The role of trait reversal in evolutionary diversification: A test using song loss in wild crickets

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The mechanisms underlying rapid macroevolution are controversial. One largely untested hypothesis that could inform this debate is that evolutionary reversals might release variation in vestigial traits, which then facilitate subsequent diversification. We evaluated this idea by testing key predictions about vestigial traits arising from sexual trait reversal in wild field crickets. In Hawaiian *Teleogryllus oceanicus*, the recent genetic loss of sound producing and amplifying structures on male wings eliminates their acoustic signals. Silence protects these 'flatwing' males from an acoustically orienting parasitoid and appears to have evolved independently more than once. Here we report that flatwing males show enhanced variation in vestigial resonator morphology under varied genetic backgrounds. Using laser Doppler vibrometry, we found that these vestigial sound-producing wing features resonate at highly variable acoustic frequencies well outside the normal range for this species. These results satisfy two important criteria for a mechanism driving rapid evolutionary diversification: sexual signal loss was accompanied by a release of vestigial morphological variants, and these could facilitate the rapid evolution of novel signal values. Widespread secondary trait losses have been inferred from fossil and phylogenetic evidence across numerous taxa, and our results suggest that such reversals could play a role in shaping historical patterns of diversification.

acoustic communication | diversification | evolutionary rate | sexual signal | trait loss

Introduction

One of the most contentious debates to have arisen in evolutionary biology centres on the rate at which diversification proceeds (1). In particular, the mechanisms responsible for driving rapid bursts of macroevolution remain unresolved despite decades of study (2-4). Here we evaluate an overlooked mechanism that could cause rapid diversification: the release of cryptic variation following secondary loss of a mate recognition signal, which exposes a widened range of vestigial signalling structures to the action of selection. If novel or variable signal values subsequently evolve, they could play a key role in speciation.

Secondary trait losses are common (5) and in several studies have been suggested to precede diversification, for example in stick insects and in plethodontid salamanders (6, 7). Loci involved in functional traits important for diversification, such as spectral tuning of the visual system in cichlids, are known to be evolutionarily labile (8), and when such traits are lost, functionless vestigial structures or behaviours are left behind which could facilitate the re-evolution of new functions or trait values (2, 9-11). Sexual traits involved in mate recognition systems are particularly prone to reversal (12). Their reduction under pressure from countervailing natural selection is a central prediction of sexual selection theory (13, 14), and widespread sexual trait losses have been inferred phylogenetically (12). Acoustic signals play a prominent role in speciation, communication and many animal behaviours. Here we tested how their evolutionary reversal might predispose populations to diversification using a field cricket system in which the sexually-selected male acoustic signal has been recently, and abruptly, lost from multiple wild populations (15, 16).

Male crickets produce calls by stridulating: they rub modified forewings together to generate mechanical vibrations (**Fig. 1*A***). An individual producing an advertisement, courtship, or aggressive song will draw a thickened ridge of tissue (the scraper) on one wing across a corrugated vein (the file) on the opposing wing. In many species, the resulting vibrations are amplified by resonating membranes formed from modified wing cells. When coupled with wing motor behaviours that repeat this movement in succession, the pulse rate, pattern, and carrier frequency of chirps can convey information about mate location, identity, quality, or aggressiveness. We studied the widely-distributed Austro-Pacific cricket *Teleogryllus oceanicus*. Hawaiian populations of this species overlap with an acoustically-orienting endoparasitoid fly (*Ormia ochracea*) which responds to male songs and infests them with destructive larvae. A mutation(s) showing Mendelian segregation on the X chromosome appeared in a population on the island of Kauai approximately two decades ago, and it silences males by erasing or dramatically reducing the stridulatory apparatus and sound resonators on their forewings (15). Females have undifferentiated wings and do not sing. Males carrying the *flatwing* genotype develop wings resembling those of females, so are referred to as 'flatwing males' (**Fig. 1*B***). Flatwing males are protected against parasitoid infestation (15), and the flatwing phenotype rapidly spread and now appears on more than one Hawaiian island (16, 17). In all cases investigated, *flatwing* segregates as a single-locus trait on the X (16, 18), but the degree to which affected male wings are feminised varies noticeably between islands, and several lines of evidence suggest that independent *flatwing* mutations have arisen convergently (16). On Kauai, flatwing male wings tend to be almost completely feminised and lack identifiable resonators characteristic of grylline species, whereas flatwing males from the neighbouring island of

Significance

Bursts of rapid evolutionary diversification are widely observed, but their underlying causes are controversial. We tested whether secondary loss of sexual traits could play a role in rapid diversification, by releasing variation in vestigial signalling structures which then facilitates the rapid evolution of novel signal values. We found evidence to support such an evolutionary model in the field cricket *Teleogryllus oceanicus*, which has recently lost the ability to sing. Trait reversals are widespread, and may play an underappreciated role in determining the pattern and rate of macroevolutionary change.

Reserved for Publication Footnotes

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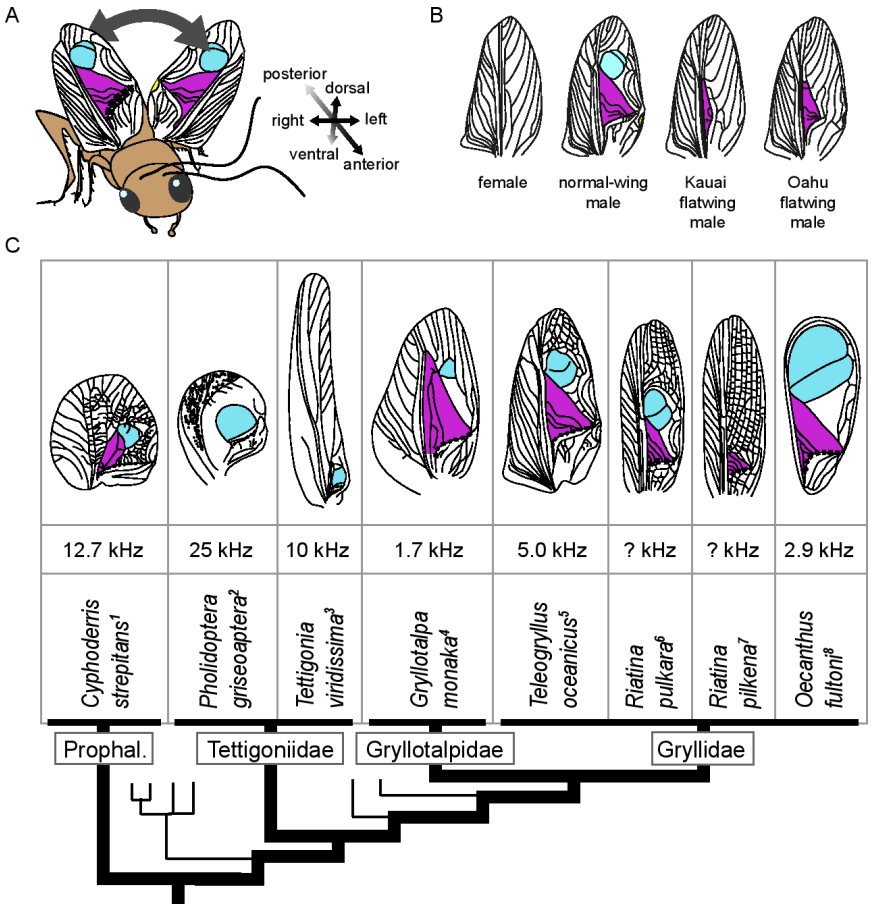

Fig. 1. Diversity of wing venation and acoustic signals in crickets and katydids. (*A*) Forewing stridulation in a normal-wing *Teleogryllus oceanicus* male (anterior dorsal view with cricket's directions indicated), with mirror, harp and scraper highlighted in turquoise, purple, and yellow, respectively. The dashed black line indicates the stridulatory file present on the ventral surface of the upper (right) wing, and the solid gray line indicates the direction of forewing movements during singing.(*B*) Representative Hawaiian *T. oceanicus* forewings, showing differences in the degree to which Kauai and Oahu flatwings are feminised. Resonators and corresponding vestigial structures are highlighted as above. Adapted from (16). (*C*) Male forewings from exemplar orthopteran species (not to scale). Sampled clades are labelled on the phylogeny (Proph. = Prophalangopsidae), and approximate carrier frequencies reported in the literature ("?" if unknown) are shown above species names. Shaded regions of the wing visually illustrate taxonomic variation in sound resonator morphology across this group. In this simplified phylogeny adapted from (30), branch lengths do not scale to divergence time. Thin branches represent groups that do not sing or are not represented here. Sources from which figures were drawn and carrier frequencies obtained: [1][figure: (S. K. Sakaluk); Cf: (68)], [2][figure: (69); Cf: (70)], [3][figure: (28); Cf: (70)], [4][figure: (33); Cf: (33)], [5][figure: (S. Pascoal); Cf: (33)] [6][figure: (33)] [7][figure: (33)], [8][figure: (71); Cf: (72)].

Oahu retain approximately one third to one half of their harp and often possess a scraper (**Fig. 1*B***) (16).

Research examining acoustic signal function and diversity in ensiferan singing insects (crickets and katydids) has mostly focused on the behavioural components of song, i.e. the pattern of sound pulses produced during wing movement (19, 20). However, a major source of variation in acoustic signals is their carrier frequency, which is increasingly recognised as an important signal feature distinguishing closely-related species (21, 22). Frequency is primarily determined by the morphology of sound resonating structures (23, 24), and in some species can be varied by mechanically shifting between different resonant modes (25-27). Resonator morphology most likely evolved from the modification and specialisation of structural wing venation (28-30), subsequently elaborated and diversified through coevolution with receivers (31). **Fig. 1*C*** illustrates the diversity of wing resonators across taxa: morphological variation over macroevolutionary timescales shows suggestive parallels to the morphological variation observed among the wings of flatwing *T. oceanicus* males from different Hawaiian islands. We took advantage of the recent, repeated loss of signalling in *T. oceanicus* to examine whether secondary signal loss can generate variation in morphological signal components that recapitulates this deeper macroevolutionary variation. Our study addressed two objectives focused on the early stages of such a process. The apparently different underlying genetic causes of the loss-of-function flatwing phenotype, coupled with the incomplete erasure of resonating structures in some populations, allowed us first to identify and measure the variability of vestigial structures remaining on flatwing males' wings. We specifically evaluated whether background genetics could lead to expression of decanalized variation following trait loss (32). Our results indicated that trait loss is associated with the predicted increase in variation of vestigial acoustic resonators, so we next used laser Doppler vibrometry (LDV) to characterise acoustic resonances of these new wing areas and assess their potential to influence the evolution of new signal values.

Results

Despite possessing wings that lack functional sound-producing structures, flatwing males still produce the motor patterns associated with song: they elevate their forewings and silently move them in a precise pattern characteristic of male sexual advertisement song (11). The persistence of what appear to be partially-formed resonating structures (hereafter referred to as 'vestigial resonators') on flatwing males' forewings, coupled with the persistence of wing motor behaviour associated with song, is consistent with the idea that trait loss could potentiate the evolution of novel signal variants. The only requirement for the evolutionary origin of a new or re-evolved signal is invasion of a genotype that re-engages the residual file and scraper mechanism currently expressed in a reduced, functionless state in some flatwing males (**Fig. 1*B***). Developmental constraints could influence signal evolution following such a reversal, but the existence of sister *Teleogryllus* species with different male carrier frequencies (21) suggests that such constraints would not necessarily cause re-evolution of the exact original configuration of resonating structures. The existence of wide variation in song carrier frequency and wing venation suggests that such constraints are either weak, or have been broken repeatedly during the evolutionary history of many ensiferan taxa (33).

To test whether variability in flatwing vestigial resonator morphology has been released following loss of male-typical wing

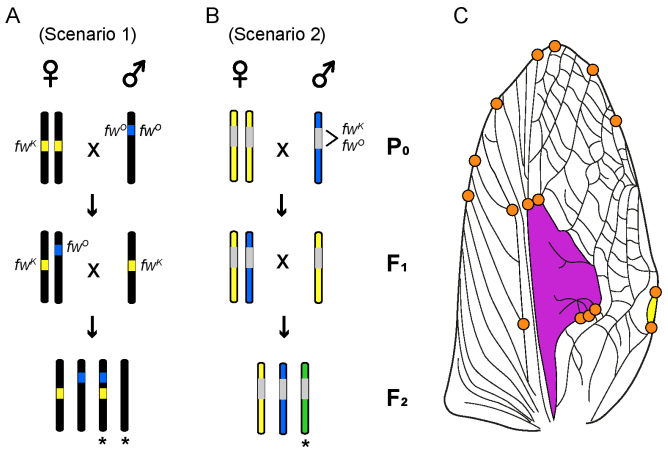

Fig. 2. Cross design for complementation test and geometric morphometrics. For each test family, a parental *flatwing* male from Oahu (fw^O) was crossed with a homozygous *flatwing*-carrying female from Kauai (fw^K). Recombination could potentially occur in the resulting heterozygous F_1 females. A full-sib mating was then performed to produce F_2 offspring. F_2 males were expected to represent either parental or recombinant (asterisks) genotypes, assessed using landmark-based geometric morphometrics. The same crossing scheme was followed using fw^K sires and fw^K dams as a control.Two genetic scenarios are illustrated. (*A*) If fw^K and fw^O are sufficiently physically distant on the X (hypothetically illustrated with yellow and blue colour, respectively), rare recombinant males with a restored normal-wing phenotype might be detected in the F_2 generation. The phenotype of the other recombinant progeny (fw^K/fw^O) is unknown. (*B*) If fw^K and fw^O are distinct loci but sufficiently tightly linked (represented by the gray region), recombination between *flatwing* loci is unlikely to occur. In this case, genomic background effects (indicated by the yellow and blue shaded chromosomes) might be expected to predominate, and recombinant F_2 offspring would represent a mix of recombinant backgrounds (green shaded chromosome). Under this scenario, variation in flatwing morphology is predicted to reflect the release of cryptic genetic variation that epistatically interacts with wing venation loci, despite not producing obvious recombinant phenotypes. The two scenarios are not mutually exclusive, but make distinct predictions about whether normal-wing recombinants or release of cryptic variation should predominate patterns of variation among F_2 flatwing males. (*C*) Exemplar flatwing male forewing showing the 16 landmarks used in this study (orange dots). Colour scheme for vestigial resonator follows **Fig. 1**.

structures, we performed a series of crosses with crickets known to carry *flatwing* genotypes derived from either Kauai or Oahu. We tested whether we could recover rare normal-wing recombinants in a complementation-like assay, whether the genetic background of different populations affected expression of vestigial wing structures, and whether family-level variation was detectable for flatwing morphology. The crossing design allowed us to examine two genetic scenarios. Under the first, background effects are minimal and variation following trait loss is mainly caused by the expression of independent loss-of-function *flatwing* mutations (**Fig. 2*A***). Under the second, background effects play a more significant role in generating variability among flatwing crickets (**Fig. 2*B***).

Sex determination is female homogametic (XX/XO) in *T. oceanicus*, and in both populations used, the flatwing phenotype segregates as a single-locus trait on the X chromosome (16). Using pure-breeding Kauai lines and Oahu flatwing males, two generations of crosses were performed to introduce *flatwing*-carrying X chromosomes from Kauai and Oahu populations (fw^K and fw^O, respectively) into the same female to allow potential recombination on the X ("test" condition). Simultaneously, the same crossing design using only Kauai genotypes was undertaken separately ("control" condition). We performed visual assessments for the presence or absence of scrapers and mirrors, and used landmark-based geometric morphometrics and multivariate

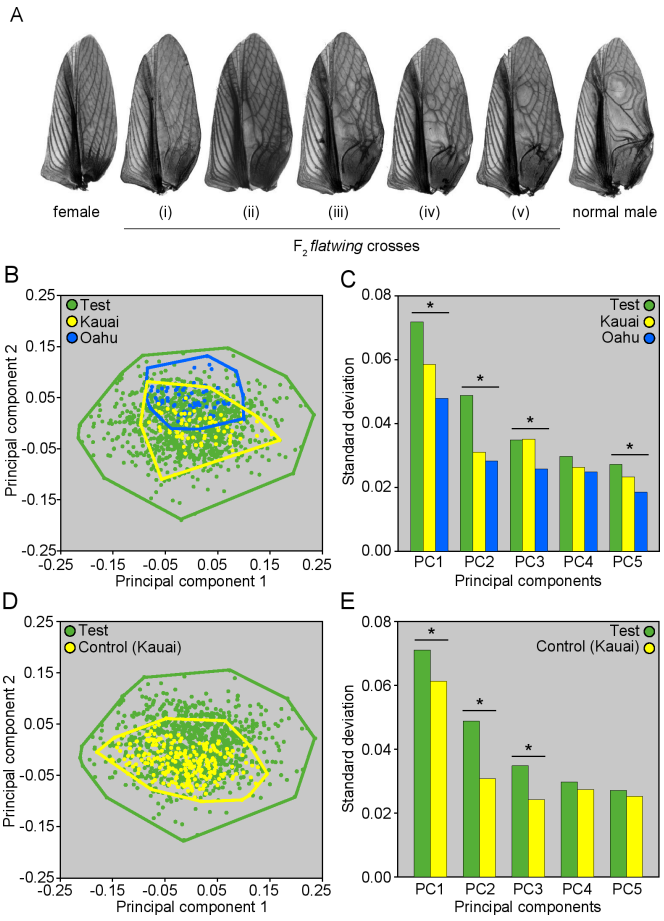

Fig. 3. . Flatwing *T. oceanicus* wing venation. (*A*) Variable feminisation of vestigial sound-producing structures. Selected wings (i) through (v) illustrate the range of variation in F_2 individuals, from no scraper, no mirror and minimal harp area in (i), to prominent scraper, ca. ½ sized harp, and almost complete mirror in (v). Female and normal male wings are shown for comparison. CorelDraw v.12 was used to adjust contrast and remove background. (*B*) Principal components describing flatwing venation among the two island subtypes (data from (16)) and F_2 test wings. Polygons indicate the data range for each group. (*C*) Variability of wing venation, contrasting groups in *B*. (*D*) Principal components describing test and control F_2 flatwings; the former are the same samples as in *B*. Polygons indicate the data range for each group. (*E*) Variability of wing venation, contrasting groups in *D*. Asterisks indicate that group variation differed significantly (see **Table 1** for statistics).

analyses to quantify variation in wing venation among the test and control crickets (**Fig. 2*C***).

A total of 1,067 F_2 test crickets and 245 F_2 Kauai control crickets were scored. Visual classification of scraper and mirror presence revealed that 63.7% (n = 680) of test crickets possessed a residual scraper, 1.2% (n = 13) possessed a definable, partial mirror, and a further 4.5 % (n = 48) possessed incomplete mirror-like structures (e.g. enlarged but not completely enclosed wing cells). Examples of the range of flatwing phenotypes recovered are provided in **Fig. 3*A***. Among Kauai control crickets, 32.2% (n = 79) possessed a vestigial scraper, and one (0.4%) possessed a partial mirror. We validated our visual scoring system by assigning a randomly-selected subset of 100 wings to a sample-blind scorer, and proportions carrying scrapers were consistent with the original dataset for both control crickets (Fisher Exact Test: p = 1.00) and test crickets (Chi-square test with Yates' correction: χ^2 = 0.30, p = 0.584). Across all 100 validation samples, concordance between scorers was 96% for the presence or absence of scrapers, and 100% for mirrors.

Table 1.

Table 1. Principal components describing variation in forewing venation among groups of flatwing males (*A*) F_2 complementation test, Kauai, Oahu (*B*) F_2 complementation test, F_2 Kauai controls). Explained variance and eigenvalues are given for the leading 5 components of PCAs, and statistics are from Levene's tests for homogeneity of variances performed separately for each component. Significance is indicated by bold text.

	Principal component	PCA variance (%)	PCA eigenvalue	F[1] (homogeneity)	P (homogeneity)
A. Test vs. Oahu and Kauai flatwings	PC1	42	0.00491	7.76	**<0.001**
	PC2	20	0.00235	25.38	**<0.001**
	PC3	11	0.00133	4.59	**0.010**
	PC4	7	0.00086	2.71	0.067
	PC5	6	0.00074	10.22	**<0.001**
B. Test vs. Kauai control flatwings	PC1	43	0.00494	6.98	**0.008**
	PC2	19	0.00224	60.73	**<0.001**
	PC3	9	0.00112	40.53	**<0.001**
	PC4	8	0.00087	1.87	0.172
	PC5	7	0.00076	1.97	0.161

[1] degrees of freedom (num,den) are (2,1212) and (1,1310) for (A) and (B), respectively.

We recovered no obvious recombinant, i.e. normal-wing, phenotypes, though among the test crickets, the 13 males possessing partial mirrors were classified as nearly-normal. These nearly-normal forewings possessed partial to complete scrapers, reduced but clearly distinguishable mirror membranes bounded by thickened venation, and a distinctive harp that extended significantly across the wing, but did not fully reach the distal wing margin as occurs in normal-wing males. An example is given in **Fig. 3*A***, and photographs of all 13 are provided in *SI Appendix,* Fig. S1. This suggests that any mutation(s) independently controlling the expression of flatwing phenotypes may be too closely linked on the X chromosome, or contained within a non-recombining region, to allow double recombinants to arise readily. However, the surprising level of morphological variation recovered from these crosses suggests that background or modifier effects are superimposed upon the effects of *flatwing* itself.

Consistent with the idea that trait loss leads to the expression of uncanalised or cryptic variation, the forewings of F_{2-}flatwing males from the complementation test showed greater variation than those previously reported from Kauai and Oahu laboratory populations and measured using the same methods by the same scorer (S.P.) (16). The range of phenotypic variation among F_{-2} males fully encompassed that of both island types (**Fig. 3*B***). Forewing morphology differed among the three groups of flatwing males (MANOVA: Wilks' $\lambda = 0.786$, $F_{10,2418} = 30.95$, $p < 0.001$), and pairwise *post-hoc* tests between groups for each principal component describing landmark-based wing morphology (with eigenvalue > 1) revealed that this was largely driven by Oahu, which was involved in 12 out of 16 significant *post-hoc* comparisons (*SI Appendix*, Table S2). Crucially, the amount of variation in wing venation differed among groups, and was largest for test crickets for 4 of the 5 principal components analysed (**Fig. 3*C*** and **Table 1**).

To exclude the possibility that minor variation in the genetic composition of lab stocks or methodology between this and the previous study could have influenced the differences we observed between test flatwings and Kauai and Oahu flatwings, we performed a separate analysis of Kauai control flatwings which were simultaneously produced using the same crossing protocol, contrasted with the same set of test flatwings. This analysis revealed patterns of variation in flatwing venation consistent with the previous result. A separate principal components analysis (PCA) showed that phenotypic variation of test crickets' wing venation exceeded that of the controls, again fully encompassing it (**Fig. 3*D***). Flatwing venation was significantly different between the two groups (MANOVA: Wilks' $\lambda = 0.849$, $F_{5,1306} = 46.59$, $p < 0.001$). Also as before, morphological variation was greater for test than control crickets in all 5 principal components analysed, and significantly so for the first three (**Fig. 3*E*** and **Table 1**). As a final analysis of the potential for background effects to interact with the *flatwing* genotype, we examined family-level variation among the test crickets. Significant family-level variation in wing shape among F_2 flatwing males in the complementation test provided confirmation of our interpretation of genetic background effects superimposed on different *flatwing* genotypes (MANOVA: Wilks' $\lambda = 0.763$, $F_{20,3510} = 14.89$, $p < 0.001$) (*SI Appendix*, Fig. S2).

Pre-existing morphological traits that permit the evolution of new signal variants are difficult to identify and characterise, and reconstructing the sequence of evolutionary events that coupled behavioural and morphological components of signals in ancestral lineages represents a major challenge. Characterising ancestral behaviours is in many cases impossible (though see (10)), and often the critical morphological structures involved in sound production are comprised of soft tissue that does not persist in the fossil record (though see (34)). Most work on signal macroevolution has therefore relied on comparative analyses across extant taxa (35-38). An alternative approach is to predict and characterise signal values on the basis of relevant morphological features, before the signalling traits themselves evolve. To test whether vestigial harp and mirror structures that we identified on the surface of flatwing crickets are a) capable of producing acoustic resonances, b) likely to produce a more varied range of signal values than the typical 4-5 kHz carrier frequency produced by this species, and c) to characterise these acoustic resonances, we performed a second experiment using micro-scanning LDV (**Fig. 4*A***). Adult flatwing male crickets were selected from three pure-breeding Kauai flatwing lines and four pure-breeding Oahu lines that had been subsequently produced (see Methods). For comparison, we also selected adult normal-wing males from two lines from each island. The objective was to achieve a breadth of sampling across different, naturally-occurring flatwing backgrounds, rather than a design balanced across morph types. After a pilot experiment to assess the feasibility of the approach, we successfully recorded data from 16 male cricket wings.

Analysis of wing resonances revealed acoustic resonators on flatwing males' forewings, and **Fig. 4** provides examples. Our

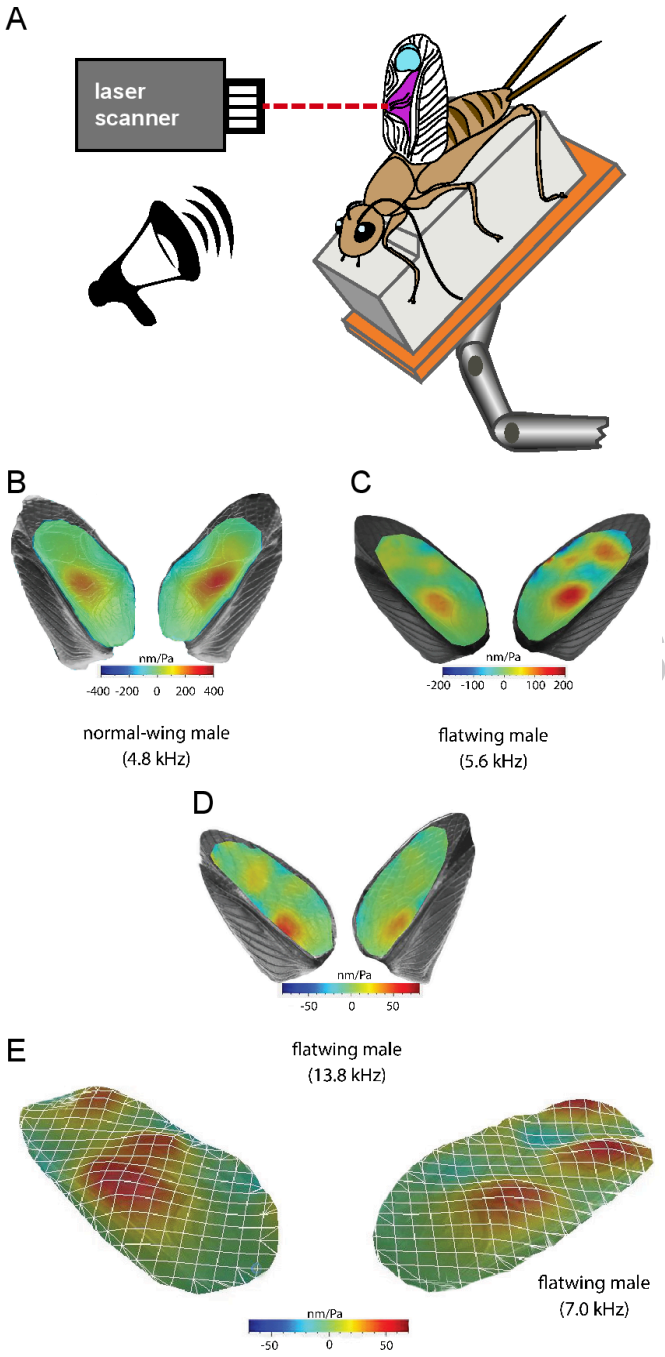

Fig. 4. Vibration maps of male forewings obtained using LDV. (*A*) Diagram of experimental set-up, showing lateral view of a normal-wing male cricket, with mirror and harp of the extended left hindwing highlighted in turquoise and purple, respectively. During scans, a male is positioned in front of the laser, which is aimed perpendicular to the plane of the wings (red line). The laser scans pre-defined grid points while a broadband signal is played back. (*B-D*) Illustrative vibration maps (displacement / sound pressure) showing resonant wing areas at the frequencies indicated (not necessarily peak resonances, see **Table 2**) for: (*B*) Normal-wing male with typical resonant frequency at 4.8 kHz. (*C*) Oahu flatwing male with vestigial harp producing a resonance at 5.6 kHz. (*D*) Kauai flatwing male with a resonance at 13.8 kHz. (*E*) Enlarged grid format of data collected from an Oahu flatwing male's left forewing, with a pronounced acoustic resonance at 7.0 kHz centred over the vestigial harp area.

main analysis focused on the harp area of the wing as it is a key determinant of the carrier frequency of male song in ensiferan insects (22). **Table 2** reports the peak resonance of the harp (or vestigial harp) for each measured individual. We confirmed that normal-wing males produced acoustic resonances characteristic of this species between ca. 4.5-5.5 kHz. In contrast, flatwing males produced a large range of peak resonant frequencies that almost exclusively did not overlap with normal-wing males (**Fig. 5**). Peak resonance frequencies differed between Kauai and Oahu flatwing crickets, with a higher average peak frequency in the former (left forewings: $t = 7.10$, $p < 0.001$; right forewings: $t = 2.88$, $P = 0.016$) (**Figs. 5*A*, *B***). Animations of wing resonances for exemplar flatwing and normal-wing males are provided in the *SI Appendix* (Movies S1-S3).

Discussion

Sexual signals play a major role in speciation (20, 39, 40), so any factor that increases the likelihood of new signal values evolving is likely to have an impact on the rate of macroevolutionary diversification (41, 42). The morphological and functional outcomes of evolved silence in field crickets support our predictions about the role of trait loss in rapid diversification. We found that secondary loss of male song in Hawaiian *T. oceanicus* is associated with substantial variation in vestigial morphological traits, susceptible to genomic background effects. Analysis of vestigial wing structures identified a broad range of acoustic resonances, which could facilitate the evolution of new cricket songs with carrier frequencies that extend well beyond the typical narrow range centred around 5 kHz for this species [$\bar{x} = 5.02$ kHz ± 0.017 s.e. reported in (43)].

The venation which has been left behind on the disrupted forewings of silent flatwing crickets includes a wide range of morphological features: more than one occurrence of genetic mutation appears to have driven convergent loss of song with noticeably different morphological consequences (16; **Fig. 1*B***), and we have found that these loss-of-function *flatwing* genotype(s) also interact with background genetic variation to produce a suite of wing structures with sharp acoustic resonances but impaired signalling capability. Peak frequencies of vestigial harps on flatwing *T. oceanicus* wings spanned a range from approximately 4.0 – 16.5 kHz in this study. The range of morphological variation we detected among flatwings is suggestively similar to that which characterises variation in wing resonators across deep evolutionary divisions within the Ensifera (**Fig. 1*C***). Acoustic signalling is thought to have facilitated rapid speciation and radiation in crickets and katydids, has evolved independently on multiple occasions, and has been secondarily lost in several lineages (33, 44). Our results raise the intriguing possibility that secondary losses of song through male wing feminisation could have played a key role in evolutionary radiations involving this group.

The existence of a suite of pre-existing morphological variants that could underpin the evolution of new signal values does not guarantee the evolution of such new signal values or subsequent diversification; these vestigial resonators may be best thought of as a facilitating, yet not sufficient, requirement for such a mode of diversification. For new signals to evolve, receiver structures and physiology must also coevolve. On a trivial level, that this has happened repeatedly throughout the evolution of sexually signalling taxa is demonstrated by the existence of divergent mate recognition systems across extant groups. The singing insects, for example, produce an exceptionally broad range of species-diagnostic carrier frequencies (27, 33, 35). One well characterised system involves the genus studied here, in which females of the sister species *T. oceanicus* and *T. commodus* filter male advertisement songs differing in carrier frequency by approximately 1 kHz, to discriminate against heterospecific calls that might be experienced in sympatry (21, 45). In another group of calling insects, lebinthine crickets, both signal and receiver shifts have occurred not only across frequency spectra (audible to ultrasonic), but also

Table 2.

Table 2. Kauai and Oahu male wing resonances. Peak resonances are provided for the harp area[1] of each specimen's right and left forewing (forewings show a dominant right-over-left overlap in this species). Normal-wing males from each population are included as verifications of the technique and to aid comparison with flatwings, and full frequency spectra of all specimens are given in Fig. 5.

Origin	Morph[2]	PL[3] (mm)	RHFL[3] (mm)	Peak ƒ (kHz) left wing	Peak ƒ (kHz) right wing
Kauai	*fw*	4.23	11.45	10.80	16.54
	fw	3.90	9.80	11.09	11.64
	fw	4.04	10.82	10.35	10.37
	fw	4.09	10.65	12.86	10.69
	nw	4.24	11.61	5.66	5.16
	nw	3.85	10.65	4.58	4.78
Oahu	*fw*	3.95	10.06	6.13	6.77
	fw	3.94	10.44	5.13	7.66
	fw	3.87	10.05	4.06	6.53
	fw	3.75	9.93	7.05	6.14
	fw	3.83	10.83	6.05	12.8
	fw	3.75	10.50	7.89	5.16
	fw	3.92	10.30	5.66	8.35
	fw	3.85	10.28	7.08	9.24
	nw	4.62	11.85	5.02	5.02
	nw	4.44	11.55	4.95	4.81

[1] in flatwings, refers to either the vestigial structure, or the area in which it would otherwise be located [2] *fw* = pure-breeding *flatwing* genotype, *nw* = pure-breeding *normal- wing* genotype [3] pronotum length (PL) and rear hind femur length (RHFL): mean of three measurements

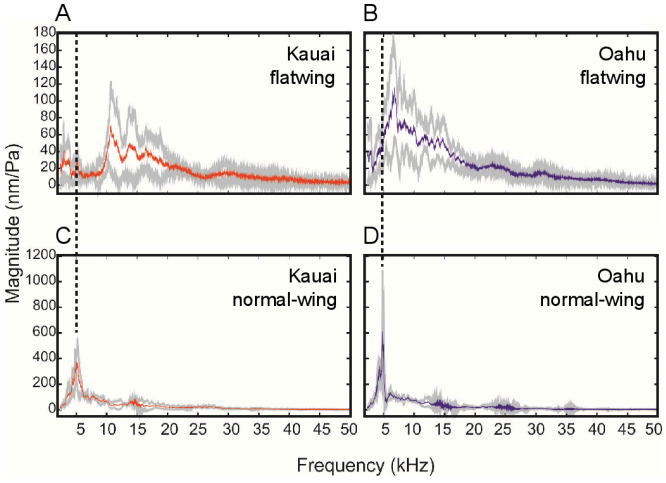

Fig. 5. Wing resonance plots for flatwing males from Kauai (*A*) and Oahu (*B*) populations, with normal-wing comparators (*C, D*). Coloured lines indicate average spectra for each group, with ±1 standard deviation shown in grey. Dashed lines indicate peak frequencies of normal-wing males recorded from each population to aid comparison with flatwing resonances. Sample sizes are provided in **Table 2**.

across modalities (from acoustic to vibratory mate localisation) (38). We note that although *T. oceanicus* females discriminate males on the basis of call frequency, with a selectivity peak at approximately 5 kHz, they will also respond to artificial song playbacks ranging from 2.5 to 7.0 kHz (21). The plausibility of a scenario involving co-option and elaboration of vestigial resonators via sexual selection is supported by the recent observation that female *T. oceanicus* from a population on Molokai preferentially associate with attenuated acoustic stimuli produced by some flatwing males, compared to silence (46). It is unclear whether these flatwing males' acoustic emissions result from engagement of a residual file and scraper mechanism or friction affecting other wing structures; amplitude of the acoustic stimuli is orders of magnitude lower than that of singing normal-wing males and likely to be close to the auditory detection threshold (47), and their frequency spectra are relatively flat (46). Nevertheless, this finding confirms observations that auditory neurons in grylline crickets show broad frequency tuning (48) and suggests that female responses to novel acoustic frequencies may be less of a barrier to signal evolution than are the biomechanical constraints imposed by morphological adaptations for sound production.

The release of variation in *T. oceanicus* following secondary loss of song satisfies a key requirement for models of rapid diversification following trait loss (2, 3, 7). Some variation among flatwing males, for example those derived from different island populations, appears to reflect different genetic causes (16), but the background and family-level effects that we found to release further morphological variation is characteristic of decanalization under different genetic backgrounds (32). Genetic control of canalisation has been characterised in other contexts, for example the heat shock protein Hsp90 in *Drosophila melanogaster* (49), and our results support the idea that a reduction in canalisation following the evolutionary loss of song in field crickets can generate a broad phenotypic substrate of male forewing variants that could facilitate the evolution of new signals. Another intriguing, non-mutually exclusive possibility is that developmental plasticity contributes to the variation in wing morphology we observed, raising the possibility that signal diversification following trait reversal could involve a simultaneous combination of selection on genetic variation and canalization of developmentally plastic phenotypes (50). Analysis of flatwing resonances revealed that vestigial resonators have the potential to generate acoustic signals at frequencies outwith the range of ordinary calling song in *T. oceanicus,* and more variable. It remains to be seen (perhaps not in our lifetimes) whether a radiation of sexual signals in *T.*

oceanicus will evolve from this broad substrate of vestigial wing structures and contribute to establishing new species boundaries. The predictions we tested about patterns of vestigial signal traits and their design features are focused on the earliest stages of such a process, and our results lend empirical support to the idea that trait loss could precede and facilitate bursts of diversification (2, 51-53).

Methods

Cricket lines and crosses. Laboratory stocks of crickets were established from eggs laid by approximately 20-30 wild-caught females. Collections were made in 2012 from populations near Wailua, Kauai and La'ie, Oahu. In the complementation experiment, we used Kauai lines breeding pure for flatwing or normal-wing morphology. The establishment of these lines using two generations of standard Mendelian crosses to identify homozygous *flatwing* and homozygous *normal-wing* genotypes has previously been described in detail (54). Crickets were reared within a temperature-controlled chamber at 25 °C on a 12h:12h photo-reversed light:dark cycle following established protocols (55). They were maintained in 16L translucent plastic tubs at a density of approximately 30-50 individuals, with cardboard egg carton for cover and *ad libitum* Burgess Excel Junior and Dwarf rabbit food and water. Maintenance was performed twice weekly.

The crossing design for the complementation test followed the schematic in **Fig. 2*A*-*B***. We set up five individual crosses using *flatwing* Kauai P_0 dams and *flatwing* Oahu sires. We did not have pure-breeding Oahu lines at the time of the complementation test, so we performed the inter-island cross in one direction only. As a control, five crosses between *flatwing* Kauai females and *flatwing* Kauai males were simultaneously performed. At the F_1 generation, ten individual full-sibling crosses for the five test and three of the control crosses were performed. All offspring were reared under common garden conditions as described above.

Wing morphometrics. Landmark-based geometric morphometrics was performed as previously described (16, 56). For n = 1067 F_2 test crickets and n = 245 F_2 control crickets, male forewings were removed and immediately mounted between two slides. They were then photographed using a Leica DFC295 digital camera affixed to a Leica M60 dissecting microscope. The 16 landmarks illustrated in Fig. 2*C* were placed using the programme tpsDIG v.2.16 (57). Software from the Integrated Morphomerics Package suite of morphometrics programmes (58, 59) was used to superimpose landmark data from all samples and quantify variation in wing venation shape using Procrustes distances (60). For each comparison performed, a common dataset comprising landmark data from all the individuals required for the comparison was assembled and Procrustes distances were produced using CoordGen6f (58). Principal components and scores for all landmark data were generated using PCAgen6n (58).

Wings of Kauai and Oahu *flatwing* males from a previous study (16) were used as a reference comparison for the F_2 male wings produced in the complementation crosses. The same worker (S.P.) scored wing features and landmarks in both studies. We visually assessed all F_2 cricket wings from the complementation experiment to classify them as Kauai-like or Oahu-like. Given the potential subjectivity of this qualitative classification, we also recorded the presence or absence of full or partial (i.e. vestigial) scrapers and mirrors. We verified this approach using a randomly selected subset of 100 wing photographs from the test and control crosses. A separate scorer (N.W.B.) blinded to sample identity scored whether each of the wings in the validation subset had scraper and a mirror. The proportion of scrapers in the test vs. control individuals from both datasets was compared, and the original scorer (S.P.) then blindly rescored the validation subset as well. Concordance between scorers was found to be highly reliable, providing confidence in our method of visually classifying wing traits.

A MANOVA was run using the first 5 principal components from a PCA in which all F_2 test crickets were pooled with the previously-published set of flatwing males from Kauai and Oahu, to test whether wing morphology of flatwing males arising from the test complementation crosses differed from flatwings from either or both island populations. A *post-hoc* homogeneity of variance analysis was performed on the MANOVA residuals for each of the five principal components, to assess whether wing variation among complementation F_2 crosses differed from that of the original Kauai and Oahu flatwing males. We re-ran the PCA and MANOVA analyses to compare the same set of test crickets against the n = 245 control wings produced using the same crossing procedure. Subsequently, we ran a separate MANOVA on scores of the first n = 5 principal components from a PCA of the complementation test F_2 crickets only, here assessing family-level variation in wing venation. The purpose of using five test families for the complementation analysis was to provide a sufficient sample size of F_{-2} flatwing males for analysis and identification of potential recombinant phenotypes. The crossing design was insufficient to formally estimate heritability of wing patterning, but quantifying family-level variation provided an indication of genetic variation underlying flatwing male wing venation, as this full-sib cross design included genetic and common environmental effects (61). Statistical analyses were performed in SPSS v.23.

Laser Doppler vibrometry. Biophysical analyses of male forewing acoustic resonances were performed using an additional three pure-breeding Kauai lines that had been re-established following outcrossing and re-crossing, plus pure-breeding Oahu lines that were later established following the same crossing procedures as described in (54). Each sampled cricket's pronotum length and right hind femur length was measured to the nearest 0.01 mm three times and then averaged. Crickets were anaesthetized using FlyNap (Carolina Biological Supply), then mounted whole with forewings extended dorso-laterally, fixed with a mixture of beeswax (Fisher Scientific) and Colophony (Sigma-Aldrich). Following Chivers et al. (62), we measured vibrating-producing regions of the mounted wings and characterised associated frequency spectra using a micro-scanning LDV (Polytec PSV-500; Waldbronn, Germany) with a close up attachment. The wings of mounted specimens were positioned perpendicular to the lens of the laser unit, and an acoustic stimulus was broadcast from a loudspeaker (Ultrasonic Dynamic Speaker Vifa, Avisoft Bioacoustics, Glienicke, Germany) positioned above the laser unit and facing the specimen (**Fig. 4*A***). The stimulus consisted of periodic chirps (1-50 kHz) generated using Polytec software (PSV 9.2), passed to an amplifier (A-400, Pioneer, Kawasaki, Japan), and sent to the loudspeaker. We flattened the periodic chirp stimulus so that all frequencies were presented at 60 ± 1.5 dB (SPL re. 20 μPA) at the position of the wings. A 1/8 inch condenser microphone (Brüel & Kjær, Denmark) was positioned dorsally between the outstretched wings to monitor and record the stimulus as a reference. Using the laser in scan mode, the extended wings were scanned using 250-300 scan points, averaging 3 times to obtain the value for each point. For each point, a fast-Fourier transform was generated using a rectangular window at a sampling rate of 512,000 samples/second, a 64 ms sampling time, and a frequency resolution of 15.63 Hz.

Raw vibrometry data was analysed using Polytec software (v. 9.2) and custom MATLAB (The MathWorks Inc., Natick, MA, USA) scripts. Vibrometry frequency spectra were normalised to the playback signal received by the microphone using a transfer function (63). To estimate the amount of unrelated noise, we also computed the magnitude-squared coherence between the vibrometer and microphone signals for each data point (64). Coherence ranges between zero and one, where one indicates no unrelated or external noise. Our aim was to identify sharply-tuned resonant peaks on crickets' forewings, which we assessed using the dimensionless index *Q* (65). We calculated *Q* by dividing the peak frequency by the bandwidth at 3 dB below the peak amplitude (66), identifying the sharpest peak (highest *Q*) on the surface of each pair of wings in the centre of the harp (in the case of normal-wing controls) or vestigial harp area (in flatwings) to report the dominant resonant frequency for each. Two-tailed t-tests were used to compare peak frequency differences between Kauai and Oahu flatwing male resonators. Although sample sizes were small, the large effect sizes (Cohen's D for left wing comparison = 4.75, for right wing comparison = 2.66) provide a measure of confidence in this approach (67). Right wing comparisons involved samples with heterogeneous variances so we performed a nonparametric test to verify the inference that Kauai flatwings produce higher peak resonances than Oahu flatwings (Mann-Whitney U test: U = 3, P = 0.028).

Data accessibility. Any data not presented in the *SI Appendix* will be archived on the Dryad Digital Repository upon acceptance.

Author contributions. N.W.B. conceived the study. N.W.B., S.P. & F.M.-Z. designed the experiments. S.P. performed complementation and morphometric experiments. N.W.B. and F.M.-Z. performed laser Dopper vibrometry experiments. All authors analysed data. N.W.B. wrote the manuscript with input from S.P. and F.M.-Z.

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