Female response and male signals in the acoustic communication system of the field cricket, *Gryllus bimaculatus* (De Geer)

by

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ABSTRACT

Sexual selection is a frame of reference that attempts to explain exaggerated signaling traits, including acoustic signals between male and female animals. Contemporary studies in the field of sexual selection are focused on the evolution of female mating preferences, with particular emphasis being placed on the good genes models of sexual selection. Here I investigate whether sexual selection is in operation in the acoustic communication system of the field cricket, Gryllus bimaculatus. Through development of new methodology I show that female crickets have a distinct and repeatable preference and selectivity for certain male song traits. For sexual selection to operate in acoustic communication systems, males must advertise some aspect of their phenotype that will influence female choice. I demonstrate that the basis for arguments invoking sexual selection for spectral song traits in a sister species, G. campestris, which is that tegmen harp area predicts song frequency, is an invalid assumption for sound production in G. bimaculatus. As a result of this finding I investigated what aspects of male song were condition- and morphology-dependent. Temporal and spectral male song traits did not convey information regarding body condition, body size or the ability to withstand developmental instability (as indicated by fluctuating asymmetry). I was unable to detect handicap sexual selection for spectral characteristics of male song despite repeatable female preference for male song frequency. Furthermore, female preference for spectral bandwidth of male song, thought to be a sexually selected trait, was shown to be governed by preference for frequency and therefore not a distinct preference. The lack of detectable sexual selection, together with observed patterns of phenotypic variation in signals and the equivalent response system, suggest that some of the male song traits function for mate recognition. However, sexual selection for call traits not considered here (e.g. duration of calling) is probable.

Key words: Female preference, acoustic signals, sexual selection, mate recognition, cricket

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Chapter 1

Introduction

The theory of sexual selection was proposed by Darwin (1871) to explain, in part, the occurrence of exaggerated secondary sexual traits. Secondary sexual traits differ between the two sexes and are not directly involved in the mechanical role of insemination, e.g. exaggerated sex-specific plumage in birds. Sexual selection, a subset of natural selection, occurs when there is selection of trait(s) by one sex that results in differential reproductive success of the other sex due to mate competition related to the expression of the trait(s) (Andersson 1994). Sexual selection is normally strongest in the sex with the greatest reproductive potential whereas mate choice is usually exerted by the other sex (Andersson & Iwasa 1996). Since male reproductive potential is normally limited only by the number of females he can fertilise (Bateman 1948), males have greater reproductive potential than females and therefore the traits involved in sexual selection are usually male advertising traits that do not necessarily improve survival, but rather enhance male ability to attract and/or fertilise a female (Andersson 1994). Consequently, females are more often the sex that exerts mate choice based on mating preferences that may be genetic or environmental in origin. It is the evolution of costly female preferences for male ornaments that is the focus of contemporary research in the field of sexual selection.

Female mating preferences for direct benefits, such as nuptial gifts in some insect mating systems, cannot explain the exaggeration of male traits and behaviour alone. It is female preference for indicators of male genetic quality that are more likely to result in extravagant male secondary sexual traits. The three main ideas of how female preferences for indicators of male quality evolve are the ‘good genes’ or ‘handicap’ hypothesis, ‘Fisherian self-reinforcing’ or ‘sexy son’ hypothesis and the ‘sensory bias’ hypothesis (Stearns & Hoekstra 2000; but see Fuller et. al. 2005 for a critical review of the sensory bias hypothesis). Mead & Arnold (2004) argue that it is misleading to distinguish between the Fisher process and ‘good genes’ hypothesis as it is unlikely that the ‘good genes’ situation exists separately from the basic Fisher process. Rather, they suggest that the question is whether the ‘good genes’ process is operative alongside the inevitable

Fisher process. The 'good genes' models of sexual selection suggest that females gain indirect genetic benefits (offspring fitness) by selecting males whose superior survival ability is displayed through honest costly signals (Stearns & Hoekstra 2000) although cost is not a prerequisite to maintain honesty of the signal (Johnstone 1995). Costly female mate preference (which reduce female viability) can evolve if male signals reveal differences in male quality (handicap principle, Zahavi 1975). This can occur either through the 'good genes' effect if male quality is heritable or through the 'good parent' effect, when males directly improve female reproductive success e.g. by providing parental care or sperm quality or quantity (Iwasa & Pomiankowski 1999). Both 'good genes' and 'good parent' processes create selection favouring stronger mate preferences and require condition-dependence of male indicator traits to work (Iwasa & Pomiankowski 1994; 1999). Few empirical studies have demonstrated conclusive evidence for a particular sexual selection hypothesis in operation.

It is crucial to the development of any theory that it be empirically verified through rigorous scientific testing of the hypotheses put forward by the theory. While many studies have claimed to detect sexual selection in a range of taxa (Andersson 1994), they did not always specify the particular hypothesis under investigation and failed in many instances to even suggest a proposed mechanism of how such female preferences could evolve. For example, although Simmons (1988a) did not suggest that his data show evidence for sexual selection, let alone argue for a particular hypothesis of how female preference may have evolved, Andersson (1994) considers the findings of this study sufficient quantitative evidence of sexual selection, despite the fact that Simmons (1988a) did not demonstrate a fitness benefit to females as a result of choice. It is the main aim of this thesis to begin thoroughly investigating whether sexual selection is in operation in the acoustic communication system of the field cricket *Gryllus bimaculatus* (Orthoptera: Gryllidae; De Geer).

The choice of *G. bimaculatus* as the study animal is based upon several key properties of this insect (see Huber et al. 1989 for greater detail). Briefly, these crickets have a cosmopolitan distribution (Africa, Europe and Asia), occur in relatively large numbers in

and around human settlements and are multivoltine. This facilitates their capture, which is accomplished easily by tracking calling males and capturing phonotaxing females near the calling males. Field crickets breed easily in captivity allowing the production of a large stock from which experimental animals may be drawn and they have a relatively short life cycle (±90 days at 28°C). If undisturbed, males readily produce calling song and females phonotax toward male song or speakers broadcasting artificial male song. A large knowledge base already exists (and is growing rapidly) on many aspects of field cricket (*Gryllus* species) biology ranging from physiology to behavioural ecology. The combination of the tractability of the acoustic communication system and the large knowledge base from which inference can be drawn makes this insect an ideal study subject.

Andersson (1994) showed that acoustic signals and songs of animals are the traits most often subject to sexual selection. Animal song, defined here as long-range acoustic signals, may have several functions including repulsion of same-sex rivals or mate attraction, the latter being the focus of this thesis. In addition to mate recognition parameters, acoustic signals must convey information regarding the phenotype of the signaler in order to become sexually selected by the receiver. Therefore, to determine if sexual selection is in operation in an acoustic communication system, researchers seek acoustic signal parameters that correlate with some aspect of male quality, fitness or condition. Once this has been accomplished, it needs to be demonstrated that females select males of high quality, fitness or condition based on the acoustic parameter(s) which was shown to be correlated with the male phenotype in question. Since female preference is far more difficult to quantify than male signals, it is usually this step of empirical verification of sexual selection that has proved problematic in the past (Wagner 1998). In Chapter 2, I develop new methodology allowing the accurate quantification of female preference at the population level for *G. bimaculatus* and demonstrate that female preference for certain song parameters is highly repeatable, another prerequisite before sexual selection can be demonstrated.

Chapter 1: Introduction

Male crickets attract females with long-range acoustic signals produced by the stridulation of their structurally modified forewings (tegmina) (Alexander 1961). Despite decades of research, the mechanism of sound production in crickets is still not fully understood (Bennet-Clark 2003). However, several biologists have suggested the possibility of sexual selection (Simmons & Ritchie 1996) as well as condition-dependence (see Cotton et. al. 2004a for a review) of spectral aspects of cricket song, based on morphology of the structures thought to be instrumental in producing the sound. In Chapter 3 I demonstrate that a fundamental assumption regarding tegmen morphology and sound production in grylline crickets is probably not valid for male *G. bimaculatus*. Specifically I show that the harp, which is the structural area of the tegmen long thought to be the primary resonator, is in fact not required for resonance of the wing and therefore sound production. This questions the broad-scale applicability of findings from studies on closely related taxa such as *G. campestris* where it has been demonstrated several times that harp area is correlated with song frequency. For this reason, Chapter 4 investigates whether the spectral characteristics of male song are sexually selected in *G. bimaculatus* as suggested for *G. campestris*. Firstly I demonstrate that male body size, fluctuating asymmetry (thought to reveal male quality, e.g. Mallard & Barnard 2004; Rantala et al. 2004) and body condition are not detectable from temporal and spectral parameters of male song. Secondly, using techniques developed in Chapter 2 to quantify female preference I show that spectral parameters of male acoustic signals are unlikely to be sexually selected. Finally, Chapter 5 places these new findings within the theoretical framework introduced above and discusses the direction of future research arising from these findings. Chapters 2-4 are pre-formatted for journal submission.

Chapter 2

Phonotactic response of female crickets on the Kramer treadmill: methodology, sensory and behavioural implications.

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Summary

Female mating preferences can shape intraspecific communication systems. It is therefore essential to describe these preferences and their variation if we are to understand the evolution of communication within the context of sexual selection. In this paper we calculate individual female response functions for four male calling song traits in the chirping field cricket, *Gryllus bimaculatus*, from phonotaxis experiments on a Kramer treadmill. Firstly we developed methodology for quantifying phonotactic accuracy by describing the sources of variation acting on it and correcting for phenomena that adversely affect the quantification thereof. Specifically, we discuss the effect of auditory asymmetry on measures of phonotactic accuracy. Secondly, we developed methodology for the characterisation of individual female response functions from phonotactic response and demonstrate the applicability of our method with respect to a recently developed method for calculating parameters of female response. We show that female phonotactic response for the same stimulus on different occasions is highly repeatable. Furthermore, for certain male signal traits, female preference and selectivity are highly repeatable. The effect of age on female response is quantified and we show that although the accuracy with which females track a sound source deteriorated with age, female preference remained the same. Finally, the limitations of phonotactic response data generated with locomotor compensators are described and discussed with regard to the estimation of the selectivity of female response.

Key words: Female preference, phonotaxis, auditory asymmetry, selectivity, response function

Chapter 2: Phonotaxis in *G. bimaculatus*

Introduction

Female mating preferences have the potential to alter the evolution of male traits. For understanding the mechanisms of female preference within the context of sexual selection (Ryan and Rand, 1993; Ritchie, 1996) it is essential to describe variation in female response within populations (Gerhardt and Huber, 2002). Female preference is, however, difficult to quantify because females often do not have graded and easily interpreted responses. In order to understand the evolution of sexual selection by female choice it is necessary to quantify the shape of individual female preference, preference variation between females as well as the repeatability of female preference (Jennions and Petrie, 1997; Wagner, 1998). Due to the amenability of acoustic communication systems to experimentation (Gerhardt and Huber, 2002) many studies investigating female preference have focused on amphibians and insects. The majority of these studies conducted experiments where only a qualitative response was required i.e. a yes/no answer to the question: "Did the female track the sound?" (e.g. Doherty 1985a; Loher et al. 1992; Murphy and Gerhardt, 2000; Grace and Shaw, 2004; but see Wagner et al., 1995). This results in binomial preference data that are often complex to interpret biologically (Kime et al., 1998; Wagner, 1998) and provides less accurate information about the strength of preference than a quantitative measure of preference would (Murphy and Gerhardt, 2000). The phonotactic responses of certain insects, e.g. crickets, appear to be sufficiently quantitative in order to characterise female preference efficiently, evident from intracellular recording of identified auditory neurons that closely correspond to phonotactic response (Schildberger et. al, 1989). A large body of information on animal communication has been generated by phonotactic measurements on insects using locomotor compensators, specifically the Kramer spherical treadmill (Kramer, 1976; Weber et al., 1981; Thorson et al., 1982). Briefly, this equipment allows the free, untethered movement of an insect towards a sound source, while remaining at a fixed distance from that source, assuring constant sound pressure level during the experiment.

Experiments attempting to quantify female preference on locomotor compensators have used several measures of phonotactic response e.g. % time tracked (Thorson et al., 1982, Doherty, 1985a), relative vector length (Loher et al., 1992), 'vector score' (Wagner et al., 1995), relative distance run (Hedrick and Weber, 1998), 'net vector

scores' (Gray and Cade, 1999). These diverse methods impede comparison between studies on female preference. To date, no study on a locomotor compensator has investigated the sources of variation affecting measures of phonotactic accuracy, a crucial first step in developing a reliable measure of phonotactic reponse. For example, although several studies have suggested the occurrence of auditory asymmetry (Boyan, 1979; Schul et al. 1998; Schul 1998), the relevance of thereof to sound localization in insects has only recently become topical (Faure and Hoy, 2000; Bailey and Yang, 2002). Moreover, as far as we are aware, only a single study has attempted to compensate for auditory asymmetry (relative phonotactic response [PR], Schul 1998). Only once a reliable measure of phonotactic response has been developed can individual female preference be characterised quantitatively using phonotactic response.

A female's *response function* describes response across a range of different male signals (Brooks and Endler, 2001). To calculate phonotactic response functions in acoustically orientating insects, the measure of phonotactic response must quantify the directional accuracy with which a female tracks a sound source. Female *preference* (see Wagner, 1998 for a discussion on the definition of female preference) is then defined as the male trait that elicits the greatest phonotactic accuracy (see Reinhold et al., 2002 for a similar definition). Female *selectivity* (synonymous to female choosiness; Jennions and Petrie, 1997; Gray and Cade, 1999; Brooks and Endler, 2001; Reinhold et al., 2002) is the sensitivity of a female to departure of the male trait from her preference. The *response magnitude* of a female is her degree of phonotactic accuracy at her preference.

Commonly used methods for describing the shape of female response functions can be inadequate to evaluate within-and between-female variation in preferences (Wagner, 1998), as well as repeatability of response. Repeatability (Falconer and Mackay, 1996) provides a measure of the consistency of a trait within individuals and sets an upper limit to the heritability of this trait (Boake, 1989). Knowledge of repeatability of female response is crucial to our understanding of how female response and male signals co-evolve in a population (Kime et al., 1998; Wagner, 1998; Widemo and Saether, 1999; Murphy and Gerhardt, 2000). It is therefore important to determine the repeatability of female phonotactic response as well as that

of female response functions. To date, the resolution of phonotactic data has been too coarse to achieve this (but see Shaw and Herlihy, 2000; Grace and Shaw, 2004) and appropriate methodology would represent an advance towards a better understanding of the evolution of communication systems.

Few studies have successfully described female response functions at the level of the individual. This is important since studies of female response at the population level can mask individual variation in response (Kime et al., 1998). Reinhold et al. (2002) proposed an approach for describing individual female response functions using non-linear regression. They measured the response calls of female grasshoppers to artificial courtship signals varying in a temporal characteristic and then estimated a Gaussian function that best fitted the female response. In addition, they separated female choice into female preference (B, the specific stimulus that elicits the greatest response), female response rate (R, the magnitude of response at the preference value B) and female selectivity (C, the parameter determining the width of the Gaussian function). Their methodology (hereafter RRJ-method) has not been applied in other studies of female preference, nor has its applicability been critically evaluated in other communication systems. Some studies have used cubic splines (Schluter, 1988) to describe female response functions at the population level (Ritchie, 1996; Brooks and Endler, 2001; Ritchie et al., 2001; Simmons et al., 2001). As far as we are aware, the efficacy of this approach has to date not been evaluated for describing the shape of an individual females' response function.

Female phonotactic response can be affected by factors such as developmental environment (Grace and Shaw, 2004), resource acquisition (Hunt et al., 2005) and age (Prosser et al., 1997; Gray, 1999; Reinhold et al., 2002; Olvido and Wagner, 2004). This could have important implications for understanding sexual selection. Reinhold et al. (2002) found that female age had no significant effect on female preference or selectivity but did affect female response rate significantly. Similarly, Olvido and Wagner (2004) demonstrated an age-related decline in female responsiveness to chirp duration in *Allonemobius socius*. Gray (1999) argued that female age, fecundity, reproductive investment and nutritional condition may affect the acoustic preferences of female crickets (*Acheta domesticus*) but found that only age significantly affected female selectivity, older females being less selective. Consequently, selection on

mating behaviour at older ages is thought to be weak. Studies on *A. domesticus* have shown that juvenile hormone III levels affect the sensitivity of auditory neurons (Stout et al., 1989a,b, 1991; Walikonis et al., 1991; Henley et al., 1992), causing older females to respond to a wider range of stimuli than young females. Since these studies showed no principal effect of age on female response, it is necessary to quantify the effect of age on phonotactic response and preference within a species.

In this paper we calculate individual female response functions for four male calling song parameters in the chirping field cricket, *Gryllus bimaculatus*, De Geer (Orthoptera: Gryllidae), using "no-choice" sequential-stimulus phonotaxis experiments.

The specific aims of this study were:

- To develop methodology for the quantification of individual female phonotactic response functions, suitable for the analysis of and comparison of populations of females.
- To quantify the effects of auditory asymmetry as well as fatigue on phonotactic accuracy
- To quantify the different levels of within-individual and between-individual variation in phonotactic response to an identical stimulus
- To quantify the repeatability and the effect of age on female phonotactic response and preference

Materials and Methods

Collection and captive care: We collected wild-living penultimate instar female field crickets from seven locations across South Africa as well as from seven captive colonies (F_1) originating from wild caught animals of a specific location and allowed them to eclose in captivity. All females were virgins since female *G. bimaculatus* do not exhibit phonotaxis after being inseminated (Loher et al., 1992). Individuals were kept in a climate-controlled chamber (25±1°C; 12:12 LD) in individual containers (500 ml) and provided *ad libitum* food (high protein cereal and fish flakes) and water (cotton-plugged vial filled with water). Individuals were randomly selected for one of four experiments described below.

Female preference: We quantified female preference through untethered phonotactic response in total darkness at a temperature of 25±1°C using a Kramer spherical treadmill (Kramer, 1976) in an anechoic chamber (>2 kHz). We conducted three experiments, each consisting of four trials. For each trial, we presented a female with a series of stimuli by manipulating a single call parameter (Table 1). Trials began with one minute of silence allowing females to become accustomed to the movement of the sphere. Each stimulus thereafter was presented twice, played back alternately from two different loudspeakers for a minute at a time respectively. Speakers were situated at 210° (speaker 1) and 90° (speaker 2) respectively from a predefined zero point in the room. Stimuli followed consecutively and were not separated by a silent pause (e.g. Doherty, 1985a). This forced females showing phonotaxis to switch direction when a stimulus changed from one speaker to the other. Furthermore, this allowed for the quantification of within-individual variation in phonotactic response to the same stimulus. We randomised the order of the trials presented to individual females. A female's phonotactic response to a stimulus may be affected by previous stimuli (Wagner, 1998). However, previous experiments (Doherty, 1985b; Hedrick and Weber, 1998) and pilot trials revealed no effect of stimulus order on female response and therefore we presented stimuli in the same order for each trial (Table 1). Nevertheless, we did investigate the effect of sequence order on female preference by reversing the order of stimuli for the syllable period trial, the trial with the longest duration (hereafter SP(Rev)). Before quantifying either phonotactic response or preference we visually inspected a females' phonotaxis by creating a trace diagram to indicate her movements throughout the duration of a trial (Fig. 1).

Acoustic stimuli: Male *G. bimaculatus* produce a calling song by stridulating their structurally modified tegmina. Each chirp comprises three to five syllables, each resulting from the closure of the tegmina. We generated synthetic acoustic stimuli played back at a sound intensity (i.e. maximum of the carrier envelope) of 70 dB SPL (measured at the top-center of the treadmill; re. 2×10^{-5} Pa). All syllables had 2 ms linear rise-fall times. We designed four trials where we manipulated either call frequency (hereafter FQ), spectral bandwidth (hereafter BW), duty cycle (hereafter DC) or syllable period (hereafter SP) (Table 1). Pre-experimental trials as well as previous experiments on *G. bimaculatus* (Doherty, 1985b) revealed a species-specific mean preference for stimuli conforming to 5 kHz frequency, 43 ms syllable period, 50% duty cycle, 4 syllables/chirp and 2 chirps/second (250 ms chirp duration). We maintained this standard, which served as the predicted preferred signal for this species, hereafter referred to as the standard (STD) stimulus, for each trial (except for SP; see below) such that only one acoustic property (e.g. frequency) was manipulated. Three of the trials (BW, DC and FQ) had an identical STD stimulus (Table 1). Following Thorson et al. (1982) and Doherty (1985a), we maintained a duty cycle of 50% while keeping the chirp duration approximately constant across all SP's tested (23-81 ms; Table 1) by varying the number of syllables. For the syllable period of 43 ms in the SP trial, stimulus characteristics for STD were as follows: 6 syllables with a chirp duration of 237 ms and an inter-chirp-interval of 349 ms, identical to that of Thorson et al. (1982). In order to quantify the magnitude of any tiring effect and to determine whether the female was still responsive to the acoustic stimuli we added an additional control stimulus (hereafter CTRL) at the end of each trial, having exactly the same characteristics as the STD stimulus.

Phonotactic response: We quantified phonotactic response for each stimulus (both speakers individually) of every trial, using a measure of phonotactic accuracy which relies on the calculation of a relative vector length (e.g. Loher et al., 1992), where

relative vector length (r)=displacement / total distance run. (1)

Displacement represents the straight-line distance between the females' starting and end positions for a particular stimulus. We calculated the angular variance (hereafter

Batschelet deviation-BatD; Batschelet, 1981), a measure of dispersion for a circular distribution as follows:

$$\text{BatD}=\sqrt{2(1-r)} \qquad (2)$$

Female crickets characteristically move with an angular error of up to 60° towards a sound source and the more accurately a female moves towards a sound source, the lower the BatD. To demonstrate the effect of auditory asymmetry on measures of phonotactic accuracy incorporating a term for angular deviation from the sound source we calculated the "sound directed component" (Schmitz, 1985) or "vector score" (Wagner et al., 1995) for the females of experiment 1, and the FQ trial. These two measures are computationally identical (hereafter CosV):

$$CosV = Cos(\alpha - S) \times r \qquad (3)$$

where α=the mean vector angle, S=angular direction of the speaker and r=relative vector length (Eq. 1). We calculated CosV as above (S=speaker direction) and also where S=the mean angle of movement (i.e. taking a females' angular offset into account).

Different approaches to analysing phonotactic response function:

1) Polynomial regression. We generated phonotactic response functions for each female and each trial by obtaining a high order regression equation from the phonotactic data (BatD) (Fig. 2). Tests for the efficiency of the regression analysis for deriving the response function indicated that, for BW and DC, 3rd order polynomials should be used and for FQ and SP, 6th order polynomials.

2) Non-linear regression (RRJ method). Following Reinhold et al. (2002), we used non-linear regression and fitted a Gaussian function to describe the response functions for each female and each trial. Since the phonotactic data were initially not suitable for normal distribution fitting (lower BatD denotes greater preference), we transformed the data (BatD') as follows:

$$BatD' = (-1) \times BatD + maxBatD + minBatD \quad (4)$$

where BatD=original phonotactic accuracy for a particular stimulus, maxBatD=the largest BatD value of a trial and minBatD=the lowest BatD value of a trial. This meant that the original BatD value with the lowest value would now have the largest value (and *vice-versa*) and that the original scaling of the data was maintained (Fig. 2). Non-linear regression (NLREG dynamic link library version 5.2; Copyright Phillip H. Sherrod 1992-2001) employing the Levenberg-Marquardt algorithm, was used to estimate the Gaussian function,

$$f(x) = R \times e^{-0.5 \times [(X-B)/C]^2} \quad (5)$$

that best fitted the transformed phonotactic data. Following Reinhold et al. (2002), we interpreted B and C, as estimates of female preference and selectivity respectively. Our measure of phonotactic response (BatD) was not directly comparable to 'response rate' but served as a quantitative measure of female response magnitude (R), since females were expected to have a small BatD when showing strong phonotaxis towards their preferred stimulus. The start value for R was always 90 while B and C were respectively set as follows: BW: 0.4, 0.3; DC: 50, 20; FQ: 4, 1.5; SP and SP(Rev): 43, 20. If we obtained a value for B that was smaller than the starting value for the trial (Table 1) we set B to the starting value of the trial and recalculated the values for C and R (this happened four times in the BW and once in the SP trial). We never obtained a value for B that was greater than the largest value for a trial.

3) Cubic spline method. We generated response functions for each female and each trial by using cubic splines (Schluter, 1988) with a smoothing parameter (λ) of-5 (Fig. 2). Schluter (1988) cautions against the use of extreme λ values as it can either cause the resulting curve to be too smooth (large λ) or the curve will simply fit each data point (small λ). We tested a large range of smoothing parameters and chose the smoothing parameter which satisfied a combination of the following criteria: minimised the general cross-validation (GCV) score (Schluter, 1988) and maximized between-individual differences in preference and repeatability of preference. Two

hundred bootstrap replications allowed each response function to be fitted with errors (± 1 SE), although these were always very small due to the relatively small number of unique stimuli presented within a trial (Schluter, personal communication). We did not require the addition of a random error to break ties (e.g. Ritchie, 1996; Ritchie et al., 2001; Simmons et al., 2001) as no female yielded identical phonotactic response (BatD) for both speakers of a stimulus. The generation of a cubic spline does not provide an equation for predictive value. We therefore obtained a high order regression equation (same order as for the polynomial regressions above) from the predicted values ($\hat{y}$) from the spline analysis and used this equation to calculate B', C' and R' (see below). These equations fitted the $\hat{y}$ values extremely well (mean r^2=0.98±0.03).

Comparison of methodology: In order to compare the quantification of female response from the polynomial regression and spline methods with the RRJ-method, we required analogous measures for estimating female preference (B), selectivity (C) and response rate (R) (Reinhold et al., 2002). We achieved this by taking female response magnitude (R') as the best phonotactic response (lowest BatD) during a trial; female preference (B') as the call parameter value (on the x-axis) corresponding to R', and female selectivity (C') was taken as the width of the regression equation at 10° above R' (Fig. 2).

Body size: After successfully completing an experiment, females were killed and digital images of the pronotum were generated with the Creative Laboratories VideoBlaster FS200 utility program. Using the same program, we measured the pronotum length and width (resolution=15.8 μm). The surface area of the pronotum (pronotum length x pronotum width; mm^2) was taken as a mass-independent measure of body size.

We conducted the following four experiments:

Experiment 1) Auditory asymmetry, fatigue and levels of variation in phonotactic experiments on the Kramer treadmill: Females of equal age (10 days post adult ecdysis) were measured in groups of 20 with each individual (n=130) subjected to three trials (BW, DC and FQ) in random order over a period of no longer than 2 days with a minimum of 10 hours rest between trials. This experimental design allowed

firstly for the quantification of female auditory asymmetry and it's effect on the calculation of phonotactic accuracy. Secondly, we were able to quantify the effect of tiring on phonotactic response since the position of the STD and CTRL stimuli varied over a large range across the three trials (0-19min, Table 1). We quantified the following hierarchical levels of variation in phonotactic response for these identical stimuli (STD and CTRL): between-female, within-female between-trials, within-trials between-stimuli and within-stimulus between-speaker. We performed a nested random-effects ANOVA in order to quantify these different levels of variation using the PROC NESTED procedure of the SAS V8.02 statistics package (SAS Institute, Cary, N.C., USA).

Experiment 2) Repeatability of phonotactic response and response function: Four groups of females of equal age (10 days post adult ecdysis) were selected, with each group subjected to either BW, DC, FQ or SP twice. This meant that each female within a group was subjected to two identical trials with at least 10 hours of rest between trials. This experimental design allowed firstly, for the calculation of repeatability of phonotactic response to an identical stimulus. Repeatabilities of phonotactic response for both the STD and CTRL stimuli were calculated separately. Secondly, the repeatability of female preference (B and B'), female selectivity (C and C') and female response magnitude (R and R') was calculated. Repeatability ± standard error was calculated following Becker (1984).

Experiment 3) The effect of stimulus sequence on SP preference: Females of equal age (10 days post adult ecdysis) were each subjected to the SP trial (Table 1) and an additional SP trial where we reversed the sequence of stimuli so that the syllable period was varied from long to short (81-23 ms). This experiment was similar in design to that of Doherty (1985b) and allowed for the calculation of repeatability of preference by comparing preference between the different trials. Low repeatability of preference in this experiment when compared to that of experiment 2 (above) would indicate an effect of stimulus sequence on phonotactic response.

Experiment 4) The effect of age on phonotactic response and preference: Females of equal age (10 days post adult ecdysis) were each subjected to four trials (BW, DC, FQ and SP) presented in random order within two days, with at least 10 hours rest

between trials. These females were subjected to the four trials every 10 days until death, allowing for the quantification of the effect of age on phonotactic response.

Results

Experiment 1a) Female auditory asymmetry and phonotactic accuracy: Nearly all females had an angular offset, i.e. a constant difference between the female's direction of movement and the direction of the speaker. Fig. 3 shows phonotaxis for a female with an angular offset of approximately -30°. A female with an auditory deficiency on her right side, has an angular offset to the left (negative offset in Figs 3 and 4) because she adjusts her movement direction in order to receive an equal sound pressure level (SPL) on both tympani (Bailey and Thomson, 1977; Thorson et al., 1982; Schmitz, 1985). By moving the more functional tympanum away from the sound source (left tympanum of female in Fig. 3), the SPL on both tympani can be equalized. The magnitude of the angular offset is determined by the degree of auditory asymmetry and affects measures of phonotactic accuracy that incorporate a term for angular deviation from the sound source. The CosV calculated around the true speaker direction (0.86±0.11) was significantly smaller than the CosV calculated around the females perceived speaker direction (0.89±0.09) (paired t-test, t_{259}=6.05, P<0.001) and therefore indicated poorer phonotactic accuracy.

The magnitude and sign of a female's angular offset affect the time taken for a female to switch direction between speakers. In Fig. 3, time taken to switch between speakers differs as the female switches almost instantly from speaker 1 (210°) to speaker 2 (90°) by following the shortest angular difference between speakers. However, when switching from speaker 2 to speaker 1 this female took more time and the greater angular difference between speakers was followed when switching from 4.5 to 5 kHz. For the females in experiment 1 (n=130), we calculated a female's mean angular offset for the STD and CTRL stimuli of each of the three trials she was subjected to. We also calculated the time in seconds from the onset of the stimulus until a female moved to within 60° of her perceived speaker direction for that stimulus. We then calculated the mean difference in duration for locating speaker 1 and speaker 2 respectively, for each female. The signed difference between these two durations was plotted against a female's mean angular offset (Fig. 4) revealing a highly significant relationship ($F_{1,128}$=63.86, r^2=0.33, P<0.001).

Experiment 1b) Effect of tiring on phonotactic accuracy: For each female of experiment 1 and each of the three trials, we calculated the difference between the distance run while tracking the STD and the CTRL stimuli. This difference served as a measure of tiring since a female is expected to run similar distances toward the same stimulus. Similarly, for each trial, the signed difference between the phonotactic accuracy (BatD) toward the STD and CTRL stimulus served as a measure of degradation of phonotactic accuracy. No significant correlation between the difference in phonotactic accuracy and the difference in distance run was found for any of the three trials. The strongest effect was found for the BW trial where the mean difference in phonotactic accuracy (BatD) was 2.04±4.69º and the mean difference in distance run was 102.7±89.94 cm (Δ BatD vs. Δ distance run: Pearson r=-0.06, P=0.47). This suggests that phonotactic accuracy is not affected by fatigue, even though there was a large mean difference in distance run between the STD and CTRL stimuli.

Experiment 1c) Sources of variation in phonotactic accuracy for an identical stimulus: We performed two nested random-effects ANOVA's on phonotactic accuracy. The variance hierarchy for the first ANOVA was nested as stimulus within trial within individual. In order to quantify the amount of variation resulting from phonotaxis towards different speakers, we required an error term and therefore performed a second ANOVA that was nested as speaker within trial within individual. This was justified since the estimates of variation due to ID and TRIAL did not differ between the two analyses and the variation due to different stimuli was not significant ($F_{390,780}$=0.94, P=0.77, 0% variation explained). Table 2 therefore presents the results from the second ANOVA. A large proportion of variation in phonotactic accuracy was attributable to the significant between-individual differences, suggesting that different females have different innate abilities to track a sound source well. A females' mean phonotactic accuracy at the STD stimulus was not affected by her body size ($F_{1,128}$=3.80, r^2=0.02, P>0.05) or the absence of a hind leg (n_1=112; n_2=18; t_{128}=0.82, P>0.41). The proportion of variation in phonotactic accuracy due to the different trials and the different stimuli (STD or CTRL) respectively, were non-significant (Table 2). A large and highly significant proportion of variation in phonotactic accuracy was due to between-speaker differences, revealing that females

always tracked speaker 1 better than speaker 2. However, the magnitude of difference of BatD was only 3.3° (Table 2).

Experiment 2a) Repeatability of phonotactic response to a standard stimulus: The first four columns of Table 3 show the repeatability estimates of phonotactic response for the STD and for the CTRL stimuli respectively for the females of experiment 2 that were presented with two identical trials (either BW, DC or FQ). The repeatabilities calculated are high and significant, indicating that a females' phonotactic response to a specific stimulus (near the predicted preference for this species) is similar between trials. We pooled the phonotactic response data for the STD and CTRL stimuli respectively across the three trials to estimate the repeatability of phonotactic response for an identical stimulus, independent of the trial. Repeatabilities of phonotactic response calculated in this manner were both high and significant (STD: 0.78±0.7, $P<0.001$; CTRL: 0.72±0.09, $P<0.001$).

Experiment 2b) Repeatability of response function: For experiments 2 and 3, we generated response functions using the three different methods described above and then calculated the repeatability of female preference (B and B'), female selectivity (C and C') and female response magnitude (R and R') (Table 3). The polynomial regression and the spline methods yielded high and significant female preference (B') repeatabilities which were very similar. Since the response functions for each trial were unimodal, their shape could potentially be approximated by the shape of a normal distribution (a prerequisite for the RRJ-method). The repeatabilities calculated from polynomial regression and the spline method were similar except for the repeatability estimates of R'. For each trial in Table 3 the R' calculated from the spline method was significantly greater (following a Bonferroni correction; Rice 1989) than the R' calculated from the polynomial regression (paired t-tests; smallest $t=3.33$ for the BW trial; $P<0.01$ for all trials). The RRJ-method did not yield comparable results and does not appear to be suitable for this type of data as the Gaussian function fitted to the phonotactic response data explained significantly less of the variation than the polynomial regression did (Table 4).

Experiment 3) The effect of stimulus sequence on SP preference: For the females subjected to the SP trial and the SP(Rev) trial (reversed sequence of stimuli), SP

preference (B') and selectivity (C') was highly repeatable and was indeed slightly greater than that calculated for the females of experiment 2 (Table 3), suggesting that the sequence of stimulus presentation has a negligible effect on female response.

Experiment 4) The effect of age on phonotactic accuracy and preference:

1) Phonotactic accuracy. Each female in experiment 4 was subjected to six identical stimuli at every age category (STD and CTRL stimuli for BW, DC and FQ trials respectively), allowing us to quantify the effect of age on the phonotactic accuracy towards a specific stimulus. We performed a repeated measures ANOVA on the phonotactic accuracy (BatD) for the STD and CTRL stimuli respectively for all females surviving to 30 days of age (Table 5). We found no difference in phonotactic accuracy attributable to age, trial or their interaction. We performed an additional repeated measures ANOVA, this time with only two age classes namely at 10 days old and the last set of measurements performed before the female died ("final age"). This "final age" ranged from 20-40 days of age, depending on the individual females' longevity. Table 5 shows that for this comparison, a significant effect of age was found for both the STD and CTRL stimulus, suggesting that females track a particular stimulus consistently throughout their lives until a few days before they die.

2) Preference. Using the spline methodology described above, we determined individual female preference (B') for each trial completed at each age class. We performed several repeated measures ANOVA in order to determine whether preference changed with the age of a female. Table 6 shows the results of the repeated measures ANOVA for ages of 10, 20 and 30 days. Preference during a specific trial did not differ between the age classes. Although with a much reduced sample size, we repeated these exact analyses but additionally included the age class of 40 in order to determine if these results were consistent across a greater age range. Again, we found no effect of age. We performed additional paired t-tests with two age classes, 10 days old and the last set of measurements performed before the female died. These analyses were performed in order to determine if preference changes and becomes unreliable just before a female dies in a similar manner to phonotactic accuracy (see Table 5). Preference did not differ between these two age classes (Table 6).

Limitations of phonotactic experiments conducted on the spherical treadmill: To determine whether female field crickets are consistent in their response to stimuli outside of their preference range, we calculated the absolute difference in phonotactic accuracy (BatD) between identical stimuli of the trial that was repeated for females of experiment 2. Duplicate trials for each female meant that for each stimulus of a trial we had a measure of the consistency of phonotactic accuracy, which differed between stimuli (e.g. FQ trial: Repeated measures Anova, $F_{8,88}$=4.23, P<0.001). We also calculated the repeatability of the phonotactic response for each stimulus. Fig. 5 shows the mean BATD difference between repeat trials for each stimulus for the FQ trial (n=12) and the repeatability estimates of phonotactic response for each stimulus. Clearly the smallest mean difference between repeats, the smallest amount of variation around the mean difference and also the greatest repeatability is close to the preferred stimuli (4.5 or 5 kHz). This pattern was similar for the other trials. These large and inconsistent differences in phonotactic response between identical stimuli at the lower and upper extremes of the signal range suggest that females do not respond consistently to stimuli outside of their preference range. This means that the degree of female indifference to a male signal cannot be quantified accurately through phonotactic response. Furthermore, the phonotactic response at the lower and upper extremes of the male trait affect the position of the mode of the Gaussian function fitted to the data (RRJ-method) and therefore the estimate of female preference. To demonstrate this we applied the RRJ-method to the FQ trial data of experiment 2 but this time we excluded the phonotactic response data for the two lower and upper extreme stimuli such that the range of frequencies now spanned from 4-6 kHz. We did this since the shape of the response function in this range resembled a Gaussian function (Fig. 2). We compared the estimates of female preference (B) obtained in this manner to the estimates of female preference obtained previously from all three methods (B and B'; Table 3) using a repeated measures ANOVA. Since we were interested in comparing the estimated female preference between methods we treated the repeats of each female as independent and thereby doubled the sample size (n=24). There was a significant difference between the preference estimates determined by the four different methods ($F_{3,69}$=22.65, P<0.001) which was solely due to the preference estimates from the original RRJ-method differing from the preference estimates of every other method (Tukey HSD *post-hoc* comparison;

P<0.001). In fact, the correlations between the preference estimates obtained from all methods except the original RRJ-method were high and significant (Table 7).

Discussion

Measuring phonotaxis in females with asymmetrical hearing: Previous studies on female crickets reported strong phonotaxis but at an erroneous angle (e.g. Fig. 3) to the sound source (Thorson et al., 1982; Doherty, 1985a; Schul et al. 1998; Schul 1998), yet only a single study has attempted to correct for this phenomenon (Schul 1998). Measures of phonotactic response that include a term for angular deviation from the sound source underestimate the performance of a female with an asymmetrical auditory system. Our results show that Batschelet Deviation, a measure independent of the angle of the mean vector, is an efficient tool for population level analysis of phonotaxis, since we were not interested in a females' absolute ability to locate a speaker but in her tracking accuracy of where she perceived the speaker to be located. While this ensured that a female's angular offset due to an asymmetrical auditory system would not affect the calculation of her phonotactic response, a potential problem arises if a female walks constantly in any random specific direction for the entire duration of a stimulus, yielding low angular deviation and falsely indicating accurate tracking. However, this problem is a feature of any measure of phonotactic response that does not include a term for angular deviation from the sound source e.g. 'relative vector length' (Eq. 1, *r*, Loher et al., 1992) or 'relative distance run' (Hedrick and Weber, 1998) and is not restricted to our measure. After viewing female phonotactic response for more than 10000 stimuli, we could not identify a single occurrence where a responsive female walked in an incorrect direction and maintained that direction throughout the presentation of a stimulus. Nevertheless, we recommend that phonotactic response during trials are visualised (as in Fig. 1) and screened before the measurement thereof.

Several females shown in Fig. 4 took up to 10 seconds longer on average to locate one of the speakers due to their auditory asymmetry. The significant effect of angular offset on the time taken to switch between speakers (Fig. 4) affected quantification of within-stimulus between-speaker variation in phonotaxis. Furthermore, calculation of phonotactic response over the entire duration of the stimulus (1 min) underestimates the phonotactic accuracy to one of the speakers for a female with asymmetrical

hearing. Since the delay in orientation to a speaker is not the same for both speakers, between-speaker differences in phonotaxis can arise. We corrected for this problem by quantifying phonotactic response (BatD) only after the female had orientated to within 30° of her perceived speaker direction. However, if a female did not orientate to within 30° of her perceived speaker direction at all during a particular stimulus then the BatD was calculated for the entire duration of the stimulus (1 min). Variation in the magnitude of auditory asymmetry between repeated trials for the same female could arise if tiny objects (e.g. dust) impair the function of one of the tympani (e.g. horizontal error bars in Fig. 4) for one of the trials. It is unlikely that such temporary auditory asymmetry can be completely avoided and therefore, where applicable, experiments should control for auditory asymmetry to ensure accurate measurement of phonotaxis (e.g. Schul 1998).

Constraints of the Kramer treadmill:

The significant proportion of variation in BatD attributable to between-speaker differences in phonotaxis (Table 2) was due to the mechanical arrangement of the experimental equipment, specifically the position of the speakers relative to the two motors driving the sphere and the acceleration of each motor. Motor A was positioned at 45° and motor B at 135°. This arrangement meant that when a female was moving towards speaker 1 (situated at 210°), motor A was mainly responsible to compensate for the forward acceleration of the female and motor B was mainly responsible for the angular correction of the female's course. However, when a female was moving toward speaker 2, both motors were simultaneously responsible for forward acceleration compensation and angular corrections. Since no two motors have identical acceleration, failure of one motor to accelerate equally when a female moved directly toward 90° would result in a small angular error towards the direction of the slower of the two motors. Females would then have to correct for this angular error and therefore the resulting BatD would be greater for speaker 2 as is evident in Table 2 and visually in the trace diagram of Figs 1 and 3. When calculating female response for a male trait however, the phonotactic accuracy toward both speakers was used in the generation of the response function (Fig. 2) and therefore conclusions about female response are not affected by these small yet significant differences.

Female indifference to a male signal cannot be quantified to the same level of accuracy as female preference. Doherty (1985b) showed that the tracking scores (their measure of phonotactic response) calculated for *G. bimaculatus* females in "no-choice" sequential experiments on syllable period had minimal variability in the effective range (40-45 ms) while stimuli with syllable periods at the margins of the effective range (30-35 and 50-60 ms) yielded variable scores within and between individuals. Also, Fig. 2 in Gray and Cade (1999) shows increased standard deviations for female net vector scores (their measure of phonotactic response) to both the upper- and lower extreme values for syllables per trill that *G. integer* females were exposed to. Our findings on female indifference are therefore not new, but the significance thereof have not been discussed to date. For example, Gray and Cade (1999) report a lack of heritable variation in female selectivity for syllables per trill in *G. integer* but do not discuss the effect of unreliable female response at the extreme male trait values on their measure of female selectivity. Consequently, their inability to detect heritable variation in female selectivity for pulses per trill may be tentative.

Phonotaxis towards a standard stimulus: Females from experiment 1 did not show reduced phonotactic accuracy or become unresponsive after the presentation of many stimuli (first nested ANOVA). We could also not detect any effect of reduced phonotactic accuracy due to fatigue *per se*. While this indicated reliability of phonotactic response within a trial, the small proportion of variation in phonotactic response to a standard stimulus between different trials (Table 2) showed that females tracked this stimulus with similar accuracy, irrespective of the stimulus setup of the trial. This was not because all females tracked the stimulus with the same accuracy since significant between-individual variation in phonotactic accuracy was found (Table 2), which was independent of body size or the absence of a hind leg. Furthermore, individual phonotactic response to a standard stimulus is highly repeatable between replicates of the same trial (Table 3). Given the reliability of phonotactic response in this species it is therefore valid to infer preference from phonotactic walking behaviour.

Methodology comparison

We do not believe the RRJ-method appropriate for estimating parameters of female response from our data since the response function derived from this method

explained significantly less of the variation in BatD than did a polynomial regression (Table 4). The reason for this is two-fold. Firstly, imposing a Gaussian shape on the female response function reduces the efficacy of that function to explain the variation in the data, even if the distribution of data deviates only slightly from the imposed shape. There is no *a priori* reason to believe that our phonotactic data should yield a response function that can be approximated by the shape of a Gaussian curve. In fact, the shape of the response function for frequency derived from the polynomial regression and spline methods (Fig. 2) remarkably resemble the shape of auditory tuning curves derived from auditory thresholds of low frequency neurons in the prothoracic ganglia which function as narrow band-pass filters (Schildberger et al., 1989). Secondly, the male trait range over which we tested female response affects the efficacy of the RRJ-method. The unreliable phonotactic response at the extreme male trait values affects the position of the maximum of the Gaussian function and therefore the estimate of female preference for the RRJ-method, whereas the estimates of female preference from spline and polynomial regression methods are not greatly affected as evident from their significant correlation (Table 7). Since female phonotactic response is reliable close to the predicted species preference for field crickets and for some male traits has a shape similar to that of an inverted Gaussian function, the RRJ-method may be useful if applied to data comprising phonotactic response to many stimuli situated close to the predicted species preference for that trait. For example, there is strong congruence between the preference estimates for FQ derived from the spline, polynomial regression and RRJ-method where we excluded the phonotactic response data for the two lower and upper extreme stimuli (min2; Table 7).

Female preference (B) derived from the RRJ-method was unlikely to reflect true female preference for reasons discussed above, and therefore the estimates of repeatability for B are not biologically relevant. Measurements of response magnitude (R) at B are consequently equally irrelevant. This can be visualized by the poor correspondence between R and the raw phonotactic response data (Fig. 2). The two significant repeatability estimates of R from the RRJ-method (BW and FQ) are therefore interpreted as spurious (Table 3). Furthermore, the single significant repeatability estimate for FQ selectivity (C) derived from the RRJ-method is also likely to be spurious since both the spline and polynomial regression methods failed to

detect repeatable selectivity for FQ (Table 3). We therefore do not continue discussion of repeatability estimates derived from the RRJ-method.

Repeatability of response functions:

Female selectivity (C'). There was almost no difference in the repeatability estimates of C' calculated from the polynomial regression and the spline method (Table 3). The absence of repeatable selectivity for DC arises since females responded well to all duty cycles except the upper and lower extremes (10% and 90%). Consequently, almost no between-individual variation in selectivity for duty cycle was detected and therefore duty cycle is unlikely to be an important signal trait for sexual selection in *G. bimaculatus*. Conversely, females were highly selective for frequency and no between-individual variation (and repeatability) in selectivity could be detected. This does not rule out the possibility of heritable variation in selectivity for this species (see Brooks and Endler, 2001). The significant selectivity for BW probably reflects a combination of repeatable frequency preference and similar frequency selectivity between females, since frequency preference is likely to determine bandwidth preference in crickets (Simmons and Ritchie, 1996). Females showed significant repeatability of selectivity for the SP and SP(Rev) trials (Table 3). Doherty (1985b), using similar equipment to ours but a crude measure of phonotactic response (% time tracked), showed that the syllable period range tracked by *G. bimaculatus* females differed between to and fro sequential trials. He did not calculate individual response functions but used population-level data to arrive at this conclusion. Furthermore, he did not provide a measure of the range that was tracked, nor statistical evidence for this conclusion. Our results show that SP selectivity is repeatable, even if the sequence of stimuli is reversed and further illustrate how population-level analyses can mask relevant individual variation in response (Kime et al., 1998). To our knowledge, the only other evidence for repeatability of female selectivity comes from guppies (Brooks and Endler, 2001) and grasshoppers (Reinhold et al., 2002), probably since very few studies have investigated female selectivity.

Response magnitude (R'). The phonotactic response magnitude (R') at the peak preference (B and B') was not always repeatable (Table 3). The smoothing effect of the spline reduced between-individual variation in R' and repeatability was not significant. Conversely, the waviness of the polynomial regression (see Schluter, 1988) inflated between-individual variation and yielded significant repeatabilities for

three of the trials. Consequently, we do not believe that R' is a reliable surrogate for response rate as measured by Reinhold et al. (2002). Although variation in responsiveness can mask variation in preference in some systems (Brooks and Endler, 2001), it is not likely to affect the calculation of female preference here as we quantified preference from phonotactic response data independently of the response magnitude.

Female preference (B'). We believe that the female preference (B') repeatabilities calculated from the polynomial regression and spline methods for the four male song traits in Table 3 approximate the true repeatabilities. Evidence for this comes from strong congruence between preference estimates derived from three different methods (Table 7). For all of the trials, repeatability estimates for B' were significant, indicating the possibility for quantitative genetic variation in preference for these male traits (see Brooks and Endler, 2001). The near lack of preference for a particular duty cycle decreased the between-individual variation in preference for duty cycle and is therefore only marginally significant. However, the other estimates of preference repeatability (BW, FQ and SP) are far greater than the preference repeatabilities for number of pulses per trill (0.50) and the inter-trill interval (0.59) reported for *G. integer* by Wagner et al. (1995) who used similar experimental equipment to ours.

Effect of age on female preference

Female preference was not affected by age (Table 6). Despite the significant degradation of phonotactic accuracy just before a female dies (Table 5), her preference is still measurable which is similar to the findings of the majority of studies (Gray, 1999; Reinhold et al., 2002; Olvido and Wagner, 2004) investigating the effect of age on female preference. Generally, no effect of age on female preference was detectable while responsiveness and selectivity appear to degrade with age. Although our measure of phonotactic response is not directly comparable to a measure of responsiveness, the decline in accurate tracking ability is likely to reflect both neurological changes as well as changes in motivational level.

Shape of response functions in G. bimaculatus: Female response is a complex interaction between environmental, neurological and behavioural components and is therefore not easily quantified. The quantification of the shape of female response is not simple and requires complex techniques such as cubic regressions (Olvido and

Wagner, 2004), cubic splines (Ritchie, 1996; Brooks and Endler, 2001; Ritchie et al., 2001; Simmons et al., 2001) or non-linear regression (Reinhold et al., 2002). Whenever possible, experiments to determine female preference for a particular male trait should test an abnormally wide range of stimuli, i.e. extreme trait values that do not occur in the natural population on either side of the trait distribution (e.g. Shaw and Herlihy, 2000; Grace and Shaw, 2004). Female response functions based on such tests will mostly be unimodal (except in the case of strong directional selection) and therefore the shape of the response function is relatively easy to approximate with a high order polynomial equation or a nonlinear technique such as the RRJ-method (Reinhold et al., 2002). However, habituation or fatigue due to multiple testing may mask variation in some response functions (Brooks and Endler, 2001). A way to avoid this problem is to limit the number of stimuli presented to a female by subjecting females to stimuli differing at a relatively course-scale. Fine-scale preference can then be estimated as demonstrated above. The applicability of these methods for inferring fine-scale preference from course-scale experiments is not limited to the field of bioacoustics.

Our experimental procedure to determine female preference has several advantages over other methods. Firstly, the use of the Kramer treadmill has numerous advantages over binomial choice tests in an arena, constant SPL of the sound source being the most crucial. Also, the exact path of the female can be investigated after completion of the experiment. Sequential presentation of stimuli has many advantages over choice tests (Wagner, 1998; Murphy and Gerhardt, 2000) although carry-over effects may sometimes affect phonotactic response (Doherty, 1985b; Wagner, 1998). However, the high and significant preference (B') and selectivity (C') repeatability of the SP(Rev) trial, where we reversed the sequence of stimuli (Table 3), suggests that it is unlikely that the sequence of stimuli affects the response of female *G. bimaculatus*.

Conclusion:

Phonotactic response in *G. bimaculatus* varies between individuals, is repeatable within individuals if the signal is close to the predicted species preference, and is a reliable measure for inferring preference. We have developed suitable methodology to quantify female preference and selectivity from phonotactic response that is independent of asymmetrical auditory systems or the effects of fatigue. The spline

method for estimating female preference may be directly applied to the study of female preference in other taxa where a quantitative measure of female response is generated. We have shown that female preference for certain male traits is highly repeatable and that although age effects a females' phonotactic accuracy while tracking a sound source, it does not affect her preference. Since we randomly selected females for our experiments, the observed repeatable variation in female preference may be due to either environmental effects during development or heritable differences between females. Low estimates of female preference repeatability reported in the past may have been a result of the lack of appropriate tools and methodology for quantifying female preference accurately (Wagner, 1998). The combination of significant between-individual variation and the high repeatability of female preference for BW, FQ and SP in *G. bimaculatus* creates opportunities for new experiments in the field of sexual selection.

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Chapter 2: Tables

Table 1. Description of the four different phonotaxis trials that female crickets (*G. bimaculatus*) were exposed to. Each stimulus was played back for a duration of one minute per speaker from two different speakers situated at different locations (210º and 90º respectively). Each stimulus was played back for a duration of one minute per speaker. All trials except SP (see text) had a standard (STD) and control (CTRL) stimulus with the following specifications: 5 kHz frequency, 43 ms syllable period, 50% duty cycle, 4 syllables/chirp and 2 chirps/second. The CTRL stimulus was the final stimulus of a trial (not indicated in the table).

Trial	# Stimuli	Stimuli presented	STD stimulus position
Bandwidth (BW)	7	0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 kHz	1
Duty cycle (DC)	7	10, 30, 40, 50, 60, 70, 90%	4
Frequency (FQ)	9	3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7 kHz	5
Syllable period (SP)	10	23, 28, 35, 39, 43, 48, 53, 58, 67, 81 ms	-

Table 2. Hierarchical levels of variation in phonotactic response to an identical stimulus (5 kHz frequency, 43 ms syllable period, 50% duty cycle, 4 syllables/chirp and 2 chirps/second) for 130 *G. bimaculatus* females. Nested random-effects ANOVA was performed with Speaker nested within Trial, which was nested within ID. Percentage values in brackets indicate the proportion of variation attributable to that source. BatD is the angular variance (°), a measure of dispersion for a circular distribution. Mean ± StdDev of BatD are presented below the F-values.

Source	df	Phonotactic accuracy (BatD)
ID	129,260	F=4.50 *** (22.4%)
TRIAL	260,390	F=0.77 (0%)
BW		23.20 ± 5.84
DC		21.68 ± 5.25
FQ		22.25 ± 5.90
SPEAKER	390,780	F=1.81 *** (22.4%)
SP1		20.71 ± 5.22
SP2		24.04 ± 5.68

*** P<0.001

Table 3. Repeatability estimates (± SE) of phonotactic response in *G. bimaculatus*. a) Significant repeatability of phonotactic response (Phonotactic accuracy; BatD [°]) for an identical stimulus (STD and CTRL, see text for details) shows that females respond similarly to an identical stimulus on different occasions. Repeatability of female response function calculated using three different methodologies (1-3). Female response function is partitioned into preference (B), selectivity (C) and response magnitude (R), calculated from the non-linear regression (Reinhold et al. 2002). For both polynomial regression and cubic spline methods the analogous measures of female choice was calculated where B'=preference, R'=BatD at the preference and C'=the width of the curve at 10° above minimum BatD (see Fig. 2).

			a) Phonotactic accuracy		1. Polynomial Regression			2. Non-linear regression			3. Cubic spline		
Trial	n	df	STD	CTRL	B'	C'	R'	B	C	R	B'	C'	R'
BW	9	8,9	0.79±0.13**	0.69±0.18**	0.79±0.139 **	0.84±0.10 ***	0.95±0.03 ***	0.70±0.18 *	0.01±0.35	0.57±0.24 *	0.83±0.11 ***	0.845±0.10 ***	0.28±0.33
DC	9	8,9	0.60±0.23*	0.77±0.14**	0.53±0.259 *	0.26±0.33	0.80±0.139 **	0.63±0.21 *	0.52±0.26	0.10±0.35	0.53±0.25 *	0.26±0.33	0.46±0.28
FQ	12	11,12	0.77±0.12***	0.74±0.14***	0.69±0.16 **	-0.11±0.30	0.067±0.30	0.47±0.24 *	0.64±0.18 *	0.53±0.22 *	0.72±0.15 **	-0.22±0.29	0.30±0.27
SP	8	7,8	-	-	0.75±0.17 **	0.67±0.21 *	0.76±0.16 **	0.39±0.32	-0.14±0.37	-0.02±0.38	0.75±0.17 **	0.67±0.21 *	-0.37±0.33
SP(Rev)	9	8,9	-	-	0.78±0.14 **	0.71±0.18 **	0.14±0.35	0.32±0.32	0.44±0.29	0.29±0.32	0.78±0.14 **	0.71±0.18 **	0.15±0.35

* P<0.05

** P<0.01

*** P<0.001

Table 4. Comparison between the r^2 (mean ± Std Dev) of the polynomial regression and the non-linear regression (Gaussian function, RRJ-method) indicating that, for each trial, the polynomial regression fitted the data significantly better (paired t-tests). All comparisons remained significant following a Bonferroni correction.

		r^2			
Trial	df	Polynomial	non-linear	t	P
BW	17	0.42±0.23	0.36±0.22	3.35	0.004
DC	17	0.78±0.17	0.67±0.18	5.18	<0.001
FQ	23	0.72±0.16	0.45±0.23	8.44	<0.001
SP	15	0.71±0.18	0.43±0.26	5.4	<0.001
SP(Rev)	17	0.68±0.13	0.42±0.26	5.29	<0.001

Table 5. Effect of age on phonotactic response to an identical stimulus. Results of four repeated measures ANOVA conducted on the phonotactic accuracy (BatD) while tracking the STD or CTRL stimulus of three trials (BW, DC, FQ). Females included in the analyses were all females surviving to 30 days of age and all females between age 10 and Final Age (defined as the last trial completed successfully before the female died).

			F	
Comparison	Source	df	STD	CTRL
Age 10,20,30	Age	2,14	2.92	1.75
	Trial	2,14	2.03	3.27
	Age*Trial	4,28	1.92	1.28
Age 10-Final	Age	1,9	8.39*	5.96*
	Trial	2,18	0.19	1.85
	Age*Trial	2,18	0.69	0.79

* $P<0.05$

Table 6. The effect of age on female *G. bimaculatus* preference for four male song traits. Repeated measures ANOVA results for females completing four different trials at age 10, 20 and 30 days and paired t-tests conducted on preference for the two age goups; Age 10 days and Final Age. Final Age is defined as the last trial completed successfully before the female died.

	ANOVA			t-test		
Trial	df	F	P	df	t	P
BW	2,16	0.59	0.57	13	-0.33	0.75
DC	2,16	0.16	0.85	12	-1.39	0.19
FQ	2,16	0.21	0.81	12	0.16	0.88
SP	2,16	1.59	0.23	10	-1.36	0.20

Table 7. Correlation matrix (Pearson r) of female preference (B and B') estimates obtained from four different methodologies for the FQ trial of experiment 2 (n=24). RRJ refers to the non-linear regression (Reinhold et al., 2002) and min2 refers to the application of the RRJ-method while excluding the phonotactic response of the two upper and lower extreme frequencies so that the preference estimates were derived from phonotactic response data that spanned 4-6 kHz.

	RRJ	Spline	Polynomial
min2	0.35	0.91***	0.79***
RRJ		0.39	0.24
Spline			0.93***

*** P<0.001

Chapter 2: Figures

Fig. 1. Trace diagram of a female's movement during quantification of her phonotactic response to different syllable periods. Each stimulus was played back for a duration of one minute per speaker from two different speakers; speaker 1 was situated at 210° and speaker 2 at 90°. Horizontal gray lines show the active speaker. Notice how the female does not respond to syllable periods >48 ms but resumes phonotaxis at the control stimulus (43 ms).

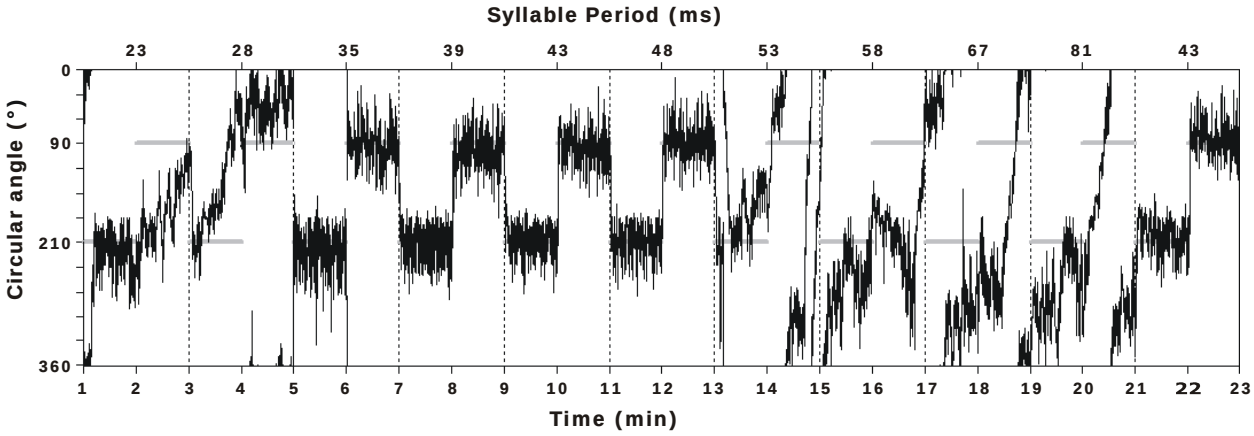


Fig. 2. Three methods used in this study to calculate female response function for the field cricket, *G. bimaculatus*. Phonotactic response (open squares) to two different speakers per frequency tested are shown for the frequency trial. A Gaussian function (dashed black line) was used to calculate preference (B), response magnitude (R) and selectivity (C) from inverted phonotactic response data (open triangles). The polynomial regression generated from the original data is presented as the solid gray line and the polynomial regression generated from the $\hat{y}$ values (filled triangles), obtained from cubic spline analysis, is presented as the solid black line. These two equations were used to calculate measures of female preference analogous to those calculated from the Gaussian function (B', R', C'). B' and C' are shown for the equation generated from spline data only.

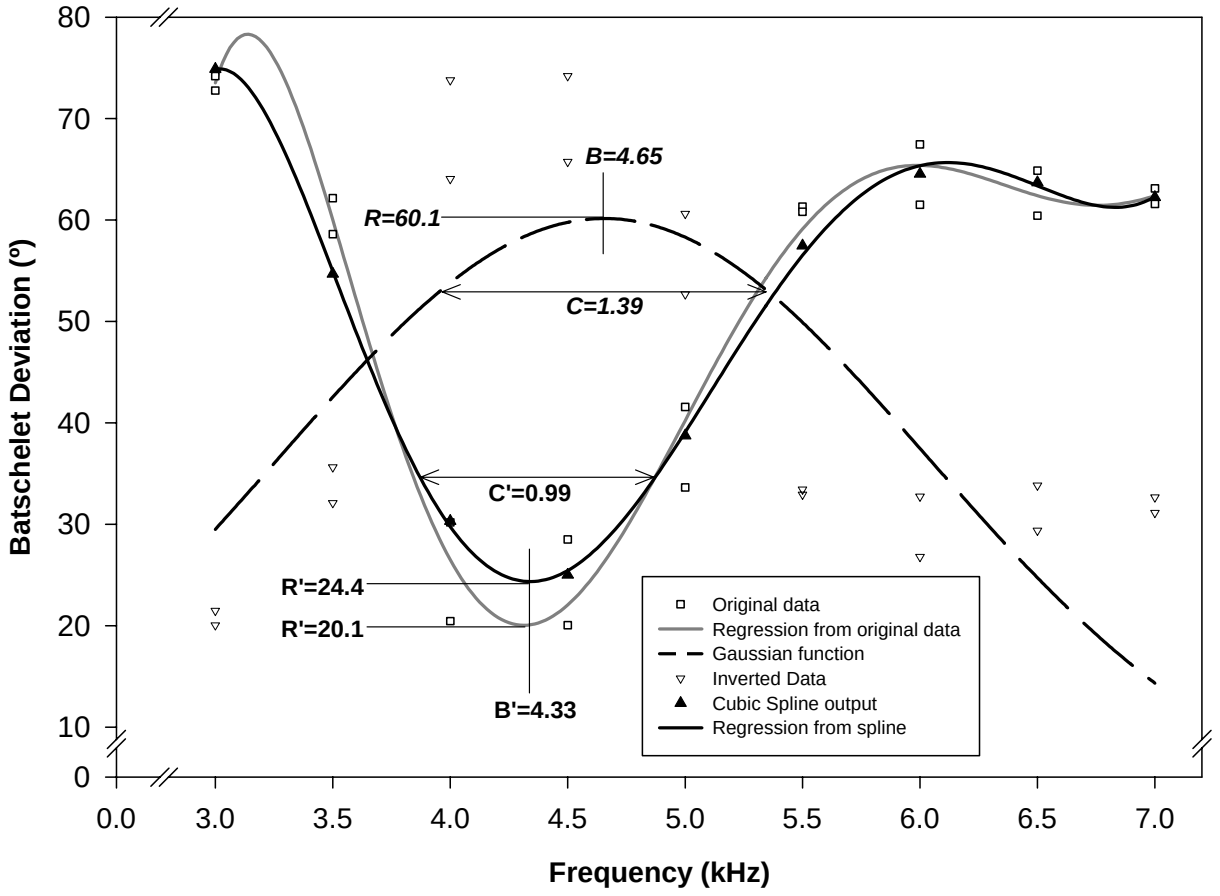


Fig. 3. A segment of a female's trace diagram showing perfect phonotaxis at an erroneous angle due to auditory asymmetry. Gray bars indicate the position of the active speakers. The angular offset is calculated as the mean difference between the female's perceived direction of the speaker and the actual direction of the speaker. In this case the angular offset was -31.4° for the 5 kHz stimulus (last 2 minutes).

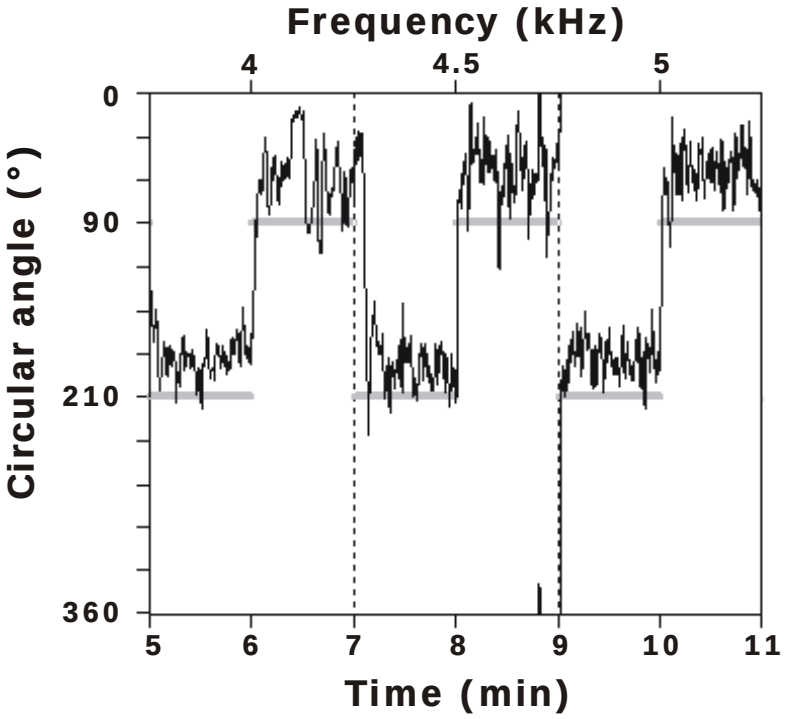


Fig. 4. The effect of an asymmetrical auditory system (angular offset) on the time taken to locate a speaker for 130 *G. bimaculatus* females. Angular offset is calculated as the mean difference between the female's perceived direction of the speaker and the actual direction of the speaker (see text for more detail). Delay difference is calculated as the difference between the time taken to locate speaker 2 and the time taken to locate speaker 1. Values are presented as mean ± SE for three trials (BW, DC and FQ) and two stimuli (STD and CTRL) per trial.

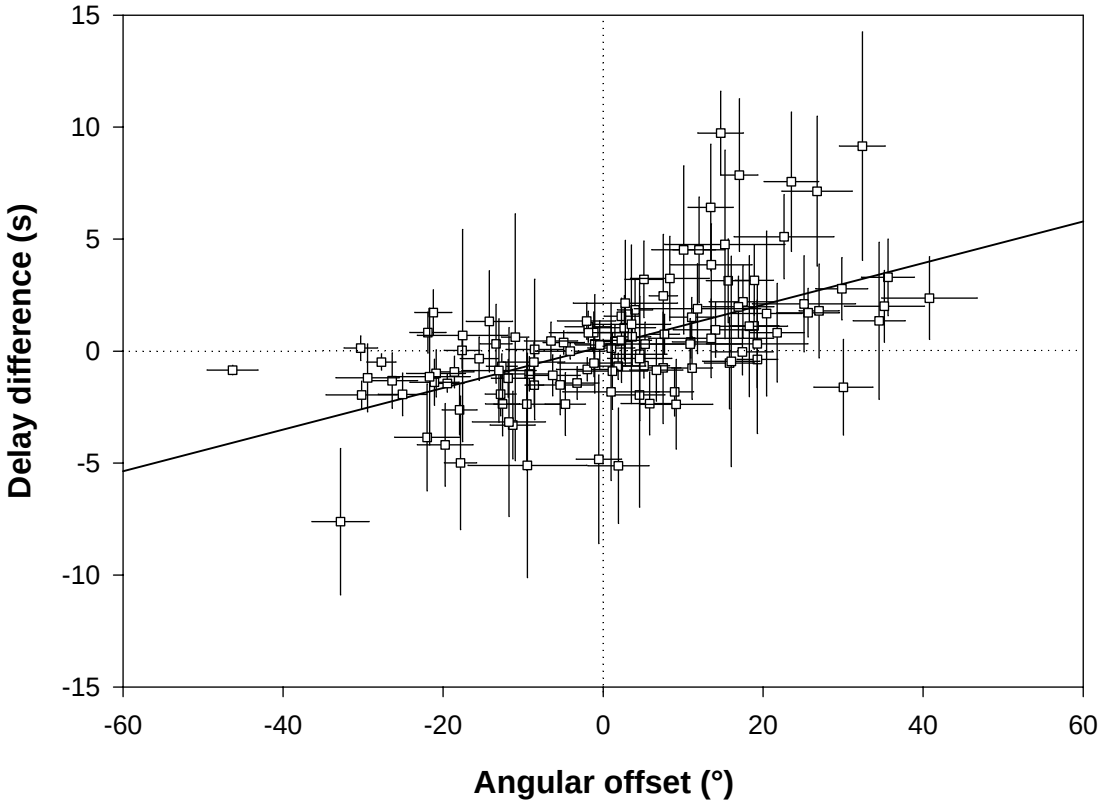
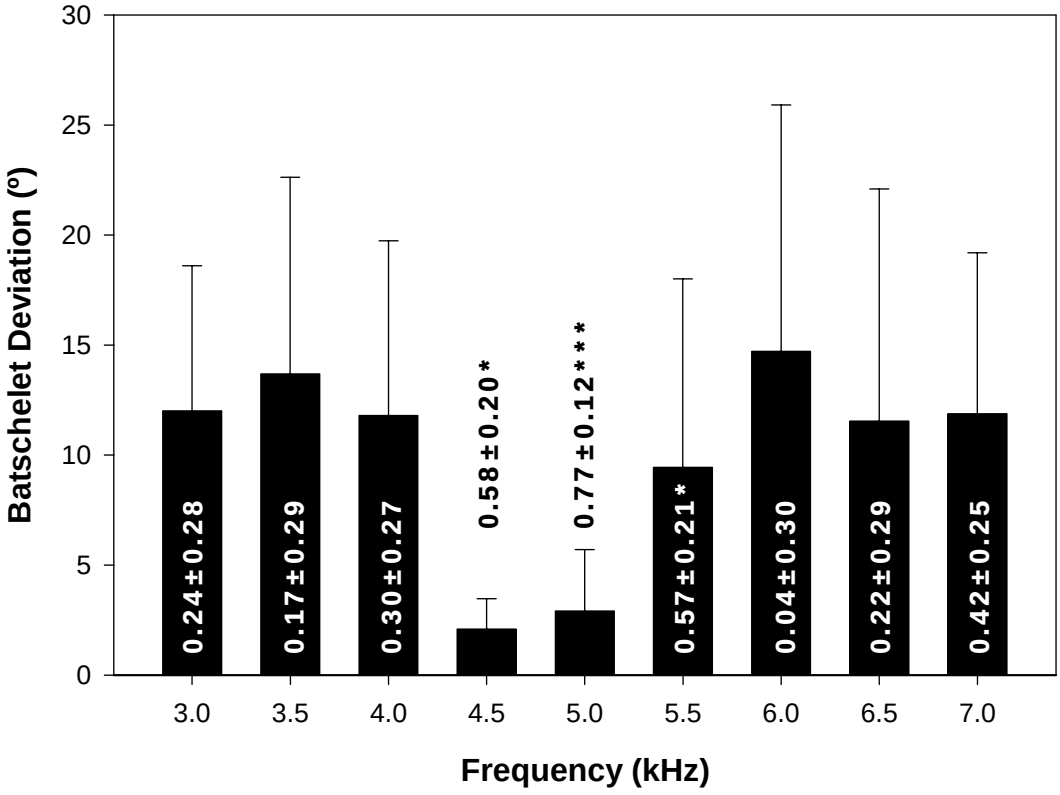


Fig. 5. Inconsistency of female phonotactic response (BatD) for male trait values at upper and lower extremes from the predicted preferred signal (4.5-5 kHz) for 24 *G. bimaculatus* females. Bars are the mean ± StdDev BatD difference between the first and 2nd repeat of the trial frequency for the females of experiment 2. Values at each bar show the repeatability ± SE of the phonotactic response (* P<0.05; *** P<0.001).



Chapter 3

The role of the tegmen harp structure during sound production in the field cricket, *Gryllus bimaculatus* (De Geer).

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Abstract

Male field crickets produce calling song by stridulating their tegmina. The harp structure on the tegmina has long been thought to be the primary resonant structure responsible for the predominant resonant frequency produced. However, a recent study has questioned the role of this structure during sound production, suggesting instead that other areas of the wing may be responsible for the resonant frequencies. We manipulated the tegmina of male field crickets, *Gryllus bimaculatus*, through sequential ablation experiments and recorded the resulting song. Cycle-by-cycle frequency analysis revealed that neither the cross veins of the harp, the harp membrane or the tegmen distal to the Cu2 vein (file) are required for resonance during the syllable. Using equations describing a simple resonance system, we were able to closely predict the results due to mass change of the system only, further questioning the role of the harp in producing the dominant frequency. Furthermore, we suggest that the left and right wings both play different roles during the syllable but that it is not as simplistic as previously reported. Finally, we demonstrate between-individual as well as within-individual variation in sound production in animals with manipulated tegmina. This suggests that ways of muscle contraction (independent of the wing structure) radically affect sound production in crickets. This area of bioacoustics deserves future research.

Introduction

Female field crickets, G*ryllus bimaculatus*, (Orthoptera: Gryllidae) are attracted to male calling song produced by raising the sclerotized forewings (tegmina) and scraping the plectrum of the left wing (posterior edge) over a series of teeth (file) on the underside of

the Cubitus 2 (Cu2) vein of the right wing (see Bennet-Clark 2003 for a detailed description of sound production in *Teleogryllus oceanicus*, similar to *G. bimaculatus*). Each tooth impact produces a sound oscillation (Elliot & Koch 1985; Koch et al. 1988). The harp area of the wings (bounded distally by the Cubitus 1 (Cu1) and proximally by the Cu2 vein; Figure 2 in Verburgt & Ferguson 2006, Chapter 4) has been suggested to be the primary resonant structure which determines the frequency of the song produced by acting as a pendulum and regulating tooth impact rate where the file and plectrum act as an escapement mechanism (Clock escapement model; Elliot & Koch 1985; Koch et al. 1988). Nocke (1971) demonstrated that the resonant frequency of the harp, when driven by sound, closely corresponds to the peak frequency of the calling song and that the harp vibrates with greater amplitude than other regions of the tegmen. The harp can be likened to a vibrating plate, the lowest resonant frequency (f_0) of which depends greatly upon the size and thickness of the membrane (Bennet-Clark 1989),

$$f_0 = 0.9342 \times \left(\frac{thickness}{2\Pi (radius)^2} \right) \times \sqrt{\frac{Young's\ \mathrm{mod}ulus}{density \times [1 - (Poisson's\ ratio)^2]}} \qquad (1)$$

or the mass and stiffness of resonant system in which a spring and a mass interact (Bennet-Clark 2003);

$$f_0 = \frac{1}{2\Pi} \times \sqrt{\frac{stiffness}{mass}} \qquad (2)$$

Analogous measures of stiffness, mass, plate size and thickness are all likely to scale allometrically with body size since larger crickets have larger wings. Therefore, if body size determines harp size and harp size determines song frequency, then females can potentially select males of preferred body size on the basis of song frequency.

Field cricket wings are predisposed to directional asymmetry (DA) in wing size (right larger than left) due to the right-wing-over-left- wing method of stridulation (Masaki et al. 1987). Simmons & Ritchie (1996) demonstrated a significant relationship between

song frequency and harp size (area) for *G. campestris* and also showed that the directional difference in harp size affects the fall in frequency ('glissando') across a syllable. From wing ablation experiments, they concluded that the left harp controls the frequency for the first part of a syllable while the right controls the latter part. They also concluded that the larger right harp introduces low frequency elements (due to its size) towards the end of the syllable and that the degree of directional asymmetry of the harp area was significantly correlated with frequency modulation of the syllables.

Two main criticisms of the Simmons & Ritchie (1996) study warrant re-investigation of their findings regarding song production in crickets:

1) Although the authors and others (Bennet-Clark 2003) referred to their ablation treatment as 'harp ablation", it was in fact "wing ablation" treatments as the entire tegmen was ablated distally to the file. Therefore, their conclusions regarding the role of individual harps during song production are tenuous since areas of the wing other than the harp may have confounded the results.

2) Although Simmons & Ritchie (1996) showed that bandwidth of *G. campestris* calls correlated with difference in harp size when the right harp was larger than the left harp, the same did not hold when the left harp was larger than the right harp. All else being equal, if the size of the harp were mostly responsible for the song frequency (simple resonator model; Nocke 1971), one would expect a similar degree of frequency modulation irrespective of the sign of the difference between left- and right harp area. The role of the harp as the primary resonator in grylline crickets has recently been brought into question through a series of elegant experiments on *T. oceanicus* (Bennet-Clark 2003). Wing resonance persisted after harp ablation although at a higher frequency. Bennet-Clark (2003) concluded that the harp frame and the wing region including the file are major components of the resonator and that the harp membrane acts as a sound-radiating surface driven by the vibration of the file via the cross veins. Additionally, no difference in harp area or harp mass was found between the left and right harp of *T. oceanicus*, although the resonant frequency of the left wing was significantly higher (~350 Hz) than that of the right wing when the resonances were driven by sound or by an electric vibrator at different parts of the wing (Bennet-Clark 2003). The calling song of *T.*

oceanicus displays the typical glissando of grylline cricket song and although not reported, is seen to be approximately 800 Hz from figures in this study. Bennet-Clark (2003) suggests that the glissando is partly due to the flexible region distal to the anterior end of the harp, since the resonances when driven in this area are lower than resonances when driven near the middle of the file, probably because this part of the file is less stiff than the center of the file.

In this study we manipulated the tegmina of male *Gryllus bimaculatus* through three sequential ablation experiments to investigate the role of the harp during sound production. Specifically we aimed:

- To determine whether *a*) the cross-veins running through the harp, *b*) the harp membrane and *c*) the whole tegmen distal to the file are required for sound production (resonance) for both the left and right wing
- To determine whether the left or right harp\tegmina predominantly controls resonant frequency and sound amplitude for a specific part of the syllable
- To determine whether the glissando effect requires both intact tegmina
- To describe between-individual and within-individual variation in sound production in animals with manipulated tegmina

Material and Methods

Collection and captive care: Males were randomly selected from laboratory-reared stock originating from seven locations across South Africa and a location in Europe. Animals were reared in a climate-controlled chamber (27±1°C; 12:12 light-dark regime) in large containers (100-liter) with a density of ±100 individuals per container. Food (high protein cereal and fish flakes) and water (cotton-plugged vials filled with water) were provided *ad libitum*.

Song recording: Males were placed singly in sound damped recording chambers where their calling song was recorded for 30 s at 25°C±1 °C with a Beringer XM200S dynamic microphone (50 Hz-10 kHz ± 3 dB; Behringer Spezielle Studiotechnik GmbH, Willich-

Münchheide II, Germany). Male calling song was recorded directly to a computer (sampling rate = 22050 kHz) after a male had been calling continuously for five minutes.

Sequential tegmen manipulations:

We recorded the calling song during one night for two *intact* (control) males (A & B). Thereafter, these males were sequentially subjected to three treatments varying in the degree of wing manipulation. Only one of the wings (left or right) was manipulated per male. The calling song was recorded for each male after each treatment to determine the effect of the treatment on the sound produced.

Holed treatment: We attempted to destroy the resonance of the harp by removing only the area of maximal vibration of the harp shown for *T. oceanicus* (Bennet-Clark 2003). This was achieved by melting a small circular hole (±1.5mm diameter) through the cross veins of the harp with a small diameter soldering iron (Figure 1A).

Harpless treatment: We ablated as much of the harp membrane (within the harp frame) as possible although some tiny fragments remained around the edges of the harp frame (Figure 1B).

Cut treatment: We cut off the wing (distal to the file) with the ablated harp (Figure 1C).

Within-individual variation in sound production over time:

We continued to record the two males (A & B) for an additional three nights (although male A only called for an additional two nights) after the *cut* treatment to determine if any changes in sound production took place over time, for example if males adjusted to the change in wing mass.

Between-individual variation in sound production:

To investigate between-individual variation in sound production after each treatment, several additional males were subjected to only two treatments, either *intact* and *harpless* (males C-F; n=4) or *intact* and *cut* (males G-L; n=6), since it proved difficult to obtain recordings for all three treatments per male.

Sound analysis:

From each recording, we selected the third syllable of a randomly-selected chirp for analysis after filtering the recording (high-pass=1 kHz). The shape of the syllable amplitude envelope was calculated from the absolute values of individual cycle peaks (relative to the peak amplitude) using custom designed software (L. Verburgt). Cycle-by-cycle frequencies were measured by zero-crossing analysis, with ZC v.5 developed by K.N. Prestwich (for details see Bennet-Clark & Baily 2002).

Results

Sequential tegmen manipulations:

The effect of varying degrees of sequential harp ablation on sound frequency and the syllable amplitude envelope are shown in Figure 2. Burning a circular hole through the center of the harp, destroying the main cross veins (*holed* treatment; either left or right tegmen), didn't cause an observable difference in the shape of the sound amplitude envelope during the course of the syllable. However, a slight increase in the frequency of the sound produced during nearly the entire course of the syllable was observed, irrespective of the wing treated. The inset in Figure 2A shows an expanded portion of the frequency plots comparing *intact* and *holed* sound. This increase in frequency appeared to be more marked when the right wing was treated (Figure 2B). Ablating a large proportion of the harp within the harp frame greatly affected the sound produced. The first half of the syllable increased in frequency while the latter half decreased in frequency for male A (*harpless* left tegmen) (Figure 2A). The shape of the syllable amplitude envelope showed large changes in relative amplitude during the course of the syllable. For male B (*harpless* right tegmen), the first half of the syllable also increased in frequency but the second half rapidly changed in frequency and was also much higher in frequency (Figure 2B). The syllable amplitude envelope clearly shows a decrease in amplitude for the second half of the syllable. Ablating the wing distally to the file yielded unexpected results. The first part of the syllable decreased further in amplitude (compared to the *harpless* treatment) while the latter half increased greatly in frequency for male A with a *cut* left tegmen. For male B with a *cut* right tegmen, the syllable amplitude envelope remained similar. However, the frequency decreased steadily during

the first half of the syllable and remained approximately stable for the latter half of the syllable, albeit at a much lower frequency than previously.

Within-individual variation in sound production over time:

Syllable amplitude envelopes remained similar for three nights for male A with a *cut* left wing, although the different positions of the peaks suggest that the wings did not engage at the same place on the file for each recording (Figure 3A). Frequency plots, although very distorted, showed a consistent pattern, especially for nights 1 and 3. Both of these syllables start at a relatively low frequency and then rapidly change to a higher frequency. Finally both syllables terminate with a short sequence of sound at roughly the same frequency as the start of the syllable. The scattered frequencies on night 2 are difficult to interpret, but the terminal sequence is very similar to that of night 1. Male B with a *cut* right tegmen showed remarkable consistency in frequency plots between nights (Figure 3B). Despite the almost indistinguishable frequency plots, the syllable amplitude envelope for night 1 is the opposite shape to that of the subsequent nights.

Between-individual variation in sound production:

We found large between-individual differences in frequency plots for males subjected to either *harpless* or *cut* treatments (Figures 4 and 5). Although all of the frequency plots showed an elevated mean frequency, there was no similarity in the pattern of frequency change over the syllable duration for males treated on the left wing with either *harpless* or *cut* treatments (Figures 4A and 5A). However, both Male C (*harpless*; Figure 4A) and Male G (*cut*; Figure 5A) maintained resonance throughout the duration of the syllable at a higher frequency than *intact* song. Moreover, both of these sounds show the typical glissando effect although the sound produced by Male C becomes disrupted toward the terminal part of the syllable. Males with ablated right harps or wings (Figures 4B and 5B) showed a similar pattern, in that the first part of the syllable was similar to that of the intact animal albeit at a higher frequency while the latter part of the syllable comprised much higher and irregular frequencies. There are however notable exceptions. Male E (*harpless*; Figure 4B) and Male L (*cut*; Figure 5B) both regained proper sound production (resonance) for the latter part of the syllable but at a higher frequency. Male B (discussed

above for Figures 2B and 3B) showed no effect of the *cut* treatment during the latter part of the syllable and Male J appeared to terminate the syllable prematurely.

Assuming that the stiffness of a resonant system remains constant, it is possible to calculate the effect of mass change on the resonant frequency in the system (from Eq 2) as follows:

$$f_{0(new)} = f_{0(original)} \times \frac{1}{\sqrt{c}} \qquad (3)$$

where f_0 is the resonant frequency and c is the proportional change in mass of the system, $(\frac{new\,mass}{old\,mass})$. Assuming that harp mass is uniformly distributed across the harp area we calculated c from the reduction in harp area due to the *holed* (Males A & B) or *harpless* (Males C & E) treatments. From the change in frequency due to each treatment, we calculated an expected value for c from Eq 3. Furthermore, we calculated the expected resonant frequency from the observed c and show the discrepancy between the observed and expected resonant frequencies in Table 1. In general, we were able to achieve relative similarity between observed and expected results, despite the obviously flawed assumptions concerning tegmen structure.

Discussion

Requirements for sound production (resonance):

As expected, ablating large areas of the harp or wing resulted in the production of much shorter syllables due to the increased speed of wing closing described in detail by Koch et al. (1988). The *holed* treatment failed to destroy tegmen resonance suggesting that the cross veins are not essential for the correct functioning of the tegmen during sound production. This area of the harp has been shown for *T. oceanicus* to be the area of maximal vibration when driven by sound (Bennet-Clark 2003). Furthermore, neither an intact harp membrane nor the tegmen distal to the file was required to maintain resonance for the majority of the syllable duration for both left and right wings. This supports the

hypothesis of Bennet-Clark (2003) that frequency control is unlikely to be solely due to the harp area in grylline crickets.

Using a simple resonance model (Eq 2), we were able to calculate resonant frequencies similar to that expected for *holed* and *harpless* treatments based on the mass change of the system (Eq 3) (Table 1). This further supports the suggestion that the harp is unlikely to be the primary resonant structure of the tegmen since the ablation thereof (*harpless*) would be expected to destroy resonance completely and not simply alter the resonant frequency in a predictable manner due to the mass change of the system. It is clear however, that our calculations for Male C (*harpless* left wing) are inaccurate and represent the only case where the expected mass change of the system was underestimated. This could be due to structural differences between the left and right harps.

Control of frequency and sound amplitude during the syllable:

We found only indirect evidence that the left harp controls the frequency for the first part of the syllable. Right harp or wing ablation resulted in normal sound production but at a higher frequency during the first part of the syllable followed by increased and irregular frequencies for the latter part (Figures 2B, 4B & 5B). This suggests that intact left wings were responsible for the control of frequency during the first part of the syllable. However, ablation of the left harp or wing altered the frequency plots for the entire syllable. Only two out of seven males showed a possible return to normal sound production during the latter part of the syllable (Figure 4A, Male A and Figure 5A, Male I) as shown for *G. campestris* (Simmons & Ritchie 1996). Male A produced a much smaller increase in frequency (± 800 Hz) during the first part of the syllable compared to ± 5 kHz for *G. campestris*. Furthermore, lower frequencies for the latter part of the syllable were produced compared to intact sound production (*harpless* Male A; Figure 4A) where *G. campestris* showed an increased resonant frequency (Simmons & Ritchie 1996).

It may be argued that partial ablation of the left wing caused the amplitude at the start of the syllable to be indistinguishable from background noise. However, the similar position of three peaks in the syllable profile of *harpless* and *cut* treatments (Figure 2A) suggests that this was not the case. Ablation of the left harp caused a decrease in sound amplitude for the entire syllable duration while wing ablation reduced amplitude further at the start of the syllable (Figure 2A).

For *cut* male B there was a remarkable reversal of the syllable amplitude envelope shape during the last three nights of recording where the amplitude was greatest towards the latter part of the syllable (Figure 3B). Experimental error can be ruled out since the frequency plots for these same syllables are nearly identical. Although we are presently unable to explain this reversal of the syllable amplitude envelope, we suggest that this may be an instance were individuals adjust their stridulation behaviour after becoming accustomed to mass changes of the wing.

Several unexpected results were produced by the ablation of large areas of the right harp and wing. Firstly, Male B (Figure 2B) shows that although *harpless* produced a similar result expected from that of Simmons & Ritchie (1996), the *cut* treatment (this particular males' treated wing is shown in Figure 1C) resulted in an opposite effect, with sound production being apparently normal during the latter part of the syllable. This pattern was repeatable over four different nights (Figure 3B). Further evidence that the right harp or wing is not solely responsible for frequency control of the latter part of the syllable comes from Male E (Figure 4B) and Male L (Figure 5B) where apparent regain of sound production (but at a higher frequency) during the latter part of the syllable can be seen. The sound production mechanism suggested by Simmons & Ritchie (1996) for *G. campestris* is clearly not in operation in *G. bimaculatus* and we suggest further investigation into the sound producing properties of the anal area of the wing, suggested by Bennet-Clark (2003) to be of importance during sound production in *T. oceanicus*.

Although it is tempting to invoke the explanation that the left and right harp/wing play different roles during syllable sound production by viewing syllable amplitude envelope

shapes and frequency plots, it is not completely justified. For example, the complete reversal of the syllable amplitude envelope shape for male B (Figure 3B) despite the exact same wing structure suggests that it is perhaps the muscular contractions and subtle changes in tegmen angles that control sound amplitude. It still remains to be empirically demonstrated that the ablated harp/wing is solely responsible for the loss of resonance or amplitude during a portion of the syllable.

Requirements for glissando effect:

The glissando effect is not likely to be caused by left/right differences in harp area. Firstly, we demonstrated above that harp area is unlikely to be the sole determinant of song frequency. Secondly, there is a clearly visible glissando (±1000 Hz) for the first part of the syllable before sound disruption occurs for *cut* males treated on the right wing (Figure 5B) and a clear glissando over the entire syllable duration for Male G (*cut*; Figure 5A). Also, Male E (Figure 4B) displays a glissando for the latter part of the syllable despite the loss of 48% of his right harp membrane. *Harpless* males C and D treated on the left wing show a decrease in frequency for the first part of the syllable (Figure 4A). These observed glissandos can therefore not be attributed to directional asymmetry of harp area as suggested by Simmons & Ritchie (1996).

Between-individual variation in sound production:

The large between-individual differences in frequency plots for *harpless* and *cut* males suggests variation in the manner which individuals use their tegmina to produce sound. While the general mechanism is the same (Clock escapement model; Elliot & Koch 1985; Koch et al. 1988), highly variable wing morphology (e.g. variable number and position of cross veins) may require variation in applied forces due to muscular contraction on the tegmina which could result in slightly different stridulation behavioural patterns. The observed between-individual variation in the mechanics of sound production is an area of bioacoustics that deserves future research.

Conclusion:

Although our experiments were far too crude to fully understand the mechanism of sound production in crickets, we have clearly demonstrated for *G. bimaculatus* that the harp area of the tegmen is not likely to be the primary resonator and that the size difference between the left and right harps is not likely to cause the glissando effect. These findings suggest that the anal area of the wing plays a more important role in sound production than previously thought and questions the findings of studies showing sexually-selected spectral aspects of male song based on the structure of the harps. Large between-individual differences in sound production (within-treatment) suggest that the mechanism of sound production is far more variable than suggested in the literature. Within-individual variation in sound production demonstrated here alludes to the possibility that individuals may be able to adjust their stridulatory behaviour due to mass changes of the tegmina and provides further evidence that the mechanics of sound production is not completely stereotyped and only determined by wing morphology. This study highlights the necessity for a comprehensive understanding of sound production in grylline crickets.

Acknowledgements

We would like to thank U.K. Verburgt for her valuable assistance during the experimental phase. L. Verburgt was funded by the Deutsche Forschungsgemeinschaft (DFG) and the National Research Foundation (South Africa, NRF).

Chapter 3: Tables

Table 1. Observed and expected (Eq 3) resonant frequencies (f_0; Hz) and proportional change in the mass of the resonant system (c) due to *holed* or *harpless* treatments. Resonant frequencies were measured as the peak frequencies for the entire syllable. Change in mass was estimated from proportion of ablated harp area. Letters in brackets (A-E) correspond to the male ID in figure 4.

	Intact vs. *Holed*		*Intact* vs. *Harpless*	
Variable	left (A)	right (B)	left (C)	right (E)
$f_{0\ original}$	5027	4836	5165	4949
$f_{0\ new}$	5344	5440	6414	7745
c observed	0.93	0.92	0.49	0.48
c expected	0.88	0.79	0.65	0.41
$f_{0\ new}$ expected	5210	5030	7354	7108
f_0 discrepancy	-134	-410	940	-637

Chapter 3: Figures

Figure 1. Three cricket forewings showing harp ablation treatments to investigate the role of the harp during sound production. A) Treatment where a circular hole was melted into the right harp through the cross veins (*holed*). B) Treatment where the entire left harp area (within the harp frame) was removed by melting the membrane inside the harp frame (*harpless*). C) Treatment where the entire right wing was ablated distal to the file (*cut*).

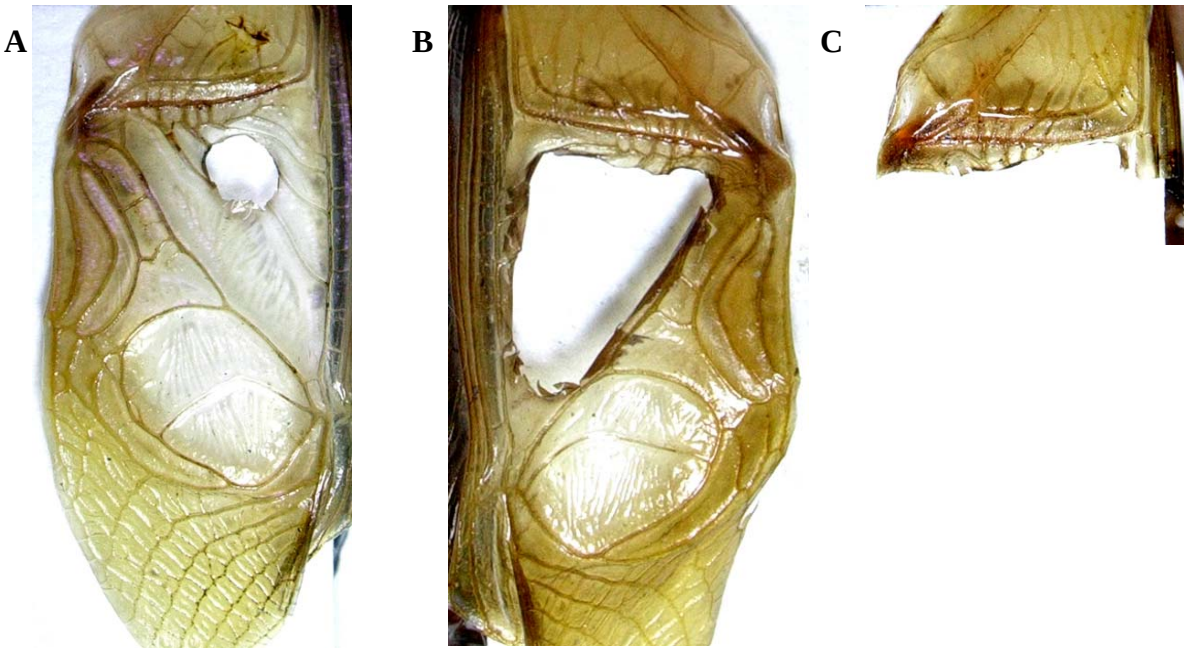


Figure 2. Cycle-by-cycle frequency plots and syllable amplitude envelopes for single syllables of two male field crickets subjected sequentially to increasing degrees of harp ablation. A) Male A with left wing treated. Inset shows magnified portion of graph to illustrate the magnitude of difference between *intact* and *holed* sound. Dashed lines represent approximate mean frequency for each series during the interval shown. B) Male B with right wing treated.

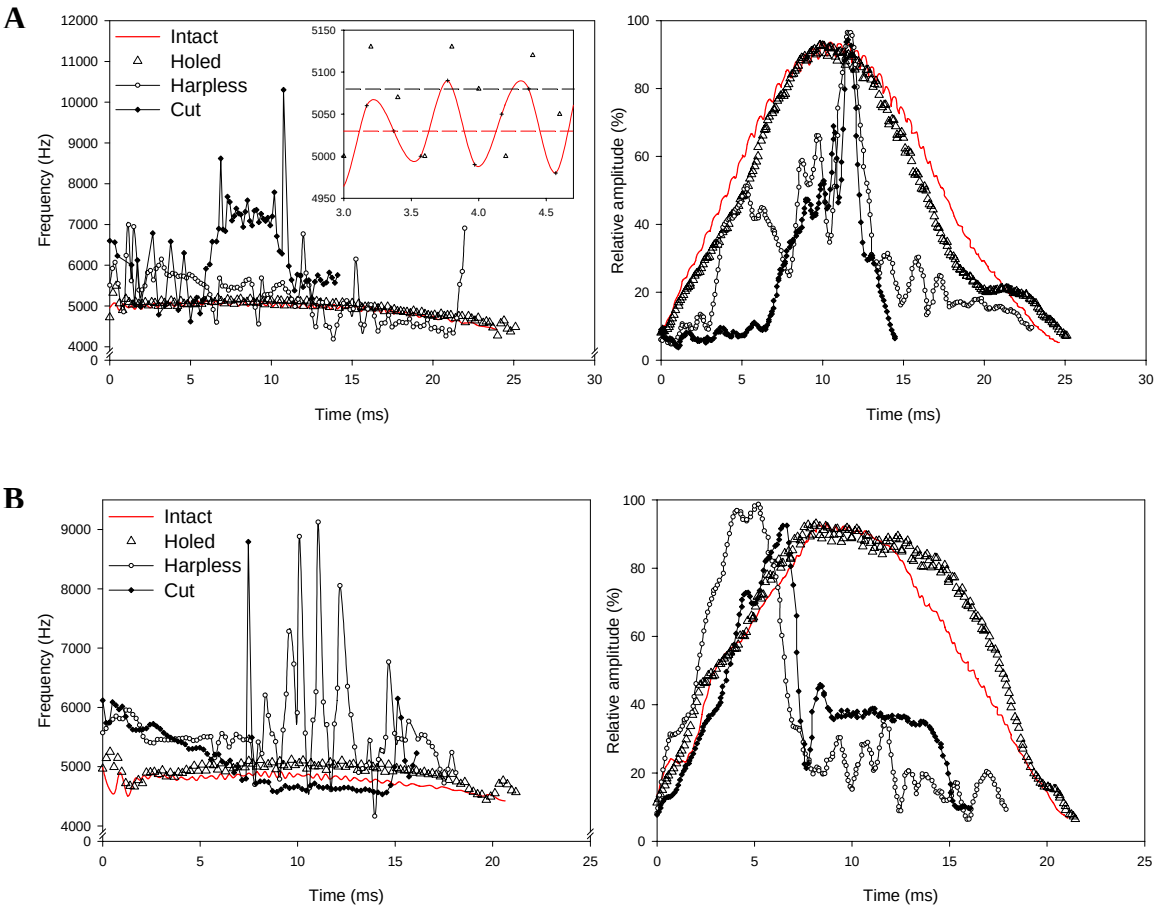


Figure 3. Sequential cycle-by-cycle frequency plots and syllable amplitude envelopes for single syllables of two male field crickets (Males A & B) with either the left (A) or right (B) tegmen ablated distally to the Cu2 vein (file) respectively. Each series represents a syllable recorded on a different night. Night 1 for both A and B are the same data as the *cut* series in figure 2 A and B respectively.

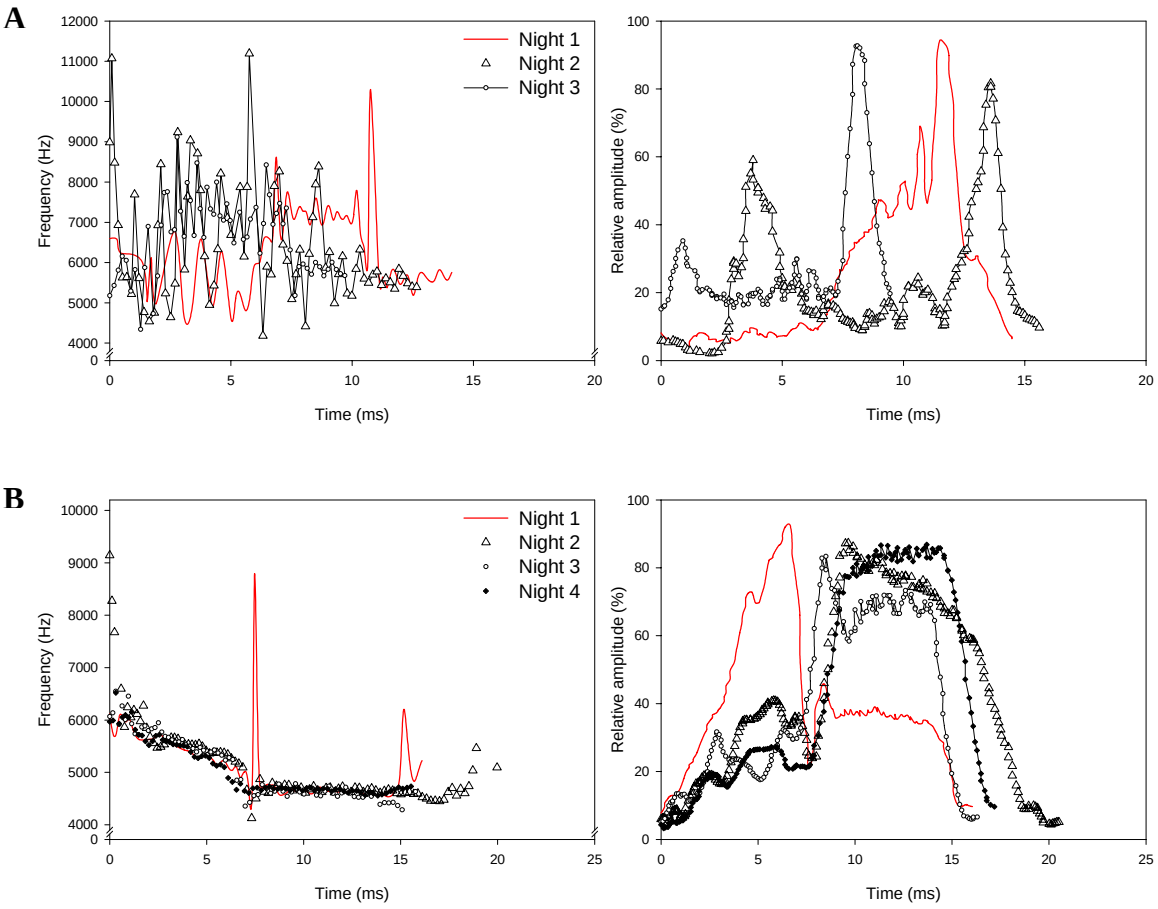


Figure 4. Cycle-by-cycle frequency plots of six male field crickets with either the left (A) or right (B) harp ablated (*harpless*). Male song was first recorded from *intact* (Control) males and thereafter with the harp completely ablated within the harp frame (Treatment). Graph insets show a scaled schematic representation of the treated wing for males B and E respectively. Arrows link the treated wing and the sound produced. Gray areas show the absence of harp membrane (see Figure 1). Note the glissando effect for the latter part of the syllable for the treated Male E.

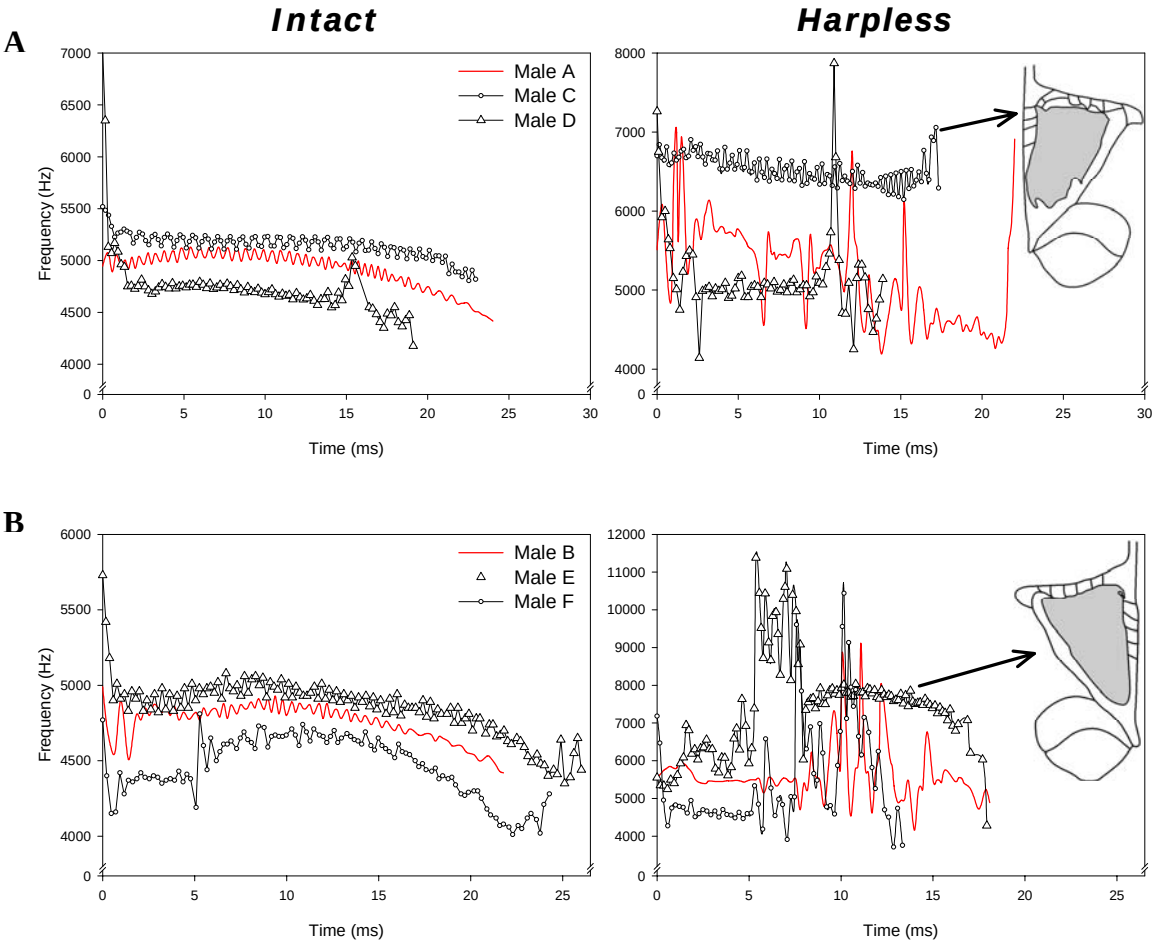
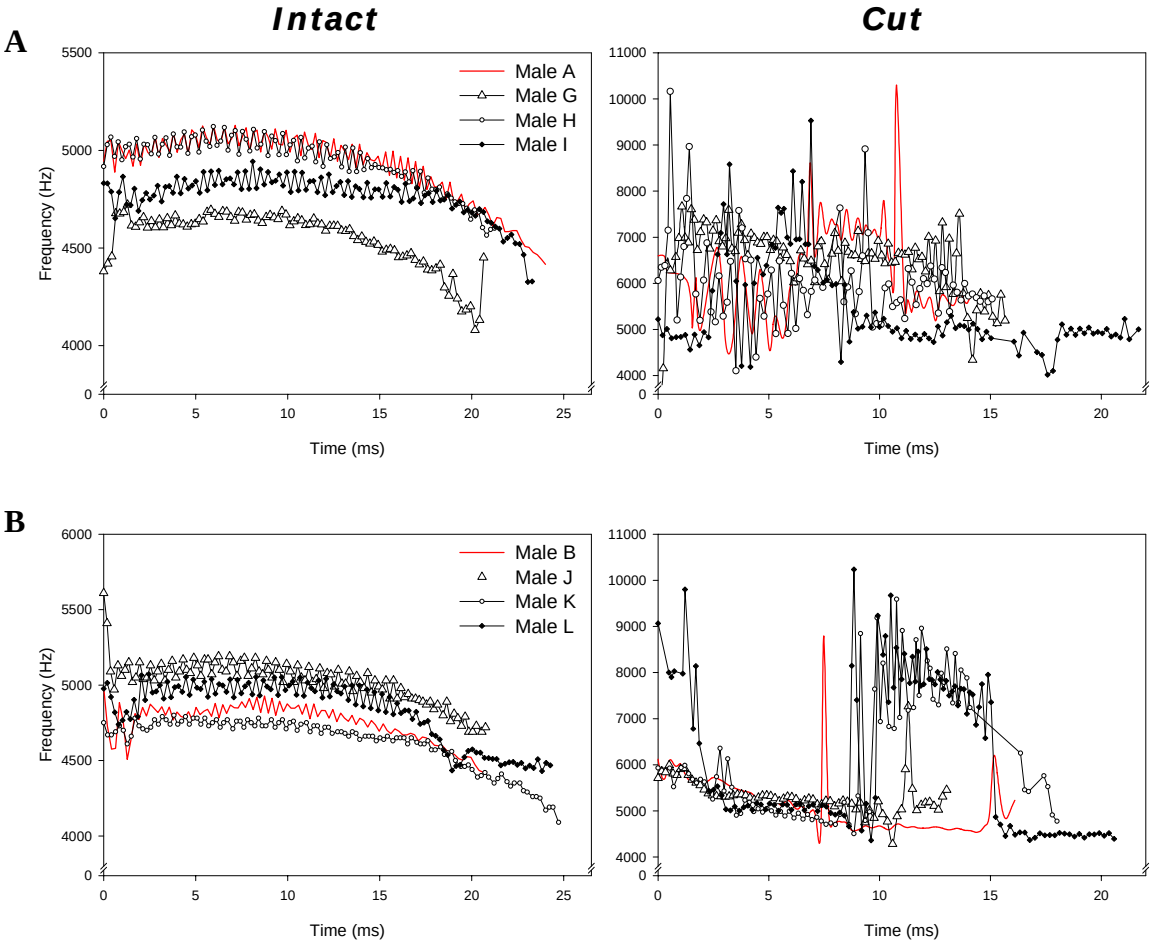


Figure 5. Cycle-by-cycle frequency plots of eight male field crickets with either the left (A) or right (B) wing ablated distally to the file. Male song was first recorded from *intact* (Control) males and thereafter with the wing ablated (Treatment).



Chapter 4

No evidence of morphology- or condition-dependent acoustic signaling in the field cricket, *Gryllus bimaculatus* (De Geer).

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Abstract

Females can potentially choose high quality males in good condition by evaluating male secondary sexual traits as proposed by the handicap hypothesis of sexual selection. In field crickets, male size influences mating success and fluctuating asymmetry (FA) has been suggested to indicate individual quality or fitness. However, no direct evidence for field crickets exists to show that males can advertise their size or quality (FA) via their calling song. Females are attracted to male calling song and it has been suggested that male quality (FA) may indirectly be advertised via the frequency modulation (bandwidth) of song. Although females have been shown to prefer certain degrees of bandwidth, suggesting selection for male quality, it has not been demonstrated that this preference is distinct from frequency preference. In this study we failed to find acoustic correlates of male size, condition and quality. Furthermore, we demonstrate that female bandwidth preference is not a distinct preference but rather determined by frequency preference. We conclude that females can not judge male phenotype based on the temporal and spectral properties of male song. We suggest that it is likely to be the energetically costly aspects of male song (not measured here) that may be condition dependent.

Introduction

A prediction of the handicap hypothesis of sexual selection is that expression of male secondary sexual traits is condition-dependent and therefore ensures the honesty of male signals (Zahavi 1975; Cotton et al. 2004b). Only males of high quality are therefore likely to produce conspicuous ornaments. Fluctuating asymmetry (FA), defined as subtle random deviations from symmetry of bilateral body structures (Ludwig 1932) is thought to reflect developmental instability (DI), which occurs when the development of a trait is

disturbed by random stochastic perturbations along the developmental trajectory (Van Dongen et al. 2005). Recently, several studies have provided evidence that FA may indicate individual quality or fitness (e.g. Mallard & Barnard 2004; Rantala et al. 2004; but see Simmons et al. 1999; Bjorksten et al. 2000a & b). These studies posit that if FA reliably indicates DI then it should indicate environmental and genetic stress (e.g. homozygosity or hybridization), and therefore covary negatively with body condition, which declines under these unfavourable conditions (see also Møller & Pomiankowski 1993). Males of high quality should therefore display good condition and produce conspicuous (large), symmetrical secondary sexual traits. Acoustic signals generated with these secondary sexual traits can influence female mate choice, specifically if a property of the signal were correlated with a male trait that could directly or indirectly benefit the female (Andersson 1994; Gerhardt & Huber 2002). Male field crickets produce calling song by stridulating their sexually dimorphic tegmina. The harp area of the male tegmin (bounded distally by the Cubitus 1 (Cu1) and proximally by the Cu2 vein) is suggested to be the primary resonant structure which determines the carrier frequency of the song produced (Nocke 1971; but see Bennet-Clark 2003; Verburgt & Ferguson 2006, Chapter 3). If harp area scales with body size and if harp area also determines song carrier frequency, then females can potentially select males of preferred body size on the basis of song carrier frequency.

The findings of studies investigating the relationship between male body size, harp area and song carrier frequency for crickets of the subfamily Gryllinae are summarised in Table 1. As far as we are aware, the only evidence that female grylline crickets could potentially select for larger males by selecting lower carrier frequencies originates from a single species (*G. campestris*). Despite studies on crickets showing female preference for males of large size (Simmons 1986; Simmons 1992; Brown et al. 1996; Bateman et al. 2001) there is no clear evidence that female crickets choose large males on the basis of carrier frequency (Gerhardt & Huber 2002). Despite several studies that have inferred a relationship between body size and song carrier frequency from a significant correlation between harp size and body size, no significant direct relationship between body size and song carrier frequency has been reported for any grylline cricket to date (Table 1). Based

on the known correlations between harp area and body size, and carrier frequency and harp area, we calculated the expected correlation (r^2) between carrier frequency and body size for the studies on *G. campestris* that did not report the correlation for this relationship (Table 1), the upper limit of which is given by the product of the two known correlations (Sokal & Rohlf 1981). Although the calculation of the expected r^2 is flawed due to different sample sizes for the two known correlations, it nevertheless serves to indicate that the expected correlation between carrier frequency and body size is very low. Indeed, for *G. bimaculatus*, no study has found a significant relationship between body size and carrier frequency although none of these studies investigated the relationship between harp size and carrier frequency or body size (Table 1). This necessitates a comprehensive study where the relationship between these three male characteristics is explicitly determined.

In addition to several studies reporting a significant relationship between song carrier frequency and harp area for *G. campestris* (Table 1), Simmons & Ritchie (1996) showed that the spectral bandwidth of male calling song was correlated with the directional asymmetry (DA; left-right<0) in harp size (only when the right harp is larger then the left) for this species. Moreover, they showed that FA of the hind tibia was correlated with DA of harp area suggesting that females could potentially choose high quality males (if FA reflects male quality) on the basis of frequency modulation of calling song. Unfortunately the authors did not report whether the degree of hind tibia FA was detectable from frequency modulation in the song (similar to the absence of results showing a direct relationship between body size and carrier frequency). The underlying DI variance of an individual is estimated with limited confidence if based on the FA of a single trait (Palmer & Strobeck 2003). Combining estimates of DI from several traits increases confidence and effectively adds a degree of freedom per trait added (Palmer 1994; Leung et al. 2000; Palmer & Strobeck 2003). The implied relationship between FA and frequency modulation of the song for *G. campestris* (Simmons and Ritchie 1996) is therefore tenuous since FA was measured from only a single trait in this study.

Female *G. campestris* were shown to prefer songs with greater frequency modulation if the starting frequency was high, but preferred pure tone songs if the starting frequency was low (Simmons & Ritchie 1996). The authors suggested that the observed pattern was due to female preference for low carrier frequency, where large degrees of modulation moved song to within the frequency range preferred by females if the song started at a high frequency. If the song started at a preferred frequency, modulation would move the song out of the preferred frequency range. No empirical evidence exists to support this proximate explanation of bandwidth preference although a recent study has shown that female preference for bandwidth is highly repeatable (Verburgt & Ferguson 2006, Chapter 2).

By employing a similar protocol to the study of Simmons & Ritchie (1996) but with new techniques and methodology, this study aims:

- To determine the relationship between body size, harp area and calling song carrier frequency in the field cricket *G. bimaculatus*
- To quantify variance in DI through FA analysis and determine whether DI correlates with body condition, morphological traits and song parameters
- To determine whether female preference for spectral bandwidth is determined by her frequency preference

Material and Methods

Collection and captive care: We collected wild-living penultimate instar male field crickets from seven locations across South Africa as well as one location in Europe (Table 2) and allowed them to eclose in captivity. Female crickets were collected previously for another experiment (Verburgt & Ferguson 2006, Chapter 2) and originated from seven different populations in South Africa. Since female *G. bimaculatus* do not exhibit phonotaxis after being inseminated (Loher et al. 1992), all females were virgins. Individuals were kept in a climate-controlled chamber (25±1°C; 12:12 light-dark regime) in individual containers (500 ml) and provided food (high protein cereal and fish flakes)

and water (cotton-plugged vials filled with water) *ad libitum*. Males and females were kept in separate chambers to ensure acoustic isolation of females.

Experiment 1. Relationship between male morphology and song traits:

Song recording. Males were placed singly in sound damped recording chambers where their calling song was recorded for 30 s at 25°C±1 °C with a Beringer XM200S dynamic microphone (50 Hz-10 kHz±3 dB; Behringer Spezielle Studiotechnik GmbH, Willich-Münchheide II, Germany). Male calling song was recorded directly to a computer (sampling rate=22050 kHz) after a male had been calling continuously for five minutes. Since a large proportion of variation in *G. bimaculatus* call characteristics is due to between-night differences within a male (Ferreira 2006, MSc Thesis), two recordings, each from a different night, were recorded for every male. Custom-designed software was used to analyse three randomly chosen consecutive chirps per recording, after initially filtering the recordings (high-pass filter=1 kHz). We measured syllable duration (SD), syllable period (SP) an inter-syllable interval (ISI) for every syllable; and chirp duration (CD), chirp period (CP) and inter-chirp interval (ICI) for every chirp (Figure 1). Since we obtained multiple measures of the same trait per recording and per night, we calculated their respective mean values and subjected these values to further analysis. Carrier frequency (analogous to emphasized- or peak frequency) and frequency bandwidth at –10 dB and at –20 dB below peak frequency were the spectral characteristics measured. Post-hoc analysis revealed that bandwidth at –10 dB and at –20 dB were highly correlated (r^2_{60}=0.71, P<0.001) and all tests conducted with both measures of bandwidth yielded similar results. We therefore do not present results for bandwidth at –20 dB.

Morphological characteristics. After successful song recording, males were killed and weighed (resolution=0.001 g). Both wings were removed and digital images (resolution=15.8 μm) of both left- and right wings for each male were generated with the Creative Laboratories VideoBlaster FS200 utility program. Custom-designed software (L. Verburgt) was used to measure the position of 17 landmarks on each wing (Figure 2). Since a right-angled triangle generated from points H_2, H_4 and H_8 only poorly reflects the shape of the harp, we calculated harp area as the area of a polygon using points H_1-H_{10}. It

is possible that other wing variables besides those acting directly on the harp may affect the sound produced and we therefore included measurements of three additional distances (e.g. A_1-A_3, see Figure 2). We also measured the pronotum length, pronotum width, hind femur length (both left and right), hereafter FEM, and hind tibia length (both left and right), hereafter TIB. The surface area of the pronotum (length x width; mm^2) was taken as a mass-independent measure of body size. Following Holzer et al. (2003), Scheuber et al. (2003a) and Jacot et al. (2005) we measured male body condition as the residuals of the regression of body mass on body size. This index of body condition has been shown to reflect energetic fat reserves of *G. texensis* under controlled feeding conditions (Gray & Eckhardt 2001). To quantify measurement error (hereafter ME) each wing, FEM and TIB was measured three times on three different days, each day of measurement separated by at least 48 hours. To exclude observer bias, all measurements were made blind of any previous measurements on the same individual and all measurements were made by the same person (L.V.). For the fluctuating asymmetry analyses, we followed the methodology of Palmer (1994) and Palmer & Strobeck (1986; 2003).

Experiments 2 and 3. Female bandwidth response and frequency preference:

Female preference. We followed the no-choice sequential stimulus experimental procedure of Verburgt & Ferguson (2006, Chapter 2) to quantify female preference. Briefly, we quantified female preference for spectral bandwidth (hereafter BW) and carrier frequency (hereafter FQ) through untethered phonotactic response in total darkness at a temperature of 25±1°C using a Kramer spherical treadmill (Kramer 1976; Weber et. al. 1981; Thorson et. al. 1982) in an anechoic chamber (>2 kHz). We conducted two experiments consisting of four and two trials respectively, each trial consisting of a series of stimuli. Each trial began with one minute of silence allowing females to become accustomed to the movement of the sphere. Each stimulus thereafter was presented twice, played back alternately from two different angularly separated speakers (210° and 90° respectively) for a minute at a time respectively. We randomised the order of the trials presented to females. We measured phonotactic response of females toward each stimulus presented as the angular variance (hereafter Batschelet deviation - BatD; Batschelet 1981), a measure of dispersion for a circular distribution, and corrected

for possible auditory asymmetry as described by Verburgt & Ferguson (2006, Chapter 2). Female response functions for FQ and BW were calculated using cubic splines (Schluter 1988) with a smoothing parameter (λ) of -5. Errors were fitted to the splines from 200 bootstrap replications. We obtained a polynomial regression equation (BW=3rd order and FQ=6th order) from the predicted values ($\hat{y}$) from the spline analysis. Preference for both BW and FQ, respectively, was calculated as the call parameter value (on the x-axis) corresponding to the best phonotactic response (lowest BatD) during a trial.

Acoustic stimuli. Acoustic stimuli played back at a sound intensity (i.e. maximum of the carrier envelope) of 70 dB SPL (measured at the top- center of the treadmill) were computer generated. All syllables had 2 ms linear rise-fall times. Following Verburgt & Ferguson (2006, Chapter 2) we maintained a standard stimulus of 5 kHz frequency, 43 ms syllable period, 50% duty cycle, 4 syllables/chirp and 2 chirps/second (250 ms chirp duration). We appended a control stimulus of exactly the same specifications as the standard stimulus at the end of each trial to determine if females were still responsive to the acoustic stimuli.

Experiment 2. Effect of different bandwidth characteristics on female preference for bandwidth:

To investigate the effect of different types of spectral bandwidth on female preference, we subjected 34 randomly selected females to four different BW trials, three of which differed only in the type of spectral sweep while the remaining trial differed in the starting frequency. Following a minute of silence, each trial started with a pure tone (i.e. no modulation) after which subsequent stimuli were modulated in increments of 200 Hz. Each trial consisted of seven different stimuli (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 kHz modulation) and a control stimulus (no modulation), each played back from two speakers, so that each trial duration was 17 minutes. For the down sweep trial (BW-D) and the high start trial (BW-H), spectral sweep was a linear modulation down from the starting frequency (5 and 6 kHz respectively), while the up-sweep trial (BW-U) was a linear modulation up to 5 kHz. For the noise trial (BW-N), white noise (power density constant over selected frequency range) was band-pass filtered to produce syllables with the

desired spectral bandwidth but no sweep. Figure 3 illustrates a single chirp for the 200 Hz modulation stimulus for each of the four different BW trials females were subjected to. These different trials allowed us to determine if female bandwidth response is affected by starting frequency, sweep direction and sweep presence. Linear regression quantified the slope for the BW preference functions.

Experiment 3. Relationship between frequency preference and bandwidth response:
Individual female preference functions for both BW and FQ for 71 randomly selected wild-caught females were calculated. As captive crickets are generally smaller due to crowding effects we specifically chose only wild-caught females to avoid any size-dependent preferences confounding our results. Nevertheless, we measured the surface area of the pronotum (length x width; mm^2) for females to confirm that body size did not affect preference. Additionally, selecting females randomly from several populations increases the probability of obtaining females with markedly different preferences if between-population differences in preference exist. The BW trial was identical to the down sweep trial (BW-D) of experiment 2. The FQ trial was identical to that of Verburgt & Ferguson (2006, Chapter 2) and consisted of nine different stimuli (3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7 kHz) and a control stimulus (5 kHz), each played back from two speakers for a minute at a time, so that the trial duration was 21 minutes. To obtain comparable measures of female selectivity for both the BW and FQ trial, each preference function was standardized by setting the best phonotactic response to zero. We then calculated the area under the preference functions by integration; for BW we calculated the definite integral $\int_{0}^{1.2} f(x)\,dx$ and for FQ $\int_{3.8}^{5} f(x)\,dx$. The definite integral for the FQ preference function served as a measure of FQ selectivity in the same range that we tested BW (starting at 5 kHz, 1.2 kHz downward linear modulation ends at 3.8 kHz). A small area would therefore denote low FQ selectivity in this signal range. The area under the FQ preference function (FQ selectivity) and the FQ preference were used as predictive variables for multiple regression with BW preference and area under BW preference function (BW selectivity) as the dependent variables respectively.

Statistical analyses: We used the SAS system (SAS Institute, Cary, NC, USA), Sigmaplot (Systat Software Inc. Richmond, CA, USA) as well as Statistica V5.5 (Statsoft Inc. Tulsa, OK, USA). Repeatabilities±SE were calculated following Becker (1984). We used sequential Bonferroni corrections to correct for multiple comparisons between traits (Rice 1989), hereafter indicated as SqB where appropriate.

Results

Correlations between male traits can be confounded by between-population differences (Simmons 1995; Ferreira & Ferguson 2002). To confirm that the populations did not differ markedly in any respect, a discriminate function analysis (DFA) was performed on six of the populations (each containing more than four individuals) using femur length, tibia length, harp area and four other wing traits as dependent traits. We found no significant ability to discriminate between populations ($F_{25,150}$=1.31, P>0.05) and further found no difference in body size between populations ($F_{5,49}$=1.10, P>0.05).

Experiment 1. Male asymmetry:

Of the original sample size of 82, eleven males had one or more hind femur missing and were excluded from the data set. Scatter plots of the remaining 71 individuals revealed no outliers due to measurement error. However, a further eleven individuals were removed from the data as they were found to be significant FA outliers, mostly due to prior injury (six femur, one tibia, four due to one or more wing trait). R-L for all traits was normally distributed (Kolmogorov-Smirnov). The following wing traits showed a significant departure (SqB) of mean (R-L) from 0 (Z-test, 2-tailed probability) indicating DA: A_1-H_3, H_{7-8}, H_3-M_2, M_2-H_9, harp area, H_{8-9}, H_2-A_1, H_3-A_2, M_4-H_9, H_{4-8}, H_{1-10}. The following traits were either significantly lepto- or platykurtic: H_{2-3}, A_1-H_3, H_{3-4}, H_3-M_2, H_8-M_4.

A 2-way mixed-model ANOVA (sides x individuals) revealed significant DA for M_2-H_9, harp area, H_{8-9}, H_2-A_1, H_3-A_2, M_4-H_9, H_{4-8}, H_{1-10}, confirming the results of the Z- test for departure of mean (R-L) from 0 and were therefore discarded from the dataset. A_{1-2} did not show significant between-individual variation ($F_{59,59}$=1.49, P=0.07; after SqB) and

was therefore discarded from the dataset. The between-sides variation (FA) was significantly > ME for all traits.

The FA |R-L| of all traits measured showed no dependency on trait size [(R+L)/2] after a SqB. A Levene's test on R-L revealed that $H_{7\text{-}8}$ and H_3-A_2 were heteroscedastic. An ID x TRAITS 2-way ANOVA using |ln(R)-ln(L)| as dependent trait revealed that FA (relative to trait size) differed between individuals and between traits (Table 3). A correlation matrix (R-L) was created for the traits which adhered to all the above requirements for FA ($H_{2\text{-}4}$, $H_{5\text{-}6}$, $H_{6\text{-}7}$, $M_{1\text{-}2}$, $M_{3\text{-}4}$, $H_{9\text{-}10}$, H_{10}-A_3, FEM, TIB) since highly correlated traits will not contribute to an organism's overall FA when combined. $H_{9\text{-}10}$ was significantly correlated with $H_{6\text{-}7}$ (r=0.457, P=0.001) and with H_{10}-A_3 (r=-0.818, P=0.001) after a SqB and were therefore discarded. Since traits examined for use in FA studies should be geometrically independent (Palmer & Strobeck 2003), linear measurements that shared a common endpoint were discarded, ($H_{6\text{-}7}$ was discarded as it shares an endpoint with $H_{5\text{-}6}$) as variation in the position of a shared endpoint will affect both dimensions.

The remaining traits were: $H_{2\text{-}4}$, $A_{1\text{-}2}$, $H_{5\text{-}6}$, $M_{1\text{-}2}$, $M_{3\text{-}4}$, H_{10}-A_3, FEM and TIB. These traits were subjected to a Levene's test in order to determine whether their ME was homoscedastic. Since we measured all traits 3 times we could not use a simple measure of ME as described by Palmer & Strobeck (2003) (ME=$|M_1\text{-}M_2|$). We therefore calculated the Standard Deviation of the measurements as follows for each trait and used this value as the dependent variable for the Levene's test:

$$\sqrt{\frac{\sum_{i=1}^{3}(l_i-\bar{L})^2+\sum_{i=1}^{3}(r_i-\bar{R})^2}{n}} \qquad (1)$$

where l_i=measurement i on the left side and $\bar{L}$ =mean left measurement, n=6 in each case. The ME of all traits was not comparable ($F_{7,466}$=48.02, P<0.001). Only once FEM and TIB were discarded was the ME homoscedastic ($F_{5,354}$=2.15, P=0.06) meaning that

these traits could be combined if their FA was also homoscedastic. ME for FEM and TIB on their own was also heteroscedastic ($F_{1,112}$=16.21, P=0.001).

The FA of all remaining traits were heteroscedastic, irrespective of the trait combination or whether FA1 (mean |R-L|) or FA8a (mean |ln(R/L)|) was used as dependent variable. The mean FA1 and mean FA8a where highly correlated with their respective standard deviations (r^2=0.98 and r^2=0.99 respectively). When Levene's test for HOV was conducted on all remaining traits except FEM and TIB with cFA 2 (Leung et al. 2000) where

$$cFA_j = \frac{|FA_{ij}|}{mean|FA_j|} \quad (2)$$

for (j=1 to k), the FA was homoscedastic ($F_{5,354}$=0.95, P>0.45) therefore allowing for the combination of these traits for a composite measure of overall individual FA (FA14; Palmer & Strobeck 2003);

$$FA_{14} = \frac{\sum|FA_{ij}| / mean|FA_j|}{N_t} \quad (3)$$

where N_t=number of traits.

Experiment 1. Relationship between male morphology and song traits:

Size-corrected DA of harp area, (R-L)/[(R+L)/2], was highly correlated with the uncorrected DA of harp area (r^2_{60}=0.98, P<0.0001) and we therefore present results for the size-corrected DA of harp area only. Additionally, harp area calculated as a polygon (19.52±2.14 mm^2) was significantly larger than harp area calculated as a right-angled triangle (16.60±1.89 mm^2) (Students t-test, t_{60}=48.01, P<0.001). However, the two areas were highly correlated (r_{60}=0.98, P<0.001), indicating the validity of measuring harp area as a right-angled triangle. We present results from the polygon method.

We performed a principle component analysis (PCA) on the signal traits to collapse them into fewer axes that describe independent uncorrelated patterns of variation (Brown et al. 1996; Zuk et al. 1998; Holzer et al. 2003; Simmons et al. 2005). Three factors with eigen values greater than 1.0 explained 71.3% of the total variation in song traits (Table 4). We

used forward stepwise multiple regression analysis to determine the relationship between morphological and song characteristics. All variables entered into this analysis were normally distributed (Kolmogorov-Smirnov; $d>0.06$, $P>0.05$). The song principle component (PC) values for each individual cricket were not significantly correlated with any of the dependent morphological variables (Table 5). A separate analysis with only the song PC values as independent variables also failed to significantly explain variation in the dependent morphological variables. DA of harp area was significantly negatively correlated with body condition (SqB) and harp area was significantly correlated with body size (SqB) (Table 5). There was a tendency for FA14 to be negatively correlated with body condition although this was non-significant after a SqB. For direct comparison with other studies, we investigated univariate relationships between song traits and morphological traits. Mean harp area was highly correlated with body size ($r^2_{60}=0.80$, $P<0.001$). Carrier frequency (FQ) was not correlated with body size ($r^2_{60}=0.03$, $P=0.22$) or harp area ($r^2_{60}=0.05$, $P=0.08$). The upper limit of the expected correlation (r^2) between FQ and body size (see Introduction and Table 1) was 0.04. Bandwidth of a males' call was not correlated with the size-corrected differences in harp area ($r^2_{60}=0.02$, $P=0.25$) or the uncorrected DA of harp area ($r^2_{60}=0.02$, $P=0.27$, Figure 4). When only considering individuals with larger right harps, the relationship was similar ($r^2_{52}<0.01$, $P=0.83$). No relationship between the FA ($|R-L|$) of FEM or TIB and the DA of harp area or bandwidth of a males' call was observed ($r<0.14$, $P>0.05$). We found no relationship between a males' overall FA (FA14) and the DA of his harp areas ($r^2_{60}<0.001$, $P=0.82$) or the bandwidth of his call ($r^2_{60}<0.001$, $P=0.84$).

Since sexually selected traits are expected to be larger in good quality individuals in good condition (Cotton et al. 2004b), we used a general linear model to test whether harp area (controlling for body size) is predictable from male condition and FA14 (quality). We found no difference in size-corrected harp area based on either body condition ($F_{1,57}=0.33$, $P>0.05$) or FA14 ($F_{1,57}=0.35$, $P>0.05$). This relationship was similar when grouping individuals into two condition groups (good condition: residuals>0; poor condition<0) and two quality groups (good quality: FA14<mean FA14; poor quality: FA14>mean FA14). Furthermore, none of the morphological traits measured differed in

absolute size or when trait size was controlled for body size between the condition and quality groups respectively (t-tests, $P>0.05$).

Experiment 2. Effect of different bandwidth characteristics on female preference for bandwidth:

Repeated measures ANOVA revealed that BW preference differed significantly between the four trials ($F_{3,99}=8.00$, $P<0.001$) which was due to the preference in the BW-H trial differing from that of every other trial (*post-hoc* analysis; Tukey HSD). This indicated that a females' BW preference is only affected by the starting frequency of the sound and not the sweep direction or the presence of a sweep. Furthermore, individual female BW preference was significantly repeatable over the BW-N, BW-U and BW-D trials ($F_{33,68}=1.71$, $P<0.05$, $r=0.19\pm0.11$). The slope of the linear regression describing a females' BW response differed significantly between the four trials ($F_{3,99}=19.45$, $P<0.001$). Again, this was solely due to the preference of the BW-H trial differing from that of every other trial, (post-hoc analysis; Tukey HSD). The slope of the linear regression describing a females' BW response was also repeatable across the BW-N, BW-U and BW-D trials ($F_{33,68}=3.8$, $P<0.001$, $r=0.48\pm0.10$)

Experiment 3. Relationship between frequency preference and bandwidth response:

The FQ preference of a female was not dependent on her body size ($F_{1,68}=0.01$, $r^2<0.01$, $P=0.93$). FQ preference and the area under the standardised FQ preference function did not predict BW preference ($F_{2,68}=1.31$, $r^2=0.01$, $P=0.28$) or the area under the standardised BW preference function ($F_{2,68}=1.96$, $r^2=0.03$, $P=0.15$).

Since the resolution of the phonotactic data was too low to predict the exact BW preference from FQ preference and FQ selectivity, we grouped the females according to the FQ stimulus that elicited the best phonotactic response (hereafter FQ-group). Only four females did not prefer 4.5, 5 or 5.5 kHz and these females were discarded from subsequent analyses (one female per FQ-group). We performed a linear regression for each female describing her BW response and used both the slope (m) and the intercept (c) as dependent variables in two separate one-way ANOVA's with FQ-group as categorical

predictor. We found a significant difference between FQ-groups for *m* ($F_{2,64}$=14.65, P<0.001) but not for *c* ($F_{2,64}$=0.16, P=0.85). Post-hoc analyses (Tukey HSD, followed by SqB) showed that *m* differed for each comparison between groups (P<0.014). We calculated individual cubic splines for the BW response and an overall cubic spline for each FQ-group by pooling the phonotactic response data for all females in a group. Since each of the data points of the overall splines were separated by more than two standard errors the splines differed significantly between groups. The individual and grouped splines as well as the mean linear regressions for each group are presented in Figure 5.

Discussion

Sound production

Spectral bandwidth was not caused by the DA of harp areas (Figure 4) even though males in our study showed a greater prevalence of DA than Simmons & Ritchie (1996) showed for *G. campestris* (88% of *G. bimaculatus* had larger left harps compared to 61% for *G. campestris;* n=60 and 61 respectively). The relationship between body size, harp size and carrier frequency (FQ) for *G. bimaculatus* was shown to be incomplete since body size was highly predictable from harp size but not from FQ despite the strongest correlation between body size and harp area reported to date for grylline crickets (Table 1). No relationship between harp size and FQ was found, contrary to the findings of four other studies on a sibling species (*G. campestris*, see Table 1). These findings are not surprising since Verburgt & Ferguson (2006, Chapter 3) demonstrated that the harp area is not likely to be the primary resonant structure responsible for the FQ in this species. In general, the FQ of insect song shows an inverse relationship with body size due to the physical constraints of sound production (Gerhardt & Huber 2002). Despite the lack of a significant relationship between FQ and body size shown here, large intraspecific differences in body size can yield a similar relationship with FQ compared with that of interspecific comparisons. For example, taking the three largest and the three smallest individuals in our male data set, yields a significant relationship between FQ and body size (r^2=0.79, $F_{1,4}$=14.6, P=0.02), but only because the large males were more than twice the size of the small males. This may be explained by Eq 1 (Verburgt & Ferguson 2006, Chapter 3) where, within a certain range, the thickness of the vibrating plate may

decrease in relation to its size so that different sized plates will vibrate with a similar resonant frequency. However, decreasing the size of the vibrating plate is likely to decrease the amplitude (intensity, dB) of the sound produced. Large *G. bimaculatus* males produce sound of greater amplitude than small males (Simmons 1988b) and females have been shown to prefer larger males in this species (Simmons 1986; 1988a Bateman et al. 2001) and in *G. campestris* (Simmons 1992). Females may therefore not need to discriminate between males of different sizes based on call FQ. However, Simmons (1988b) suggests that the unpredictable manner in which signal amplitude attenuates in the field renders song amplitude a poor indicator of male size.

Frequency tuning curves based on auditory thresholds of identified neurons show that females become less selective towards FQ as the sound intensity decreases (Schildberger et al. 1989). Bailey et al. (1990) suggested that *Requena verticalis* females are passively attracted to the closest calling male, since females always chose the loudest of two identical songs when differences in sound amplitude was 3 dB. Moreover, 10 out of 11 females chose the closer (and therefore louder) male in choice trials in the field suggesting that differences in signal amplitude are likely to outweigh any differences based on FQ in this species. Weber & Thorson (1988) showed for *G. bimaculatus* that amplitude differences as small as 1 dB are sufficient to change the attractiveness of a signal. Therefore, even if male body size was detectable from FQ it is unlikely that females could select for large males where several males are situated closely together and calling simultaneously (e.g. Simmons 1988b), as females are expected to track the loudest (usually the closest) male. Female *G. bimaculatus* show significant repeatable between-individual variation in FQ preference (Verburgt & Ferguson 2006, Chapter 2) and females in this study did not show uniform preference for low FQ as is evident from the different FQ preference groups (Figure 5). It is therefore unlikely that there is a principal benefit to preferentially mate with males producing low frequency song as strong directional selection would then be expected. However, the unimodal preference function for FQ in this species (Verburgt & Ferguson 2006, Chapter 2) does not necessarily rule out the possibility of directional selection as the unimodal preference function for song FQ in *Drosophila montana* effectively exerts directional selection on

contemporary males of the species based on the current distribution of male song FQ (Ritchie et al. 2001).

We found no evidence that any song trait measured in this study conveyed information regarding body condition, body size or genomic ability to buffer DI (FA14). FA is presently thought to be largely environmentally determined during development. It is therefore unlikely that females selecting symmetric males provide support for 'good genes' models of sexual selection (such as the handicap hypothesis) but that they rather gain direct benefits (Swaddle 2003). Consequently, it is crucial to understand the relative roles of the environment and genes in producing sexually selected traits before the role thereof can be considered in sexual selection (Polak & Starmer 2005).

Interestingly, DA of harp area was negatively correlated with body condition. Although DA is generally rejected as an indicator of stress since it has an underlying genetic basis (Palmer & Strobeck 1986), Simmons & Ritchie (1996) showed that DA of harp areas was correlated to FA and they argued that DA might reveal DI. Since we did not find a correlation between FA and DA of harp area, we suggest that the DA of harp area may provide an independent measure of DI. This is demonstrated by the inclusion of FA in the equation where DA of harp area significantly explained body condition (Table 5). If FA reliably indicates individual quality, then it is expected to covary negatively with body condition (Møller & Pomiankowski 1993). Although we found the expected negative correlation between FA and body condition, it was not significant. It may be argued that our measure of FA was problematic as it was composed of several wing measurements. Since perturbations early in development affect an entire structure or because different dimensions of the same structure (e.g. an insect wing) are more highly integrated developmentally, different dimensions of the same structure may not yield independent estimates of DI (Leamy 1993; Klingenberg & Zaklan 2000; Palmer & Strobeck 2003). However, the combination of these traits into a composite measure of FA was justified, despite the potential problem of developmental dependency because the FA of these traits was not correlated with each other and therefore provided different estimates of DI. Additionally, we may not have sampled sufficient traits that could be combined into a

composite measure of FA as only six traits adhered to all the requirements for combination. However, Mallard & Barnard (2004) were able to detect an increase in FA with decreasing condition using only a single trait (hind femur) in the same species.

High quality males are supposed to produce sexual traits that are both large and symmetric under either the 'quality heterogeneity' hypothesis or the 'environmental heterogeneity' hypothesis (Polak & Starmer 2005). However, we found no difference in trait size between any of the measurements in this study depending on either male condition or male FA. This suggests that these traits are currently not under handicap sexual selection which is not surprising as none of these traits were correlated with characteristics of male song. This is in contrast to recent studies showing both present (Scheuber et al. 2003b) and past (Scheuber et al. 2003a) condition-dependence of certain aspects of male song which have been argued to be a multicomponent sexual signal (Scheuber et al. 2005). Interestingly, these laboratory results could not be reproduced directly in the field and only the energetically costly aspects of male calling song (calling rate) were condition-dependent and were shown to affect male attractiveness (Holzer et al. 2003). The condition of *Teleogryllus commodus* as measured by immune function response was correlated with syllable duration, another energetically costly aspects of male song (Simmons et al. 2005). Similarly, in *Acheta domesticus*, immune function was correlated with number of syllables per chirp, yet another measure of the energetic cost of signaling (Ryder & Siva-Jothy 2000). In *G. integer* males, calling-bout duration, which is a preferred male trait, was shown to be condition-dependent despite high additive genetic variance for the trait (Hedrik 2005). To date, the only aspect of *G. bimaculatus* signals shown to be condition dependent (through immune function) are the energetically costly components of male courtship song (tick rate and high-frequency tick duration; Rantala & Kortet 2003). Since several calling song traits are probably required for mate recognition, it is not surprising that the emergent pattern is that energetically costly components of male signals vary with body condition. Although we did not find a male signal correlated with body condition, we did not measure total calling bout length. This aspect of signaling is likely to be restricted by body condition as it is for *T. commodus* (Hunt et al. 2004).

Females did not discriminate between different types of bandwidth (BW) sweep nor did they require the presence of a sweep. Only the starting frequency of the sound influenced BW preference. These results are similar for *G. campestris* females that preferred a large degree of modulation when songs started at high frequency but preferred less modulation when songs started at low frequencies (Simmons & Ritchie 1996). We further showed that apparent preference for BW is governed by FQ preference (Figure 5). The BW preference functions for females preferring frequencies of 4.5 and 5kHz (Figure 5) reveal a very low degree of discrimination over the BW range tested. We believe this reflects the limitations of the auditory system to resolve spectral modulation of sound, because each stimulus presented contained both preferred and non-preferred frequency elements. When the spectral modulation of presented stimuli did not contain any of the preferred frequency elements (Figure 5, 5.5 kHz frequency preference) there was clear discrimination in female response between the different stimuli. It is therefore unlikely that *G. bimaculatus* females have a preference for BW *per se*. In fact, the low frequency elements of calling song produced by males of this species as well as the frequency range (analogous to bandwidth) degrades significantly with increasing distance from the calling male (Simmons 1988b). Females may therefore not be able to detect BW at low sound intensities. We suggest that females will tolerate a certain degree of BW rather than prefer a specific BW, which depends both on the starting frequency of the stimulus and the females' FQ preference. The largest degree of spectral modulation observed for males in this study was ±700 Hz and females did not discriminate largely between stimuli of >1000 Hz modulation. We therefore support the argument of Simmons & Ritchie (1996) that female bandwidth preference is a by-product of FQ preference and not a distinct preference.

In conclusion, this study suggests that calling song frequency does not predict body size in *G. bimaculatus* since harp area is unlikely to control song FQ. Therefore, spectral bandwidth was not correlated with DA of harp areas and can not relay information on male quality. Furthermore, male condition was not correlated with any aspect of song measured, although it is likely to be reflected in energetic parameters of song not

quantified. The absence of uniform female preference for low frequency song and the fact that bandwidth preference is determined by FQ preference indicates that females probably do not select for male size and male quality on the basis of spectral characteristics of male song. In fact, we found no evidence of sexual selection based on the measured male song parameters in this species.

Acknowledgements

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Chapter 4: Tables

Table 1. The relationship between harp area, body size and song carrier frequency for four grylline cricket species (Orthoptera; Family Gryllidae; Subfamily Gryllinae).

Species	Measure of male body size	Harp area vs body size	Frequency vs body size	Frequency vs harp area	Reference
Gryllus bimaculatus	Pronotum width	-	B=0.141 (df=3,28)	-	Simmons (1988)
"	Pronotum width	-	r_b=-0.293 (n=51)	-	Simmons & Zuk (1992)
"	Pronotum width	-	no correlation found (n=75)	-	Ferreira & Ferguson (2002)
"	Pronotum width	-	r=0.07 (n=269)	-	Bateman et al. (2004)
Gryllus campestris	Pronotum width	$F_{1,55}$=43.44, r^2=0.44 ***	r=0.106 (n=15)	$F_{1,13}$=17.01, r^2=0.57 ***	Simmons (1995)
"	Pronotum width	r_s=0.748 (n=116) ***	- *(r^2_{exp}=0.12)*	$F_{1,71}$=20.30, r^2=0.22 ***	Simmons & Ritchie (1996)
"	Pronotum area	r_s=0.47 (n=68) ***	- *(r^2_{exp}=0.03)*	r=0.35 (n=60) **	Scheuber et al. (2003a)
"	Pronotum area	$F_{1,111}$=37.95 *** *(r^2_c=0.26)*	- *(r^2_{exp}=0.07)*	$F_{1,57}$=21.97 *** *(r^2_c=0.28)*	Scheuber et al. (2003b)
"	Initial weight	$F_{1,107}$=32.92 *** *(r^2_c=0.24)*	- *(r^2_{exp}=0.03)*	$F_{1,82}$=10.86 * *(r^2_c=0.12)*	Jacot et al. (2005)
Gryllodes sigillatus	Body mass	r=0.52 (n=244) ***	-	-	Sakaluk et al. (1992)
Acheta domesticus	Weight	-	r_s=0.01 (n=46)	-	Gray (1997)
"	Weight	-	$F_{1,38}$=1.61, r^2=0.02	-	Ryder & Siva-Jothy (2000)

-=Results not presented
r_b=Partial correlation coefficient
r_s=Spearman correlation
r=Pearsons correlation
B=Beta from multiple regression
r^2_c=Unadjusted r^2 calculated as [F-value/(F-value +df_{error})] for unreported cases (applicable for one-way ANOVA only)
r^2_{exp}=Expected r^2 calculated as ($r^2_{harp\ vs.\ size}$ x $r^2_{frequency\ vs.\ harp}$) or ($r_{harp\ vs.\ size}$ x $r_{frequency\ vs.\ harp}$)^2

* P<0.05
** P<0.01
*** P<0.001

Table 2. Sample sizes of male crickets drawn from the different South African populations and one European (Sevilla, Spain) population.

Population	n
Dulstroom	4
Dundee	2
Hotazel	1
Machado	10
Paarl	4
Pretoria	12
Queenstown	2
Sevilla	19
Wolmaranstad	6
Total	60

Table 3. Results of an ID x TRAITS two-way ANOVA using fluctuating asymmetry (FA) relative to trait size (|ln(R)-ln(L)|) as dependent variable for male *G. bimaculatus* morphological traits (n=60).

Source	df	F	P
ID	59	1.65	0.002
TRAIT	18	42.03	<0.001
TRAIT*ID	1056	111.94	<0.001
Error	2268		

Table 4. Principle component analysis of male *G. bimaculatus* calling song traits (n=60). Values are factor loadings (Pearson's correlation coefficient between components and the original variable). Variables accounting for less than 25% of the variance are not shown.

	Component		
Variable	1	2	3
Carrier frequency	-0.52	-	-
Bandwidth (-10dB)	-0.54	-	-
Syllable duration	-	0.82	-
Syllable period	0.77	-	-
Inter-syllable interval	0.59	-0.76	-
Chirp duration	0.53	-	0.66
Chirp period	0.84	-	-
Inter-chirp interval	0.71	-	-
Eigen values	3.0	1.7	1.0
Cumulative variance explained (%)	37.7%	58.9%	71.3%

Table 5. Partial regression values from three forward stepwise multiple regressions indicating the significant relationships between body condition, body size, FA14 and several independent variables. § indicates the variables that remained in the equation upon completion of the analysis. FA14 is an individuals' overall fluctuating asymmetry and DA is the directional asymmetry. PC1-3 are principle components derived from eight male signal traits (see Table 3). n=60 for each regression.

Variables	Body condition	Body size	FA14
FA14	§ -0.24	0.07	-
Harp Area	§ 0.23	§ 0.90 ***	§ 0.14
PC1	0.14	0.07	§ 0.19
PC2	-0.04	-0.12	0.05
PC3	§ 0.26	0.07	-0.07
Body size	§ -0.17	-	0.03
Body condition	-	-0.12	§ -0.26 *
DA of harp area	§ -0.39 **	-0.08	-0.12
r^2	0.24	0.81	0.11
df	5,54	1,58	3,56
F	3.44	240.29	2.34
P	<0.01	<0.001	0.08

* P<0.05
** P<0.01
*** P<0.001

Chapter 4: Figures

Figure 1. Song structure of the chirping cricket *G. bimaculatus*. Temporal characteristics measured in this study are indicated. Each chirp typically comprises 3-5 syllables, followed by a period of silence (inter-chirp-interval). Each syllable is produced by a single closure of the forewings. The opening of the forewings does not produce any sound (ISI).

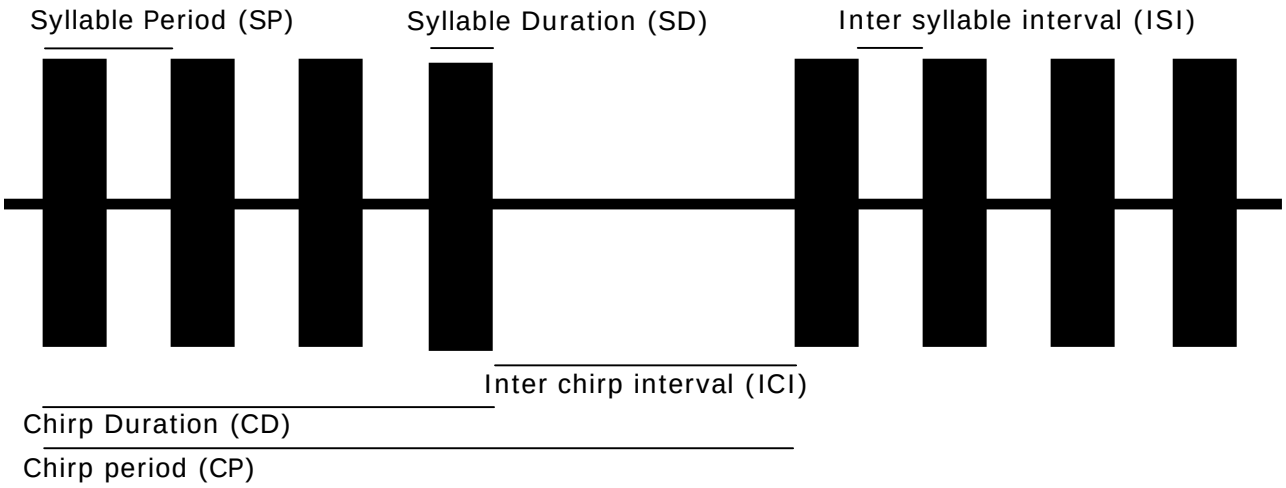


Figure 2. Schematic diagram of the major venation of a male *G. bimaculatus* tegmen. The gray area enclosed by landmarks H_1-H_{10} is the harp area, while the circular area enclosed by M_1-M_4 + H_9 represents the mirror area. A_1-A_3 are additional arbitrary landmarks measured. All landmarks were measured at the junction of two veins except for H_2 and H_4, the latter being derived from a hypothetical junction (dotted line) of the Cu2 vein. Arrows indicate the specific vein junctions measured.

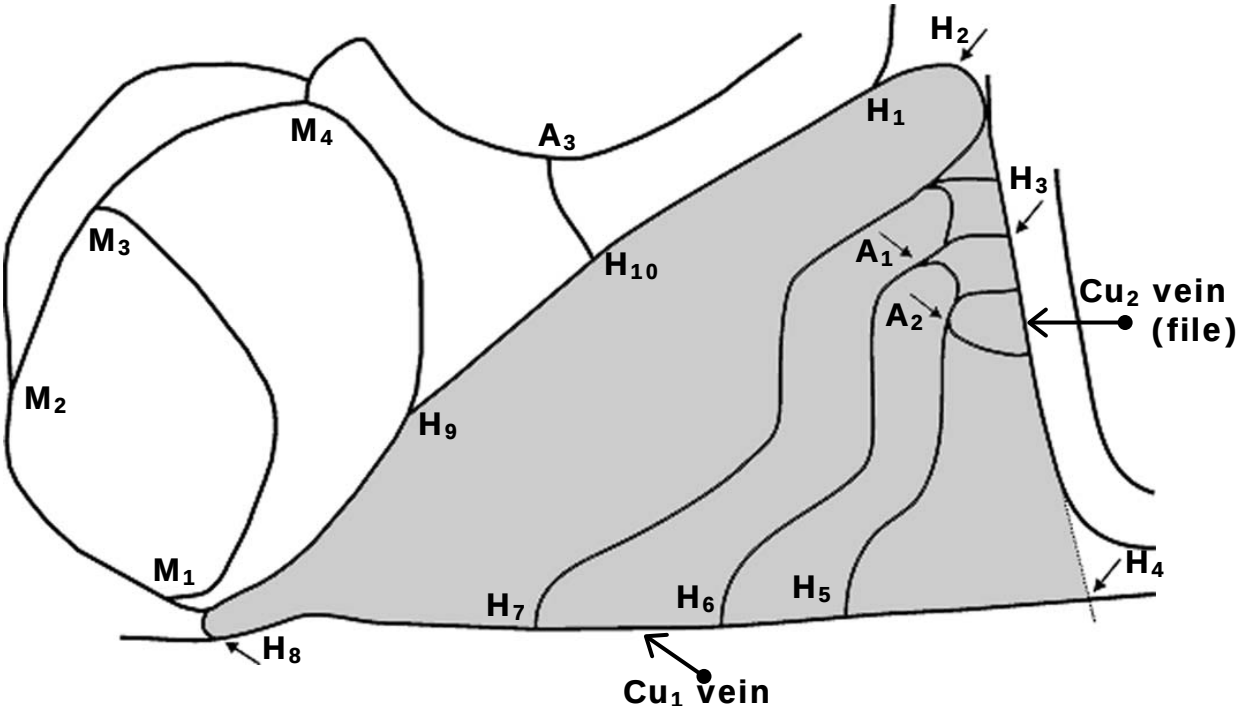


Figure 3. A single synthetic chirp (4 syllables) from the 200Hz modulation stimulus for each of the four bandwidth trials presented to females in this study. BW-H and BW-D were both linearly modulated down so that at the end of each syllable (syllable duration=22ms) the desired degree of modulation was reached. BW-U was linearly modulated upward while BW-N was generated from band-pass filtered white noise.

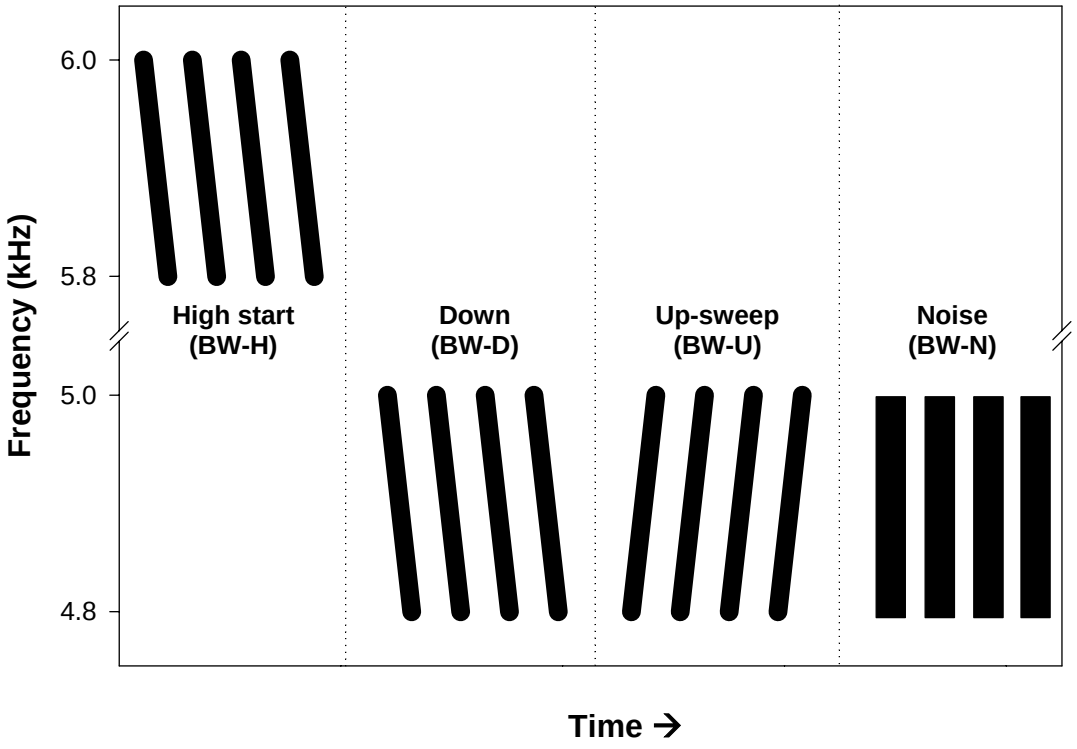


Figure 4. Relationship between spectral bandwidth (-10dB) and harp area asymmetry in *G. bimaculatus* (n=60). This figure further illustrates that there is directional asymmetry (DA) of harp area (mean R-L>0) and provides information regarding the distribution of both bandwidth and DA for *G. bimaculatus*.

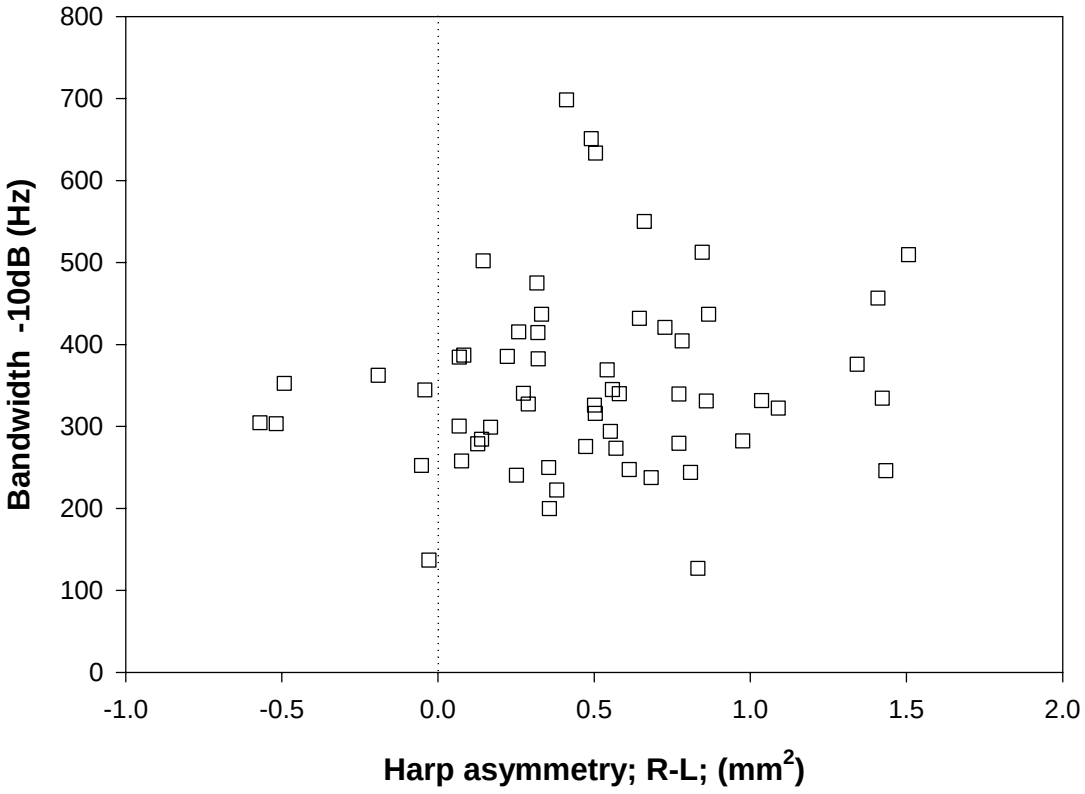
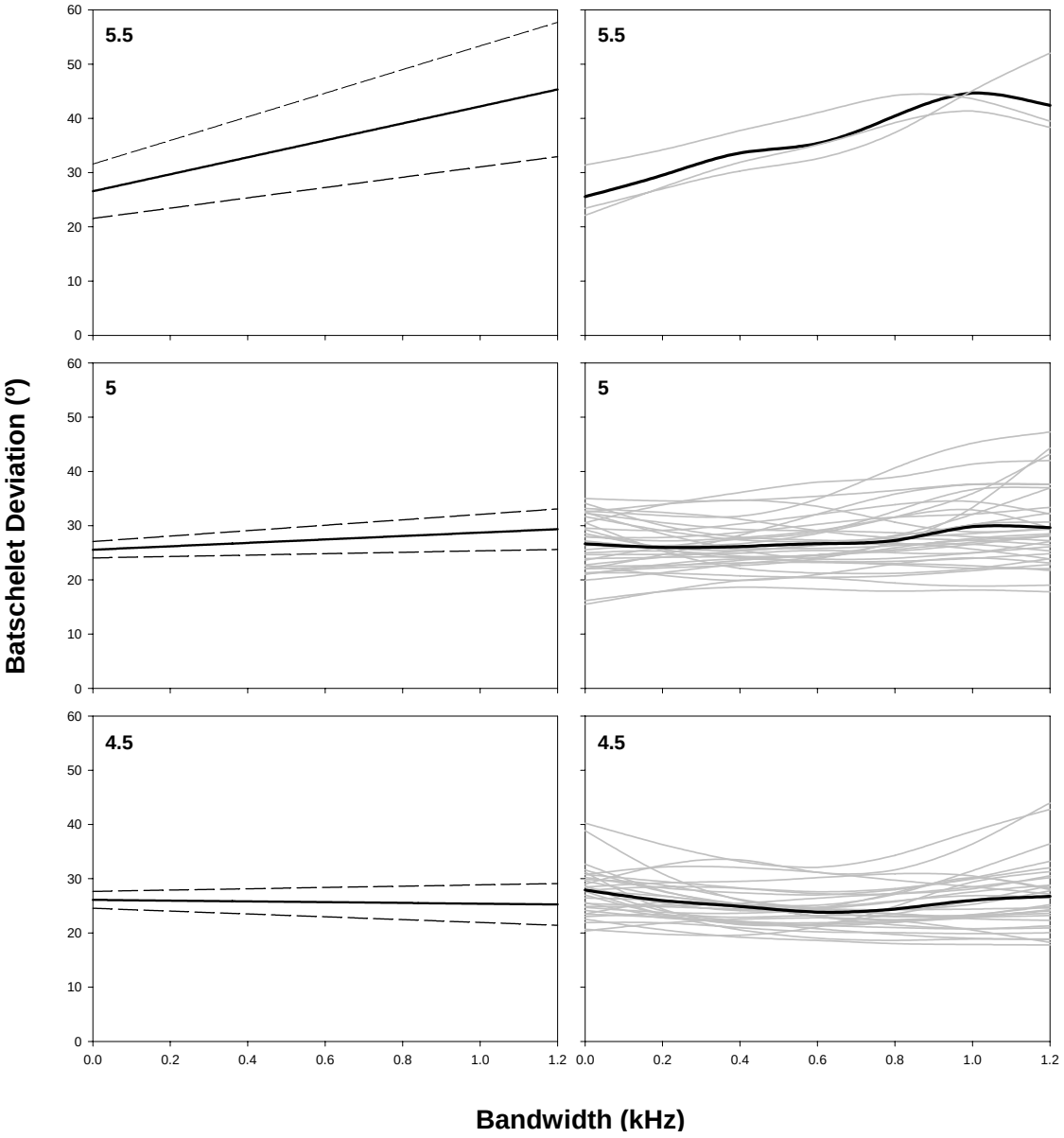


Figure 5. Linear regressions and cubic splines of bandwidth response for females preferring three different frequencies (4.5, 5 and 5.5kHz). Linear regression slopes and overall cubic splines (thick black lines) differed significantly between groups. Dashed lines represent 95% confidence intervals. Gray cubic splines are individual female responses functions.



Chapter 5

Conclusion

This work demonstrated three major findings. Firstly, female *Gryllus bimaculatus* have a distinct and repeatable preference and selectivity for certain male song traits, notably carrier frequency, syllable period and spectral bandwidth (Verburgt & Ferguson 2006, Chapter 2). However, preference for spectral bandwidth was shown to be a consequence of frequency preference and most likely frequency selectivity and is therefore not a distinct preference *per se* (Verburgt & Ferguson 2006, Chapter 4). Secondly, the basis for arguments invoking sexual selection for spectral song traits in the sister species *G. campestris*, which is that harp area predicts song frequency, was shown to be an invalid assumption for sound production in *G. bimaculatus* (Verburgt & Ferguson 2006, Chapter 3). Thirdly, contrary to the findings of several other studies (Verburgt & Ferguson 2006, Chapter 4, Table 1), temporal and spectral male song traits did not convey information regarding body condition, body size or the ability to withstand developmental instability (as indicated by fluctuating asymmetry). Although we were unable to detect handicap sexual selection for spectral characteristics of male song despite repeatable female preference for frequency (Verburgt & Ferguson 2006, Chapter 2), it does not rule out the possibility of sexual selection on other aspects of song not measured.

Gryllus bimaculatus male calling song traits show large degrees of between-individual variation which is thought to be largely environmentally determined as there are large amounts of gene flow among populations (Ferreira & Ferguson 2002; Ferreira 2006, MSc Thesis). We found no clear distinction between six populations based on seven morphological traits of *G. bimaculatus* males and could also not detect any difference in body size between these populations (Verburgt & Ferguson 2006, Chapter 4). Significant between-individual variation in all of the song traits measured was found and these differences were highly repeatable within-individuals (Table 1). However, the magnitude of difference between individuals was rather small as indicated by the low coefficient of variation (CV) for most of the call traits (Table 1). In Túngara frogs, the trait crucial for mate recognition (whine) was shown to have low CV between populations while the mate

choice trait (chuck) exhibited large between population differences and consequently large CV (Ryan et al. 1996). Mate recognition traits allow a female to recognize an appropriate mate of the same species (Butlin et al. 1985; Paterson 1985; Templeton 1989). It has been suggested that syllable period (SP) and carrier frequency (FQ) are mate recognition traits in the song of *G. bimaculatus* and that these traits show insufficient variation (possibly due to stabilizing selection) to allow sexual selection to operate (Huber et al. 1989; Ferreira & Ferguson 2002). SP and FQ were the most repeatable traits within males and had the smallest CV's (Table 1). This suggests that these traits are subject to stabilizing selection and involved in mate recognition (Paterson 1985; Rivero et al. 2000), since selection pressure on the reliability of mate recognition signals for effective intraspecific communication should reduce within-individual variation (high repeatability) and between-male variation (low CV) (Butlin 1995). Our results are very similar to that of Rivero et al. (2000) who found that mate recognition traits (syllable rate and syllable rate change) in wolf spiders, *Hygrolycosa rubrofasciata*, had high repeatability (0.63–0.84) and low CV (3-8%) whereas a mate choice trait (signal length) showed high repeatability (0.55–0.66) and large amounts of between-individual variation (CV 14-30%). Since SP and FQ appear to fit the criteria for mate recognition traits in *G. bimaculatus*, it is not surprising that we were unable to detect significant morphological correlates of these song traits.

Several studies have shown weak correlations between an aspect of male phenotype and an acoustic parameter only for another study to show a completely different result later. For example, in *G. bimaculatus*, Simmons (1998a) showed that syllable rate was significantly positively correlated with male body size (partial correlation=0.435; n=60) while Simmons and Zuk (1992) showed that syllable rate was significantly negatively correlated with male size (partial correlation=-0.512; n=51) in the same species. Since the same author was involved in both studies, it is unlikely that methodological errors are responsible for this discrepancy. This example suggests that caution is required when interpreting the results of correlational studies, as they do not demonstrate cause and can therefore be misleading. Our results showing the lack of any relationship between male morphology and song traits are not purely correlational (Verburgt & Ferguson 2006,

Chapter 4) since we previously demonstrated the lack of a causal relationship between harp area and tegmen resonance in *G. bimaculatus* (Verburgt & Ferguson 2006, Chapter 3). Furthermore, we recorded males on two different nights to account for between-night variability in song production which has recently been shown to account for a large proportion of variation in *G. bimaculatus* calling song (Ferreira 2006, MSc Thesis). Finally, we indirectly demonstrated the implausibility of song FQ as an indicator of male body size through the lack of uniform female preference for low frequency songs (Verburgt & Ferguson 2006, Chapter 4). We therefore have confidence in our conclusions regarding the role of FQ in the acoustic communication system of *G. bimaculatus*.

According to Paterson (1985), reciprocal recognition leads to stabilizing selection exerted by each sex on the signals or responses of the other. Therefore female preferences for mate recognition traits are expected to be highly repeatable and show low degrees of between-individual variation if the male signals involved in mate recognition do. Female preference for FQ was shown to be highly repeatable (Verburgt & Ferguson 2006, Chapter 2), and the CV for female FQ preference (n=71, experiment 3; Verburgt & Ferguson 2006, Chapter 4) was 7.49%, a value similarly low to that for male song FQ (Table 1). The low preference CV and low signal CV for FQ supports the suggestion of Ferreira and Ferguson (2002) that FQ of male *G. bimaculatus* calling song does not show sufficient variation for sexual selection to operate. Absence of detectable sexual selection on male song frequency (Verburgt & Ferguson 2006, Chapter 4) provides additional empirical support for this hypothesis. Since SP of male song was highly repeatable with a low CV (Table 1) and female SP preference was shown to be highly repeatable (Verburgt & Ferguson 2006, Chapter 2), it is expected that female SP preference will also have a low CV if SP is indeed a mate recognition trait. This prediction requires empirical verification.

This study raises an important question regarding female phonotactic response (here taken to mean the preference and selectivity) to several male song traits: What are the causative agents responsible for the observed variation in female response? Significant

between-individual variation in FQ preference was demonstrated in Verburgt & Ferguson, Chapter 2 (2006) and suggested by the different frequency groups in Verburgt & Ferguson, Chapter 4 (2006). The females in Verburgt & Ferguson, Chapter 4 (2006) were wild-caught individuals originating from seven geographically separated populations so the possibility exists that geographical variation in environmental factors may influence female preference. For example, different vegetation types place constraints on the efficacy of male acoustic signaling (e.g. in katydids *Neoconocephalus* species, Schul & Patterson 2003 and in the cricket frog, *Acris crepitans*, Ryan & Wilczynski 1991). Variation in female preference and selectivity may therefore simply reflect environmentally induced optimization of signal receiving rather than sexual selection *per se*. Alternatively geographical variation in female response may have a genetic component and reflect restricted gene flow among populations. Restricted gene flow is unlikely in *G. bimaculatus* due to the large distances that females can fly (up to 930km out to sea; Ragge 1972) and the high estimates for migration rates and gene flow among populations within South Africa and between South African and European populations (Ferreira 2006, MSc Thesis). Quantifying geographical variation in female *G. bimaculatus* response (such as performed for *G. bimaculatus* male signals by Ferreira & Ferguson 2002; Ferreira 2006, MSc Thesis) is the next step to gain better measurements of the CV in female response to different male song traits and to determine whether female response curves exert stabilizing or directional selection on male song traits. This work is now possible with the advent of appropriate methodology for quantifying female preference (Verburgt & Ferguson 2006, Chapter 2).

A quantitative genetic analysis of the acoustic communication system of *G. bimaculatus* is also required. Quantifying the contributions of additive genetic variation and environmental variation in producing the observed phenotypic variation (Falconer & Mackay 1996) in female phonotactic response and male song traits will greatly enhance our understanding of this communication system. Selection can only operate on additive genetic variation, the magnitude (relative to environmental variation) of which will influence the rate of evolution (Lynch & Walsh 1998). Furthermore, quantitative genetic analysis will enable measurement of the genetic covariance between female preferences

and male traits. Both Fisherian and 'good genes' models of sexual selection require the presence of a genetic correlation between a female preference and male trait in order to work although such a positive genetic correlation can arise under the sensory bias model from assortative mating due to genetic variance in preferences (Lande 1981).

Chapter 5: Tables

Table 1. Coefficients of variation (CV) and repeatability (r) estimates (±SE) for male *G. bimaculatus* calling song traits. All F-values were highly significant (P<0.001). r was calculated from male calling song traits recorded on two separate nights. n=60 in all cases.

Trait	CV (%)	r	$F_{59,60}$
Chirp Duration	16.9	0.62±0.08	4.25
Chirp Period	15.0	0.56±0.09	3.52
Inter-Chirp-Interval	20.7	0.55±0.09	3.45
Syllable Duration	10.5	0.40±0.11	2.32
Syllable Period	7.4	0.76±0.05	7.46
Inter-Syllable-Interval	18.0	0.62±0.08	4.25
Frequency	6.3	0.76±0.05	7.43
Bandwidth (-10)	38.7	0.62±0.08	4.2

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