Multivariate sexual selection on male tegmina in wild populations of sagebrush crickets, *Cyphoderris strepitans* (Orthoptera: Haglidae)

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Abstract

Although the strength and form of sexual selection on song in male crickets have been studied extensively, few studies have examined selection on the morphological structures that underlie variation in males' song, particularly in wild populations. Geometric morphometric techniques were used to measure sexual selection on the shape, size and symmetry of both top and bottom tegmina in wild populations of sagebrush crickets, a species in which nuptial feeding by females imposes an unambiguous phenotypic marker on males. The size of the tegmina negatively covaried with song dominant frequency and positively covaried with song pulse duration. Sexual selection was more intense on the bottom tegmen, conceivably because it interacts more freely with the subtegminal airspace, which may play a role in song amplification. An expanded coastal/subcostal region was one of the phenotypes strongly favoured by disruptive selection on the bottom tegmen, an adaptation that may form a more effective seal with the thorax to prevent noise cancellation. Directional selection also favoured increased symmetry in tegminal shape. Assuming more symmetrical males are better able to buffer against developmental noise, the song produced by these males may make them more attractive to females. Despite the strong stabilizing selection documented previously on the dominant frequency of the song, stabilizing selection on the resonator that regulates dominant frequency was surprisingly absent. Nonetheless, wing morphology had an important influence on song structure and appears to be subject to significant linear and nonlinear sexual selection through female mate choice.

Introduction

Sound communication plays an essential role in the mating systems of a wide array of taxa including birds, anurans, mammals and insects (Andersson, 1994). Song is an especially conspicuous feature of male crickets and other ensiferan Orthoptera and normally functions as the primary means by which males attract female conspecifics (Alexander, 1962; Huber *et al.*, 1989). In addition to its role in pair formation, various components of male song structure are known to be important in female mating preferences. Females have been shown to be more strongly attracted to males that sing at an increased calling rate (Stout *et al.*, 1983; Wagner, 1996; Wagner & Reiser, 2000) and, in many species, temporal features of song are important in determining attractiveness. For example, in the Pacific field cricket (*Teleogryllus oceanicus*), the male's song consists of a long chirp of 3–8 pulses followed by a sequence of short chirps, and females prefer songs that have a greater proportion of long chirps (Simmons *et al.*, 2001; Bailey, 2008). In Hawaiian sword-tail crickets (*Laupala cerasina*), there is significant linear sexual selection on male pulse rates and pulse durations, with females showing increasing positive phonotaxis towards songs with slower pulse rates and longer pulse durations (Oh & Shaw, 2013). Male tree crickets that

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produce louder calls are able to attract females over longer distances (Symes *et al.*, 2015). In most cricket species, female hearing is tuned to a species-specific frequency, and males that are able to more closely match this song frequency are more attractive (Stout *et al.*, 1983).

It is not surprising, then, that studies which have measured the strength and form of sexual selection on song have found evidence of both linear and nonlinear selection. Females exert directional sexual selection for increased male calling effort (French & Cade, 1989; Hunt *et al.*, 2004; Bentsen *et al.*, 2006). Evidence of stabilizing selection has been found for call structure (Brooks *et al.*, 2005; Bentsen *et al.*, 2006; Hunt *et al.*, 2007) and dominant frequency (Oh & Shaw, 2013; Ower *et al.*, 2013), both of which may be important in species recognition.

Although numerous studies have examined the role of song traits in mate attraction (Ryan & Keddy-Hector, 1992; Ritchie, 1996), few have examined selection on the morphological structures that underlie variation in males' song (Pitchers *et al.*, 2014). Measuring selection on morphology is important because song is both a product of behaviour and morphology (Montealegre-Z *et al.*, 2011; Pitchers *et al.*, 2014). Male crickets produce song through stridulation of specialized regions of their forewings (i.e. tegmina). During stridulation, a plectrum located on one tegmen strikes, in succession, a number of teeth forming part of a modified vein on the other, the file. As the plectrum strikes each tooth on the file of the opposing tegmen, the harp, the main sound radiator, vibrates, which cyclically compresses and rarefies air, producing song (Bennet-Clark, 1990). The dominant frequency (DF) or pitch of the song depends on the tooth strike rate, which, according to the clockwork cricket model, is regulated by the harp (Elliott & Koch, 1985; Koch *et al.*, 1988; Bennet-Clark, 1990). Vibration of the harp acts like a clock escapement mechanism, releasing the plectrum from the file and allowing it to strike the next tooth, which deforms the harps on both tegmina upwards and sets them into oscillation. A single wing closure produces a pulse, and the pulse duration (PD) is determined by the length of the file. The interpulse duration (IPD) is the amount of time it takes for the male to open his tegmina and reset the plectrum to the beginning of the file. A series of tegminal closures produces a pulse train. Train duration (TD) is a measure of the length of time spent producing a pulse train, whereas intertrain duration (ITD) is the length of time spent resting between pulse trains (see Fig. 1 in Ower *et al.*, 2013).

Although the form and intensity of sexual selection on song in male crickets have been studied extensively, the extent to which sexual selection drives evolution of the underlying morphological traits has received less attention, and remains virtually unexplored in wild populations. Comparative studies suggest that trade-offs
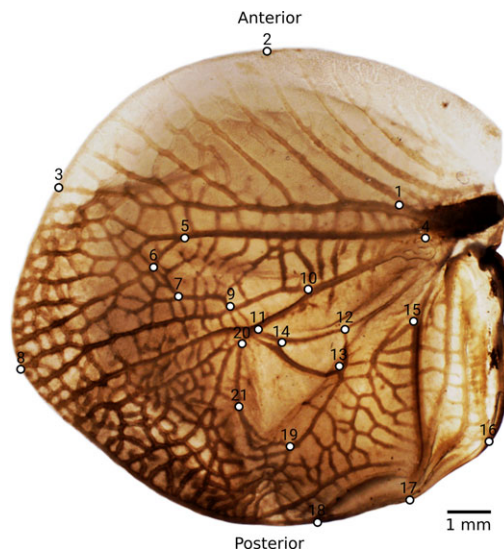

Fig. 1 Landmarks 1–20 were based on homologous venation among tegmina, making them type I landmarks under Bookstein's classification. Landmark 21 was placed mid-way between landmarks 19 and 20 due to highly variable venation posteromedially, making it a type III landmark. Scale bar = 1 mm.

between morphology and energy expenditures may shape interspecific variation in song features (Symes *et al.*, 2015). For example, the number of teeth on the file may constrain an individual's pulse rate and individuals with greater body mass can produce louder signals, but are unable to endure long bouts of calling (Symes *et al.*, 2015). In the current study, we assess the strength and form of sexual selection acting on the morphological traits involved in song production in wild populations of free-living male sagebrush crickets, *Cyphoderris strepitans*, mating in the field under natural conditions.

Measuring the mating success of insects in nature poses a unique challenge because it is rarely possible to observe all the copulations occurring in a population (but see Rodríguez-Muñoz *et al.*, 2010). Sagebrush crickets offer an ideal model system in this regard because mating imposes an unambiguous phenotypic marker on males that results from an unusual form of nuptial feeding by females. As in other ensiferan Orthoptera, male sagebrush crickets sing to attract sexually receptive females (Sakaluk & Snedden, 1990; Snedden & Sakaluk, 1992; Snedden & Irazuzta, 1994). When a calling male succeeds in attracting a female, she mounts the male dorsally to initiate a 3–5 min mating, which ends with the transfer of a spermatophore (Dodson *et al.*, 1983; Eggert & Sakaluk, 1994a). While mounted, the female feeds on the tips of males' fleshy hind wings and ingests hemolymph seeping from the open wounds that result from her feeding. Thus, it is possible to ascertain whether or not a male has mated

merely by inspecting his hind wings for the wounds inflicted by females at mating.

Time-lapse video studies have revealed that copulation never occurs without wing feeding; thus, none of the males lacking wing wounds in our field study and, hence, classified as virgins, can be considered 'false negatives' (i.e. males that actually mated). Mounting of the female and wing feeding on the male can occur without spermatophore transfer, when females feed for a brief time and then dismount before the male succeeds in transferring the spermatophore (Eggert & Sakaluk, 1994a; Sakaluk *et al.*, 1995). However, a male bearing wing wounds represents a male that has succeeded in attracting a female regardless of whether he succeeds in consummating the mating. Because the traits under consideration in our study are those that are used in mate attraction (male song parameters and their morphological underpinnings), the incidence of wing wounding represents a powerful and reliable proxy for measuring a male's ability to attract a mate, which makes it an appropriate fitness measure for a study of this sort. Moreover, we can rule out any effect of age based on an earlier mark–recapture study designed to determine whether there is an association between first appearance in the population and the likelihood of mating by males in the field (see 'Validating Our Measurement of Sexual Selection in the Wild' in Ower *et al.*, 2013).

In an earlier companion study, sexual selection was measured on the song of *C. strepitans,* and longer pulse durations, train durations and intertrain durations were associated with increased likelihood of mating (Ower *et al.*, 2013). Increased pulse and train durations likely improve the chances of attracting a female, but selection for longer intertrain duration suggests that long pulse and train durations are energetically costly and that males may need 'time outs' after bouts of singing. Indeed, playback trials confirmed that females prefer songs with long pulse and train durations, but not long intertrain durations. Additionally, evidence of strong stabilizing selection was found on dominant frequency (Ower *et al.*, 2013), and because the resonator regulates the song pitch, we would expect to find strong stabilizing selection on the size and shape of the resonator.

The objective of this study was to measure sexual selection on song-producing structures in male *C. strepitans,* and the extent to which variation in morphological traits is tied to variation in song parameters. Although numerous studies have examined correlations between linear or area measurements of tegmina and single song parameters, a more comprehensive approach is needed to capture the complex geometric shape and size of the tegmina (Pitchers *et al.*, 2014). The geometry of tegmina has indeed recently been shown to be of critical importance in determining tegminal resonance (Mhatre *et al.*, 2012). Thus, we used geometric morphometrics (Zelditch *et al.*, 2012), which enabled us to capture the multivariate

shape of the wings, but also is advantageous because it statistically allows the effects of size and shape to be independently assessed.

Methods

Field collection

Sagebrush crickets were collected from three populations located in Grand Teton National Park, WY, where the high altitude sagebrush meadow habitat of sagebrush crickets is abundant (Deadman's Bar 43°45′33.91″N, 110°37′25.12″W, Pacific Creek 43°51′25.67″N, 110°31′8.41″W and Bridger-Teton 43°54′40.56″N, 110°28′20.24″W). Males were found at night by orienting to their calls and using head lamps to determine their location within the sagebrush. The mating status of males was determined by examining their hind wings for evidence of wing wounding by females. Virgin males were identified by their intact wings, whereas mated males were identified by visibly wet wounds with no discoloration, indicating that the male had mated on the night of capture, or dry, melanized wounds, indicating that the male had mated at least one night previous to the night of capture (see fig. 1 in Leman *et al.*, 2009). We continued to sample males from each population on a binightly basis until the population attained a ratio of approximately 1 : 1 virgin to mated males, at which time we collected approximately 200 males at random from the population over two successive nights. This protocol ensured that females were given ample opportunity to mate with the most attractive males in the population. Males were held individually in collecting vials and transported to the University of Wyoming-National Park Service Research Station, <30 km away, for processing.

Mounting of tegmina for microscopic examination and imaging

Similar to those of the true crickets (Gryllidae), *Cyphoderris* tegmina are mirror images of one another, possessing a harp, file and plectrum on each wing. However, unlike true crickets, that have maintained a right-over-left tegminal overlap for more than 150 million years (Masaki *et al.*, 1987), and katydids (Tettigoniidae), that maintain a left-over-right overlap, the majority of male *C. strepitans* sing with a preferred left- or right-handed tegminal orientation. Although some males are ambidextrous and capable of singing with either tegminal overlap (Morris & Gwynne, 1978), reversals in tegminal overlap rarely occur (G.D.O., unpubl. data). As approximately half of males have a left-over-right (or *vice versa*) tegminal orientation that is fixed for most males, *C. strepitans* could potentially be a good candidate for studying the link between development and function. The orientation of a tegmen likely has a strong bearing on its role in producing song, so

we chose to analyse selection on the top and bottom tegmina separately.

The tegminal orientation of each preserved male was noted, and the tegmina were removed using microscissors under a dissecting microscope. The ventral surface of the tegmina was photographed using a Nikon Coolpix 4500 camera and Wild M5A stereomicroscope. All mounting and photography was performed by a single person (GDO) to avoid introducing measurement error through subtle differences in procedure. The tegmina were precisely mounted in a standardized position in the centre of the field of view to minimize any possible effects of lens distortion (Zelditch *et al.*, 2012). The tegmina were mounted by placing them under a microscope slide submerged in a shallow dish of 70% ethanol. Because the ventral surface of sagebrush cricket tegmina is concave, the wings tended to crease if the microscope slide was pressed completely flat. This was resolved by placing 1-mm-thick spacers under either side of the microscope slide, which allowed the tegmina to be flattened without creasing.

Geometric morphometrics analysis

Prior to applying landmarks, all right tegmen photographs were batch reflected so that the landmarks could be applied in the same orientation. This eliminated potential human error introduced from applying landmarks to mirror images that in turn could result in a false positive for asymmetry.

Tegmen size and shape were analysed through the use of geometric morphometrics techniques (Zelditch *et al.*, 2012). Twenty-one landmarks were selected that could be reliably located on the wings of individuals from all three study populations (Fig. 1). Of these, 20 were based on homologous wing venation, making them type 1 landmarks under Bookstein's (1991) landmark classification. The 21st landmark was positioned on a vein precisely at the mid-point between landmarks 19 and 20 using custom software, making it a type 3 landmark (Bookstein, 1991). This was necessary because venation was highly variable posteromedially, making it difficult to locate a homologous point across all individuals, and having a landmark at the extreme edge of the resonator was desirable to give a measure of its shape and size. Prior to digitizing the landmarks, wing images were randomly shuffled across populations to avoid problems with autocorrelation, and all landmark digitizing was performed by one person (GDO) to minimize measurement error from subjective landmark placement decisions.

Detection of bilateral asymmetry requires very precise measurements because its subtlety can easily be obscured or biased by measurement error (Palmer & Strobeck, 1986; Palmer, 1994). To assess the repeatability of the tegmina mounting, photography and landmark digitizing protocols, the left and right tegmina of 60 randomly selected individuals were remounted and rephotographed in random order at two separate times. The resulting photographs were randomly shuffled, so that the landmark digitizer (GDO) was blind to previous landmark placements. Repeatability was assessed with a two-factor Procrustes ANOVA (using MorphoJ v. 1.05a, Klingenberg, 2011) with individual as a random effect, tegmen side as a fixed effect and the replicate wing measurements as the error term (Leamy, 1984; Klingenberg & McIntyre, 1998; Klingenberg *et al.*, 2002). A significant individual-by-tegminal side interaction would indicate that some fluctuating asymmetry is discernible in addition to the measurement error, but the corresponding *F* value must be large for measurement error to be negligible (Klingenberg, 2015).

Landmark configurations were superimposed with the generalized least-squares Procrustes method (Rohlf & Slice, 1990), which scaled them to a uniform centroid size of 1.0, transformed their centroids to (0, 0), and rotated them to minimize the Procrustes distance between landmark configurations. To reduce the number of shape variables, relative warps analysis was employed using TpsRelw v. 1.49 (Rohlf, 2010) with the bending energy metric α set to 0, which is equivalent to principal components analysis (Rohlf & Slice, 1990; Rohlf, 1993). This reduced the number of shape variables to three components, PC1, PC2 and PC3 (Fig. 2). Although these three principal components together explained only 44.9% of the variance, it was necessary to omit the other components to permit the inclusion of other variables of interest (centroid size, and shape and size asymmetry) in the selection analysis.

Centroid size for each tegmen was calculated as the square root of the sum of the squared distances between each landmark and the tegmen centroid $(\bar{x}, \bar{y})$. Size asymmetry was calculated for each individual as $|R - L|/((R + L)/2)$, in which the denominator corrects for size-dependent asymmetry (Palmer, 1994). While correcting a size asymmetry index for size dependence might seem counterintuitive, without this correction, larger males could have larger size asymmetry just by nature of their size alone. A measure of shape asymmetry was calculated as the square root of the sum of the squared deviations between corresponding landmarks of each individual's left and right tegmina (Smith *et al.*, 1997), which approximates the Procrustes distance between landmark configurations of each individual's left and right tegmina (Slice *et al.*, 1996). This measure of shape asymmetry does not take into account directional asymmetry and is, therefore, not intended to serve as a measure of fluctuating asymmetry (Klingenberg & Monteiro, 2005; Klingenberg, 2015).

Partial least-squares analysis of song and song-producing tegmina

Prior to being preserved for geometric morphometrics measurements, captured males were kept in mesh
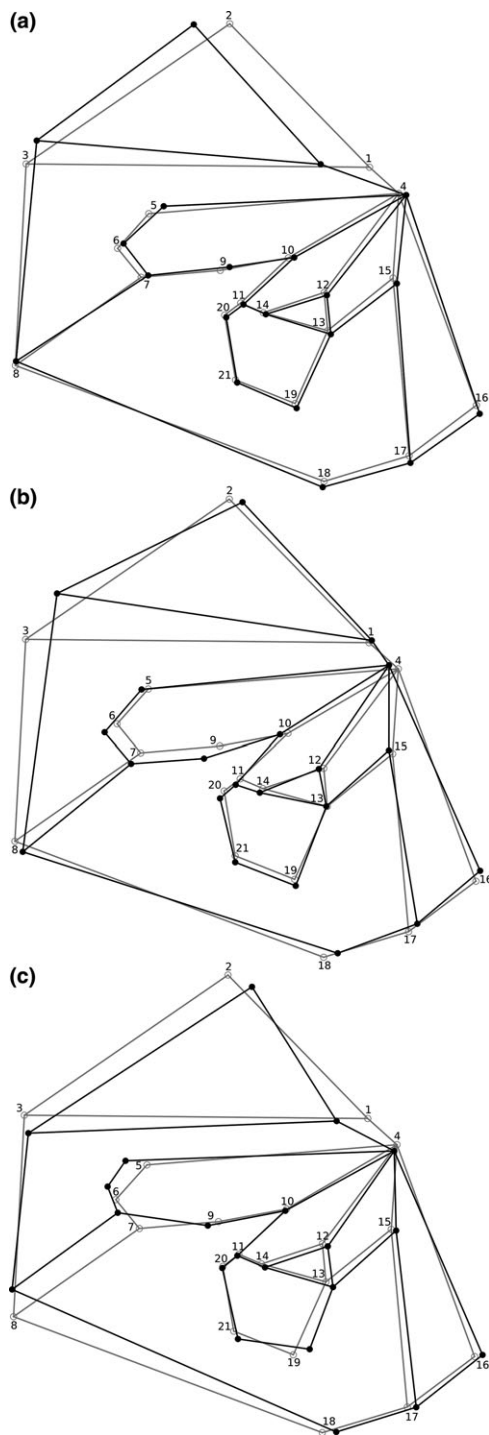
Fig. 2 Wireframes created in MorphoJ showing the shape changes of (a) PC1, (b) PC2 and (c) PC3 from relative warps analysis. Dark grey lines represent shape changes from the consensus (mean) configuration shown in light grey for comparison. The wires approximate wing venation – actual veins are more curvilinear.

recording chambers and were fed a diet of sagebrush galls and sliced apple. On subsequent evenings, males were digitally recorded to a computer for at least 2 min using a unidirectional condenser microphone (BG 4.1; Shure, Inc., Niles, IL, USA). Due to time limitations, it was only possible to digitally record a subset ($n = 389$) of the captured males ($n = 514$). Five focal song characters, pulse duration (PD), interpulse duration (IPD), train duration (TD), intertrain duration (ITD) and dominant frequency (DF), were measured using Raven Pro 1.4 (see Ower *et al.*, 2013 for more detailed procedures and a selection analysis on the song characters).

To analyse covariation between the five song characters of Ower *et al.* (2013) and the geometric morphometric shape and size characters measured in this study, we did separate two-block partial least-squares (PLS) analyses for size and shape on the top and bottom tegmina. PLS produces axes that describe the direction of covariance between two blocks of data, each with a singular value that describes the magnitude of covariance between corresponding axes (Rohlf & Corti, 2000; Zelditch *et al.*, 2012). The significance of the covariance (RV) between blocks and singular values (SVs) was assessed with permutation tests in which one of the blocks of data was shuffled, and permuted RV and SVs calculated (Zelditch *et al.*, 2012). The *P* value was calculated as the total number of permuted RV or SVs that were ≥the observed RV or SV divided by 10 000 iterations. The PLS analysis was performed with custom **R** code modified from Claude (2008) and Pitchers *et al.* (2014).

Multivariate selection analysis

The multiple-regression-based approach of Lande & Arnold (1983) was used to estimate selection on the tegmina. We assigned an absolute fitness score of 0 to virgin males and 1 to mated males, as determined by inspecting the males' hind wings for damage inflicted by hind wing feeding of females at mating (Morris, 1979; Morris *et al.*, 1989; Eggert & Sakaluk, 1994a). Following Lande & Arnold (1983), fitness scores were transformed to relative fitness scores by dividing the vector of fitness scores by its mean. The independent variables were PC1, PC2 and PC3 from relative warps analysis (Fig. 2), centroid size (CS), size asymmetry (ZA) and shape asymmetry (PA). Each of the independent variables was standardized to have a mean of 0 and standard deviation of 1 by subtracting the mean and dividing by the standard deviation (Lande & Arnold, 1983). Linear selection gradients (β) were estimated with a first-order multiple regression. Nonlinear selection gradients (γ), which represent both quadratic and correlational selection, were estimated with a second-order multiple regression. To avoid underestimating quadratic selection, the quadratic gradients were doubled (Stinchcombe *et al.*, 2008).

Correlational selection can also lead to an underestimate of quadratic selection, because it represents quadratic selection along axes not parallel to the measured traits. Canonical rotation identifies the major axes of selection and rotates the data to eliminate correlational selection (Phillips & Arnold, 1989; Blows & Brooks, 2003). Quadratic selection is strongest along the major axes identified by canonical analysis. Linear selection (θ) and quadratic selection (λ) gradients were estimated by fitting first- and second-order multiple regressions, with relative fitness as the dependent variable and the vectors of rotated data as the independent variables.

Due to non-normality of the dependent variable (fitness) and independent variables (PC1, PC2, PC3, CS, PA, ZA), randomization tests were used to assess the significance of all selection gradients (Mitchell-Olds & Shaw, 1987). The significance of linear and nonlinear sexual selection acting on the eigenvectors of γ was also assessed using randomization tests. For each iteration, the fitness scores were randomly shuffled whereas the *m*scores from the original canonical rotation were held constant (Lewis *et al.*, 2011). This procedure differs from that of Reynolds *et al.* (2010) in which a new canonical rotation is performed for each permutation and different eigenvectors are tested each time. We used this approach because we wanted to determine whether specific eigenvectors experienced more linear and nonlinear selection than would be expected by chance alone, instead of whether there was significant linear and nonlinear selection overall (Chenoweth *et al.*, 2012).

Comparing multivariate selection among populations

Linear, quadratic and correlational selection gradients among our three study populations were compared using sequential model-building procedures for response surface designs (Draper & John, 1988; Chenoweth & Blows, 2005). When traits were standardized and fitness was made relative within populations, there were no significant differences in selection gradients for top (linear: $F_{6,500} = 0.856$, $P = 0.527$; quadratic: $F_{6,488} = 1.82$, $P = 0.093$; correlational: $F_{15,458} = 0.704$, $P = 0.781$) or bottom tegmina (linear: $F_{6,500} = 0.858$, $P = 0.53$; quadratic: $F_{6,488} = 0.974$, $P = 0.442$; correlational: $F_{15,458} = 0.624$, $P = 0.856$), so the populations were pooled to achieve greater statistical power. Pooling the data across populations allowed us to meet ($n = 514$) the recommended sample size of 500–1000 needed to reliably detect nonlinear selection (Kingsolver *et al.*, 2001).

Fitness surface visualization

The fitness surfaces were visualized with thin-plate splines (Green & Silverman, 1994), using the Tps function from the *fields* package (Nychka *et al.*, 2015) of **R** (R Core Development Team, 2015).

Results

Relative warps analysis

Relative warps analysis identified three principal components that explained 20.8%, 14.6% and 9.5% of the variance, respectively, and which are graphically represented with wireframes in Fig. 2. The landmarks in the resonating region of all three principal components showed little difference in position from the consensus (mean) landmark configuration, although PC3 had a slightly broadened cell in the cubital region (surrounded by landmarks 11, 14, 13, 19, 21 and 20; Fig. 2c). The principal components mostly represented differences in shape on the anterior edge of the tegmina. PC1 described an expansion of the subcostal and costal tegmen region, which displaced the first branch in the radial vein distally (landmark 1, Fig. 2a). The medial region was broader in PC2, which displaced the terminal radial vein anteriorly (landmark 3, Fig. 2b). PC2 also had a broader frame around a region of the tegmen that is roughly the same size as the resonating region (enclosed by landmarks 4, 5, 6, 7, 9 and 10; Fig. 2b). PC3 represents tegmina that are slightly shortened distally (displacing landmarks 5, 6, 7 and 8) and anteriorly (moving landmarks 2 and 3).

Measurement error and fluctuating asymmetry

The individual-by-side interaction was highly significant for both tegmen size and tegmen shape, indicating that variance from fluctuating asymmetry was larger than measurement error (Table 1). The ANOVA also revealed directional asymmetry in the subsample of tegmina, because the main effect of side was significant (Table 1). Additionally, with asymmetry partitioned out by the ANOVA, there was significant variation in symmetric shape among individuals (Table 1).

PLS analysis between song and song-producing tegmina

There was significant covariation between the song and size of the tegmina (top tegmina: RV = 0.0435, $P = 0.0001$; bottom tegmina: RV = 0.0431, $P = 0.0001$). Singular Axis 1 was significant for both top and bottom tegmina, and both were weighted positively by centroid size and pulse duration, and negatively by dominant frequency (Table 2).

Song and tegminal shape also significantly covaried (top tegmina: RV = 0.0194, $P = 0.037$; bottom tegmina: RV = 0.0201, $P = 0.0279$). The top tegminal singular axes explained 62.2%, 36.9% and 9.1% of the covariation, respectively (Table 3A), but only the 2nd axis was

Table 1 The individual-by-side interaction was significant for both tegmen size and shape, indicating that variation from fluctuating asymmetry was significantly greater than measurement error. The main effect of side, which was represented as the top minus the bottom tegmen, was significant, indicating directional asymmetry. With asymmetry partitioned out by the two-way ANOVA, there was significant symmetric variation among individuals. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Tegmen size	DF	SS	MS	F	P
Individual	56	572113.461	10216.312	15.31	<0.0001***
Side	1	3336.747	3336.747	5.00	0.0294*
Individual*Side	56	37376.939	667.445	6.36	<0.0001***
Measurement Error	112	11752.791	104.936		

Tegmen shape	DF	SS	MS	Pillai's trace	P
Individual	2138	0.596	2.80×10^{-4}	24.17	<0.0001***
Side	38	0.00521	1.37×10^{-4}	0.83	0.0195*
Individual *Side	2138	0.268	1.26×10^{-4}	23.22	<0.0001***
Measurement Error	4256	0.140	3.28×10^{-5}		

Table 2 Partial least-squares analysis of song vs. size for both the (A) top and (B) bottom tegmina.

	Axis 1
(A) Top tegmina	
PD	0.628
IPD	0.082
TD	0.261
ITD	−0.031
DF	−0.728
CS	1.000
Singular value	0.320
P value	0.0001
(B) Bottom tegmina	
PD	0.560
IPD	0.044
TD	0.164
ITD	−0.033
DF	−0.810
CS	1.000
Singular value	0.319
P value	0.0001

Table 3 Partial least-squares analysis of song on shape for both the (A) top and (B) bottom tegmina

	Axis 1	Axis 2	Axis 3
(A) Top tegmina			
PD	−0.898	−0.096	−0.370
IPD	0.148	−0.762	0.015
TD	−0.107	0.361	0.584
ITD	0.146	−0.408	−0.021
DF	0.372	0.336	−0.722
PC1	−0.045	0.150	0.988
PC2	0.966	0.259	0.005
PC3	−0.255	0.954	−0.156
Singular value	0.222	0.171	0.027
P value	0.078	0.009	0.882
(B) Bottom tegmina			
PD	−0.752	−0.398	0.168
IPD	−0.083	0.559	0.438
TD	0.437	−0.330	−0.434
ITD	0.249	0.410	0.142
DF	0.419	−0.502	0.756
PC1	0.408	0.846	−0.344
PC2	0.911	−0.401	0.095
PC3	−0.058	−0.352	−0.934
Singular value	0.227	0.142	0.102
P value	0.063	0.080	0.018

significant. Singular Axis 2 was most strongly weighted positively by shape PC3 and negatively weighted by song interpulse duration and to a lesser extent, intertrain duration (Table 3A). For the bottom tegmina, singular axes 1–3 explained 62.7%, 25.6% and 12.7% of the covariance, respectively (Table 3B). Singular Axis 3 was significant and was negatively weighted predominantly by shape PC3 with a smaller negative contribution from train duration (Table 3B). In the positive direction, singular Axis 3 was weighted most strongly by dominant frequency with a smaller contribution from interpulse duration (Table 3B).

Multivariate selection analysis

For both top and bottom tegmina, there was significant directional selection for increased shape symmetry (Table 4). There was a significant positive correlation between PC1 and size asymmetry on the bottom tegmina, indicating the tendency for greater size asymmetry to be associated with an enlarged costal/subcostal region on the bottom tegmen (Table 4B). No other linear, quadratic or correlational gradients were significantly different from zero.

Canonical rotation of the γ matrix for the top tegmen revealed significant disruptive selection along the $\mathbf{m_1}$ eigenvector, which was most heavily influenced by a positive weighting from PC3, and to a lesser extent by positive weightings from PC1, size asymmetry and centroid size (Table 5A). No other eigenvectors had significant linear or nonlinear selection for the top tegmen (Table 5A).

Canonical analysis on the bottom tegmen identified three eigenvectors with significant nonlinear sexual selection ($\mathbf{m_1}$, $\mathbf{m_5}$ and $\mathbf{m_6}$, Table 5B). The dominant

Table 4 Linear (β) and nonlinear (γ) selection gradients for the principal components from relative warps analysis (PC1-3), centroid size (CS), size asymmetry (ZA) and shape asymmetry (PA). Randomization tests: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

		γ					
	β	PC1	PC2	PC3	CS	ZA	PA
(A) Top tegmina							
PC1	−0.020 ± 0.043	0.048 ± 0.035					
PC2	−0.016 ± 0.043	−0.024 ± 0.042	−0.038 ± 0.033				
PC3	−0.016 ± 0.042	0.057 ± 0.045	0.005 ± 0.041	0.104 ± 0.033			
CS	−0.009 ± 0.043	0.012 ± 0.045	−0.020 ± 0.045	0.030 ± 0.044	−0.025 ± 0.034		
ZA	0.017 ± 0.045	−0.010 ± 0.050	0.026 ± 0.056	0.052 ± 0.052	0.040 ± 0.055	0.023 ± 0.023	
PA	−0.120 ± 0.046**	0.004 ± 0.038	−0.019 ± 0.050	−0.018 ± 0.048	0.077 ± 0.054	0.007 ± 0.047	−0.023 ± 0.036
(B) Bottom tegmina							
PC1	−0.015 ± 0.043	0.108 ± 0.033					
PC2	0.019 ± 0.043	−0.061 ± 0.040	−0.104 ± 0.030				
PC3	0.019 ± 0.043	0.031 ± 0.042	0.018 ± 0.048	−0.010 ± 0.031			
CS	−0.020 ± 0.043	0.017 ± 0.044	0.009 ± 0.044	0.020 ± 0.047	−0.097 ± 0.033		
ZA	0.016 ± 0.046	−0.179 ± 0.058**	−0.005 ± 0.054	−0.063 ± 0.043	−0.054 ± 0.040	0.018 ± 0.021	
PA	−0.114 ± 0.046*	0.012 ± 0.044	0.022 ± 0.043	−0.003 ± 0.045	0.068 ± 0.051	−0.026 ± 0.045	0.004 ± 0.035

Table 5 Standardized linear (θ_i) and nonlinear (λ_i) selection gradients for the major axes identified by canonical analysis and their eigenvectors. Randomization tests: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

			M					
	θ	λ	PC1	PC2	PC3	CS	ZA	PA
(A) Top tegmina								
m_1	−0.023 ± 0.043	0.081 ± 0.032*	0.406	−0.014	0.809	0.250	0.341	0.048
m_2	0.051 ± 0.039	0.032 ± 0.025	0.402	0.004	0.192	−0.548	−0.456	−0.542
m_3	−0.061 ± 0.044	0.029 ± 0.029	0.589	−0.411	−0.238	0.284	−0.419	0.414
m_4	−0.018 ± 0.044	−0.013 ± 0.031	−0.564	−0.290	0.480	0.114	−0.582	0.124
m_5	0.062 ± 0.048	−0.030 ± 0.036	−0.095	−0.858	−0.075	−0.094	0.370	−0.321
m_6	−0.068 ± 0.043	−0.055 ± 0.027	−0.009	−0.103	0.124	−0.731	0.157	0.644
(B) Bottom tegmina								
m_1	−0.035 ± 0.044	0.138 ± 0.032***	0.737	−0.090	0.215	0.152	−0.604	0.120
m_2	0.088 ± 0.047	0.023 ± 0.033	0.286	−0.282	−0.136	−0.417	0.078	−0.800
m_3	0.062 ± 0.040	−0.002 ± 0.016	−0.269	0.252	0.857	−0.013	−0.130	−0.336
m_4	−0.032 ± 0.042	−0.051 ± 0.025	0.205	−0.599	0.446	−0.118	0.532	0.322
m_5	−0.012 ± 0.042	−0.065 ± 0.027*	0.222	0.497	0.045	−0.775	0.079	0.308
m_6	−0.024 ± 0.047	−0.083 ± 0.033*	−0.460	−0.493	−0.020	−0.434	−0.568	0.184

eigenvector of disruptive sexual selection (m_1) was strongly loaded by a positive weighting from PC1 and a negative weighting from size asymmetry. The dominant eigenvector of stabilizing selection (m_6) was loaded with relatively equal negative contributions from PC1, PC2, centroid size and size asymmetry (Table 5B). The opposing signs of the m_1 and m_6 eigenvalues are indicative of a multivariate saddle-shaped fitness surface, which can be visualized in the thin-plate spline (Fig. 3). Significant stabilizing selection was also detected along the m_5 eigenvector, which was most strongly influenced by a negative weighting from centroid size, and to a lesser extent by positive weightings from PC2 and shape asymmetry. Multivariate stabilizing selection can be visualized on the fitness surface of the m_5 and m_6 major axes (Fig. 4).

Discussion

PLS analysis of morphological traits and song characters

There was significant covariation between song-producing structures and song characters shared in common between the top and bottom tegmina (Table 2). Not surprisingly, centroid size and dominant frequency negatively covaried, such that as the size of the tegmen increased, the area of the harp increased and correspondingly the frequency of the song decreased (Table 2). The negative correlation between harp size and dominant frequency has been found in a large number of orthopteran taxa (Simmons, 1995; Simmons & Ritchie, 1996; Pitchers *et al.*, 2014). Pulse duration
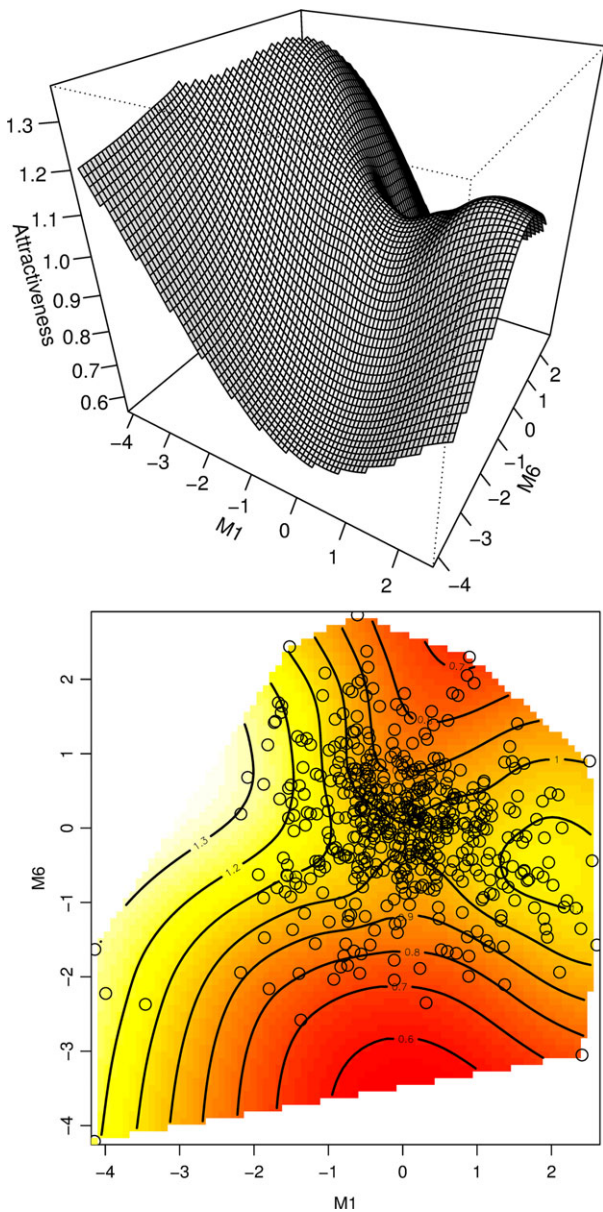
shape PC3 with negative contributions from interpulse duration and to a lesser extent intertrain duration (Table 3A). In contrast, Axis 3 of the bottom tegmina was negatively loaded by PC3 and positively loaded by dominant frequency, with smaller contributions positively from interpulse duration and negatively from train duration (Table 3B). The proximate cause of this covariation between tegminal shape and song structure remains unclear. There was a slight increase in the area of the resonator, which could correspond to the negative covariation with dominant frequency. The shape of PC3 could also alter the engagement of the file and plectrum, which could change the strike rate and dominant frequency. It is also possible that the shape of PC3 allows for more efficient wing closure cycles, reducing both interpulse duration and intertrain duration for the top tegmen, and interpulse duration and train duration for the bottom.

Influence of shape and size symmetry on male mating success

The song-producing tegmina of male sagebrush crickets captured from wild populations were under significant linear and nonlinear sexual selection. Both top and bottom tegmina were under directional selection for increased tegminal shape symmetry (Table 4). Despite directional selection for shape symmetry, selection seemed to be operating mostly on the bottom tegmen, with only one significant selection gradient (m_1) found for the top tegmen (Tables 4 and 5). This is a curious result, because directional selection for shape symmetry might be expected to place both tegmina under similar selective pressures. However, the bottom tegmen could conceivably play a more important role in sound production, and could, therefore, be subject to more intense sexual selection if the subtegminal airspace acts as an acoustic resonator or amplifier (Bailey & Broughton, 1970; Stephen & Hartley, 1995; Sakaluk & Ivy, 1999; but see Prestwich *et al.*, 2000). The top tegmen is at least partially obstructed from interacting with the subtegminal airspace by the bottom tegmen during tegminal closure, and consequently, it may have less influence on song production and, therefore, be under weaker sexual selection.

Sexual selection on size asymmetry was more complex, because it incorporated elements of both disruptive (m_1) and stabilizing (m_6) selection. These two axes formed a saddle-shaped fitness surface rising towards two peaks, the highest of which occurred at low values of m_1 and intermediate values of m_6, which corresponds with increased size asymmetry. If selection continued to operate in this direction, it would likely promote a consistent left- or right-handed tegminal orientation with potential for increased specialization of the song-producing morphological features of the top and bottom tegmina. However, selection for
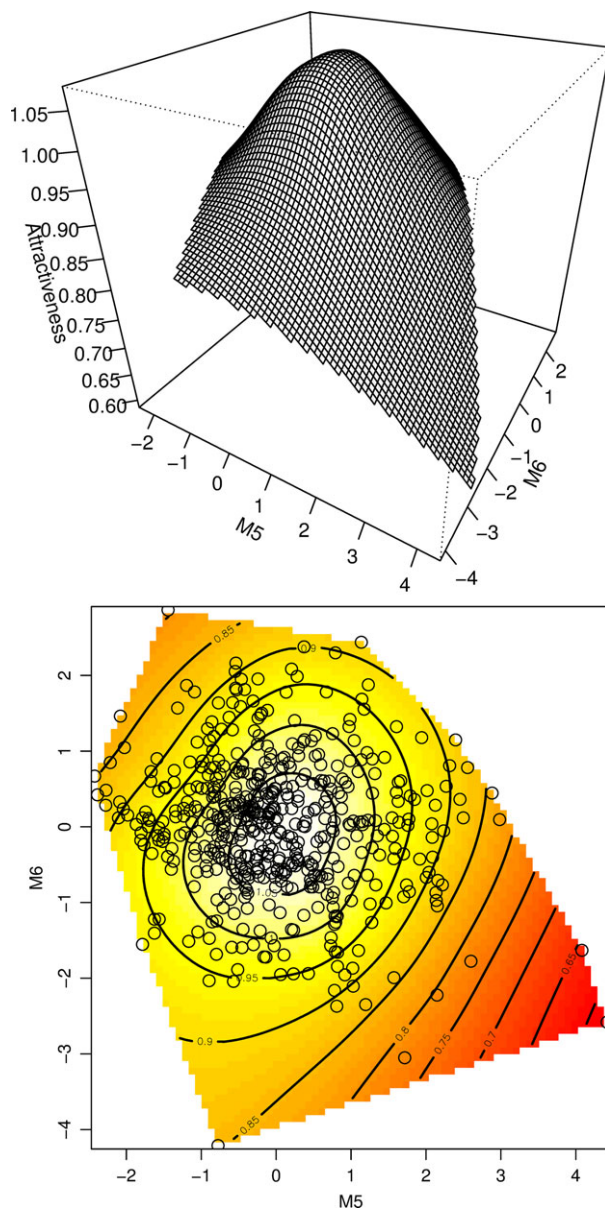

Fig. 3 Thin-plate spline of the $m_1 \times m_6$ major axes identified by canonical analysis with each of the bottom tegmina data points plotted on the accompanying contour plot.

positively covaried with centroid size in both top and bottom tegmina (Table 2). As the size of the tegmen increased, the length of the file also likely increased, which presumably lengthens the time the plectrum remains in contact with the file, resulting in longer pulse durations.

There was significant covariation between song and tegminal shape, but the direction and magnitudes of covariation differed for the top and bottom tegmina. Axis 2 of the top tegmina was loaded positively by

Fig. 4 Thin-plate spline of the $m_5 \times m_6$ major axes identified by canonical analysis with each of the bottom tegmina data points plotted on the accompanying contour plot.

handedness was opposed by the lower adaptive peak at high values of m_1 and intermediate values of m_6, which corresponded with increased size symmetry (Fig. 3). This, in addition to the directional selection for increased shape symmetry, is likely to prevent handedness and tegminal specialization in sagebrush cricket populations until such time as female preferences change.

Males with symmetric tegmina could benefit from being able to sing equally well with either tegminal orientation. The role that muscles play in tegminal stridulation might be expected to differ depending upon the tegminal overlap. The ability to switch tegminal orientation once muscles have become fatigued could allow symmetric males to sing more relative to fatigued asymmetrical males that cannot sing as effectively with both tegminal orientations. Alternatively, the increased mating success of males with symmetric tegmina could be due to their greater intrinsic attractiveness to females. In the subsample of tegmina used to estimate measurement error, the two-way ANOVA interaction between individual and side was significant for both tegminal size and shape, which indicates that these traits could be influenced by fluctuating asymmetry (Table 1). Theoretically, males of higher genetic quality might be better able to buffer against developmental noise (Møller & Thornhill, 1998), which could, in turn, make fluctuating asymmetry of tegmina (or more specifically, any aspects of male song it influences) a condition-dependent indicator of quality. However, the result of significant fluctuating asymmetry should be interpreted with caution, because for both traits (tegminal size and shape), the main effect of side was also significant, indicative of directional asymmetry (Table 1). Because fluctuating asymmetry results from the random process of developmental noise, right minus left samples for traits exhibiting fluctuating asymmetry are expected to be normally distributed with a mean of zero (Palmer & Strobeck, 1986). Directional asymmetry indicates that the right minus left sample mean is skewed and it often is due to a heritable departure from symmetry. The majority of tettigoniids and gryllids maintain a consistent tegminal overlap that has resulted in directional asymmetry (Masaki *et al.*, 1987; Eggert & Sakaluk, 1994b), which is likely a heritable specialization of their tegmina for producing more efficient song. Because the tegminal overlap within *C. strepitans* populations is approximately 50% left-over-right and 50% right-over-left, it seems unlikely that the directional asymmetry is heritable, but this requires further investigation.

Selection against noise cancellation

Tegmina act as dipolar sound sources: as the tegminal resonators vibrate upwards, air is compressed above the wing and rarefied below the wing, so that the song produced on either side of the wing is out of phase with one another (Forrest, 1982; Bennet-Clark, 1990). Crickets sing with their tegmina raised at a 45° angle, and if the anterior edges of their wings are $> \lambda/3$, they can act as a baffle to prevent noise cancellation along the edge of their tegmina where the antiphase songs would otherwise destructively interfere (Forrest, 1982; Bennet-Clark, 1990). In *C. strepitans*, an effective baffle would be larger than 8.8 mm, whereas their baffle is actually only 3–5 mm. However, the anterior edge of their wing could still act as a baffle because it loosely seals with the

thorax. In the top three shape components identified by relative warps analysis, most of the variation in shape was concentrated on the anterior edge of the tegmina (Fig. 2). An expanded coastal/subcostal region was one of the phenotypes favoured by disruptive selection on the bottom m_1 eigenvector (Table 5B, Fig. 3), an adaptation that may form a more effective seal with the thorax to prevent noise cancellation.

PC1 represented an enlarged costa–subcosta region, which could be advantageous for preventing noise cancellation. However, the resulting costa–subcosta flap is not reinforced with radial venation and may be more susceptible to creasing. It is not uncommon to find males with their baffle crumpled between their tegmen and thorax (G.D.O. and S.K.S, pers. obs.). As long as the enlarged baffle does not crease, it might offer an advantage of additional noise cancelling ability, but if it creases, the tegmen often becomes inflamed with a fluid hemolymph bubble that could decrease the quality of males' song. This might partially explain the disruptive selection found on PC1 (bottom m_1, Table 5B).

Multivariate stabilizing selection on song frequency

Significant stabilizing selection was found on the bottom tegminal m_5 and m_6 eigenvectors (Table 5B). The intermediate maximum for these two fitness surfaces was confined entirely within the sampled space (Fig. 4), which meets the criteria for designating it as multivariate stabilizing selection (Mitchell-Olds & Shaw, 1987). The m_5 major axis was most strongly influenced by a negative loading from centroid size and to a lesser extent a positive loading from PC2, whereas the m_6 major axis was loaded with relatively equal contributions from size asymmetry, centroid size, PC2 and PC1 (Table 5B). Both centroid size and PC3 covaried with dominant frequency (Tables 2 and 3B), which was under strong stabilizing selection (Ower *et al.*, 2013). Multivariate stabilizing sexual selection on dominant frequency has also been reported in Australian black field crickets, *Teleogryllus commodus* (Brooks *et al.*, 2005) and Hawaiian sword-tail crickets, *Laupala cerasina* (Oh & Shaw, 2013).

Relative strength of selection

In a recent meta-analysis of standardized selection gradients, Kingsolver *et al.* (2001) found quadratic selection to be relatively weak, suggesting that stabilizing selection is uncommon in nature. Disruptive selection found on the bottom m_1 eigenvector ($\lambda = 0.138 \pm 0.032$, Table 5B) was comparatively stronger than the median of $|\gamma| = 0.10$ found by Kingsolver *et al.* (2001) for quadratic selection, but the dominant eigenvector of stabilizing selection (m_6) was substantially weaker ($\lambda = -0.083 \pm 0.037$, Table 5B).

Although we only found relatively weak stabilizing selection, it may be difficult to detect strong stabilizing selection because it inevitably reduces variability in heritable traits. For example, among the three shape components identified by relative warp analysis, there was very little variation in the position of landmarks located around the presumed resonating region (Fig. 2), which suggests that the resonator could be under very intense stabilizing selection to maintain an optimized song frequency for successful mate attraction. According to the clockwork cricket model (Elliott & Koch, 1985; Koch *et al.*, 1988; Bennet-Clark, 1990), the resonator regulates the escapement mechanism between the plectrum and the file, which in turn determines the tooth strike rate and dominant frequency. Dominant frequency in *C. strepitans* has been found to be under very strong stabilizing selection ($\gamma = -0.359 \pm 0.074$, $P < 0.001$, see Ower *et al.*, 2013).

As song plays an essential role in mate attraction, it seems likely that the resonator is under extreme stabilizing selection, which ultimately could have eliminated much of the variation among resonator landmarks. However, extreme stabilizing selection would be hard to detect because there were no males with defective resonators in our sample. Although the difficulty in capturing silent males could have biased our sample, it seems unlikely that silent males are very common, because males have never been observed adopting a satellite mating strategy (S.K.S. and G.D.O., pers. obs.), in which silent males aggregate around singing males to opportunistically intercept females attracted to calling males (Van Rhijn, 1973; Cade, 1975, 1979; Zuk *et al.*, 2006). The reduced variation associated with resonator landmarks may reflect the ghost of strong stabilizing selection past, but, paradoxically, makes it more difficult to detect such selection empirically. Alternatively, even strong selection on a specific song characteristic like frequency may not necessarily lead to changes in sound-producing structures of the wing if other morphological alterations exist that can bring about the same change in song structure (Klingenberg *et al.*, 2010).

In conclusion, we show that wing morphology has an important influence on song structure in *C. strepitans* and is subject to significant linear and nonlinear sexual selection through female precopulatory mate choice. Although numerous studies have documented the strength and form of sexual selection targeting song characteristics in crickets (Brooks *et al.*, 2005; Bentsen *et al.*, 2006; Oh & Shaw, 2013; Ower *et al.*, 2013), relatively few studies have examined the pattern of selection on wing morphology (Pitchers *et al.*, 2014), especially in wild populations. As song production is both a product of behaviour and the underlying morphology of the wing, understanding the link between morphology and behaviour is likely to have important implications for how song structure evolves. It has been argued that the ability of behavioural traits to respond almost immediately to environmental stimuli means

that they have the potential to evolve more rapidly than morphological traits (West-Eberhard, 1989). However, the range of behaviours available to an individual will ultimately be constrained by its morphology (West-Eberhard, 1989). Our finding that wing morphology is a major determinant of song structure, coupled with the fact that there is much stronger sexual selection acting on song structure than wing morphology, suggests that the latter may constrain the evolution of the former. Further work is needed, however, to test this directly.

Acknowledgments

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