Vestigial singing behaviour persists after the evolutionary loss of song in crickets

Will T. Schneider[1,†], Christian Rutz[1], Berthold Hedwig[3], Nathan W. Bailey[1]

[1]Centre for Biological Diversity, School of Biology, University of St Andrews,

 St Andrews, Fife KY16 9TH, UK

[2]Department of Zoology, University of Cambridge, Downing Street, Cambridge,

 CB2 3EJ, UK

[†]Present address: Department of Physiology, Development and Neuroscience,

 University of Cambridge, Downing Street, Cambridge, CB2 3EJ, UK

Author for correspondence:

Nathan W. Bailey

e-mail: nwb3@st-andrews.ac.uk

Word count: 2,593

21 **Abstract**

22 The evolutionary loss of sexual traits is widely predicted. Because sexual signals can arise

23 from the coupling of specialised motor activity with morphological structures, disruption to a

24 single component could lead to overall loss of function. Opportunities to observe this process

25 and characterise any remaining signal components are rare, but could provide insight into

26 the mechanisms, indirect costs, and evolutionary consequences of signal loss. We

27 investigated the recent evolutionary loss of a long-range acoustic sexual signal in the

28 Hawaiian field cricket *Teleogryllus oceanicus*. Flatwing males carry mutations that remove

29 sound-producing wing structures, eliminating all acoustic signalling and affording protection

30 against an acoustically-orientating parasitoid fly. We show that flatwing males produce wing

31 movement patterns indistinguishable from those that generate sonorous calling song in

32 normal-wing males. Evolutionary song loss caused by the disappearance of structural

33 components of the sound-producing apparatus has left behind the energetically-costly motor

34 behaviour underlying normal singing. These results provide a rare example of a vestigial

35 behaviour and raise the possibility that such traits could be co-opted for novel functions.

1. Introduction

The evolutionary loss of sexual signals is a central prediction of sexual selection theory [1]. Its widespread occurrence is supported by numerous examples inferred from phylogenetic studies, but the rarity of contemporary cases makes it challenging to study its evolutionary dynamics [2]. Since sexual signalling frequently involves the coupling of multiple trait components, such as complex motor activities and specialised morphologies [3], its evolutionary loss might be predicted to occur in a stepwise fashion. If initially only one component is lost, others may be left behind as non-functional vestigial traits [4]. Characterizing such vestiges could help reveal evolutionary constraints or paths of least resistance leading to trait loss, fitness consequences of trait loss, and mechanisms by which vestigial traits might be co-opted for new functions [5].

We addressed this by studying field crickets (*Teleogryllus oceanicus*) that experienced the recent evolutionary loss of male song. Males sing to attract females for mating, but in Hawaii their song also attracts female parasitoid flies (*Ormia ochracea*) whose larvae burrow into, consume, and kill their host [6]. A novel, genetic male morph incapable of producing song (flatwing) was discovered in 2003 on the island of Kauai [7]. Currently, approximately 95% of males on Kauai and 50% of males on the neighbouring island of Oahu express the flatwing phenotype [7,8]. Crickets normally produce acoustic signals by rhythmically opening and closing their forewings, scratching the scraper of one wing against the file of the other [9], but these wing structures are severely reduced or absent in flatwings (figure 1*a*) [7,8]. We used an opto-electronic camera [10] to test whether flatwing males continue to express the stereotyped wing movements that produce sonorous calling song in normal-wing males, and if they do, whether wing movement patterns differ between morphs.

2. Material and methods

(a) Cricket origins and husbandry

Homozygous flatwing (n = 6) and normal-wing (n = 6) lines of *T. oceanicus* were established in 2012 from stock populations originating from Oahu, using the crossing methods detailed in Pascoal et al. [11]. Crickets were reared in 16 litre plastic containers, at ca. 25 ˚C on a 12:12 hour light:dark cycle. We provided cardboard for shelter, cotton wool water pads, and *ad libitum* access to Burgess Excel dwarf rabbit food. At least four days before experimentation, reproductively mature adult males were separated into single-sex containers.

(b) Experimental procedures and analysis

Wing movements were measured using an opto-electronic camera with a position-sensitive photodiode as a sensor, as described in [10] and figure 1. In this procedure, males with reflective markers adhered to their forewings are placed on a turntable in front of the camera and microphone. Red light is directed from the camera towards the cricket, and the reflection from the marker is used to measure wing position. During singing, males raise their forewings above the abdomen. The wings open and silently move downward to either side, and in normal-wing males produce a sound pulse as they return upwards to close (figure 1*b*). During this process, the camera measures the vertical position of the marker as a voltage signal, which together with a microphone signal, is recorded by a PC running Spike2 software (CED, Cambridge, UK).

Wing movements associated with singing typically occur in bouts lasting several seconds to minutes. Before recordings took place, a reflective marker (3M Laboratories, Scotchlite foil type 7610, 1.0 mm diameter) was adhered to the costal margin of each male's forewing using PVA glue, and subjects were isolated in transparent 150 mL tubs. If we observed a subject attempt to sing during incidental visual monitoring, then it was selected

87 for recording. The male was placed in front of the camera and microphone and the camera

88 was adjusted to monitor its wing movements. The system only enabled recording one male

89 at a time.

90 The number of recordings per cricket ranged between 1 and 13. We retained

91 recordings for onward analysis if they contained at least 20 seconds of continuous singing,

92 and we measured 10 consecutive songs from the earliest such bout of each recording. Of 52

93 crickets fitted with reflective markers, 16 individuals passed these criteria (9 flatwing, 7

94 normal-wing, from 9 different lines). Recording took place over 11 days, at 20 °C under low

95 light. We separately verified that flatwing males produce no sonorous signal up to ca. 48 kHz

96 (electronic supplementary material, figure S1).

97 *Teleogryllus oceanicus* calling song consists of two distinctive pulse patterns: the

98 "long chirp", containing a series of 5 to 8 pulses that typically increase in amplitude, followed

99 by "short chirps" (or trills), which are lower amplitude and contain multiple pairs of pulses [6].

100 Calling song can be further characterised by frequency and temporal components reflecting

101 pulse durations and intervals. Figures 1*c* and *d* show a simultaneous recording of the sound

102 and corresponding wing movements produced during calling song, with 16 song components

103 illustrated. We observed that both male morphs produce wing movements containing these

104 components, so we then tested for quantitative differences between morphs.

105 We ran two general linear mixed models for each song component to test for

106 differences between morphs. One model included "morph" (flatwing/normal-wing) as a fixed

107 effect, while the other did not. "Individual" was always included as a random effect to

108 account for the non-independence of within-subject recordings. A likelihood-ratio test was

109 used to compare goodness of fit and assess evidence for variation in wing movements

110 between morphs. Analyses were run using lme4 in R version 3.2.4 [12].

111

112

113

3. Results

Both male morphs expressed the overall long chirp/short chirp pattern of wing movements (figure 2a) (electronic supplementary material, figure S2). Moreover, we found no significant quantitative differences between morphs for any of the 16 song components after correction for multiple comparisons (Bonferroni correction; k = 16, α = 0.003) (figure 2b). Without correction, there was a significant difference (p = 0.036) in component 16 (figure 1c, table S1). The electronic supplementary material contains statistical details and video of a flatwing male moving his wings in a stereotyped calling-song pattern (table S1 and video S1).

4. Discussion

The evolutionary disappearance of a sexual signal in *T. oceanicus* was caused by the loss of a key morphological trait, but we found that behavioural movement patterns underlying signal generation persist in high fidelity. Silent flatwing males continue to express the stereotyped wing motor behaviours produced by normal-wing males during calling song. This "silent singing" provides a rare example of a vestigial behaviour [13] and affords three insights into the evolutionary dynamics of trait loss.

First, gradual evolutionary reduction of costly traits after selection ceases is a defining feature of vestigial characters [4], but we did not detect signs of such decay in patterns of wing movement during silent singing in *T. oceanicus*. In crickets, song-generating wing movements are energetically costly [14,15]. For example, the metabolic expenditure of singing in the species *T. commodus* is approximately four times higher than that of resting [16]. Wing stroke rate is the main factor determining energetic costs of song [17], but only 0.05% of metabolic energy is converted into acoustic energy [16]. The motor activity underlying silent singing is thus likely to incur almost all the energetic expense of sonorous signalling, but without any sexually-selected benefit, illustrating the indirect costs that can

139   affect individuals during early stages of evolutionary trait loss. In *T. oceanicus*, secondary

140   mutations that mitigate energetic costs by reducing long-term calling effort in flatwing males

141   are theoretically possible, but would need to either co-segregate with flatwing-causing

142   mutation(s), or be sufficiently beneficial to counterbalance selection against them when

143   expressed in normal-wing males. Costs of silent singing might be particularly likely to impose

144   selection for reduced calling effort in populations where flatwing males predominate.

145   Second, silent singing in flatwing *T. oceanicus* provides a counter-example to the

146   frequent observation that behavioural resistance—adaptive behavioural change under

147   stressful conditions—underlies rapid adaptation to ecological or environmental pressures

148   [18] (e.g. escape behaviour in the lizard *Anolis sagrei* [19] and parasite tolerance in the frog

149   *Hyla femoralis* [20]). In *T. oceanicus*, adaptation occurred through morphological, not

150   behavioural, change. Although behaviour has been suggested to facilitate rapid evolution by

151   enabling plasticity or relaxing selection [21], thereby accommodating indirect fitness costs of

152   adaptive mutations, the persistence of silent singing in *T. oceanicus* highlights the need to

153   also test behaviour's inhibitory effects on evolutionary adaptation.

154   Finally, remnants of lost sexual traits may represent a particularly evolvable substrate

155   upon which selection can act. Patterned wing movements specific to long-range calling in *T.*

156   *oceanicus* are not known to serve any other function, and it is unlikely that the air currents

157   and substrate vibrations they create are detectable over longer distances given their rapid

158   attenuation [22]. Flatwing males still attempt to produce courtship song, but this is also silent

159   [23]. Over longer evolutionary timescales, co-option of vestigial signal components for

160   different functions might represent a path of least resistance to the acquisition of

161   evolutionary novelties. Recent evidence that vibration-duetting courtship behaviour of

162   Lebinthine crickets may have arisen from a behaviour originally used for predator avoidance

163   is consistent with this idea [5]. Future work testing whether vestigial trait components

164   respond to different selective pressures following the loss of their original function could

165   ultimately illuminate mechanisms by which evolutionary novelties arise.

References

180

181   1.   Lande R. 1981 Models of speciation by sexual selection on polygenic traits.
182   *Proc. Natl. Acad. Sci. USA* **78**, 3721-3725.

183   2.   Wiens JJ. 2001 Widespread loss of sexually selected traits: how the peacock
184   lost its spots. *Trends Ecol. Evol.* **16**, 517-523.

185   3.   Guilford T, Dawkins MS. 1991 Receiver psychology and the evolution of
186   animal signals. *Anim. Behav.* **42**, 1-14.

187   4.   Fong DW, Kane TC, Culver DC. 1995 Vestigialization and the loss of
188   nonfunctional characters. *Annu. Rev. Ecol. Systemat*. **26**, 249-268.

189   5.   ter Hofstede HM. *et al*. 2015 Evolution of a communication system by sensory
190   exploitation of startle behaviour. *Curr. Biol.* **25**, 3245-3252.

191   6.   Zuk M, Simmons LW, Cupp L. 1993 Calling characteristics of parasitized and
192   unparasitized populations of the field cricket *Teleogryllus oceanicus*. *Behav.*
193   *Ecol. Sociobiol.* **33**, 339-343.

194   7.   Zuk M, Rotenberry JR, Tinghitella RM. 2006 Silent night: adaptive
195   disappearance of a sexual signal in a parasitized population of field crickets.
196   *Biol. Lett.* **2**, 521-524.

197   8.   Pascoal S. *et al*. 2014 Rapid convergent evolution in wild crickets. *Curr. Biol.*
198   **24**, 1369-1374.

199   9.   Koch UT, Pfau HK. 1994 The functional morphology of singing in the cricket.
200   *J. Exp. Biol.* **195**, 147-167.

201   10. Hedwig B. 2000 A highly sensitive opto-electronic system for the
202   measurement of movements. *J. Neurosci. Meth.* **100**, 165-171.

203 11. Pascoal S. *et al.* 2016 Rapid evolution and gene expression: A rapidly-
204   evolving Mendelian trait that silences field crickets has widespread effects on
205   mRNA and protein expression. *J. Evol. Biol.* **29**, 1234-1246.

206 12. Bates D. *et al.* 2015 Fitting linear mixed-effects models using lme4. *J. Stat.*
207   *Soft.* **67**, 1-48.

208 13. Carson HL, Chang LS, Lyttle TW. 1982 Decay of female sexual behaviour
209   under parthenogenesis. *Science* **218**, 68-70.

210 14. Hoback WW, Wagner WE. 1997 The energetic cost of calling in the variable
211   field cricket, *Gryllus lineaticeps. Physiol. Entomol.* **22**, 286-290.

212 15. Hack MA. 1998 The energetics of male mating strategies in field crickets
213   (O*rthoptera*: *Gryllinae: Gryllidae*). *J. Insect Behav.* **11**, 853-867.

214 16. Kavanagh MW. 1987 The efficiency of sound production in two cricket
215   species, *Gryllotalpa australis* and *Teleogryllus commodus* (Orthoptera:
216   Grylloidea). *J. Exp. Biol.* **130**, 107-119.

217 17. Prestwich KN, Walker TJ. 1981 Energetics of singing in crickets: effect of
218   temperature in three trilling species (Orthoptera: Gryllidae). *J. Comp. Physiol.*
219   A **143**, 199-212.

220 18. Sih A, Ferrari MCO, Harris DJ. 2011 Evolution and behavioural responses to
221   human-induced rapid environmental change. *Evol. Appl.* **4**, 367-387.

222 19. Losos JB, Schoener TW, Spiller DA. 2004 Predator-induced behaviour shifts
223   and natural selection in field-experimental lizard populations. *Nature* **432**, 505-
224   508.

225 20. Sears BF, Snyder PW, Rohr. 2013 Infection deflection: hosts control parasite
226   location with behaviour to improve tolerance. *Proc. R. Soc. Lond. B* **280**,
227   20130759.

228 21. Duckworth RA. 2009 The role of behaviour in evolution: a search for

229   mechanism. *Evol. Ecol.* **23**, 513-531.

230 22. Pollack G, Givois V, Balakrishnan R. 1998 Air-movement "signals" are not

231   required for female mounting during courtship in the cricket *Teleogryllus*

232   *oceanicus*. *J. Comp. Physiol. A* **183**, 513–518.

233 23. Bailey NW, McNabb JR, Zuk M. 2007 Preexisting behavior facilitated the loss

234   of a sexual signal in the field cricket *Teleogryllus oceanicus*. *Behav. Ecol.* **19**,

235   202-207.

236 24. Schneider WT, Rutz C, Hedwig B, Bailey NW. 2018 Data from: Vestigial

237   singing behaviour persists after the evolutionary loss of song in crickets.

238   Dryad Digital Repository.

239   (http://datadryad.org/review?doi=doi:10.5061/dryad.7tv59)

240   **Figure Legends**

241

242   **Figure 1.**

243   (*a*) Structural differences in the forewings of normal-wing and flatwing males. Flatwing males

244   lack, or have severely reduced, vibration-generating (file and scraper) and resonating

245   structures (mirror and harp). Yellow symbols indicate the placement of reflective markers

246   used for opto-electronic measurements of wing movement. (*b*) Vertical forewing movements

247   associated with singing. (*c*) Representative calling song from a normal-wing male (top), with

248   corresponding wing-movement recording (bottom). We measured 16 wing-movement

249   parameters corresponding to key song components (described in the electronic

250   supplementary material, table S1). Components fall into three categories: numbers of chirps

251   or pulses (1-3), long-duration features on the order of seconds (4-7) and short-duration pulse

252   or interval traits on the order of milliseconds (8-16). (*d*) Enlarged section of song from (*c*).

253

254   **Figure 2.**

255   (*a*) Five-second excerpts of wing-movement recordings for a normal-wing (top) and a

256   flatwing (bottom) male illustrating that both morphs are capable of producing the distinctive

257   two-part composition of the *T. oceanicus* calling song (a trill-like 'long chirp' followed by a

258   series of lower amplitude 'short chirps'). (*b*) Comparisons of wing-movement data between

259   male morphs for individual song components, grouped and labelled with numbers as

260   illustrated in figure 1*c*. Yellow dots, thick black bars and thin black lines indicate medians,

261   inter-quartile ranges and 95% confidence intervals, respectively, and the shaded regions

262   show probability density estimates for the data (grey: normal-wing males, red: flatwing
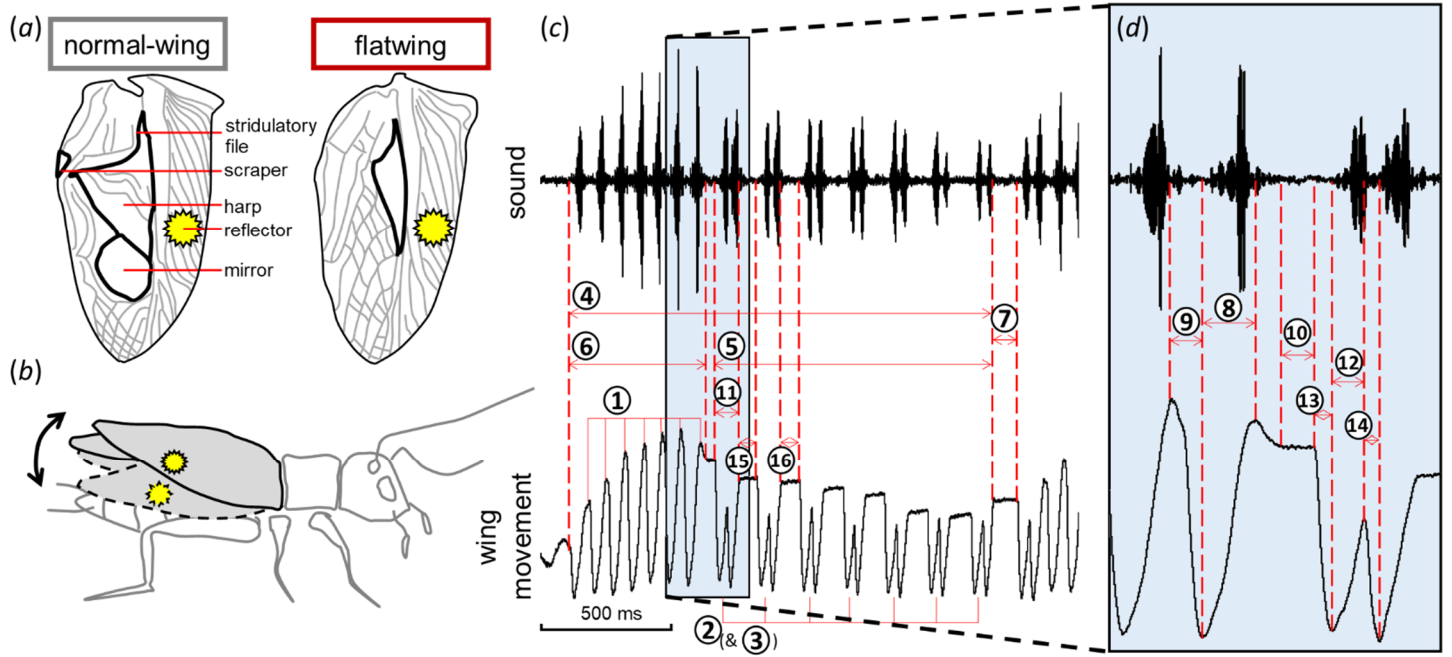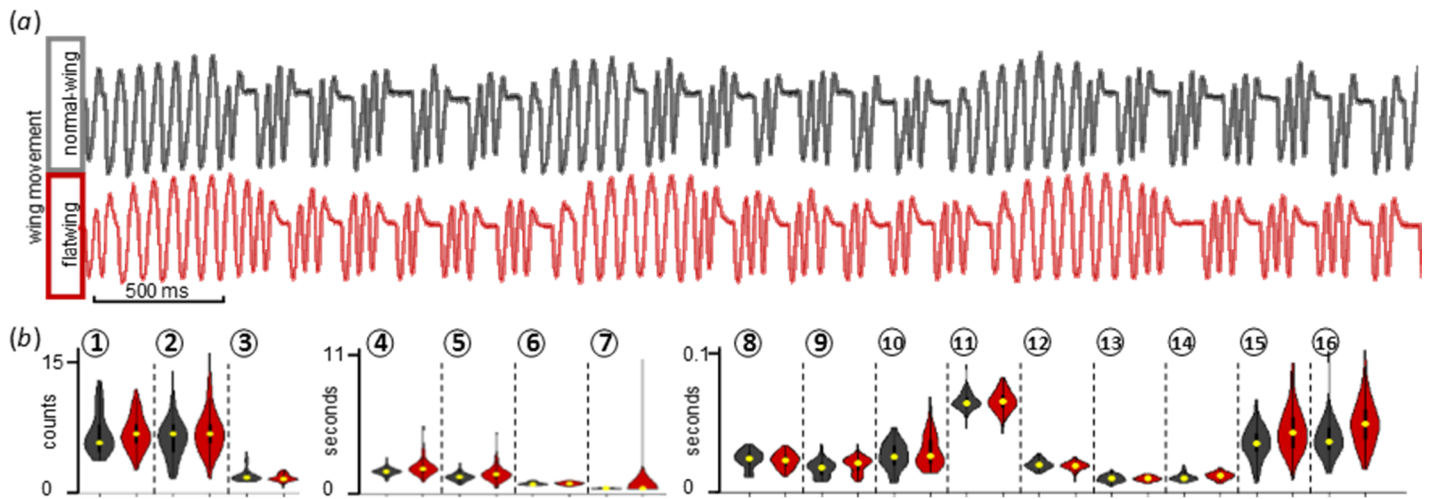
263   males).

Figure 1.

Figure 2.

Supplementary Material

Vestigial behaviour persists after the evolutionary loss of song in crickets

Will T. Schneider[1,†], Christian Rutz[1], Berthold Hedwig[2], Nathan W. Bailey[1]

[1]Centre for Biological Diversity, School of Biology, University of St Andrews,
St Andrews, Fife KY16 9TH, UK

[2]Department of Zoology, University of Cambridge, Downing Street, Cambridge,
CB2 3EJ, UK

[†]Present address: Department of Physiology, Development and Neuroscience,
University of Cambridge, Downing Street, Cambridge, CB2 3EJ, UK

Author for correspondence:

Nathan W. Bailey
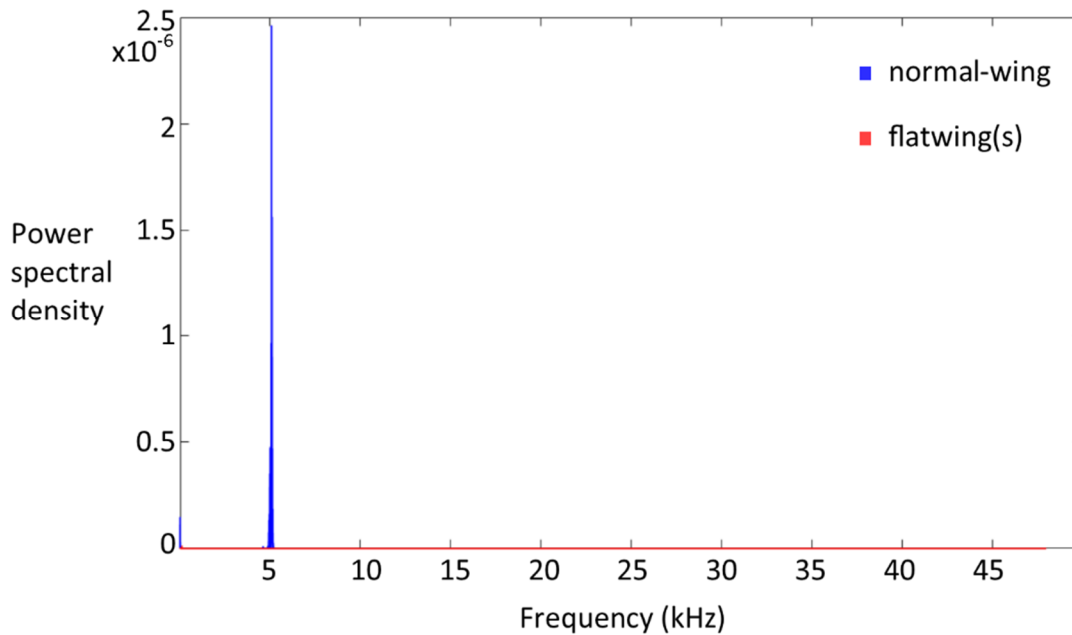
e-mail: nwb3@st-andrews.ac.uk

Figure S1. Power spectral densities of a single normal-wing male producing calling song (blue) and 4 flatwing males during silent singing (red). During wing movement trials, we never heard flatwing males produce sonorous calling songs. In flatwing male crickets, the stridulatory file is dramatically reduced in size and repositioned on the right forewing in a way that precludes engagement with the scraper on the opposite wing. It is therefore unlikely that flatwing males could produce sounds outwith the range of human hearing, but we verified this by making separate ultrasonic audio recordings. We used a Bruel and Kjaer 4135 ultrasound condenser microphone and a Sony handheld linear PCM Recorder (PCM-M10) recording at a 96 kHz sampling rate. This enabled us to detect signals up to ca. 48 kHz. Performing a Fast Fourier Transform in a 5 second time window containing song or silent singing in the case of flatwing males, we computed the frequency spectra of the first 5 seconds of all 5 subjects and compared these together. The spectra clearly indicate a high-powered, dominant peak at the characteristic carrier frequency of ca. 5 kHz for the normal-wing male, and negligible to no acoustic output for the silent-singing flatwing males. Note that the flatwing male spectra above are overlaid on top of the normal-wing spectrum to illustrate the absence of detectable peaks in the former at any frequency measured.

_____

Vestigial singing behaviour persists after the evolutionary loss of song in crickets

Will T. Schneider[1, †], Christian Rutz[1], Berthold Hedwig[2], Nathan W. Bailey[1]

[1]Centre for Biological Diversity, School of Biology, University of St Andrews, St Andrews, Fife KY16 9TH, UK
[2]Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ, UK
[†]Present address: Department of Physiology, Development and Neuroscience, University of Cambridge, Downing Street, Cambridge, CB2 3EJ, UK

Author for correspondence:
Nathan W. Bailey
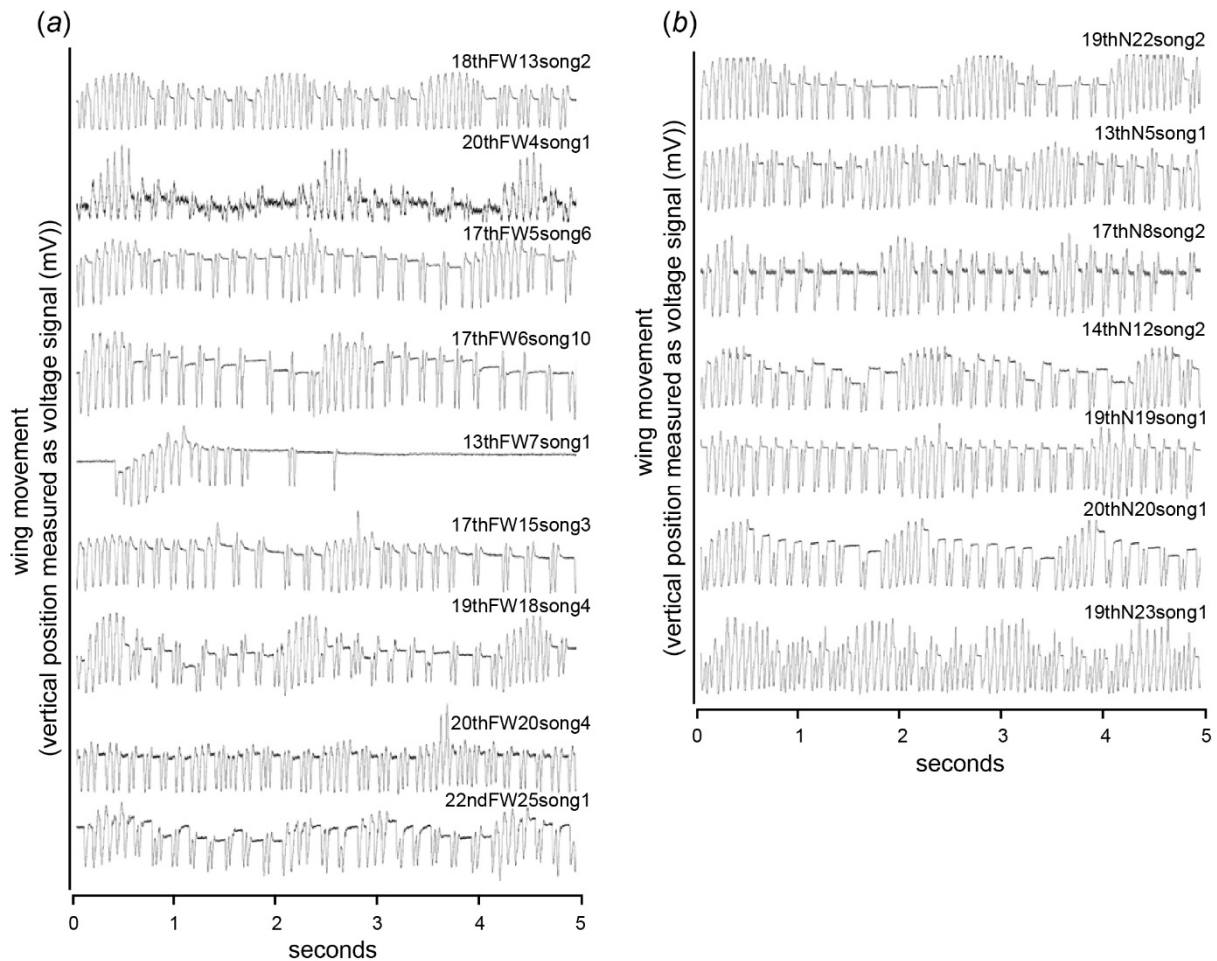e-mail: nwb3@st-andrews.ac.uk

Figure S2. Five-second excerpts of wing movement recordings from (*a*) flatwing males and (*b*) normal-wing males used in the study, demonstrating the long chirp – short chirp wing movement patterns characteristic of male *Teleogryllus oceanicus* calling song. Labels at the top right of each recording refer to sample ID. Labels and axes were manually re-drawn for clarity. The y-axes for each trace are not scaled equally, as the voltage signal reflecting wing movement also depends on the test subject's position in relation to the camera. Analyses of individual song components 1-16 were performed on a larger sample of song phrases per individual (see Main Text and Table S1).

Vestigial singing behaviour persists after the evolutionary loss of song in crickets

Will T. Schneider[1, †], Christian Rutz[1], Berthold Hedwig[2], Nathan W. Bailey[1]

[1]Centre for Biological Diversity, School of Biology, University of St Andrews, St Andrews, Fife KY16 9TH, UK
[2]Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ, UK
[†]Present address: Department of Physiology, Development and Neuroscience, University of Cambridge, Downing Street, Cambridge, CB2 3EJ, UK

Author for correspondence:
Nathan W. Bailey
e-mail: nwb3@st-andrews.ac.uk

Table S1. Results of tests for quantitative differences in wing movement between flatwing and normal-wing males for 16 calling song components. For each component, two general linear mixed models were run using lme4 in R version 3.2.4 following a visual assessment for data normality. All models contained "individual" as a random effect. One model contained a fixed effect of "morph" while the other did not, and they were compared using likelihood ratio tests (df = 4). Song components follow figure 1*c* in the main text: (1) number of pulses in long chirp, (2) number of short chirps, (3) average number of pulses per short chirp, (4) total song length, (5) length of short chirps, (6) long chirp length, (7) inter-song-interval, (8) final long chirp up-stroke, (9) final long chirp down-stroke, (10) long chirp-short chirp interval, (11) short chirp paired pulse length, (12) short chirp up-stroke, (13) short chirp down-stroke, (14) short chirp inter-pulse-interval, (15) first short chirp inter-chirp-interval, (16) second short chirp inter-chirp-interval.

	1	2	3	4	5	6	7	8
	long chirp (LC) pulses	short chirps (SC)	average # pulses in SC	song length	SC length	LC length	inter-song-interval	LC up-stroke
χ^2	0.002	1.055	0.828	3.361	3.064	0.034	1.881	0.158
p-value	0.966	0.304	0.363	0.067	0.080	0.855	0.170	0.691

	9	10	11	12	13	14	15	16
	LC down-stroke	LC-SC interval	SC paired pulse length	SC up-stroke	SC down-stroke	SC inter-pulse-interval	1st SC chirp-interval	2nd SC chirp-interval
χ^2	1.675	1.302	0.038	0.822	<0.001	1.531	2.575	4.417
p-value	0.196	0.254	0.845	0.364	0.996	0.216	0.109	0.036

_____

Vestigial singing behaviour persists after the evolutionary loss of song in crickets

Will T. Schneider[1, †], Christian Rutz[1], Berthold Hedwig[2], Nathan W. Bailey[1]

[1]Centre for Biological Diversity, School of Biology, University of St Andrews, St Andrews, Fife KY16 9TH, UK
[2]Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ, UK
[†]Present address: Department of Physiology, Development and Neuroscience, University of Cambridge, Downing Street, Cambridge, CB2 3EJ, UK

Author for correspondence:
Nathan W. Bailey
e-mail: nwb3@st-andrews.ac.uk

Video S1. "*Schneider_et_al_Silent_Singing.mp4*"

A flatwing *T. oceanicus* male exhibits stereotyped forewing movement patterns associated with calling song. A reflective disk is shown attached to the lateral surface of the right forewing, and the video is played back at 0.08x speed.

_____

Vestigial singing behaviour persists after the evolutionary loss of song in crickets

Will T. Schneider[1, †], Christian Rutz[1], Berthold Hedwig[2], Nathan W. Bailey[1]

[1]Centre for Biological Diversity, School of Biology, University of St Andrews, St Andrews, Fife KY16 9TH, UK
[2]Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ, UK
[†]Present address: Department of Physiology, Development and Neuroscience, University of Cambridge, Downing Street, Cambridge, CB2 3EJ, UK

Author for correspondence:
Nathan W. Bailey
e-mail: nwb3@st-andrews.ac.uk