africa. A more complex model is therefore required. Following the NCA, restricted gene flow with isolation by distance was the most likely explanation for the gene flow patterns for genetically differentiated populations from South Africa (Appendix 1). The inference of large-scale gene flow between some of the populations is supported by the migration estimates from AMOVA and maximum likelihood estimates (Tables 4.4 & 4.5). Geographical genetic differentiation could therefore be brought about by a combination of isolation by distance overlaid by unequal gene flow between geographical areas.

Maintenance of genetic variation in G. bimaculatus

Europe experiences severe seasonal changes that probably lead to large fluctuations in population densities, and could in turn, give rise to the small degrees of within-population genetic variation in the Italian and Spanish populations. One South African population, namely Queenstown, also has a small degree of within-population genetic variation. Crickets were not always abundant at Queenstown, and it is therefore possible that large fluctuations in this population's density might cause the small degree of within-population genetic

variation in Queenstown. On the contrary, there are large degrees of within-population genetic variation within the other South African populations. This suggests that a large gene pool of *G. bimaculatus* is potentially maintained in this region, and that the large amounts of gene flow that occur between populations probably maintain the genetic diversity across the geographical range of this species. It is also suggested that between-population variation in the phenotypic traits of *G. bimaculatus* reflect the between-population genetic variation among South African and European populations.

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CHAPTER 5

Environmentally-induced geographical variation in the calling song of the field cricket, *Gryllus bimaculatus* De Geer (Orthoptera: Gryllidae)

Abstract

I investigated geographical variation in the calling song and morphometrics of ten wild-caught and six captive-reared F_1 African and European populations of the field cricket, *Gryllus bimaculatus*. Inter-continental and regional variation in the calling song and morphometrics of *G. bimaculatus* is probably not explained by an isolation-by-distance effect. The call and morphological traits differ more within-regions than between-continents, reflecting the large amount of gene flow that occurs among the continents. A significant part of the geographical variation is probably environmentally induced, based on the following findings: (a) except for the Queenstown population, between-population differences observed in wild-caught populations are not evident in captive-reared F_1 populations, (b) captive-reared F_1 populations have smaller degrees of variances in their phenotypic traits and (c) there is no correlation of traits from wild-caught populations with traits from their captive-reared F_1 offspring. The large degree of between-population differences in the calling song of *G. bimaculatus* can compromise the communication system in this cricket. It is therefore suggested that female preference studies be conducted to investigate the evolutionary implications of between-population differences of the male calling song of *G. bimaculatus*.

Keywords: *Gryllus bimaculatus*, Calling song, Morphology, Inter-continental variation, Between-population variation, Environmental effects, Communication system

Introduction

Calling songs, emitted by male crickets, are species-specific, under neurological control (Bennet-Clark, 1989; Huber, 1962; Huber & Thorson, 1985; Schildberger *et al*., 1989) and elicit phonotaxis by females (Alexander, 1962). Despite the importance of the calling song in mate recognition, there is significant within-male, within-population and between-population variation in the calling song of the field cricket, *Gryllus bimaculatus* (Ferreira, 2006a, b, Chapters 2 & 3; Ferreira & Ferguson, 2002). Several authors have suggested that comparative information about the between-individual variability and between-population variability in calls across a species range is needed (Asquith *et al*., 1988; Zuk *et al*., 2001) and Zuk *et al*. (2001) emphasized that geographical variation is one of the most important sources of variation. The calling song is regarded as one of the most important premating barriers in crickets (Otte, 1989), and it is therefore important to have an estimate of between-population variability in this song across a species range. If differences in the sexual signaling system is due to between-population genetic differences, it could give rise to prezygotic isolation and one needs to understand the mechanisms underlying geographical differentiation in this system (Butlin, 1996). In addition, evolutionary forces act on communication systems (Ryan & Wilczynski, 1988) and Ryan & Rand (1995) suggested that it is important to have an understanding of signal variation and its influence on the behavioural response of the receiver. According to Endler & Houde (1995), geographical variation in the calling song could give rise to correlated female preferences, but Zuk *et al*. (2001) suggested that rapidly evolving populations with different signals can resist differentiation if gene flow among populations is maintained. However, this was not the case in the planthopper, *Nilaparvata lugens*, where genetic drift was suggested to be the most probable cause for male signal-female preference divergence (Butlin, 1996). According to Paterson (1985), there should be stabilizing selection on call traits involved in mate recognition. Geographical variation in these traits should be environmentally induced and not genetically induced (Butlin, 1995), since these traits are under stabilizing selection while a species occupies its normal habitat (Paterson, 1985).

Geographical variation in advertisement calls of anurans and crickets could be explained by isolation by distance effects, different environmental conditions as

well as between-population genetic differences. An isolation by distance effect was the main cause of geographical variation in the advertisement song of the túngara frog, *Physalaemus pustulosus*, (Ryan *et al*., 1996) and the mating call of the green treefrog, *Hyla cinerea* (Asquith *et al*., 1988). Different environmental conditions (open vs. forest habitat) experienced by populations of the cricket frog, *Acris crepitans*, explained between-population differences in call traits (Ryan & Wilczynski, 1991). Between-population genetic differences were the main cause of clinal variation in the advertisement call of *A*. *crepitans* (Ryan & Wilczynski, 1991) and geographical variation in the calling song of the field cricket, *Teleogryllus oceanicus* (Zuk *et al*., 2001). However, some studies could not explain geographical variation found in some frog and cricket advertisement calls. Geographical variation in the advertisement call of the frog *Leptodactylus fuscus* did not coincide with genetic variation or geographic distance (Heyer & Reid, 2003) and it is unclear whether the large degree of geographical variation found in the syllable number of the calling song of the bushcricket, *Ephippiger ephippiger*, is environmentally or genetically induced (Ritchie, 1992).

Ferreira & Ferguson's study (2002) on geographical variation in the calling song of *G. bimaculatus* mainly focused on the influence of geographical variation on species recognition and sexual selection call traits, and did not provide any explanation for between-population differences in the call traits or morphometrics. Their study only included four populations from South Africa. This study, on the contrary, investigates geographical variation in the calling song and morphometrics of *G*. *bimaculatus* on an inter-continental and regional scale by investigating wild-caught and captive-reared crickets from two European regions and eight South African localities.

The aims of this study are:

1. To relate geographical variation in the calling song and morphometrics of *G. bimaculatus* to geographic distance between populations as well as between-population genetic differences and gene flow estimates, following the results of Ferreira (2006c, Chapter 4).
2. To infer to what degree the between-population differences in the calling song and morphometrics of field crickets could be environmentally induced by comparing the variances in the calling song characteristics and morphometrics

of wild-caught populations from South Africa and Europe with their captive-reared F_1 offspring.

Materials and methods

Geographical variation in calling song characteristics and morphometric measurements of ten wild-caught populations

Sampling localities

During 2001 – 2003, wild field crickets, *G. bimaculatus*, were captured in South Africa (Figure 5.1a) at Dullstroom, Dundee, Hotazel, Makhado, Paarl, Pretoria, Queenstown and Wolmaransstad (27°23'S, 26°00'E). During 2003, wild field crickets were captured in Italy at Genova, Modigliana, Peccioli and Russi (Figure 5.1b) and in Spain at Sevilla (Figure 5.1b). Longitudes and latitudes of these localities are provided in Ferreira (2006c, Chapter 4). Due to the small sample sizes at each of the Italian localities, the four Italian populations were combined to form one Italian sample.

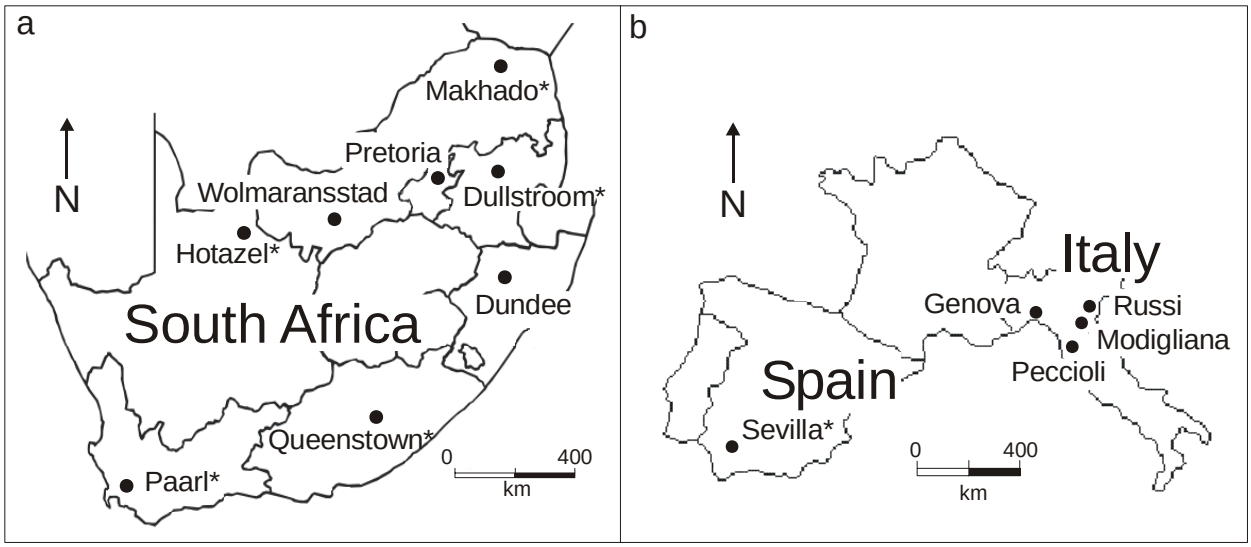


Figure 5.1. Sampling localities of wild-caught *G. bimaculatus* in (a) South Africa and (b) Europe. '*' indicates the localities of wild-caught crickets for which captive-reared F_1 offspring were reared.

Calling song recordings

Males were placed in sound damped recording chambers where the calling song of each male was recorded. Recording chambers (31 cm x 35 cm x 26 cm; acoustic isolation 50.13 ± 1.73 dB (mean ± SE) between neighbouring chambers) were maintained at 25°C ± 1°C using an electronically controlled heater. Each chamber was sound damped with a glass fibre mat (thickness 5 cm) and equipped with a Beringer XM200S dynamic microphone (50 Hz – 10 kHz ± 3 dB; Behringer Spezielle Studiotechnik GmbH, Germany). A Sony TC-D5M cassette recorder was used to record the calling songs. Each male was placed in a gauze container (11 cm x 11 cm x 12 cm) with a cardboard floor and a small piece of egg carton for shelter. High protein cereal (Pronutro®) and water were provided *ad libitum*. A 12h:12h Light:Dark (L:D) light regime was implemented using an 8000 mCd light-emitting diode above each gauze container. Three sound recordings of 30 seconds duration each were made per night for at least two nights (i.e. a total of six recordings) with between-recording intervals of at least five minutes.

Calling song characteristics and morphometric measurements

Canary V1.2.4 (Cornell Laboratory of Ornithology, Ithaca, New York) was used on an Apple Macintosh computer for spectrographic analysis of the calls. A power spectrum was generated to measure calling song frequency with maximum amplitude (F_{max}), bandwidth at 10 db below maximum amplitude (Fwidth-A) and bandwidth at 20 db below maximum amplitude (Fwidth-B) for each recording. An oscillogram was used to measure number of syllables per chirp (S_C), syllable period (SP), syllable duration (SD), inter-syllable interval (ISI), chirp period (CP), chirp duration (CD) and inter-chirp interval (ICI) for each recording. For formal definitions of these traits see Ferreira (2006a, Chapter 2). Three consecutive chirps were measured from each recording. A mean was calculated for the six recordings for each of the call traits. Each chirp comprised three to six syllables (Desutter-Grandcolas & Robillard, 2003). Calling songs of 314 males were recorded as described above (Dullstroom: *n* = 24, Dundee: *n* = 17, Hotazel: *n* = 16, Italy: *n* = 9, Makhado: *n* = 49, Paarl: *n* = 30, Pretoria: *n* = 102, Queenstown: *n* = 28, Wolmaransstad: *n* = 19 and Sevilla: *n* = 20). After killing a male with ethyl acetate its head length, head width, thorax length, thorax width, thorax area (thorax length x thorax width), length of the right hind femur (Fem-R) and length of

the right hind tibia (Tib-R) were measured using the VideoBlaster FS200 video kit (Creative Laboratories, Singapore; resolution = 16 microns). After the body measurements were taken, the wings of each male were removed and three mirror measurements (M4_M8, M6_M8 and H1_M9) and five harp measurements (H1_H2, H2_H3, H1_H3, H10_H11 and harp size) of each right wing were measured following Ferreira & Ferguson (2002) and Ferreira (2006a, Chapter 2).

Geographical variation in calling song characteristics and morphometric measurements of six captive-reared F_1 populations

Separate breeding colonies of six wild caught populations (Dullstroom, Hotazel, Makhado, Paarl, Queenstown and Sevilla) were established in a climate room at 27°C ± 2°C with a 12h:12h L:D light regime. Each breeding colony was maintained in a 100-litre plastic container and were provided with egg cartons for shelter, petri dishes filled with cotton wool serving as egg laying trays, high protein food (Pronutro® and fish flakes) and water *ad libitum*. The egg laying trays of each colony were collected on a weekly basis and placed separately in labeled 2-litre plastic containers. Each breeding colony's F_1 first instar nymphs were transferred to a 100-litre plastic container and reared until final instar was reached. Twenty-five of these penultimate instar males were randomly chosen from each of the F_1 colonies and placed individually in labeled 2-litre plastic containers. Males were checked daily and their calling songs were recorded ten days after final ecdysis.

Calling songs of 118 males were recorded as described above (Dullstroom: n = 22, Hotazel: n = 14, Makhado: n = 18, Paarl: n = 23, Queenstown: n = 21 and Sevilla: n = 20). After obtaining the recordings, call traits and morphometric measurements were measured as described above.

Statistical analyses

Analyses were performed on two separate data sets. The first data set comprised wild-caught crickets and the second comprised captive-reared F_1 crickets. Unless indicated otherwise, the wild-caught data set comprised ten populations and the captive-reared F_1 data set comprised six populations.

SAS V8.02 (Proc GLM; SAS Institute Inc., Cary, NC, USA) was used to perform a univariate analysis of variance (ANOVA) to compare the means of the

call traits as well as the means of five morphometric measurements (thorax area, thorax length, thorax width, Fem-R and harp size) between the populations. Tukey HSD *post-hoc* analyses were performed to determine which populations differed significantly from one another. A Bonferroni adjustment (Rice, 1989) was applied to the Tukey HSD *post-hoc* results of both data sets. A random effects ANOVA (Proc Varcomp; SAS) was performed on the wild-caught data set to determine the between-continent, within-continent and error variance components for the call and morphological traits used in the univariate ANOVA. Principal components analysis (PCA: varimax rotation on normalised data) was used, with Statistica V5.5 (StatSoft, Inc. (1999), Tulsa, USA), to determine which call traits and morphometric measurements (excluding thorax length and thorax width, due to the inclusion of thorax area) contributed most to the total variation of each data set. Forward stepwise discriminant function analysis (DFA), with Statistica, was used to determine which call traits could be used to discriminate between populations.

A cluster analysis was performed in Statistica to create Euclidian distance matrixes (Sokal & Sneath, 1963) for both data sets utilizing either ten call traits and five morphometric measurements (thorax area, thorax width, thorax length, Fem-R and harp size) or only ten call traits. Distance matrixes of the ten wild-caught and six F_1 populations were then compared with geographic distances between populations (in km) using a Mantel test (Mantel, 1967), within Mantel V2.0 (Liedloff, 1999) with 1000 permutations. These Mantel tests were performed to determine whether crickets from closely located populations have more similar call structures and morphometrics than crickets that are located further apart. A Euclidean distance matrix was then created for nine wild-caught populations (Dullstroom, Dundee, Hotazel, Makhado, Pretoria, Paarl, Queenstown, Italy and Sevilla). The Wolmaransstad population was excluded from this specific data set, since this population was not included in the genetic study of Ferreira (2006c, Chapter 4). The distance matrixes and F_{ST} statistics (Tamura-Nei distance parameter: Ferreira, 2006c, Chapter 4) of nine wild-caught and six F_1 populations were compared respectively, using Mantel with 1000 permutations, to determine whether populations with large amounts of between-population gene flow have crickets more similar in call structure and morphometrics than populations with smaller amounts of between-population gene flow.

Following the results of the univariate ANOVA, the overall variance and within-population variance were calculated. Overall variance was calculated for all individuals across all populations. A random effects ANOVA was performed to determine the between-population variance and within-population variance component for the call traits (Proc Varcomp; SAS).

A two-way fixed effects ANOVA was performed on each of the ten call traits and five morphometric measurements, with Statistica, of the six wild-caught populations and their six captive-reared F_1 populations, to determine the effect of locality and captivity on these traits. This was followed by Tukey HSD *post-hoc* comparisons. A Bonferroni adjustment (Rice, 1989) was applied to the significant ($P < 0.05$) *post-hoc* comparisons.

A Pearson product moment correlation was performed, with Statistica, to determine any correlation of the call traits and five morphometric measurements of six wild-caught populations (Dullstroom, Hotazel, Makhado, Paarl, Queenstown and Sevilla) with their captive-reared F_1 offspring.

Results

Ten wild-caught populations

Between-population differences in call traits and morphology of wild-caught males

Univariate ANOVA showed that there were significant between-population differences for all the morphometric measurements and the majority of the call traits (Table 5.1). Tukey *post hoc* comparison, after a Bonferroni adjustment, revealed that the call traits differed significantly among several South African populations, while the morphometric measurements mainly differed significantly between the South African and Italian populations (Table 5.2). Wild-caught populations from the northeastern part of South Africa differed significantly from those in the southwestern parts (Table 5.2). These patterns are consistent with gene flow estimates (Ferreira, 2006c, Chapter 4). For the chirp traits, the Queenstown and Paarl populations differed significantly from the other South African populations. The Queenstown population had a significantly shorter chirp duration and the significantly longer inter-chirp interval of the Paarl population gave rise to the significantly longer chirp period of this population (Figure 5.2). For the syllable and spectral call traits, the Hotazel population differed significantly from most of the other populations, while the Queenstown population differed

significantly from all of the other populations. The Hotazel population produced a significantly longer syllable duration that gave rise to the significantly longer syllable period compared with the shorter syllable periods of the other populations (Figure 5.2). On the other hand, the Queenstown population produced a significantly shorter syllable duration that gave rise to the significantly shorter syllable period (Figure 5.2). While the Queenstown population produced a significantly higher calling song frequency than the other populations, the Hotazel population produced a significantly lower calling song frequency (Figure 5.2). For the morphometric measurements, the Italian population had a significantly larger body size than the other populations, while the Queenstown and Paarl populations had significantly smaller harp sizes compared with the other populations (Figure 5.2).

Between-continent variation and within-continent variation in call traits and morphology of wild-caught males

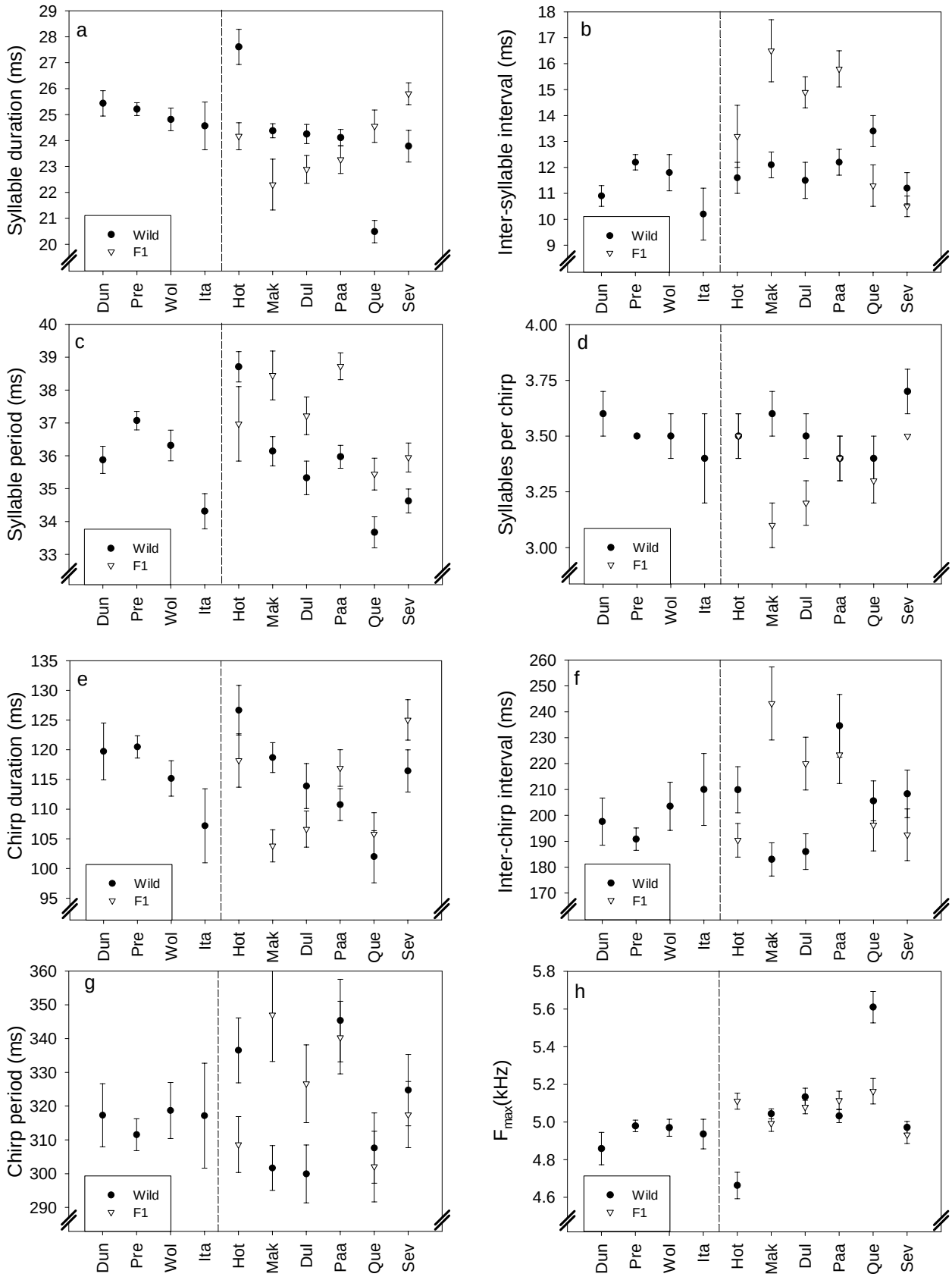
Only bandwidth and inter-syllable interval had a larger between-continent than within-continent variance component (Table 5.1). The within-population variance component (i.e. error variance %) contributed the most to the variation in the call and morphological traits (Table 5.1).

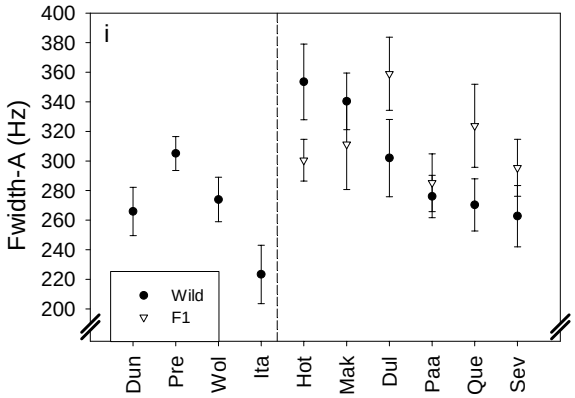
Table 5.1. Results of a univariate ANOVA and a random effects ANOVA of call traits and morphological measurements of *G. bimaculatus* from South Africa and Europe. The univariate ANOVA was performed on two data sets, the first comprised ten wild-caught populations (10 Wild) and the second comprised six captive-reared F_1 populations (6 Captive). Significance values are indicated by asterisks: $P < 0.05$ *, $P < 0.01$ ** and $P < 0.001$ ***. The random effects ANOVA was performed on ten wild-caught populations

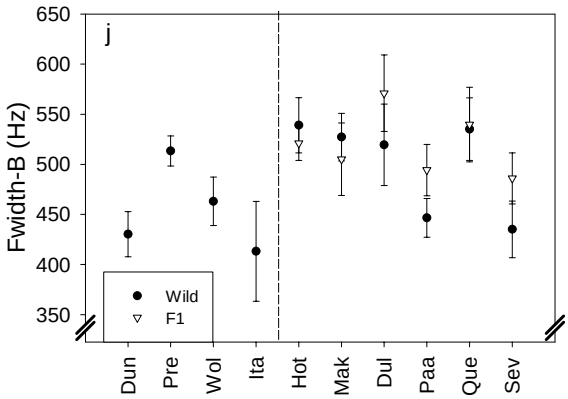
	Univariate ANOVA		Random effects ANOVA		
	F statistic		Variance components (10 Wild)		
	10 Wild	6 Captive	Between continents %	Within continents %	Error %
Variable	$F_{9,311}$	$F_{5,112}$			
S_C	1.03	4.03**	0.00	0.28	99.72
CD (s)	3.91***	6.41***	0.00	8.54	91.46
ICI (s)	3.82***	3.575**	0.00	8.77	91.23
CP (s)	2.55**	2.47*	0.00	4.13	95.87
SD (s)	14.91***	4.29**	0.00	24.13	75.87
SP (s)	7.99***	4.85***	0.66	17.21	82.13
ISI (s)	1.65	9.22***	5.85	0.28	93.87
F_{max} (kHz)	18.07***	3.056*	0.00	24.13	75.87
Fwidth-A (kHz)	2.72**	1.322	7.72	2.79	89.49
Fwidth-B (kHz)	2.37*	1.053	9.41	1.66	88.94
Thorax area (mm^2)	5.88***	9.74***	0.00	8.45	91.55
Thorax length (mm)	3.42***	9.22***	0.60	4.68	94.72
Thorax width (mm)	5.69***	7.22***	0.00	7.68	92.32
Femur-R (mm)	6.08***	$F_{5,109}$: 6.56***	5.46	17.00	77.53
Harp (mm^2)	6.87***	$F_{5,110}$: 3.52**	0.00	13.69	86.31

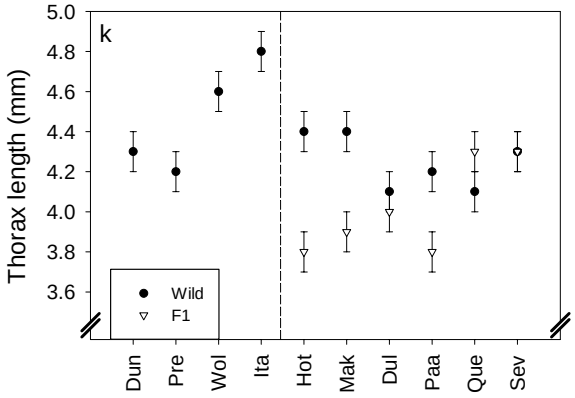
Table 5.2. Results of Tukey *post-hoc* comparison tests, after Bonferroni adjustments, showing populations that differed significantly ($P < 0.05$) for each of the variables subjected to the univariate ANOVA in Table 5.1. To save space the following abbreviations are used and only apply here: traits are referred to as being related to chirps (prefix C), frequency (prefix F), syllables (prefix S) or morphology (prefix M): chirp traits = C_1: S_C, C_2: CD, C_3: ICI, C_4: CP; syllable traits = S_1: SD, S_2: SP, S_3: ISI; frequency traits = F_1: F_{max}; morphometric traits = M_1: thorax area, M_2: thorax length, M3: thorax width, M_4: Fem-R, M_5: harp size. Comparisons above the diagonal represent comparisons between ten wild-caught populations, while comparisons below the diagonal represent comparisons between six captive-reared F_1 populations of *G. bimaculatus*. 'NS' indicates non-significant comparisons, $P > 0.05$. '-' indicates comparisons that could not be made due to the absence of captive-reared F_1 crickets of these populations

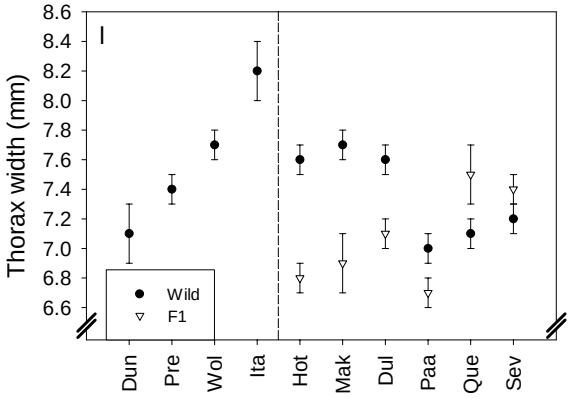
	Dullstroom	Hotazel	Makhado	Paarl	Queenstown	Sevilla	Dundee	Pretoria	Wolmaransstad	Italy
Dullstroom		S_1, S_2, F_1	M_4	C_3, M_5	S_1, F_1, M_5	NS	NS	NS	NS	M_1, M_2, M_4
Hotazel	NS		S_1, F_1	S_1, F_1	C_2, S_1, S_2, F_1	S_1, S_2	NS	F_1	NS	S_2
Makhado	NS	C_1, C_3		C_3, C_4, M_1, M_3, M_5	C_2, S_1, S_2, F_1, M_1, M_5	NS	NS	M_4	NS	NS
Paarl	NS	NS	NS		S_1, F_1	NS	NS	C_3, M_5	M_1	M_1, M_3, M_5
Queenstown	NS	M_1, M_2, M_3	C_3, S_2, S_3, M_1, M_2, M_3, M_4	S_2, S_3, M_1, M_2, M_3, M_4		S_1, F_1	S_1, F_1	C_2, S_1, S_2, F_1, M_5	S_1, F_1, M_1, M_5	S_1, F_1, M_1, M_2, M_3, M_5
Sevilla	C_2, S_1, S_3	S_3, M_1, M_3	C_1, C_2, C_3, S_1, S_3, M_2	S_2, S_3, M_1, M_2, M_3, M_5	C_2, F_1		NS	S_2	NS	M_1, M_3
Dundee	-	-	-	-	-	-		NS	NS	M_1, M_3
Pretoria	-	-	-	-	-	-	-		NS	M_1, M_4
Wolmaransstad	-	-	-	-	-	-	-	-		NS
Italy	-	-	-	-	-	-	-	-	-	

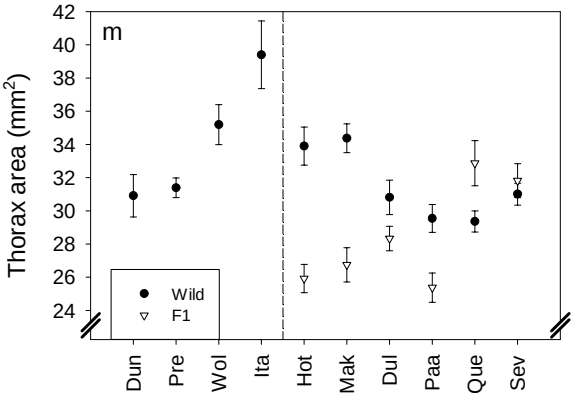

a
Syllable duration (ms)
b
Inter-syllable interval (ms)
c
Syllable period (ms)
d
Syllables per chirp
e
Chirp duration (ms)
f
Inter-chirp interval (ms)
g
Chirp period (ms)
h
F_{max}(kHz)
Wild
F1
Dun
Pre
Wol
Ita
Hot
Mak
Dul
Paa
Que
Sev

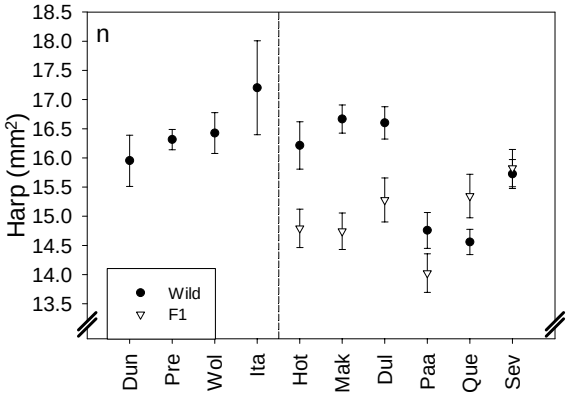

i
Fwidth-A (Hz)
400
380
360
340
320
300
280
260
240
220
200
Wild
F1
Dun
Pre
Wol
Ita
Hot
Mak
Dul
Paa
Que
Sev


j
Fwidth-B (Hz)
650
600
550
500
450
400
350
Wild
F1
Dun
Pre
Wol
Ita
Hot
Mak
Dul
Paa
Que
Sev


k
Thorax length (mm)
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
Wild
F1
Dun
Pre
Wol
Ita
Hot
Mak
Dul
Paa
Que
Sev


l
Thorax width (mm)
8.6
8.4
8.2
8.0
7.8
7.6
7.4
7.2
7.0
6.8
6.6
Wild
F1
Dun
Pre
Wol
Ita
Hot
Mak
Dul
Paa
Que
Sev


m
Thorax area (mm2)
42
40
38
36
34
32
30
28
26
24
Wild
F1
Dun
Pre
Wol
Ita
Hot
Mak
Dul
Paa
Que
Sev


n
Harp (mm2)
18.5
18.0
17.5
17.0
16.5
16.0
15.5
15.0
14.5
14.0
13.5
Wild
F1
Dun
Pre
Wol
Ita
Hot
Mak
Dul
Paa
Que
Sev

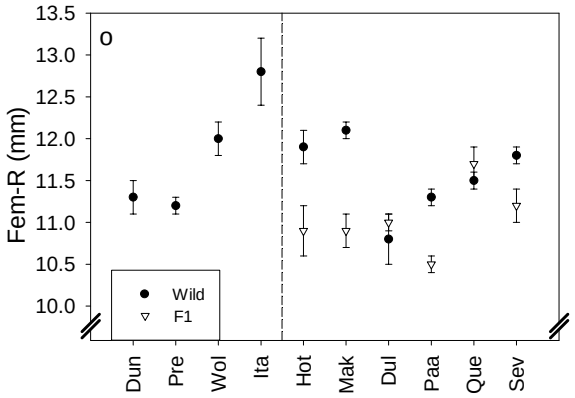

o
Fem-R (mm)
13.5
13.0
12.5
12.0
11.5
11.0
10.5
10.0
Wild
F1
Dun
Pre
Wol
Ita
Hot
Mak
Dul
Paa
Que
Sev

Figure 5.2a-o. Mean values with associated standard errors (error bars) for the calling song characteristics and morphometric traits of ten wild-caught and six captive-reared F_1 populations of *G. bimaculatus*. Populations left of the vertical broken line lack captive-reared F_1 offspring. Locality names are abbreviated as follows: Dun = Dundee; Pre = Pretoria; Wol = Wolmaransstad; Ita = Italy; Hot = Hotazel; Mak = Makhado; Dul = Dullstroom; Paa = Paarl; Que = Queenstown; Sev = Sevilla.

Variables contributing to the variation in the wild-caught populations

Principal components analysis showed that six principal components (eigenvalues > 1) explained more than 80% of the total variation in the wild-caught data set (Table 5.3). The wing measurements contributed significantly to principal component 1, representing 40% of the total variation. Bandwidth contributed significantly to principal component 2, representing some 14% of the total variation and F_{max} did not contribute in a measurable way to the total variation. The chirp traits contributed significantly to the third and fourth principal components, representing 16% of the total variation. Syllable duration was the only syllable trait that contributed significantly to any of the principal components, principal component 5, representing 6% of the total variation. The body measurements contributed significantly to principal component 6, representing only 5% of the total variation. Although there was a tendency for same-population individuals to cluster together in R-rotated PCA plots, there was no distinct clustering of the individuals into their respective populations (Figure 5.3a).

Table 5.3. Principal components (PC) showing to what degree the calling song characteristics and morphometric measurements contributed to the variation in ten wild-caught populations (W) and six captive-reared F_1 populations (F_1) from South Africa and Europe. Loadings indicated by W and F_1 are > 0.700. The following variables did not contribute in a measurable way to the variation in either of the data sets: F_{max}, M4_M8 and H10_H11

Variable	PC1	PC2	PC3	PC4	PC5	PC6
S_C			W	F_1		
CD			W	F_1		
ICI		F_1		W		
CP		F_1		W		
SD					W	
ISI		F_1				
SP		F_1				
Fwidth-A		W	F_1			
Fwidth-B		W	F_1			
Head length					F_1	W
Head width					F_1	
Thorax area					F_1	
Fem-R					F_1	W
Tib-R					F_1	W
H1_H2	W, F_1					
H1_H3	W, F_1					
H2_H3	W, F_1					
H1_M9	W, F_1					
M6_M8	W					
Harp	W, F_1					
Total variance explained % (F_1)	39.81	14.22	9.15	6.64	5.86	5.37
Total variance explained % (W)	36.64	13.46	9.19	8.62	7.19	

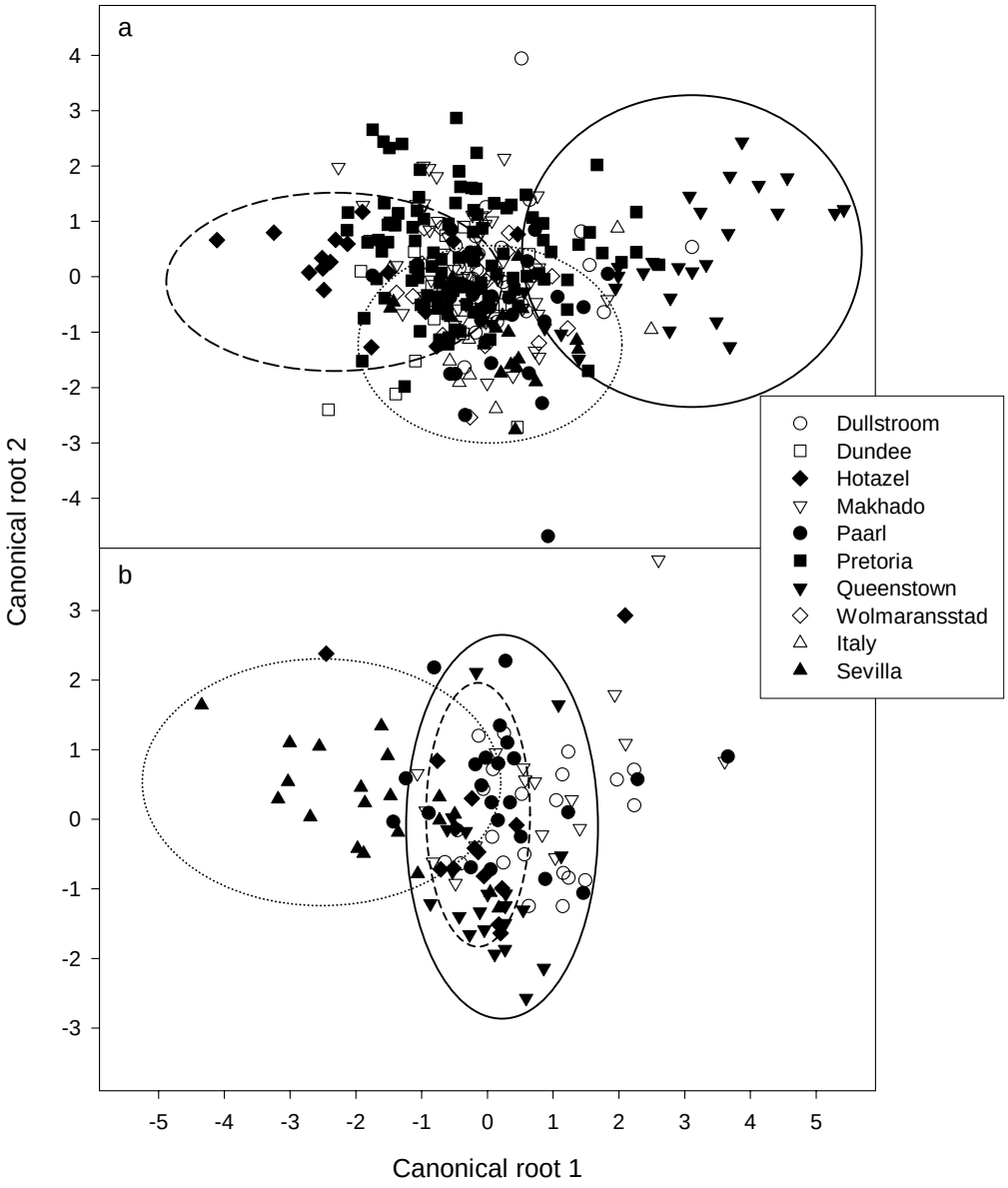


Figure 5.3. Clustering of (a) ten wild-caught populations and (b) six captive-reared F_1 populations from South Africa and Europe following discriminant function analyses that utilized ten calling song characteristics of *G*. *bimaculatus*. The Hotazel, Queenstown and Sevilla populations (circled) have distinct clusters in at least one of the analyses.

Call traits useful in discriminating between wild-caught populations

Following the DFA, calling song frequency, syllable duration, inter-chirp interval, syllable period, Fwidth-A and Fwidth-B (F_{max} = most important and Fwidth-B = least important) can be used to discriminate between populations (Wilks' Lambda = 0.34, $F_{54, 1529} = 6.66$, $P < 0.001$, $n = 314$). The significantly different values of syllable duration and calling song frequency of the Queenstown and Hotazel populations (Figure 5.2a, h), enhanced the effectiveness with which these two call traits can be used to discriminate between populations. A large degree of similarity was observed between the Dullstroom, Makhado, Pretoria and Wolmaransstad populations as well as between the Dundee, Italian, Paarl and Sevilla populations (Figure 5.3a). The Queenstown and Hotazel populations clustered separately from each other and the other populations. However, only 44% of the individuals were classified correctly into their respective populations. No individuals from Hotazel were assigned to Queenstown or *vice versa*. Four of the South African populations (Makhado, Paarl, Queenstown and Wolmaransstad) had at least one individual classified mistakenly into the Sevilla population. The Wolmaransstad and Italian populations did not have a single individual classified into them. The majority of the Wolmaransstad individuals were mistakenly classified into the Pretoria population while the Italian individuals were mistakenly classified into the Sevilla, Pretoria, Queenstown and Dundee populations. Sixteen of the 20 Sevilla individuals were mistakenly classified into the Pretoria, Paarl and Makhado populations.

Correlation with geographic distances, call traits and morphology in ten wild-caught populations

There was no significant association between geographic distances among localities and any of the call traits or morphometric measurements (Mantel test: g = 1.55, r = 0.263, $P > 0.05$, $n = 314$), suggesting that populations that are located close to each other do not have more similar crickets (based on call traits and morphological measurements) than those populations located further apart. A Mantel test performed only on call traits did not reveal any significant association between geographic distances and call traits (g = 1.55, r = 0.262, $P > 0.05$, $n = 314$), suggesting that cricket populations that are located close to each other do not have more similar calling songs, than those populations located further apart.

Correlation with gene flow, call traits and morphology in nine wild-caught populations

There was no significant association between the gene flow estimates (F_{ST} statistics) from Ferreira (2006c, Chapter 4) and any of the call traits or body measurements (Mantel test: g = 0.396, r = 1330.140, $P > 0.05$, $n = 295$). This suggests that populations with large degrees of between-population gene flow do not have crickets that are more similar in body size or call traits than populations with smaller degrees of between-population gene flow. The Mantel test also did not reveal any significant association between the F_{ST} statistics and call traits (g = 0.391, r = 1327.140, $P > 0.05$, $n = 295$), indicating that cricket populations with large degrees of between-population gene flow do not have more similar calling songs than populations with smaller degrees of between-population gene flow.

Six captive-reared F_1 populations

Between-population differences in call traits and morphology of captive-reared F_1 males

As for the wild-caught populations, the univariate ANOVA also revealed extensive between-population differences in the call traits and morphometric measurements of the captive-reared F_1 populations (Table 5.1). The Tukey *post-hoc* comparison, after a Bonferroni adjustment, showed that the Queenstown population differed significantly from Makhado and Paarl, while Sevilla differed significantly from most of the South African populations (Table 5.2; Figure 5.2). For the chirp traits, the Sevilla population had a significantly longer chirp duration and a significantly shorter inter-chirp interval compared with the South African populations. The Queenstown and Sevilla populations had significantly longer syllable durations and significantly shorter inter-syllable intervals than the other populations, which gave rise to the significantly shorter syllable period produced by these two populations. Calling song frequency was the only spectral trait that differed significantly between populations and it only differed between the Sevilla and Queenstown populations. For the morphometric measurements, the Queenstown population was significantly larger than all of the South African populations except Dullstroom. The Paarl population was significantly smaller than the Sevilla and Queenstown populations. For the wing measurements, harp size only differed

significantly between two populations, with the Sevilla population having a larger harp size than the Paarl population.

Variables contributing to the variation in the captive-reared F_1 populations

Principal components analysis showed that five principal components (eigenvalues > 1) explained 75% of the total variation in the captive-reared F_1 data set (Table 5.3). The wing measurements contributed significantly to principal component 1, representing 37% of the total variation. Two chirp (CP and ICI) and two syllable (SP and ISI) traits contributed significantly to principal component 2, representing 14% of the total variation. Calling song frequency did not contribute in a measurable way to the total variation, while bandwidth contributed significantly to principal component 3, representing 9% of the total variation. The remaining two chirp traits (S_C and CD) contributed to principal component 4, representing only 9% of the total variation. The morphometric measurements contributed the least to the total variation, only 6%, and contributed significantly to principal component 5. Same-population individuals clustered together in the R-rotated data, although there was no complete clustering of the individuals of different populations (Figure 5.3b).

Call traits useful in discriminating between captive-reared F_1 populations

Only three of the ten call traits (inter-syllable interval, chirp duration and calling song frequency: ISI = most important and F_{max} = least important) can be used to discriminate between populations (Wilks' Lambda = 0.39, $F_{25, 402} = 4.645$, $P < 0.001$, $n = 118$). The significantly different attributes of inter-syllable interval of the Sevilla and Queenstown populations (Figure 5.2) contributed to the effectiveness with which there can be discriminated between populations. Although the Sevilla and Queenstown populations clustered separately from each other and other populations, there was overlap between the Queenstown and Hotazel populations (Figure 5.3b). There was a large degree of similarity between the Dullstroom, Makhado and Paarl populations. Fifty five percent of the individuals were classified correctly into their respective populations. The Sevilla population had the highest percentage (75%) of correctly classified individuals, followed by the Queenstown population (71%). The remaining populations had less than 53% correct classifications. Five of the 20 Sevilla individuals were mistakenly classified

into three South African populations (Hotazel, Paarl and Queenstown), while three individuals from Paarl and Hotazel were mistakenly classified under the Sevilla population.

Correlation with geographic distances, call traits and morphology in six captive-reared F_1 populations

The Mantel test found no significant association between geographic distance and any of the call traits or body measurements (g = -0.259, r = -0.083, $P > 0.05$, n = 118). There was also no significant association between geographic distance and any of the call traits (g = -0.263, r = -0.084, $P > 0.05$, n = 118), reflecting the results found in the wild-caught populations.

Correlation with gene flow, call traits and morphology in six captive-reared F_1 populations

There was no significant association between the gene flow estimates (F_{ST} statistics) from Ferreira (2006c, Chapter 4) and any of the call traits or body measurements (Mantel test: g = -0.453, r = -0.134, $P > 0.05$, n = 118) or between the gene flow estimates and call traits (Mantel test: g = -0.471, r = -0.139, $P > 0.05$, n = 118) for the six captive-reared F_1 populations. This indicates that populations with large degrees of between-population gene flow do not have crickets that are more similar in call structure or morphometrics than populations with small degrees of between-population gene flow.

Comparison of wild-caught populations with captive-reared F_1 populations

Between-population variation in the calling song characteristics and body measurements

There was a significant difference in the overall and within-population variances of the majority of the call traits and morphometric measurements for both the wild-caught and captive-reared F_1 populations (Table 5.4). The overall and within-population variance of all the syllable traits was higher in the captive-reared F_1 populations than in the six wild-caught populations, while this was only true for one of the chirp traits and two of the body measurements. In contrast, the overall and within-population variances of the spectral traits of the six wild-caught populations were higher than those of captive-reared F_1 crickets.

On a finer scale, the random effects ANOVA revealed that the chirp traits and inter-syllable interval had a larger degree of between-population variation in the six captive-reared F_1 populations than in the ten wild-caught populations (Figure 5.4a). However, when comparing the six captive-reared F_1 populations with the six wild-caught populations only three call traits (syllables per chirp, chirp duration and inter-syllable interval) had a larger degree of between-population variation in the captive-reared F_1 crickets than the wild-caught crickets (Figure 5.4b). The between-individual variance component contributed the most to the variation in all the call traits in the six wild-caught populations (SD and F_{max} < 65%; other call traits > 80%) as well as in the six captive-reared F_1 populations (spectral traits and CP: > 90%; other call traits less than 90%).

The results of the two-way fixed effects ANOVA revealed a strong effect of locality on all but four traits (Table 5.5). Although the effect of captivity was mostly evident in the syllable traits and morphometric measurements, there was a significantly strong interaction between locality and captive state on the majority of traits (Table 5.5). The Sevilla population did not differ markedly from the South African populations. The Hotazel population differed from the other populations in syllable duration, while the Queenstown population differed significantly from the other populations in calling song frequency. The Paarl population differed significantly from the other populations in body size. Three call traits (syllables per chirp, inter-syllable interval and syllable period) differed significantly between the wild-caught and captive-reared F_1 populations. The morphometric measurements of the wild-caught populations differed significantly from the captive-reared F_1 populations.

Table 5.4. Overall variance and within-population variance of the call traits and body measurements for ten wild-caught populations (10 Wild), six wild-caught populations that have captive-reared F_1 offspring (6 Wild) as well as their six captive-reared F_1 populations (6 Captive). The *F* statistic was calculated for each variable, using the six wild-caught and six captive-reared F_1 populations. '*' indicates significance for $P < 0.05$

	Total variance				Within-population variance			
Variable	10 Wild	6 Wild	6 Captive	*F* statistic	10 Wild	6 Wild	6 Captive	*F* statistic
S_C	0.197852	0.203719	0.141261	$F_{166, 117} = 1.442$*	0.191979	0.194984	0.119736	$F_{166, 117} = 1.628$*
CD	0.000360	0.000368	0.000272	$F_{166, 117} = 1.354$*	0.000322	0.000319	0.000211	$F_{166, 117} = 1.51$*
ICI	0.002122	0.002421	0.002565	$F_{117, 166} = 1.059$*	0.001906	0.002073	0.002212	$F_{117, 166} = 1.067$*
CP	0.002459	0.002848	0.002592	$F_{166, 117} = 1.099$	0.002286	0.002534	0.002335	$F_{166, 117} = 1.085$
SD	0.000007	0.000008	0.000009	$F_{117, 166} = 1.099$*	0.000005	0.000004	0.000007	$F_{117, 166} = 1.648$*
SP	0.000008	0.000008	0.000008	$F_{117, 166} = 1.096$*	0.000006	0.000006	0.000007	$F_{117, 166} = 1.179$*
ISI	0.000009	0.000009	0.000018	$F_{117, 166} = 2.058$*	0.000008	0.000009	0.000013	$F_{117, 166} = 1.532$*
F_{max}	0.117454	0.131717	0.052854	$F_{166, 117} = 2.492$*	0.076520	0.065438	0.046508	$F_{166, 117} = 1.407$*
Fwidth-A	0.012097	0.013075	0.011499	$F_{166, 117} = 1.137$	0.011195	0.011909	0.010858	$F_{166, 117} = 1.1$
Fwidth-B	0.022739	0.024436	0.020461	$F_{166, 117} = 1.194$	0.021249	0.022796	0.019543	$F_{166, 117} = 1.166$
Thorax length	0.264145	0.179543	0.175609	$F_{166, 117} = 1.022$	0.239888	0.156134	0.124395	$F_{166, 117} = 1.255$*
Thorax width	0.452909	0.323587	0.399356	$F_{117, 166} = 1.234$*	0.387573	0.253523	0.302016	$F_{117, 166} = 1.191$*
Thorax area	32.445717	26.998301	27.974020	$F_{117, 166} = 1.036$*	27.633437	22.490586	19.497508	$F_{166, 117} = 1.153$
Fem-R	1.266426	0.947301	0.655581	$F_{147, 114} = 1.445$*	1.053002	0.740582	0.503853	$F_{147, 114} = 1.47$*
Harp	0.008486	2.941981	2.606636	$F_{165, 115} = 1.129$	0.058300	2.154238	2.247462	$F_{165, 115} = 1.043$*

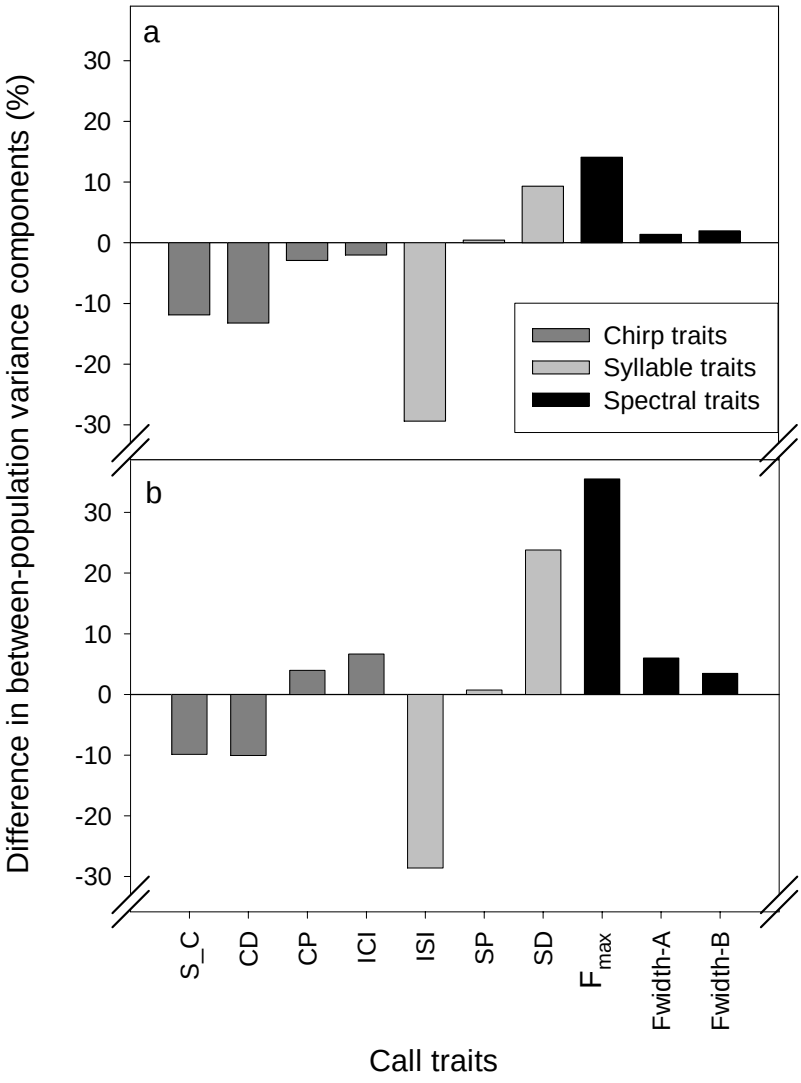


Figure 5.4. The difference between the between-population variance components of the calling song characteristics of *G. bimaculatus* for (a) ten wild-caught and six captive-reared F_1 populations and (b) six wild-caught and six captive-reared F_1 populations.

Table 5.5. Results for a two-way fixed effects ANOVA performed on the six wild-caught populations and their captive-reared F_1 offspring for two treatments (locality and wild/captive) and the interaction between them (interaction). The degrees of freedom of the *F* statistic for Fem-R are given directly after the value of its *F* statistic. Results for Tukey *post-hoc* comparisons, after Bonferroni adjustments, are indicated with asterisks. Empty cells indicate non-significant comparisons ($P > 0.05$). Asterisks indicate significance values as follows: $P < 0.05$ *, $P < 0.01$ ** and $P < 0.001$ ***

	Two-Way ANOVA			*Post-hoc* analyses													
	Locality	Wild/Captive	Interaction	Locality													
	F statistic	*F* statistic	*F* statistic	Hot-Mak	Hot-Dul	Hot-Paa	Hot-Que	Hot-Sev	Mak-Dul	Mak-Paa	Mak-Que	Mak-Sev	Dul-Paa	Dul-Que	Dul-Sev	Paa-Que	Paa-Sev	Que-Sev
Call trait	$F_{5, 273}$	$F_{1, 273}$	$F_{5, 273}$															
S_C	2.498*	11.162***	2.435*															
SD	9.069***	0.829	14.256***	***	***	***	***											***
ISI	6.752***	17.467***	7.538***									***			***		***	
SP	11.079***	18.851***	3.32**				***	***			***	**				***	**	
CD	6.733***	0.902	3.665**				***											***
ICI	2.878*	1.2	5.733***															
CP	3.193**	0.482	3.051*													**		
F_{max}	21.848***	0.11	13.275***		**		***				***			***		***		***
Fwidth-A	2.15	0.753	1.71															
Fwidth-B	2.575*	1.063	0.612															
Body measurements	$F_{5, 274}$	$F_{1, 274}$	$F_{5, 274}$															
Thorax length	4.19**	24.669***	8.38***															**
Thorax width	5.667***	22.929***	9.386***							***			***			***	**	
Thorax area	4.45***	25.53***	10.084***							**						**	***	
Fem-R	6.561***; $F_{5, 252}$	25.09***; $F_{1, 252}$	6.275***; $F_{5, 252}$						**	**				**		***	**	
Wing measurement	$F_{5, 271}$	$F_{1, 271}$	$F_{5, 271}$															
Harp	7.491***	15.62***	5.2***							***			***				***	

Correlations between wild-caught and captive-reared F_1 crickets' call traits and morphometric measurements

No significant correlations between the call traits and morphometric measurements of the six wild-caught populations and six captive-reared F_1 populations were found (S_C: r = 0.107, CD: r = 0.335, CP: r = -0.06, ICI: r = -0.344, SD: r = -0.215, SP: r = 0.411, ISI: r = 0.162, F_{max}: r = 0.369, Fwidth-A: r = 0.063, Fwidth-B: r = 0.712, thorax area: r = -0.472, thorax length: r = -0.332, thorax width: r = -0.315, Fem-R: r = 0.015, harp: r = 0.157; $P > 0.11$ in all cases, $n = 6$).

Discussion

Inter-continental and regional differences in male calling song and morphometrics

This study found only a small degree of differentiation among European and South African populations in the calling song and morphometrics of wild-caught *G. bimaculatus* (Tables 5.1 & 5.2; Figure 5.3). The large amounts of gene flow that occur between Europe and South Africa (Ferreira, 2006c, Chapter 4) should increase the degree of similarity in the calling songs and morphometrics between these populations, especially since this study could not find any evidence of clinal variation. The between-population differences within South Africa were more evident than in Ferreira & Ferguson's (2002) study. This is probably explained by the increase in sampling localities and sample sizes at each locality. In addition, the present sampling localities represent various climatic regions of South Africa (Preston-Whyte & Tyson, 1988; Schulze, 1994) and are therefore more representative of the range of environmental conditions that these crickets may experience in the wild. There was a larger degree of regional variation and between-population differences in the call traits and morphometrics of *G. bimaculatus* (Figure 5.2; Tables 5.1 & 5.2) compared with inter-continental variation and inter-continental differences (i.e. between South Africa and Europe). Zuk *et al*. (2001) found similar results: there were larger degrees of between-region (i.e. within-continents) than between-continent variation in the calling song of the field cricket, *Teleogryllus oceanicus*. The mate recognition system of *Drosophila melanogaster* is also very stable among populations worldwide (Henderson & Lambert, 1982). My findings suggest that there is large-scale stability in the communication system of this cricket, consistent with Paterson's (1985) prediction of stabilizing selection in mate recognition systems among populations.

Although there was variation in the song traits within South Africa, the call traits important for mate recognition (calling song frequency and syllable period; Bennet-Clark, 1989; Schildberger *et al*., 1989) had less between-population differences (Table 5.2) and contributed less to the total variation in the data set (Table 5.3) compared with other call traits, supporting the findings of Ferreira & Ferguson (2002). Ryan *et al*. (1996) also found that although there was significant between-population variation in the advertisement call of *P*. *pustulosus*, the traits important for mate recognition had smaller degrees of variation than other call traits.

Environmentally induced variation in the male calling song and morphometrics

I did not conduct a quantitative study (e.g. calling song of *G*. *integer*: Gray & Cade, 1999; morphology of *G. bimaculatus*: Simmons, 1987; morphology of *G. pennsylvanicus*: Simons & Roff, 1994) and my results can therefore not provide a precise estimate of the amount of environmental variance that contributes to variation in the calling song and morphometrics of *G. bimaculatus*. However, my conclusions based on the comparative analyses of the wild-caught and captive-reared F_1 populations, estimates part of the environmental component of geographical variation in the call and morphological traits. When Mousseau & Howard (1998) compared wild-caught and laboratory-reared populations of two species of ground crickets, *Allonemobius fasciatus* and *A. socius*, as well as a hybrid population, they found a significant effect of the environment on call traits. A significant part of the geographical variation in the call traits and morphometric measurements of *G. bimaculatus* is probably environmentally induced based on the following results. Firstly, overall and within-population variances were larger in the wild-caught than the captive-reared F_1 populations for six of the ten call traits and two of the morphometric measurements (Table 5.4). Secondly, there was a decrease in the between-population variances of seven call traits in six captive-reared F_1 populations compared with six wild-caught populations (Figure 5.4b). Thirdly, no significant correlation of the call traits and morphometric measurements of the captive-reared F_1 populations with the wild-caught populations could be found. Fourthly, there was a strong interaction effect of locality and captivity on eight call traits and all of the morphometric measurements (Table 5.5). Lastly, with the exception of the Queenstown population, significant between-population differences in the call traits

and morphometric measurements of the wild-caught populations were not evident in the captive-reared F_1 populations (Table 5.2). The wild-caught and captive-reared F_1 Queenstown populations differed significantly from the other populations in call structure and body size (Tables 5.2 & 5.5; Figure 5.2). The wild-caught Queenstown population also clustered separately from the other populations in the discriminant function analysis (Figure 5.3a). The phylogeographical study of Ferreira (2006c, Chapter 4) on *G. bimaculatus* revealed (a) that only small amounts of gene flow occurred among the Queenstown population and other South African populations, (b) that the Queenstown population has a small degree of within-population genetic differentiation and (c) that it differs significantly from the other populations. This could imply that part of the distinctive genetic characteristics of the Queenstown population are reflected in this population's phenotypic traits.

Correlation of calling song and morphometric differences with geographical distance and gene flow

This study could not find any evidence of an isolation-by-distance effect in the call traits and morphometric measurements (see results) as were found in other studies (Asquith *et al*., 1988; Ryan & Wilczynski, 1991; Ryan *et al*., 1996; Zuk *et al*., 2001). There was also no significant association between the call and morphometric traits with gene flow (see results), suggesting that populations with large amounts of gene flow among them are not necessarily more similar in call structure or morphology. Indeed, there were fewer differences in the song and morphometrics of the individuals from the Queenstown and Sevilla populations compared with Queenstown and other South African populations (Table 5.2). More individuals of these two populations share a specific haplotype (h2; Ferreira, 2006c, Chapter 4) compared with the other populations, which could explain why the call and morphological traits of the Queenstown and Sevilla populations do not differ to such a great extent from each other. Call traits and morphometric measurements of wild-caught populations in the northeastern parts of South Africa differed significantly from those of the wild-caught populations in the southwestern parts of the country (Table 5.2), thereby reflecting the gene flow patterns observed in Ferreira (2006c, Chapter 4). However, this pattern is not observed in the captive-reared F_1 populations, suggesting that geographical variation in the call traits and morphometric measurements of this

cricket is not solely explained by between-population genetic differentiation, but rather by different environmental conditions.

Estimates of the magnitude of between-population variances

Between-individual variances were larger than between-population variances in the call traits of the six wild-caught and six captive-reared F_1 populations (see results). The within-population variance component was also larger than the inter-continental and between-population variance components in the call and morphological traits of the ten wild-caught populations (Table 5.1). These results are consistent with the findings of Ferreira (2006b, Chapter 3) who showed that between-individual and within-individual variances contribute largely to the variation in the call traits of two populations of *G. bimaculatus*. These findings suggest that there are not large degrees of between-population differences in the call structure and body sizes of *G. bimaculatus* crickets among the European and South African populations.

Implications for the communication system: efficiency of communication

This study showed that variation in the calling song and morphometrics of *G. bimaculatus* crickets is more evident within-populations than among European and South African populations of wild-caught and captive-reared F_1 crickets. Diverse environmental effects experienced by populations could act as noise affecting the communication system of *G. bimaculatus*. This could, in turn, affect the reliability with which males signal their qualities (e.g. body size and health; Simmons, 1988). However, these effects on the sexual selection process need to be investigated by in-depth studies on the communication system of these crickets.

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CHAPTER 6

General conclusion

Mate recognition and sexual selection in the calling song of Gryllus bimaculatus

The acoustic communication system of various orthopteran and anuran species is involved in sexual selection (e.g. Gray, 1997; Simmons, 1988; Simmons & Ritchie, 1996; Simmons & Zuk, 1992; Simmons *et al*., 2001). Simmons (1988) suggested that chirp duration, syllable rate and chirp rate convey information relating to male size in *G*. *bimaculatus*, and Bateman *et al*. (2001) and Simmons (1986a, b) showed that females prefer large males. In addition, it has been shown that older *G. bimaculatus* males have a significantly higher daily mating rate than younger males, and one of Simmons & Zuk's (1992) hypotheses was that females use syllable rate as an indicator of male age.

On the other hand, authors such as Boake *et al*. (1997), Ryan *et al*. (1996) and Schildberger *et al*. (1989) suggested that the communication system is also involved in species recognition. Bennet-Clark (1989) and Schildberger *et al*. (1989) showed through neurological studies that calling song frequency and syllable period are important mate recognition signaling traits in the calling song of *G*. *bimaculatus*.

Calling song traits under sexual selection and mate recognition

During the first 49 days after adult eclosion, males produced significantly longer chirp periods as they aged, suggesting that chirp traits can be used by females to judge male age. When the age and body size effects on the call traits were combined for the period of ten to 140 days post adult eclosion, it was shown that an older, large male called at a slower chirp and syllable rate than a younger, small male. Female preference studies that control for male size and male age respectively, need to be conducted to determine whether females use male song as an indicator for male size and/or male age. I therefore suggest that, while the chirp traits are potentially involved in sexual selection in *G*. *bimaculatus*, the

syllable traits and calling song frequency are potentially involved in mate recognition. Evidence below supports this interpretation.

Variation in the mate recognition and other call traits of G. bimaculatus

It has been predicted that mate recognition and sexual selection traits have different degrees of variation. Sexual selection has diversifying effects on mating signals (Lande, 1981; Panhuis *et al*., 2001), leading to large degrees of between-individual and between-population variation in these signals (Etges, 2002). Mate recognition, on the other hand, is suggested to exert stabilizing effects on mating signals across the geographic range of a species (Paterson, 1985), and one would expect these signals to have small degrees of between-individual and between-population variation. This study focused on several sources of variance that potentially contribute to the variation in the calling song of *G. bimaculatus*. Traits implicated in mate recognition were shown to have smaller degrees of variation than those traits implicated in sexual selection as follows:

a) Ferreira (2006a, Chapter 2) revealed that ageing effects contributed the least to the variation in the mate recognition traits (Table 2.1). The compensatory interaction between syllable duration and inter-syllable interval resulted in an extremely stable syllable period over the first 49 days of male ageing (Figures 2.4 & 2.5). The large degree of between-individual variation in the mate recognition traits compared with the chirp traits, are probably an artifact of the ANOVA since, when within-individual variation decreases (constant signal) then one would expect a larger between-individual variance component (Table 2.1).
b) Sources of variance that could contribute to within-population variation in the call traits were investigated in two South African populations, Makhado and Pretoria (Ferreira, 2006b, Chapter 3).
    i) Calling song frequency and syllable period had smaller degrees of between-chirp variation compared with the other call traits, suggesting a large degree of stability in the mate recognition traits of a particular male between chirps.
    ii) Principal components analysis (PCA) indicated that syllable traits contributed the least to the total within-population variation, while calling song frequency did not contribute to the total variation in either data set

(Table 3.4). On the other hand, the chirp traits contributed to a large degree to the total variation.

c) Supporting evidence was also found when investigating geographical variation in call traits (Ferreira, 2006c, Chapter 5).

   i) Although there was a large degree of between-population variation in call traits, PCA indicated that calling song frequency and syllable period did not contribute to the total variation in the data set consisting of ten wild-caught populations (Table 5.3). In a captive-reared F_1 data set (consisting of six populations) calling song frequency did not contribute to the total variation in the data set and the syllable traits contributed less to the total variation than the other call traits (Table 5.3).

   ii) The large degrees of inter-continental stability in the mate recognition traits (Table 5.2), supports the findings of Henderson & Lambert (1982) who found considerable stability in the mate recognition system of worldwide populations of *Drosophila melanogaster*. This suggests that inter-continental variation forms only a subset of the total variation that exists in the signaling traits of *G. bimaculatus*.

d) For mate recognition traits, captive-reared F_1 offspring had a smaller degree of between-population variance than their wild-caught parents. This suggests that environmentally-induced variance is more important for the mate recognition traits and also implies that genetic variation may be less for these traits.

The above results are consistent with the idea that mate recognition traits and sexual selection traits are separate aspects of communication. One could therefore predict that the mate recognition traits show stabilizing selection across a significant part of the geographical range of *G. bimaculatus*, and are therefore consistent with the prediction of Paterson (1985). In addition, the mate recognition traits also have smaller degrees of variation compared with other call traits, which is consistent with the predictions of Etges (2002), Lande (1981) and Panhuis *et al*. (2001). On the other hand, the geographical study (Ferreira, 2006c, Chapter 5) showed that the mate recognition traits of two South African populations (Hotazel and Queenstown) differed significantly from the other South African and European populations (Table 5.2). This could be consistent with Fisher's runaway process that predicts no predictable between-population differences in courtship signals

(Fisher, 1958). However, one should expect to see larger degrees of between-population differences within South Africa, if the Fisher process is operational in South Africa. In order to conclude whether geographical variation in the communication system of *G. bimaculatus* is consistent with Paterson's or Fisher's predictions, inter-population female preference studies where females are subjected to male calling songs from different populations need to be conducted. The outcome of the female preference studies will be important for understanding the evolution of communication for this species. For instance, if Queenstown females only prefer local males (i.e. Queenstown males), then this might be suggestive of covariance between the male signal and the female preference. On the other hand, if the Queenstown females do not have a preference for native males above foreign males, then stabilizing selection is probably acting on this signaling system and the species is probably not susceptible to speciation.

Effect of the environment on mate recognition traits of G. bimaculatus

Based on the comparative results of wild-caught populations from South Africa and Europe with their captive-reared F_1 offspring (Ferreira, 2006c, Chapter 5), this study suggests that a significant part of the geographical variation in mate recognition traits is probably caused by environmental effects (Table 5.2; Figure 5.4). These environmental effects could act as noise affecting the acoustic communication system of *G. bimaculatus*. Ferreira (2006b, Chapter 3) revealed large seasonal differences with large between-season variance components in the mate recognition traits. However, these results were not consistent between the two populations (Makhado and Pretoria), suggesting that the environmental component contributing to variation in signaling traits is not predictable and that several factors such as food availability, temperature, rainfall and population density probably contribute collectively to this variation. If environmental effects largely comprise temperature effects, it could be negated through 'temperature coupling'. Doherty (1985) showed that there is parallel coupling of female preferences to the effect that temperature has on the male signaling system in *G. bimaculatus*. A full-scale quantitative study needs to be undertaken to estimate the environmental component of variance in call traits.

Gene flow and mate recognition in G. bimaculatus

Surprisingly, the phylogeographical study (Ferreira, 2006d, Chapter 4) revealed a large degree of gene flow among seven South African populations, as well as between populations in Spain and Italy. The inter-continental estimates of gene flow were lower than for within-continent gene flow estimates. The fact that one haplotype was shared among all the populations suggest that there is ongoing inter-continental gene flow within this species. This could explain the observed stability in the mate recognition traits, and suggests that female *G. bimaculatus* from Europe should be able to recognise males from South Africa as conspecific, and *vice versa*.

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Appendix 1. Results of the nested clade distance analysis for cytocrome *b* haplotypes of *G*. *bimaculatus* from South Africa for (a) the 2-step cladogram and (b) the 3-step cladogram (see results in Chapter 4). Interior vs. tip contrasts for *Dc* and *Dn* are indicated with 'I-T' for nested clades where the interior/tip status is known. 'L' Superscript indicates the distance is significantly large at the 5% level, while 'S' superscript indicates that it is significantly small. Inferences from the nested clade distance analysis are given following the inference key of Templeton (2004)

a

2-step cladogram									
Haplotypes			1-step clades			2-step clades			
Clade	*Dc*	*Dn*	Clade	*Dc*	*Dn*	Clade	*Dc*	*Dn*	Inference
h2	490.68 S	496.95 S							
h16	0	711.95							
h18	0	920.05							
h22	0	920.05							
h24	0	367.40	1-1	528.00	530.09				1-19-20-2-3-5-6-7
h25	0	382.85							
h28	717.28 L	728.14 L							
h30	0	515.20							
I-T	509.57	-120.03				2-1	533.68	536.42	
h8	0	410.49 L							
h19	0	407.47 S	1-2	408.98	634.92				1-19-20
I-T	0	-3.02 S	I-T	119.01	-104.83				
h7	166.36	184.03							
h10	0	72.50	1-3	224.08	363.23				
h14	0	575.95							
I-T	166.36	-140.20							
h4	159.49	150.64							
h26	0	85.16	1-4	126.39	297.28				
I-T	159.49	65.48							
			1-5	0	327.11				
			1-6	0	516.92				
			1-7	0	394.88	2-2	457.56	476.02	1-19-20-2-3-4
			1-8	0	387.12				
h1	484.79	479.96							
h6	114.32 S	422.87							
h9	0	354.50							
h11	0	361.56							
h12	0	361.56							
h13	0	361.56	1-9	488.96 L	480.77 L				1-19-20-2-3-5-6-7-8
h15	0	594.67	I-T	329.89	93.46				
h17	0	594.67							
h20	0	1015.71							
h21	0	1015.71							
h27	0	594.67							
I-T	442.30	-45.35							

b

3-step cladogram

Haplotypes			1-step clades			2-step clades			3-step clades			
Clade	*Dc*	*Dn*	Clade	*Dc*	*Dn*	Clade	*Dc*	*Dn*	Clade	*Dc*	*Dn*	Inference
h2	490.68 S	496.951 S										
h16	0	711.95										
h18	0	920.05										
h22	0	920.05										
h24	0	367.40	1-1	528.00	530.10							1-19-2-3-5-6-7
h25	0	382.85										
h28	717.28 L	728.14 L				2-1	533.68	533.82				1-19-20-2-11-17
h30		515.20										
I-T	509.57	-120.03										
h8	0	410.49 L										
h19	0	407.47 S	1-2	408.98	634.92							1-19-20
I-T	0	-3.019 S	I-T	None					3-1	525.78	523.73	1-19-20-2-11-17
h7	166.36	185.41										
h14	0	581.04	1-3	251.35	249.35 L							
I-T	166.36	-395.63				2-2	224.08 S	458.57				1-19-20-2-11-17-4
			h10	0	72.50	I-T	309.60 L	75.26				
			I-T	-251.35	-176.85 S							
									3-2	471.61	481.50	
									I-T	None		

Appendix 1b (Continued)

h4	159.49	150.64						
h26	0	85.16	1-4	126.39	307.31 S			
I-T	159.49	65.48						
			1-5	0	331.8			
			1-6	0	493.69			
			1-7	0	383.74			
			1-8	0	409.81			
h1	484.80	479.96				2-3		
h6	114.32 S	422.87						
h9	0	354.50						
h11	0	361.56						
h12	0	361.56						
h13	0	361.56	1-9	488.96	486.12 L		1-19-20-2-3-5-6-7-8	
h15	0	594.67	I-T	472.48	73.23			
h17	0	594.67						
h20	0	1015.71						
h21	0	1015.71						
h27	0	594.67						
I-T	442.30	-45.35						