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Tests of the role of sexual selection in genitalic divergence with mutiple hybrid clines

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Abstract

Determining whether sexually selected characters affect reproductive isolation is key to understanding the significance of such selectively driven divergence in speciation. Divergence in the shape of male genitalia is ubiquitous in many insect taxa and can have important fitness consequences for males within a species. Yet, it is unclear whether this selectively divergent character affects gene flow among species. We test this hypothesis using explicit predictions about the clinal transition in genitalic shape across a hybrid zone between the grasshopper subspecies *Barytettix humphreysii humphreysii* and *B. h. cochisei*. Slight morphological differences in genitalic shape across their ranges raised the possibility that local processes might give misleading interpretations about sexual selection's potential contribution to reproductive isolation. Therefore, we examined multiple transects to explore the effectiveness of genitalic divergence as a barrier to gene flow on a background of potentially different extrinsic (*e.g.*, environmental conditions) or intrinsic (*e.g.*, genetic makeup) factors. If sexual selection is the predominant factor maintaining the hybrid zone, the shape of the clines between transects is expected to be concordant, thereby overriding potential dispersal differences associated with varying environmental and ecological conditions or variation in population densities that would cause cline shape to vary among localities. Furthermore, if selection against hybrids with intermediate genitalic morphologies does indeed represent a strong barrier to gene flow, the morphological transition between the adjacent subspecies is predicted to be quite steep. These predictions were supported by the geometric morphometric analyses, suggesting that genitalic divergence plays a direct role in reproductive isolation and that sexual selection plays a predominant role in the maintenance of the subspecies differences. Thus, the results of our study provide compelling evidence that postmating-prezygotic interactions can indeed limit gene flow, and consequently may play an important role in speciation. Using this framework, we suggest future studies that can address a number of remaining questions about the nature of selection, and ultimately, how sexual selection is operating in these taxa.

Key words

cryptic female choice, geometric morphometrics, introgression, postmating-prezygotic interactions, reproductive isolation

Introduction

The shape of character clines, as well as the degree of concordance and coincidence of different characters across hybrid zones, can provide valuable insights into the nature of barriers to gene exchange (Barton & Hewitt 1985, 1989, Harrison 1990). Yet, sexually-selected traits are surprisingly rarely studied in hybrid zones (Butlin & Neems 1994), despite the potential of this context to reveal whether evolutionary divergence of such characters has important consequences for reproductive isolation, as predicted by theoretical work (*e.g.*, Lande 1981, 1982, Schluter & Price 1993, Gavrilets 2003).

In a hybrid zone the steepness of character transitions between species can inform about the strength of selective forces separating the taxa (Barton & Gale 1993). The frequently observed concordance of multiple characters across hybrid zones has emphasized the role of selective pressures arising from genetic associations between alleles, or breakdown of coadapted-gene complexes, where the movement of alleles is impeded by unfavorable, or inviable, genetic backgrounds (Barton & Hewitt 1985, 1989, Harrison 1990). With such intrinsic barriers to gene flow, character transitions between the adjacent species arise as a result of the strong linkage disequilibrium generated by dispersal into the zone – that is, a steep cline reflects the strong net selective pressure of the whole genome. However, like the effects of disruptive-ecological selection, an effective barrier to gene flow can be generated if hybrids have a strong mating disadvantage (*e.g.*, Stratton & Uetz 1986, Vamosi & Schluter 1999, Cruz *et al.* 2001, Naisbit *et al.* 2001). Such factors may play a much more important role than the traditional criteria for reproductive isolation – hybrid inviability and sterility (Coyne & Orr 1989) – which are frequently absent in recently diverged taxa suggesting that such postzygotic effects are the by-products of genetic divergence rather than a driving force in speciation.

In this study we examine two clines in a morphological character – the shape of the male genitalia – and we compare cline shape between transects to examine the role sexual selection might play in the evolution of this character and in reproductively isolating the grasshopper subspecies, *Barytettix humphreysii humphreysii* and *B. h. cochisei* (Orthoptera: Acrididae: Melanoplinae). While differences in genitalic morphology in insects is widely believed to reflect divergence driven by sexual selection (Eberhard 1996, Eberhard & Cordero 2003, House & Simmons 2003, Hosken & Stockley 2004), what, if any, consequence such divergence has for speciation is not clear (*e.g.*, Oneal & Knowles 2012). By studying divergence in the genitalic character across a hybrid zone, we gain insight into how strong selection might be (*i.e.*, would genitalic divergence pose a significant barrier via a mechanism of strong selection against hybrid individuals with intermediate genitalic phenotypes) and whether it predominates (as measured by similar cline shapes across independent transects).

The hybrid zone between *B. h. humphreysii* and *B. h. cochisei* extends from southern Arizona to northern Sonora, Mexico (Cohn & Cantrall 1974). These grasshoppers are flightless and do not sing or exhibit obvious courtship displays or ecological differences. They are morphologically quite similar – the primary character distinguishing them is differences in the male genitalia, specifically

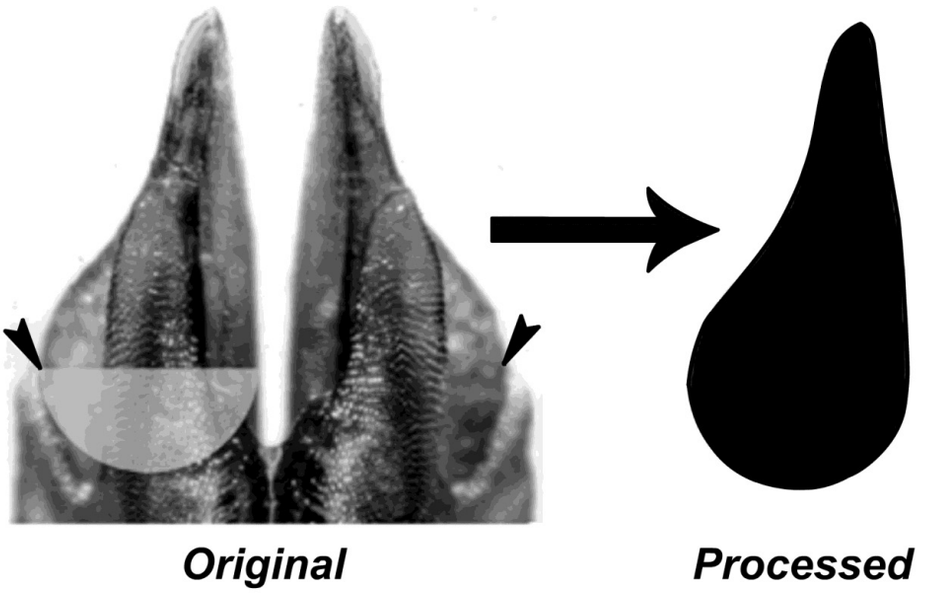

Fig. 1. Photographs of male genitalia (aedeagus) were converted to a silhouette for collecting morphometric outlines; a consistent landmark (as indicated by arrows) was used to orient the images and identify the lower boundary of the structure, which was defined as a semi-circle as shown in the grayed out region of the aedeagus.

the aedeagus, or intromittent organ. Slight morphological differences in the genitalia across the range of each subspecies (Cohn & Cantrall 1974) raises the possibility that interpretations about general hybrid zone maintenance may be misled by local processes (Butlin 1994, Shapiro 1998), including different extrinsic (*e.g.*, environmental conditions) or intrinsic (*e.g.*, genetic makeup) factors (Hairston *et al.* 1992, Buerkle & Rieseberg 2001, Morgan-Richards & Wallis 2003, Smadja *et al.* 2004). However, by studying multiple transects across the hybrid zone, we can explore whether selection is the primary factor maintaining the zone, and consequently the effectiveness of genitalic divergence as a barrier to gene flow. If sexual selection is the predominant factor maintaining the hybrid zone, we predict that the shape of the clines between transects to be concordant, thereby overriding potential dispersal differences associated with varying environmental and ecological conditions or variation in population densities that would cause cline shape to vary among localities (*e.g.*, Szymura & Barton 1991, Butlin *et al.* 1991). Furthermore, if selection against hybrids with intermediate genitalic morphologies does indeed represent a strong barrier to gene flow, then we predict the morphological transition between the adjacent subspecies to be quite steep. Results from morphometric analyses are indeed consistent with these predictions, suggesting that genitalic divergence plays a direct role in reproductive isolation and that sexual selection plays a predominant role in the maintenance of the subspecies differences, although our work does not identify the mechanism by which sexual selection might act (see Eberhard 1996). However, there are a number of remaining questions about the nature of this selection (*i.e.*, we cannot rule out selection on other correlated characters, or that the steepness of the cline is unique to genitalic characters, for example). Nevertheless, using these findings as a framework, which is informative without invoking a specific mechanism about how sexual selection might operate, we discuss additional studies that could potentially reveal how sexual selection is operating in these taxa.

Material and methods

Samples.—Adult male specimens were collected from two transects across the hybrid zone between grasshopper populations of the subspecies *Barytettix h. humphreysii* and *B. h. cochisei* (Orthoptera: Acrididae: Melanoplinae) in southern Arizona (Appendix 1). 106 and 230 museum specimens (Museum of Zoology, Insect Division, University of Michigan) were examined from two transects of 18.2 km and 22.6 km in length, which we refer to as the "Bisbee Junction" and "Don Luis" transects, respectively; specimens are deposited in the collection with genitalia extruded for digitization. The transects are approximately 4.2 km apart and oriented in an east-westerly direction (see Fig. 4 for orientation of specimens across hybrid zone). Additional collections of non-hybrid individuals were also made from two sites about 50 km from the transects (Appendix 1). Patterns of morphological variation were quantified using morphometric analysis of outlines of the genitalic structures (*e.g.*, Arnqvist 1998). In the context of clinal variation, morphological shape can be analyzed in a manner similar to the method of plotting allele frequency as a function of position along a hybrid transect to characterize cline shape across a hybrid zone. Note that while we focus on a specific trait, this does not preclude that other characters, including those of the genitalic complex, might be subject to selection (see Marquez & Knowles 2007). Whether such characters do or do not show strong clinal variation is reserved for future investigations and we highlight what such findings would imply for our results (see Discussion).

Collection of morphometric outlines.—Photographs of the extruded male genitalia taken from a dorsal perspective were digitized (16-bit grayscale). Before the shape of specimen could be quantified, each image was processed according to a standardized procedure to produce silhouettes that tracked the margin of the right ventral valve of the aedeagus (Fig. 1). To produce a closed outline so that the shape of the genitalia could be described using an elliptical Fourier analysis (Ferson *et al.* 1985), the outlines were closed with a half circle at the junction between the aedeagal sheath and the aedeagus itself – a clearly identifiable and consistent landmark (see also Marquez & Knowles 2007). The program SHAPE (Iwata & Ukai 2002) was used to conduct the elliptic Fourier analysis. Fourier analyses were made invariant of size, position and rotation (*i.e.*, the first three harmonics were not included in the statistical analyses, Rohlf & Archie 1984) using the "Chc2Nef" component of SHAPE. For each specimen, 11 harmonics were included in analyses, where each harmonic yields 4 variables or Fourier coefficients that were analyzed in a principal-components analysis (Liu *et al.* 1996, Rohlf & Archie 1984) using the "PrinComp" module of SHAPE;

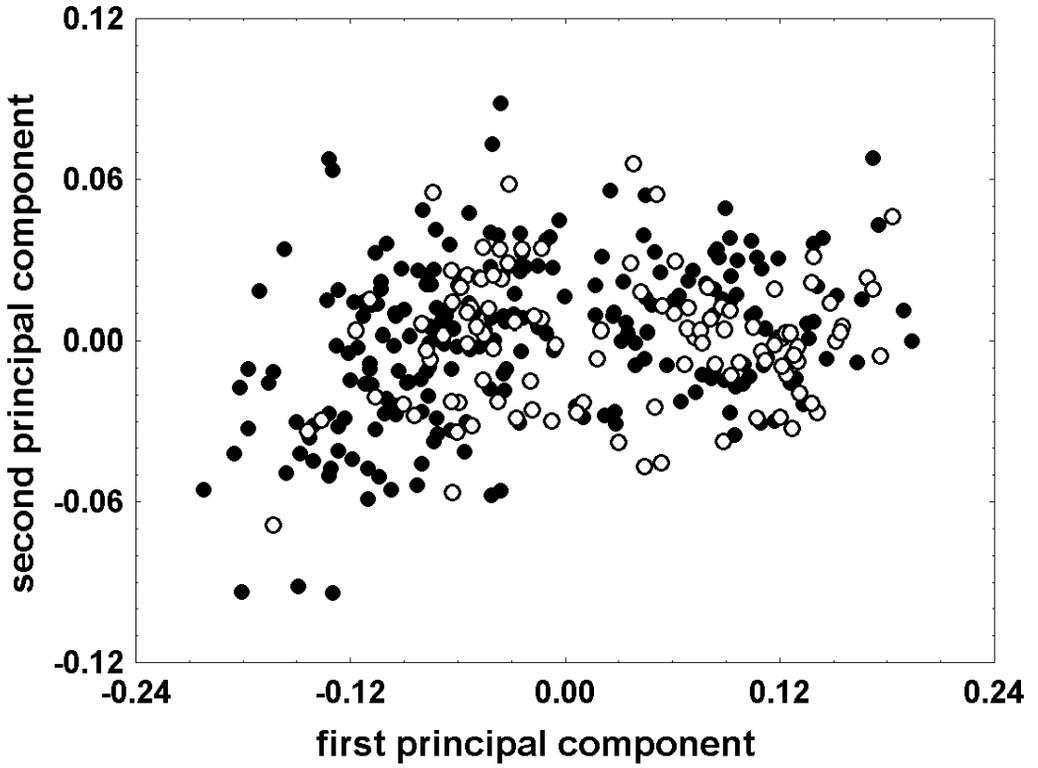

Fig. 2. Second *vs* first principal components of the pooled outline data from the "Bisbee junction" (open circles) and "Don Luis" (closed circles) transects.

ordination of the specimens indicated that the first five principal components, each of which accounted for 1% or more of the total variation of each transect, captured 96% of the variance among the specimens (scores: PC1 = 82%, PC2 = 7%, PC3 = 4%, PC4 = 2%, and PC5 = 1%). Further analyses were therefore based on the first five principal components of shape variation of each sample. We note that elliptical Fourier analysis is effective for characterizing shape variation (see Marquez & Knowles 2007), especially when landmarks are difficult to identify (although see Oneal & Knowles 2012 for an alternative based on semi-landmarks).

Statistical Analysis.—To quantify shape differences among individuals collected along a transect, a measure of morphological distance between each specimen and a parental species was used and calculated separately for each transect. Specifically, the distance *D* was computed as the Euclidean distance between each specimen and the parental (*i.e.*, non-hybrid) species at the eastern end of its respective transect (*i.e.*, *B. h. cochisei* that is geographically adjacent to the study transect), based on principal component scores, according to the equation:

$$D = [(PC1_{parental} - PC1_{hybrid})^2 + (PC2_{parental} - PC2_{hybrid})^2 + \ldots + (PCn_{parental} - PCn_{hybrid})^2]^{1\backslash 2},$$

for the *n* selected principal components. Finally, *D* was normalized so that all distances were in the interval [0,1], thus providing a scale-free representation of each specimen in a common multivariate-morphometric space, allowing the direct comparison of the two transects in terms of morphological distance.

Regression of *D* against corresponding geographical distances between each specimen and the western end of the transect, normalized to the [0, 1] interval, was used to characterize the geographic distribution of morphological change along the transects. Prior to this regression, a logit transformation was applied to morphological distances (Barton & Gale 1993). To compare the clines of the two transects, slopes from the linear portion of the regression of normalized *D* against normalized geographical distances (*e.g.*, Szymura & Barton 1986), were first calculated. Then each transect was bootstrapped (N = 10,000) to obtain standard errors and 95% confidence intervals for each of the transect slopes. To test for significant differences between slopes, data from the two transects were pooled and 10,000 bootstrap subsamples with the same size as the original samples were obtained from the pooled dataset and their slopes calculated as described above. The absolute difference between slopes was computed for each pair of bootstrap subsamples to produce an empirical distribution of the expected difference between slopes under the null hypothesis of clinal homogeneity across transects. Observed absolute slope difference was then compared to this distribution. An observed value larger than 95% of the bootstrapped differences would reject the hypothesis that both transects belong to clines with equal slopes.

Additionally, principal component scores were plotted against each other for each transect to identify trends in the relationships of the principal components among localities. Analyses were carried out in Matlab v. 6.5 (Mathworks 2003). Compiled versions of the software employed are freely available upon request.

Results

Principal-components analysis indicates that not only is the variation among specimens captured by the elliptical-Fourier analysis, but also that the character transition between *B. h. humphreysii* and *B. h. cochisei* can be precisely described in this quantitative framework. The shape of the male genitalia of the parental subspecies was similar between transects; average non-normalized morphological distance (*D*) was 0.241 (± 0.019) and 0.210 (± 0.007) for *B. h. humphreysii*, and 0.057 (± 0.005) and 0.042 (± 0.004) for *B. h. cochisei*, from the Don Luis and Bisbee transects, respectively. Scores of hybrid individuals from the two transects widely overlap in the first two principal components of the pooled data, which account for 84.5%

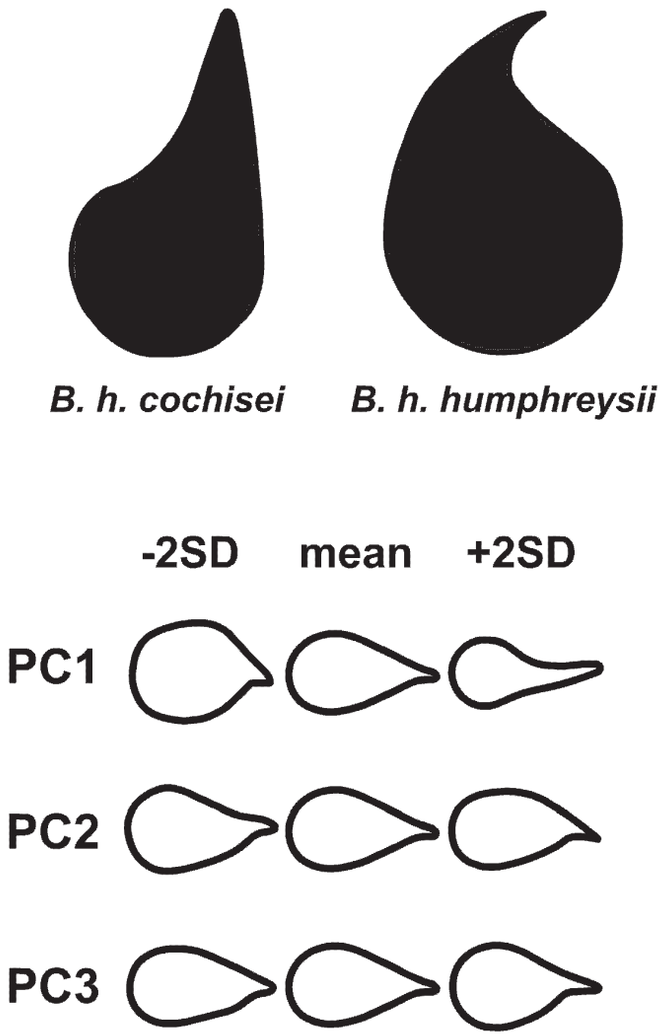

Fig. 3. Qualitative representations of the species-specific differences in the shape of the male genitalia as described by Cohn & Cantrall (1974) (upper panel) compared to the graphical depiction of the three major components of the outline shape variance across subspecies, as derived from a principal component analysis of elliptical Fourier coefficients (lower panel). Principal components (PC) 1 and 2 account for 84.5% and 7.0% of the variance, respectively. Illustrations are shown for the overall mean shape and ±2 standard deviations (SD) from the mean along each PC.

and 7.0% of total variance, respectively (Fig. 2), indicating that specimens from both transects share a large portion of their shape variation. Specific differences between the male genitalia of *B. h. humphreysii* and *B. h. cochisei*, as well as the shape of the hybrid individuals, quantified by the elliptical Fourier analysis (Fig. 3) matched the qualitative descriptions of Cohn & Cantrall (1974).

Analysis of the shape of the male genitalia across the hybrid zones revealed that both transects were characterized by sigmoid curves with a steep clinal transition between the two adjacent taxa (Fig. 4). The slope at the Bisbee Junction transect was 9.03 (95% confidence interval: 8.4635-9.4522) and did not differ significantly from the slope of the Don Luis transect, which was 9.37 (95% confidence interval: 8.8874-9.8979); the observed difference of 0.35 between the slopes is not large enough to reject the hypothesis that the transects' slopes were equal (P = 0.75) based on 10,000 bootstrap subsamples. Although we have a fairly large number of observations to calculate the slope (with more than 100 specimens per transect), it is possible that additional samples might provide power for detecting a difference in slope that was not detected here.

Discussion

The steep character transition between *B. h. humphreysii* and *B. h. cochisei* is indicative of strong selection acting on the hybrids. Moreover, the smooth cline shape and concordance between the transects confirms that this selection dominates the dynamics of the hybrid zone, overriding the effects of other potential factors such as environmental and ecological differences or varying population densities between transects (Barton & Hewitt 1985). Because the steep cline was observed in the male genitalia, a character widely postulated to be under sexual selection (Eberhard 1996), the results have a number of intriguing implications.

Even without specifying the mechanisms of selection, not only are the results consistent with the hypothesized direct role of male-genitalic shape differences in reproductive isolation, but the study also provides evidence for a predominant role of sexual selection in the maintenance of species differences (because we are studying a character involved in reproductive interactions, sexual rather than natural selection is the focus of our conclusions; see Eberhard 1994). As such, the work complements other work that aims to understand the drivers of genitalic divergence and the role of genitalic divergence in speciation. However, a number of unanswered questions about the nature of selection remain. The framework provided by these museum specimens highlights that future investigations could elucidate longstanding issues regarding the genetic basis of reproductive isolation, and ultimately how sexual selection may be involved in the isolation of species (Jiggins *et al.* 1996).

Role of genitalic divergence in reproductive isolation.—Cline widths vary in response to the levels of selection a particular character experiences (Barton & Gale 1993). If the shape of the male genitalia was not related to hybrid fitness, then a broad cline is expected. Moreover, lack of significant difference in slopes of character transition across transects implies that selection against hybrids is independent of environmental or ecological conditions (Buerkle & Rieseberg 2001). Otherwise, introgression patterns would differ between transects (*e.g.*, Butlin *et al.* 1991, Szymura & Barton 1991).

The implications of this steep clinal transition in the shape of the male genitalia across the hybrid zone are particularly intriguing. Unlike other sexually selected traits, genitalic characters are not subject to other selective influences so they can provide a clear window into sexual selection without being confounded by other factors. This contrasts with cases where sexual selection affecting divergence and reproductive isolation is mediated by ecological divergence – that is, the selection on the mating characters directly affecting reproductive isolation as a pleiotropic effect of ecological change (*e.g.*, in *Heliconius* butterflies, Naisbit *et al.* 2001; in *Littorina* snails, Cruz *et al.* 2001; in wolf spiders, Stratton & Uetz 1986). For example, divergent habitat preferences in sticklebacks bias encounter rates between hybrids and parental species producing the conditions for sexual selection to contribute to reproductive isolation because mating success is tied to habitat use (Hatfield & Schluter 1996, Vamosi & Schluter 1999).

Evidence of sexual selection acting on hybrids of *B. h. humphreysii* and *B. h. cochisei* is also uniquely significant; sexual selection in this case is operating on a postmating-prezygotic character. In studies

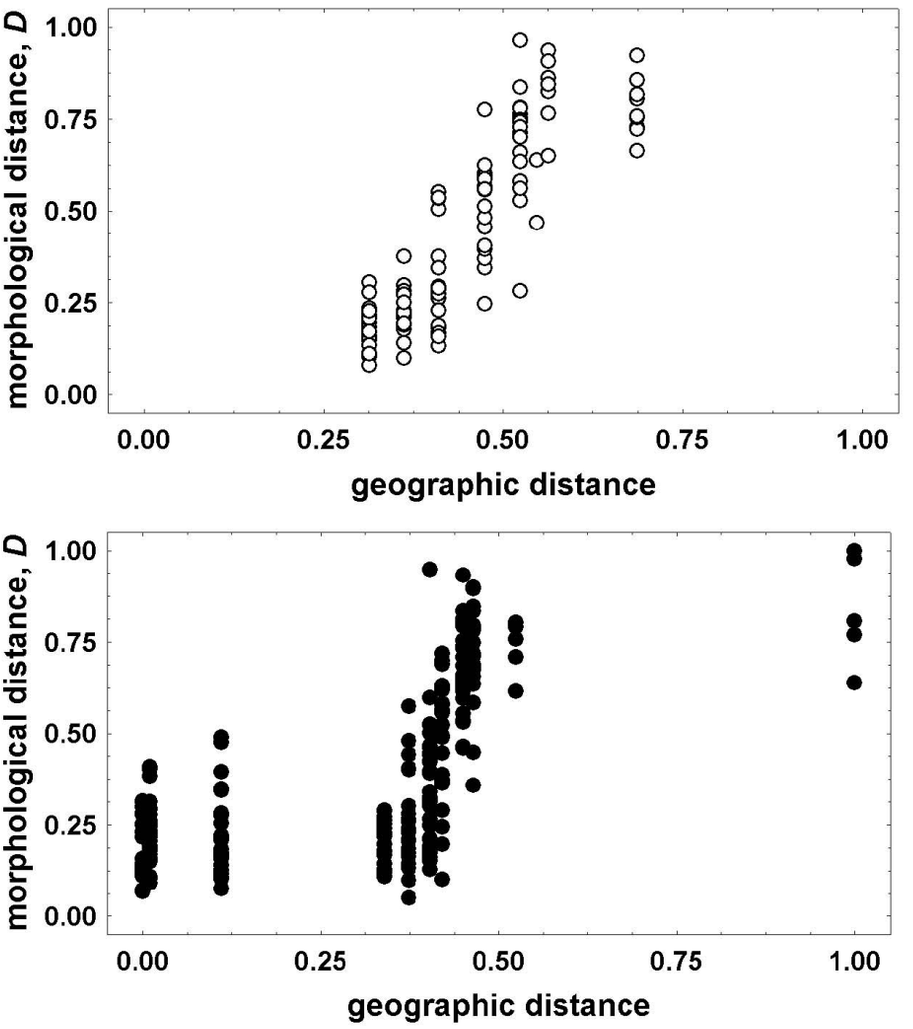

Fig. 4. Clinal transition in male genitalic shape between *Barytettix humphreysii cochisei* (east end of geographic cline; left side of the plot) and *B. h. humphreysii* (west end of cline; right side of the plot) for the "Bisbee junction" (upper panel, open circles) and "Don Luis" (lower panel, closed circles) transects. Shape differences are presented as normalized morphological (Euclidean) distances (*D*) between each specimen and a reference specimen at the eastern end of the transects, based on the first five principal components of each transect (*i.e.*, the genitalia are more *B. h. cochisei* like as the morphological distance approaches zero). Corresponding geographic distances have also been normalized (see Appendix 1 for geographic coordinates).

that have shown reproductive isolation is directly affected by sexual selection, gene flow between the hybridizing taxa is impeded by premating characters (*e.g.*, Brumfield *et al.* 2001, McDonald *et al.* 2001, Bailey *et al.* 2003, Bronson *et al.* 2003, Smadja *et al.* 2004). Attention to postmating-prezygotic characters, including male genitalia (*e.g.*, Arnqvist 1998, Eberhard & Cordero 2003), and their potential role in species divergence has increased recently, with the primary focus on antagonistic interactions between the sexes (*e.g.*, Wolfner 2002, Civetta & Clark 2000, Knowles & Markow 2001, Swanson *et al.* 2001). Yet, whether selection acting on postmating-prezygotic traits is strong enough to produce reproductive isolation remains largely unknown (Knowles *et al.* 2004a, b). The results of our study provide compelling evidence that postmating-prezygotic interactions can indeed limit gene flow, and consequently may play an important role in speciation.

The nature of selection.—How selection is acting on the male genitalia is an interesting question that requires further study. Despite the conspicuous and fairly ubiquitous pattern of genitalic divergence among insect taxa (Eberhard 1996), surprisingly little is known about how selection might operate (*e.g.*, Oneal & Knowles 2015). Similar to sexual selection on premating characters, reduced fitness of hybrids could reflect female choice (*e.g.*, Saetre *et al.* 1997, Babik *et al.* 2003, Smadja *et al.* 2004), as well as male-male interactions (*e.g.*, Brumfield *et al.* 2001, McDonald *et al.* 2001, Bronson *et al.* 2003). For example, the assortative fertilization associated with differences in the shape of the male genitalia could result from the reduced success of sperm storage of the hybrid males, possibly reflecting lowered stimulation of females to store sperm or misplacements of spermatophores by the males in the female genital tract (Eberhard 1996). Understanding how selection operates against hybrids could also provide insights into the driving forces underlying the divergence of male-genitalic characters.

While our results are consistent with models of speciation that posit a direct role for divergent sexual selection (*e.g.*, Lande 1981, 1982, Schluter & Price 1993, Gavrilets 2003), important aspects about the nature of this selection still need to be addressed. A steep cline may not necessarily reflect the strength of selection acting on any single character alone (Gavrilets & Cruzan 1998). Selection on other characters that are in linkage disequilibrium with the character under study can contribute to the shape of the cline (Szymura & Barton 1991, Barton & Gale 1993, Barton & Baird 1996, Bridle *et al.* 2001). Consequently, to determine sexual selection's specific contribution to reproductive isolation (*i.e.*, the strength of selection operating against hybrids based on differences in male-genitalic morphology), the potential contribution of the net selective pressure on the genome needs to be considered (*e.g.*, Dasmahapatra *et al.* 2002). This study provides a framework in which the clinal transitions of other characters, and specifically those not under sexual selection, can be studied to distinguish these alternative interpretations

about the nature of selection. If strong selection against hybrids of intermediate genitalic morphology constitutes the primary factor affecting reproductive isolation, then coincident clines of multiple characters are not expected since the fitness associated with each character across the hybrid zone is likely to differ (Barton & Hewitt 1985, 1989, Harrison 1990, Butlin *et al.* 1991). A steeper cline is also predicted for the male genitalia compared to non-sexually selected traits reflecting the different intensities of selection impeding the movement of alleles between the adjacent parental taxa (*e.g.*, Szymura & Barton 1991, Harrison & Bogdanowicz 1997, Brumfield *et al.* 2001, Babik *et al.* 2003). Further study of this hybrid zone will not only reveal the extent to which genitalic differences, and therefore sexual selection, contribute to reproductive isolation, but will also contribute interesting insights into longstanding questions regarding the evolution of reproductive isolation, and specifically the role of selectively driven divergence (Wu 2001, Gray & Cade 2000, Vines *et al.* 2003).

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Appendix 1. Specific locality information for specimens (Cochise Co., AZ).

specimen number	locality label	locality description	latitude	longitude
95-99	Bisbee5	1.5 mi. E. Bisbee Jct..	31.35	-109.86
100-118	Don1	12 mi. W. Don Luis (E. side of river) 0.6 mi. E. Palominas	31.40	-110.11
119-147	Don2	11.7 mi. W. Don Luis (W. side of river), 0.9 E. Palominas	31.40	-110.11
148-170	Don3	8.7 mi. W. Don Luis, 3.9 mi. E. Palominas	31.40	-110.06
171-193	Don4	1.9 mi. W. Don Luis, 10.7 mi. E. Palominas	31.40	-109.94
194-218	Don5	0.9 mi. W. Don Luis, 11.7 mi. E. Palominas	31.40	-109.93
219-245	Don6	1.9 mi. NE. Don Luis	31.42	-109.89
246-268	Don7	0.7 mi. NE. Don Luis	31.40	-109.90
304-324	Don8	4.3 mi. SW. Don Luis	31.40	-109.96
269-302	Don0	Don Luis (just N. Naco Rd. Jct.)	31.40	-109.91
524-532	Bisbee1	6.3 mi. E. Bisbee (High Lonesome Rd.)	31.34	-109.77
515-516	Bisbee2	4.8 mi. E. Bisbee Jct.	31.34	-109.80
517-523	Bisbee3	2.6 mi. E. Bisbee Jct.	31.34	-109.84
513-514	Bisbee4	2.2 mi. E. Bisbee Jct.	31.35	-109.84
349-366	Bisbee5	1.5 mi. E. Bisbee Jct.	31.35	-109.86
367-383	Bisbee6	2.0 mi. W. Bisbee Jct.	31.35	-109.91
384-398	Bisbee7	3.5 mi. W. Bisbee Jct.	31.35	-109.94
471-491	Bisbee8	3.3 mi. N. Bisbee Jct.	31.39	-109.88
492-512	Bisbee0	Bisbee Jct.	31.35	-109.88
533-537	Doug1	4.4 mi. W. Douglas	31.34	-109.61
543-546	Doug2	27.2 mi. E. Douglas	31.34	-109.08