Increased socially-mediated plasticity in gene expression accompanies rapid adaptive evolution

Sonia Pascoal[1*†], Xuan Liu[2†], Yongxiang Fang[2], Steve Paterson[2], Michael G. Ritchie[3], Nichola Rockliffe[2], Marlene Zuk[4] & Nathan W. Bailey[3*]

[1]Department of Zoology, University of Cambridge, CB2 3EJ, U.K.

[2]Centre for Genomic Research, University of Liverpool, Liverpool, L69 7ZB, U.K.

[3]Centre for Biological Diversity, University of St Andrews, St Andrews, KY16 9TH, U.K.

[4]Department of Ecology, Evolution and Behavior, University of Minnesota, St Paul, MN 55108, U.S.A.

Sonia Pascoal: scm77@cam.ac.uk; Xuan Liu: Xuan.Liu@liverpool.ac.uk; Yongxiang Fang: y.fang@liverpool.ac.uk; Steve Paterson: S.Paterson@liverpool.ac.uk; Michael G. Ritchie: mgr@st-andrews.ac.uk; Nichola Rockliffe: N.Rockliffe@liverpool.ac.uk; Marlene Zuk: mzuk@umn.edu; Nathan W. Bailey: nwb3@st-andrews.ac.uk

[*] Correspondence: scm77@cam.ac.uk (+44 (0)1223 334466) or nwb3@st-andrews.ac.uk (+44 (0)1334 463367)

[†] Equal contributors

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Conceived and designed the experiments: NWB, Sonia P, MGR, MZ. Performed the experiments: Sonia P, NR. Analyzed the data: NWB, XL, Sonia P, Steve P, YF. Contributed reagents/materials/analysis/tools: NWB, NR. Wrote the paper: NWB, Sonia P, MGR, MZ.

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Abstract

Recent theory predicts that increased phenotypic plasticity can facilitate adaptation as traits respond to selection. When genetic adaptation alters the social environment, socially-mediated plasticity could cause co-evolutionary feedback dynamics that increase adaptive potential. We tested this by asking whether neural gene expression in a recently arisen, adaptive morph of the field cricket *Teleogryllus oceanicus* is more responsive to the social environment than the ancestral morph. Silent males (flatwings) rapidly spread in a Hawaiian population subject to acoustically-orienting parasitoids, changing the population's acoustic environment. Experimental altering crickets' acoustic environments during rearing revealed broad, plastic changes in gene expression. However, flatwing genotypes showed increased socially-mediated plasticity, while normal-wing genotypes exhibited negligible expression plasticity. Increased plasticity in flatwing crickets suggests a coevolutionary process coupling socially flexible gene expression with the abrupt spread of flatwing. Our results support predictions that phenotypic plasticity should rapidly evolve to be more pronounced during early phases of adaptation.

Introduction

Adaptive mutations are likely to cause correlated phenotypic effects that extend beyond traits directly targeted by selection (Raymond et al. 2001). The fate of a new mutation during establishment and spread will therefore depend on the balance of costs and benefits of those associated effects, and phenotypic plasticity has been proposed as a mechanism that can mitigate the costs. Despite more than a century of debate focusing on how plasticity impacts rates of evolutionary change, the challenge of empirically testing the link between plasticity and the establishment of new mutations has defied resolution (Baldwin 1896; West-Eberhard 2005; Ghalambor *et al*. 2007; Scoville and Pfrender 2010; Stoks *et al*. 2015). An influential model of this process predicts that increased plasticity associated with traits directly affected by abrupt ("extraordinary") changes in selection should evolve over tens of generations, followed by a much longer period during which adaptive, previously plastic, phenotypes become genetically assimilated (Lande 2009). Increased plasticity can also increase the likelihood of adaptive evolutionary responses, even if some of the plasticity is initially counter-selected (Ghalambor *et al*. 2007; 2015).

An overlooked and unresolved question about the relationship between plasticity and rapid adaptive evolution concerns the extended phenotypic consequences of new mutations. Genomic invasion of mutations of large effect can indirectly cause major social changes that provoke plastic phenotypic responses, generating coevolutionary feedback (Bailey 2012). For example, adaptive mutations that affect social behaviour will alter the social environment as they spread, potentially altering the expression of other traits such as aggression or mating behaviour that are sensitive to the social environment (Schradin 2013). Pre-existing plasticity may enable persistence of new mutations with otherwise negative effects, but provided there is sufficient genetic variation for that plasticity, it could also

coevolve with adaptive mutations if they alter the environment that cues plastic responses (West-Eberhard 2005; Lande 2009). This scenario requires only a new genotype under selection that creates environmental feedback, plus genetic variation for plasticity, and it makes testable predictions about how plasticity modulates the rate of evolution.

We tested these predictions by capitalizing on the recent and rapid spread of a male-silencing wing morph in the Pacific field cricket (*Teleogryllus oceanicus*). Silence protects males in Hawaii from attack by an acoustically-orienting parasitoid fly, *Ormia ochracea*, and the phenotype, flatwing, segregates as a Mendelian trait on the X chromosome (Zuk *et al*. 2006; Tinghitella 2008; Pascoal *et al*. 2014). Males who carry flatwing mutation(s) develop wings that are incapable of normal sound production. These flatwing males appeared in 2003 and spread to near-fixation over approximately 20 generations, so dynamics of this system reflect the early stages of rapid adaptive evolution (Zuk *et al*. 2006). Flatwing males are protected from parasitoid attack, but they face difficulty in mate attraction because in this species, male calling song is the only known long-range mating signal and females cannot sing. Male song thus constitutes a dominant component of the social environment, and plasticity mediated by the acoustic environment appears to be advantageous in *T. oceanicus* populations that contain a large proportion of flatwing males. Females reared in environments lacking song are more responsive, which may enable them to compensate for the lack of signalling males by responding more quickly and with less discrimination to the few calling males that remain in the population (Bailey and Zuk 2008). Males reared in silence invest less in reproductive tissues but are more likely to adopt alternative reproductive tactics that increase the likelihood of encountering females (Bailey *et al*. 2010), present decreased immunity (Bailey *et al*. 2011) and show increased locomotion (Balenger and Zuk 2015).

Here, we asked whether enhanced socially-mediated plasticity is associated with the rapidly-evolving flatwing genotype, as theoretical arguments and models predict (West-Eberhard 2005; Lande 2009). We quantified transcriptome plasticity to the social environment in crickets that did or did not carry alleles for flatwing, and tested whether the genotypes respond to the social environment differently. We specifically evaluated the effects of prior social experience during development and maturation, rather than an instantaneous or short-term response as might be activated during mate choice and phonotaxis (Immonen and Ritchie 2012). We focused on longer-term effects of the acoustic environment because such exposure mimics variation that crickets would experience while developing in wild populations dominated by singing normal-wing males or silent flatwing males.

We examined socially-mediated gene expression using tissue derived from cricket heads, which comprised central and peripheral nervous tissues plus associated sensory structures contained within the head capsule, assayed during a relevant developmental interval of adulthood. In crickets, head capsule tissue contains the central brain structures, which themselves contain approximately 100 times more cells than any one of the ganglia distributed along the ventral nerve cord (Schildberger *et al*. 1989). We examined gene expression in tissue contained within head capsules (hereafter referred to as 'neural' or 'brain' tissue for convenience) because we were interested in genes and transcripts that might influence behavioural responses to the acoustic environment. Such responses need not rely exclusively on gene expression in the brain, but the tissue-specificity of our approach allowed us to exclude expression differences that might be associated with downstream effects of the obvious morphological variation between morphs (Zuk *et al*. 2006; Pascoal *et al*. 2014).

Examining neural expression allowed us to bypass difficulties that can arise from selecting and measuring plasticity of traits at the level of organismal phenotype. A growing literature focuses on how genomic approaches to the study of phenotypic plasticity can illuminate causal expression differences underlying plastic responses (Aubin-Horth and Renn 2009), or differential expression arising as a downstream consequence of earlier plastic changes (Aubin-Horth et al. 2005; Nyman et al. 2017). Others have characterized gene expression differences underlying environmentally-induced polyphenisms, as in morphs of the locust *Locusta migratoria* (Wang et al. 2014) or alternative male phenotypes in the bulb mite *Rhizoglyphus robini* (Stuglik et al. 2014). The present study had a different aim: our tests were focused on the prediction that rapid adaptation is facilitated by associated increases in phenotypic plasticity, and we focused on plasticity's relationship with a genetically-determined polymorphism evolving under selection. Thus, we tested whether flatwing and normal-wing genotypes show different neural transcriptome responses to the social environment in *T. oceanicus*, which would provide evidence that transcriptome plasticity to the social environment is coevolving with the segregating trait, flatwing, which directly alters that social environment. Our findings support the theoretical prediction that increased phenotypic plasticity characterizes early stages of rapid adaptation.

Material and methods

Crickets and acoustic environment manipulation

We used 3 replicate lines each of Kauai pure-breeding flatwing and normal-wing *T. oceanicus* to test whether neural gene expression in mutant and normal-wing crickets responds differently to changes in the acoustic environment. The lines were generated through a series of crosses to ensure homozygosity at the locus or loci causing the flatwing genotype

155 (the phenotype segregates as an X-linked, single locus trait), but the lines were not isogenic
156 (Zuk *et al*. 2006; Pascoal *et al*. 2014; Pascoal *et al*. 2016a). Stock crickets were reared in 16 L
157 plastic containers under common garden conditions in a temperature-controlled chamber at
158 25 ^{0}C with a 12:12h light:dark cycle. They were provided with moistened cotton and
159 cardboard egg cartons for shelter and fed Burgess Supa Rabbit Exel Junior and Dwarf rabbit
160 pellets *ad libitum*. When sex differences became apparent, males and females were isolated
161 in 118 mL plastic cups and thereafter reared individually and maintained twice weekly as for
162 the stock crickets. Isolated crickets were randomly assigned to one of four temperature-
163 controlled incubators under two treatments. We adapted previously-described methods
164 (Kasumovic et al. 2011; Thomas et al. 2011; Bailey and Zuk 2012; Bailey and Macleod 2014;
165 Pascoal *et al.* 2016b) to manipulate crickets' perceptions of their acoustic environment. Two
166 incubators were kept in silence ("no song" treatment mimicking a population with few or no
167 normal-wing males) and two incubators playing back two different average Kauai male
168 calling songs simultaneously ("song" treatment) mimicked a population with a high density
169 of singing males. Average calling song parameters were determined from laboratory
170 recordings made at 25 ± 2 °C of n = 24 normal-wing males from a Kauai stock population,
171 and the two average Kauai songs were artificially constructed by excising pulses representing
172 the correct length and carrier frequency from recordings, and manually arranging them into
173 the required pattern of pulse intervals (Table S1). Since *T. oceanicus* are mainly active at
174 night, we played back song only during the dark phase of the crickets' light:dark cycle. All
175 conditions other than the presence or absence of song were kept uniform in the two
176 treatments. Just after adult eclosion, the left wing scrapers were removed from all crickets
177 to prevent singing which would interfere with the silent treatment (flatwing males and

178 females cannot sing but were also clipped to control for confounding effects due to cutting).
179 One week later, cricket tissues were dissected and stored in RNALater at -20 ^{o}C.

180

181 **RNA extractions, library preparation and sequencing**

182 RNA extraction, library preparation and sequencing were performed as described in Pascoal
183 *et al*. (2016a). Briefly, we extracted total RNA from cricket heads (n=48; 3 biological
184 replicates for each sex, morph, social treatment and incubator, Table S2) using TRIzol plus
185 RNA purification kits (Life Technologies) and PureLink DNase treatment (Invitrogen),
186 followed by Qubit (Invitrogen) and Bioanalyser (Agilent) quantification and quality control.
187 We depleted total RNA with RiboZero following the manufacturer's protocol. Purified RNA
188 was checked for depletion and then libraries were constructed using the ScriptSeq protocol
189 (Epicentre). After fragmentation and conversion to cDNA, samples were purified with
190 Ampure XP beads, barcoded, PCR amplified for 14 cycles, and multiplexed. We checked
191 quantity and quality of final pools and performed qPCR using Illumina Library Quantification
192 Kits (Kapa) on a Roche Light Cycler LC480II. Denatured DNA was loaded at 9 pM with 1%
193 fragmented phage PhiX DNA spiked-in, then sequenced on an Illumina HiSeq 2000 (2×100 bp
194 paired end reads).

195

196 **RNA-seq data analysis**

197 Data analysis was conducted following the same pipeline as described in Pascoal *et al*.
198 (2016a). Briefly, CASAVA version 1.8.2 (Illumina), Cutadapt version 1.2.1 (Martin 2011) and
199 Sickle version 1.200 with a minimum window quality score of 20 were used for initial
200 processing and quality control of the data (Table S3). We used Trinity (Grabherr *et al*. 2011)

to create a combined transcriptome assembly using *in silico* normalisation of trimmed read data and a k-mer size of 25bp (Table S4). In common with other transcriptome assemblies, we recovered a large number of contigs and unitigs (Grabherr et al. 2011) (Table S4). These may relate to different isoforms or different exons deriving from the same gene, and differential expression of these transcripts between genes may therefore reflect differences in either transcription or splicing of genes, both of which may be biologically important. Quantification of transcript abundances was done with RSEM (Li and Dewey 2011): reads were mapped to the *de novo* transcriptome assembly using BOWTIE 2 (Langmead and Salzberg 2012), and expected raw read counts for downstream differential expression (DE) analysis were generated using the mapping BAM files. Prior to DE analysis, we applied a minimum expression level filter by only retaining transcripts that had non-zero counts in at least 6 samples, which is the number of samples in a group and thus the minimum number of non-zero samples likely to be biologically informative. It is possible to implement additional filtering by removing transcripts for which expression levels are lower than 1 count per million (cpm) in a specified number of groups; however, this must be balanced against the anti-conservative effect of increasing the false discovery rate when the number of DE transcripts recovered is reduced. We therefore present results based on data filtered as above, but performed additional filtering for the analysis presented in Figure 1 and verified that it does not qualitatively change the main patterns recovered (Fig. S6).

Read numbers mapping to each transcript were modelled with negative binomial error distributions using edgeR (Robinson *et al*. 2010). We implemented generalized linear models (GLMs) containing each of the three factors of interest (sex, morph and acoustic treatment) plus all two-way and three-way interactions. Normalisation factors were calculated to correct for differences in library size among samples, which might otherwise

cause bias in differential gene expression analysis. The "TMM" (Trimmed Mean M-values) method in edgeR (Robinson *et al*. 2010) was applied, with default parameters. Common, trended and tag-wise dispersion parameters were estimated. Tagwise dispersion was used for fold change estimating and significance testing. The estimated $\log_2$ fold change for the each of the models and contrasts were tested in edgeR using a likelihood-ratios (LR) test (Wilks 1938). P-values associated with logFC (log2 fold change) were adjusted for multiple testing such that genes with a false discovery rate (FDR) adjusted P-value < 5% were defined as significantly differentially expressed (Benjamini and Hochberg 1995).

Pairwise comparisons of major interest (i.e. normal-wing male song vs. normal-wing male no song; flatwing male song vs. flatwing male no song; normal-wing female song vs. normal-wing female no song; flatwing female song vs. flatwing female no song; all females vs. flatwing males and all females vs. normal-wing males) were also tested. To visualise whether and how overall patterns of gene expression separated samples by sex, genotype and acoustic treatment, a multidimensional scaling (MDS) plot was drawn using the plotMDS function in edgeR applied to all transcripts. We used Trinotate (trinotate.sourceforge.net/) to annotate the transcriptome and DE sequences and Blast2GO (http://www.blast2go.com) (Conesa *et al*. 2005) to create gene ontology outputs.

Nanostring validation

To validate the RNA-seq data, we used Nanostring technology with a subset of 32 target probes that allowed us to analyse the same 48 samples used for the RNA-seq experiment. Nanostring technology directly obtains sample read count numbers without the need for cDNA synthesis and intermediate PCRs. Each selected probe represents an individual transcript or a group of transcript isoforms with the same gene expression pattern. For the

list of probes to test (nCounter CodeSet) we included: i) gene annotations of interest, ii) transcripts that were simultaneously DE in different contrasts (referred as overlap probes), iii) up- and down-regulated transcripts for each of the individual contrasts and iv) transcripts that were not DE in RNA-seq. 100 ng of total RNA, as quantified by Qubit assay, was used for each hybridization assay in a volume of 5 µl. Hybridisation buffer, reporter CodeSet and Capture probe set was added to each sample and incubated overnight (16-18H) at 65°C, according to manufacturer's instructions. Samples were handled in groups of 12. After hybridization, the samples were washed and loaded onto an nCounter cartridge. Each prepared cartridge was loaded into the counter with the associated CodeSet definition file allowing count generation for each transcript, including the negative and positive controls.

Data analysis was performed using the NanoString software nSolver Analysis Software Version 2.5.34. Background subtraction was done using all internal Nanostring negative controls, normalization was obtained using the internal Nanostring positive controls and 3 reference transcripts that were not DE in the RNA-seq experiment, and fold change ratios were estimated using data partitioning with NormalMaleSong treatment as baseline. Different normalization (just using the internal positive controls) and fold change methods (pairwise) were also tested but did not differ from the previous results. We chose to use the portioned method for fold change analysis because the same baseline was used in the RNA-seq global GLM analysis (dataset upon which the CodeSet selection was based). A direct fold change comparison for the different contrasts (sex, morph and acoustic treatment) between Nanostring and RNA-seq datasets was performed. Regression and paired t-test sample analyses were performed in SPSS Statistics 22.

Results

Neural gene expression

We assembled and characterised *de novo* transcriptomes for *T. oceanicus* (Tables S3-S5), generating a combined assembly to facilitate differential expression (DE) analysis. *T. oceanicus* lacks an annotated reference genome and is distantly-related to commonly employed model insects such as *Drosophila melanogaster*, so we performed expression analyses *de novo* at the level of isoforms. We recovered a characteristically large number of contigs and unitigs as a result, and we collectively refer to these as 'transcripts' for convenience. Our comparisons did not depend on the presence of annotation information, so we utilised the entire set of annotated and unannotated transcripts and followed this with homology-based identity and functional categorization where possible. Nanostring analysis performed on the same 48 samples used for RNA-seq yielded consistent results (see Figs. S1 and S2).

In a model that combined data from all treatments, sex differences accounted for the largest number of differentially-expressed neural transcripts (Fig. 1). Gene expression also differed between flatwing and normal-wing genotypes, and between acoustic treatments (Fig. 1). Gene Ontology (GO) terms associated with the latter group of socially-mediated plastic transcripts included sensory perception of sound, smell, touch; locomotion; and spermatogenesis, which correspond with known behavioural, physiological and morphological responses to the acoustic environment in this species, in particular the tendency of males to strategically allocate sperm resources depending on the perceived presence of rival males (Bailey *et al*. 2010; Gray and Simmons 2013).

Flatwing and normal-wing neural transcriptomes respond differently to the acoustic

environment

There were considerable differences in neural gene expression between flatwing and normal-wing genotypes, and annotations of interest included *rhomboid*, *hedgehog*, and *wingless*. Crucially, the morph genotypes showed different neural gene expression responses to the acoustic treatments. Interaction terms in the global model of gene expression illustrated the latter point: 7,927 transcripts showed different responses across acoustic treatments in males versus females (sex*acoustic treatment interaction), and 6,982 transcripts showed different responses across acoustic treatments in flatwing versus normal-wing crickets (morph*acoustic treatment interaction) (Fig. 1).

The large number of transcripts that showed different patterns of socially-mediated transcriptome plasticity in flatwing versus normal-wing genotypes (Fig. 1) supported the prediction that socially-mediated transcriptome plasticity is coevolving with the genetic mutation(s) that cause flatwing. Given our interest in the differential sensitivity of flatwing and normal-wing crickets to the social environment, we followed up our global analysis of transcriptome variation with individual pairwise contrasts testing differential expression between “song” and “no song” treatments in each of the four classes of cricket: normal-wing and flatwing males and females. This analysis was designed to investigate whether and how sexes and morphs differ in socially-mediated plastic gene expression, and it confirmed our main result: flatwing and normal-wing genotypes show strikingly different patterns of transcriptome plasticity (Fig. 2). Very few transcripts were differentially expressed between acoustic environments in normal-wing crickets, whereas flatwing crickets showed considerable transcriptomic responses to the social environment (Fig. 2, see also Fig. S3).

Thus the dominant pattern underlying transcripts recovered from the morph*acoustic interaction term in the main GLM is differential expression in flatwings

320 across social environments, but little to negligible socially-mediated plasticity in normal-wing
321 crickets. Gene expression also responded differently to the social environment in male
322 versus female neural tissue: there was no overlap of DE transcripts between the sexes. The
323 lack of overlap is in agreement with the finding above that a significant number of
324 transcripts show sexually dimorphic responses to the acoustic environment. While flatwing
325 genotypes showed greater plasticity than normal-wing genotypes ($\chi^2 = 767.30$, df = 1, $p <$
326 0.001), flatwing males showed greater transcriptome sensitivity to the acoustic environment
327 than flatwing females ($\chi^2 = 206.32$, df = 1, $p < 0.001$). The pattern of sex differences was
328 reversed in normal-wing crickets, although this is based on a very small number of DE
329 transcripts recovered in the normal-wing comparison (n = 15 in normal-wing females, versus
330 zero in normal-wing males) ($\chi^2 = 15.00$, df = 1, $p = 0.001$).

331 In pairwise comparisons, only 15 transcripts showed socially-mediated plasticity in
332 normal-wing females. Nevertheless, GO analysis recovered annotations including response
333 to stimulus and locomotion among these, again consistent with prior findings about
334 flexibility in female mate choice and searching behaviours. Flatwing males showed 610
335 differentially expressed transcripts between acoustic treatments and 30% (n=179) had
336 annotations including GO terms such as localization, response to stimulus, signalling,
337 reproduction, reproductive process, and locomotion. Female flatwings had 201 DE
338 transcripts but only 6% (n=12) had associated annotations; this may reflect male-biased
339 availability in public datasets.

340 A final set of analyses tested how morph genotype, acoustic treatment effects and
341 their interaction impacted the transcriptomes of each sex separately. These broadly
342 supported our previous findings, and indicated that although both sexes show expression
343 variation depending on whether they carry flatwing vs. normal-wing alleles, the bulk of

plastic expression variation between morph genotypes appears to be driven by males. We interrogated patterns of socially-mediated plasticity between the morphs in greater detail by performing a clustering analysis of the 5,547 transcripts recovered in the morph*acoustic interaction term in the males-only analysis (Fig. 3). This analysis was only done for males owing to a paucity of differentially-expressed transcripts in females (see Table S6 and Fig. S4). The analysis produced 11 clusters describing differences in the way that gene expression was governed by the social environment in normal-wing versus flatwing males. Overall, expression differences appeared to be more extreme between social environments in flatwing males, although some transcripts showed reversed patterns of socially-mediated plasticity. For example, cluster 1 transcripts were downregulated in the “song” treatment compared to the “no song” treatment in flatwing males, whereas they were upregulated in the “song” treatment in normal-wing males. A similar reversal occurred in the opposite direction in cluster 3. Such patterns exemplify crossing reaction norms. In contrast, transcripts in cluster 7 and 11 appear to be downregulated in the “song” environment in flatwing males, but with little to no differential expression in normal-wing males. An assessment of functional annotations associated with transcripts in each cluster revealed several suggestive patterns related to behavioural phenotypes. For example, both clusters 7 and 11 contained transcripts with GO terms describing locomotor behaviour, and sensory perception of sound was annotated in clusters 7, 9, 10, and 11. Additional behavioural annotations included flight from cluster 6, inter-male aggression from cluster 7, and male courtship from cluster 11.

Nearly half (45%) of the 5,547 transcripts implicated in the male morph*acoustic interaction had an associated annotation. Metabolic and cellular processes were highly represented, and biologically relevant recovered GO terms include response to stimulus,

368 developmental process, reproduction, locomotion, reproductive process, behaviour,
369 immune system process and growth (Fig. S5). These enriched GO terms are suggestive of
370 differences in the mechanisms by which flatwing and normal-wing genotypes respond to
371 acoustic cues in their rearing environment. Previous experiments have provided evidence
372 that each of these processes are affected by exposure to the acoustic environment during
373 development and rearing, providing corroboration for gene expression data, and potential
374 candidates for future study of the functional genomics of socially-mediated plasticity.

375

376 **Transcriptome feminisation and sex differences in plasticity**

377 The nearly 7,000 transcripts identified as significant in the overall sex*morph interaction
378 (Fig. 1) suggested that brain transcriptomes showed different levels of sex-biased expression
379 in the two morphs. A comparison of differential expression between flatwing males versus
380 all females, and between normal-wing males versus all females, revealed that there were
381 fewer sex differences in flatwing male brain transcriptomes compared to normal-wing male
382 brain transcriptomes (Fig. 4a) ($\chi^2 = 2011.79$, df = 1, $p < 0.001$). Flatwing males thus had more
383 female-like patterns of neural gene expression. We used multidimensional scaling (MDS) to
384 plot similarities among samples in expression measured across all transcripts (Fig. 4b). The
385 first and second dimensions separated the sexes and morph genotypes, respectively. As with
386 the previous analysis, flatwing male brain transcriptomes appeared more female-like than
387 those of normal-wing males, but this feminisation was most prominent in flatwing males
388 that had been reared in silence (Fig. 4b). Thus, flatwing males not only showed the greatest
389 degree of transcriptome plasticity in response to acoustic signals in their environment, but
390 their exposure to song appeared to mitigate female-like patterns of gene expression in the
391 brain. Despite the fact that expression of the flatwing phenotype is sex-limited, female

carriers of the flatwing mutation(s) also showed altered neural gene expression compared to normal-wing females. On average, expression patterns differed the most between normal-wing males and flatwing females, although neural expression differences between genotypes were more pronounced in males than in females (Fig. 4b).

The pattern of transcriptome feminisation in flatwing males is consistent with the well-documented female-like venation patterns on their forewings (Zuk *et al*. 2006), and it is notable that both *doublesex* and *fruitless* were identified as differentially expressed between the sexes. However, female-like expression patterns of flatwing brains are not consistent with the idea that the causative mutation(s) underlying flatwing exert effects that are strictly compartmentalised to wing venation. Instead, flatwing and normal genotypes appear to constitutively differ in the expression of brain transcripts, suggesting widespread genomic effects associated with the mutation(s) arising either through pleiotropy, linkage disequilibrium, or coevolution (Pascoal *et al*. 2016a).

Discussion

There is much debate and controversy concerning the role of phenotypic plasticity in evolutionary change, and both adaptive and non-adaptive plasticity have been proposed to increase the likelihood of adaptive evolution (West-Eberhard 2005; Ghalambor *et al*. 2015). Plasticity can create opportunities for divergent selection to act, accelerate responses to selection, pre-adapt populations to respond to novel selective pressures, increase the likelihood of diversification, or deflect the effects of selection (West-Eberhard 1989; West-Eberhard 2003; DeWitt and Scheiner 2004; West-Eberhard 2005; Wund 2012; Zuk *et al*. 2014). These predictions have received mixed empirical support. Comparative work has

415 linked diversity with patterns of ancestral plasticity in spadefoot toad species (Gomez-
416 Mestre and Buchholz 2006), and patterns of plasticity have been found to recapitulate
417 macroevolutionary patterns of trait divergence in *Polypterus*, the ray-finned fishes (Standen
418 *et al*. 2014). Despite the intense interest and focus this topic has received, however,
419 plasticity is often treated as a static property, rather than an evolvable quantity. For
420 example, the idea that pre-existing phenotypic plasticity acts as a pre-adaptation is
421 appealing, and has received support in the cricket system we used here (Bailey *et al*. 2008;
422 Tinghitella *et al*. 2009; Zuk *et al*. 2014), yet we still do not understand how plasticity interacts
423 with traits under selection throughout the ongoing process of adaptive evolution. Our
424 findings in *Teleogryllus oceanicus* reveal a genetic association between a rapidly evolving
425 genotype and plasticity in neural gene expression supporting the view that plasticity itself is
426 subject to evolutionary forces, and, in particular, can increase during the early stages of
427 adaptive evolution in line with theoretical predictions (West-Eberhard 2005; Garland and
428 Kelly 2006; Lande 2009). Box 1 and Fig. 5 provide a graphical description and explanation of
429 this process.

430 Prior work has revealed acoustically-mediated plasticity in a broad spectrum of traits
431 related to mating and reproduction in *T. oceanicus* from the island of Kauai, where alleles
432 causing the erasure of sound-producing structures on male wings have rapidly spread,
433 almost always in a manner that would be expected to increase fitness in a silent
434 environment dominated by silent flatwing males (Zuk *et al*. 2006; Pascoal *et al*. 2014; Zuk *et*
435 *al.* 2014). The constitutive difference in acoustically-mediated plastic gene expression in *T.*
436 *oceanicus* crickets carrying flatwing versus normal-wing genotypes is consistent with the
437 rapid evolution of increased plasticity in neural gene expression in flatwing genotypes –
438 increased plasticity to the acoustic environment accompanied the rapid spread of flatwing.

In contrast, we recovered very few socially-mediated plastic transcripts in crickets carrying normal-wing genotypes; in individual comparisons for normal-wing males, there were none. Flatwings of either sex, however, showed hundreds of transcripts DE between social environments. While it is possible that a single, or very few, transcripts could control responses to the social environment at the phenotypic level in female crickets carrying normal-wing genotypes, for example if some genes within regulatory networks exert greater control over such plasticity than others, they nevertheless exhibited a different pattern of neural transcriptome plasticity than females carrying the recently-derived flatwing genotype. Both the order of magnitude difference in the number of socially-cued DE transcripts between morph genotypes in pairwise comparisons and the existence of nearly a dozen distinct expression clusters in the morph*acoustic environment interaction for males, indicated that numerous genetic modules are implicated in responses to acoustic social cues.

It is unclear whether the socially-mediated plasticity in gene expression we have documented is causally linked to adaptive phenotypic responses. For example, enhanced adaptive plasticity is expected following episodes of rapid adaptation to extreme environmental pressures (Lande 2009), although this may be accompanied by the release of cryptic genetic variation for both adaptive and non-adaptive plasticity (Fischer et al. 2016). In situations where non-adaptive plastic responses to environmental change enhance responses to directional selection by exposing cryptic variation, those plastic responses that persist in newly-adapted populations may be of lower magnitude, but are likely to lie along adaptive phenotypic trajectories (Ghalambor *et al*. 2015, though see Crispo et al. 2010). We note that exposure to song in the acoustic environment of *T. oceanicus* appeared not to change neural transcriptomes in the same direction as morph-associated changes, but instead predominately shifted transcriptome profiles along a sex-biased gene expression axis

463 (x-axis on MDS plot in Fig. 4b) in a male-biased direction.

464 Evidence from other systems suggests that stress responses may represent a
465 frequent underlying mechanism for acoustically-induced expression changes. Acoustically-
466 mediated plasticity has been suggested to facilitate adaptive responses to the presence of
467 signalling rivals in other cricket species (*T. commodus*; Kasumovic et al. 2011) and to
468 anthropogenic noise pollution in birds (the nightingale *Luscinia megarhynchos*; Brumm
469 2004). In *Drosophila melanogaster*, courtship song signals activate stress-related gene
470 expression pathways (Immonen and Ritchie 2012), and in the zebrafish *Danio rerio*, gene
471 expression changes in the inner ear have been linked to recovery from trauma caused by
472 over-exposure to extremely loud (179 dB) stimuli (Schuck et al. 2011). A future objective in *T.*
473 *oceanicus* is therefore to determine whether enhanced brain transcriptome plasticity
474 associated with flatwing genotypes is causally linked to adaptive phenotypic responses,
475 either as a mechanistic driver of those responses or as a consequence of them (Mateus *et al.*
476 2014; Aubin-Horth et al. 2005).

477 We would not have expected a difference in plastic responses of flatwing and
478 normal-wing genotypes if the average genotype in the population had been subjected to
479 similar selection favouring the rapid evolution of socially-mediated plasticity. It appears that
480 the initial spread of flatwing was facilitated by pre-existing plasticity, followed by further
481 differential selection on plasticity in flatwing versus normal-wing genotypes. It is important
482 to note that pre-existing genotypic variation in plasticity is necessary for plasticity to
483 subsequently evolve: the existence of reaction norm variation prior to dramatic
484 environmental change favouring increased plasticity is a key assumption of the Lande (2009)
485 model. There is evidence for such reaction norm variation in *T. oceanicus* (Bailey and Zuk
486 2012), and it seems likely that the different morphs experience distinct selective pressures

because of the differences in both parasitoid attack rates and mating tactics employed by either type of male (Zuk *et al*. 2006). Because of the short timeframe in which the evolution and spread of flatwing has taken place, the difference in plasticity between flatwing versus normal-wing genotypes strongly suggests a pleiotropic effect of flatwing allele(s) or loci maintained in linkage disequilibrium. Rapid evolution of *de novo* physical linkage is an unlikely scenario. Two intriguing possibilities are that both morphs may demonstrate plasticity at the level of observable reproductive or physiological phenotypes, yet be subject to different environmental triggers or neurogenomic mechanisms of socially-mediated plasticity, or that selection has favoured canalized responses to the social environment in normal-wing genotypes, with correspondingly different consequences for plastic changes in the brain transcriptome (Cardoso *et al.* 2015).

The constitutive differences in how flatwing and normal-wing transcriptomes respond to cues in the social environment support key theoretical predictions about the coevolution of plasticity with novel adaptations. Lande (2009) and others (West-Eberhard 2005; Garland and Kelly 2006) predict a rapid evolutionary increase in plasticity at the onset of dramatic environmental changes. In Hawaiian *T. oceanicus*, the acoustic environment underwent an abrupt and profound change because of the rapid spread of silent males: in the span of several dozen generations, the population on Kauai shifted from one in which long-range acoustic signals were the dominant mode of social communication, to a population effectively depauperate in song (Zuk *et al*. 2006). Feedback between the rapid change from a song-rich to a silent environment, and plasticity in response to the acoustic environment, appears to have created a situation favourable for the rapid coevolution of socially-cued plasticity and alleles that cause the silent flatwing phenotype. Over time, genetic assimilation is predicted to more permanently link these traits, but it is likely to

511 occur on the order of hundreds to thousands of generations, not dozens (Box 1) (Lande
512 2009). Similar feedback effects are pervasive in evolving systems (Crespi 2004), and the
513 relationship between flatwing and transcriptome plasticity in *T. oceanicus* demonstrates
514 how the general impact of phenotypic plasticity on evolutionary change in other systems is
515 likely to be inextricably linked to its own coevolution with traits under selection.

516

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525

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Figures

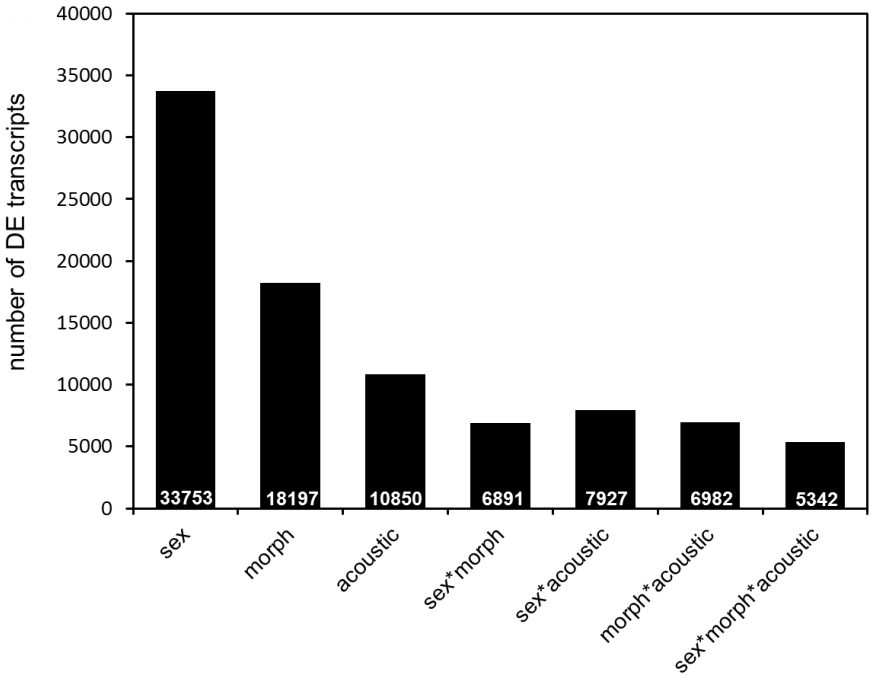
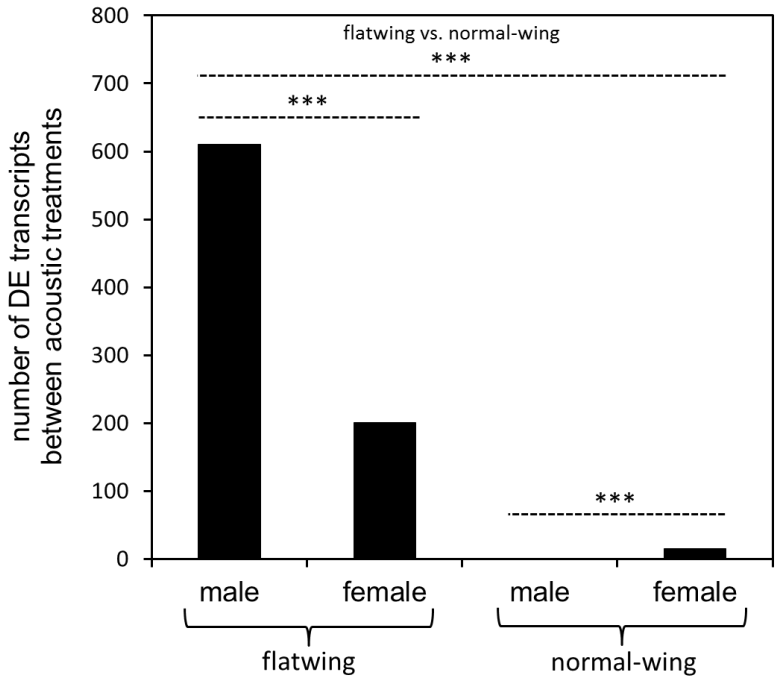


Figure 1 Differential transcript expression in cricket neural tissue. Expression differences were inferred using generalized linear models (GLMs). The bars show numbers (given in white text) of transcripts that were DE between sexes, between wing morphs, and between acoustic treatments. Interaction terms indicate transcripts whose differential expression was not heterogeneous, i.e. not in the same direction or magnitude in different groups.



670

671 **Figure 2 Socially-mediated plasticity in gene expression is constitutively different between**
672 **morph genotypes.** The number of differentially-expressed transcripts in the brains of adult
673 crickets that had been reared in song vs. silence is indicated for each morph and sex.
674 Differential expression was separately assessed for each of the four types of crickets using
675 pairwise comparisons between the “song” and “no song” acoustic treatment groups.
676 Asterisks highlight significant differences in the proportion of differentially expressed
677 transcripts for the comparisons indicated (Chi-square tests using a total of n = 1,545,564
678 observations for all groups. All $p < 0.001$ after Bonferroni correction at $\alpha = 0.0003$

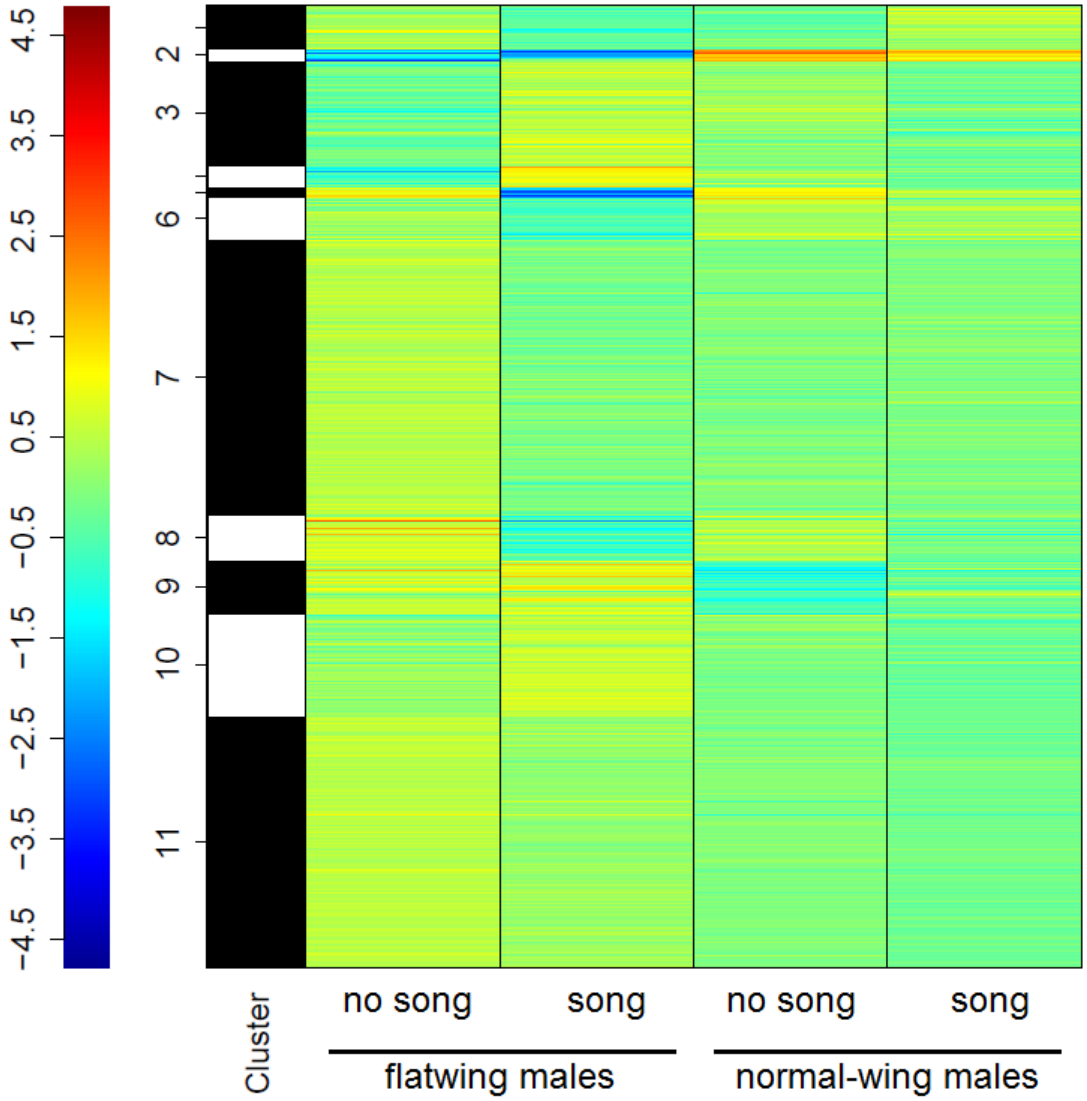


Figure 3 Comparison of socially-mediated gene expression in flatwing vs. normal-wing males. Transcripts whose expression was significant in the morph*acoustic interaction of the male-specific expression analysis are depicted. The significance of the interaction term indicated that the two morph genotypes regulate expression of that transcript differently in response to the acoustic environment. Transcripts are grouped into 11 clusters describing similar patterns of socially-mediated plasticity. The color gradient represents the difference in log2 fold change compared to the across-treatment average, with larger values (red) indicating up-regulation, and smaller values (blue) indicating down regulation. For each gene, data from all samples are zero-centred to facilitate visual interpretation.

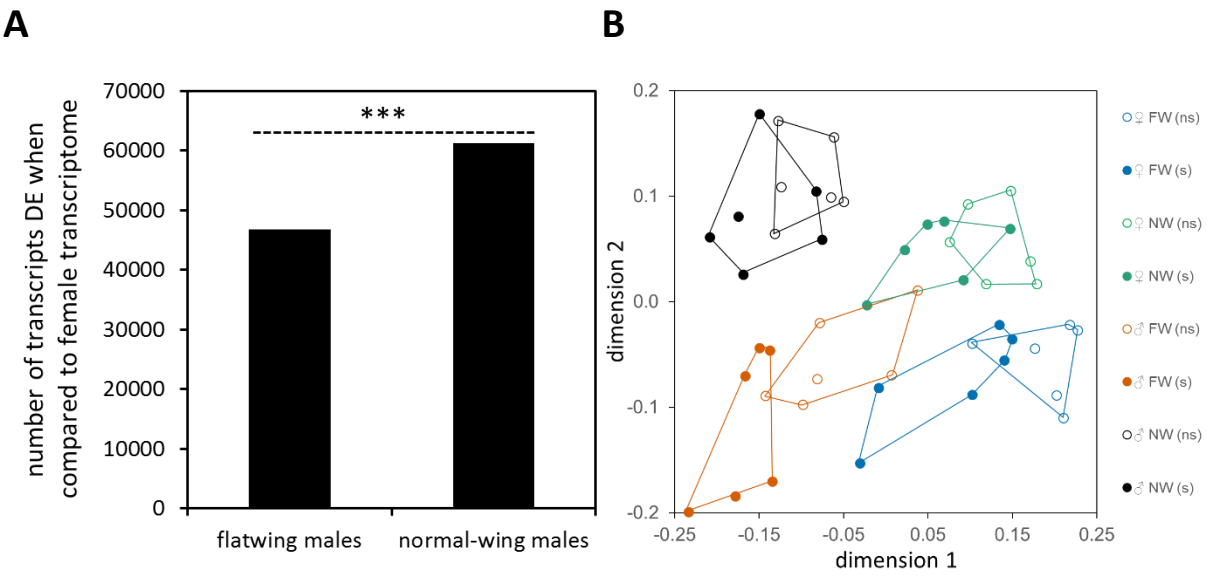
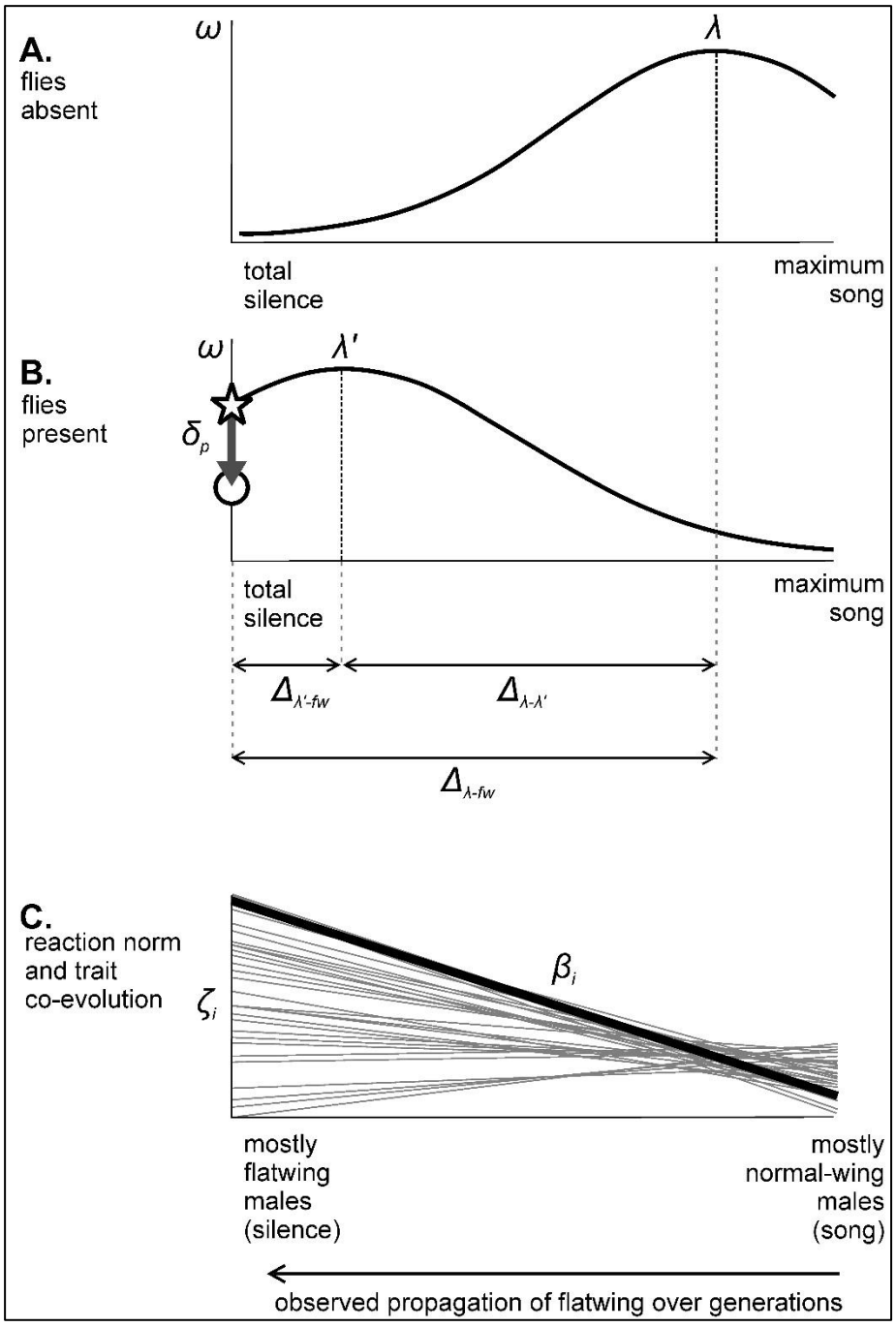


Figure 4 Neural transcriptomes are feminised in flatwing males. (a) Number of transcripts differentially expressed between flatwing males versus all females and between normal-wing males versus all females. Greater similarity between flatwing males and females than between normal-wing males and females indicates transcriptional feminisation of flatwing male neural tissue; asterisks indicate a significant difference ($\chi^2 = 2011.79$, df = 1, $p < 0.001$). **(b)** Multidimensional scaling (MDS) plot showing overall patterns of neural gene expression in each of the 48 samples, for all mapped transcripts. Open symbols represent crickets reared in silence and solid symbols represent those reared with song. Polygons have been drawn to enclose all the replicates of each type of cricket. The factors “sex”, “morph”, and “acoustic treatment” explain 8%, 4%, and 3% of the total variation (Bray distance) in transcriptome profiles, respectively.



704

705 **Figure 5 Schematic illustration depicting coevolution between phenotypic plasticity and a**

706 **novel adaptive phenotype, as described in Box 1.** Panels (a)-(c) illustrate a scenario of rapid

707 evolution of male silence in *Teleogryllus oceanicus*, and a hypothetical role for plasticity

708 based on Lande 2009.

709

Box 1. Rapid coevolution of socially-mediated plasticity and a trait under selection. The evolutionary loss of male song in *Teleogryllus oceanicus* is used as an example (Figure 5).

[A] Hypothetical Gaussian fitness function for male singing tendency in an ancestral environment. The y-axis represents relative male fitness (ω), which depends on how much males sing (x-axis). Song is advantageous owing to its role in mate attraction, courtship and aggression, but energetic and mechanical constraints reduce male fitness beyond an optimal level of song production, λ (Fig. 5A).

[B] Shift of the optimal male singing tendency when acoustically-orienting parasitoids are present. The y-axis still represents relative male fitness (ω) and the x-axis how much males sing. Song still functions in mate acquisition and thus carries a sexually selected benefit. However, optimal levels of male song production are now lower (λ') because of countervailing natural selection exerted by fatal parasitoids that use it to locate hosts. The shift in optimum male phenotype along the x-axis is indicated by $\Delta_{\lambda-\lambda'}$, and can be conceptualised as selection on quantitative variation underlying the tendency to sing, by forcing a shift in the distribution of singers vs. non-singers in the population or alternatively through a change in average behaviour across males. Early field studies found support for the latter (Cade 1975; Zuk *et al*. 1993; Rotenberry *et al.* 1996; Zuk *et al*. 1998). Despite the benefits of song reduction, complete cessation of singing still carries costs, for example because of the need to acquire mates via other means (Bailey *et al.* 2010, Rotenberry *et al.* 2015) and poorer performance in agonistic encounters (Logue *et al*. 2010).

The star indicates the phenotype of obligately silent flatwing males. The invasion of flatwing allele(s) into the population marks the emergence of a new, discrete phenotype favoured because it places males closer to the optimal phenotype when flies are present. If there were no flies, the flatwing male phenotype would carry a severe cost owing to its distance from the population optimum, $\Delta_{\lambda-fw}$, yet when flies are present it clearly confers an advantage despite having "overshot" the optimal phenotype, $\Delta_{\lambda'-fw}$. Flatwing is also known to cause a range negative pleiotropic effects in males that express it: they cannot advertise for or court females, and they experience dysfunction in agonistic encounters (Zuk *et al*. 2006; Bailey *et al*. 2008; Logue *et al*. 2010). Flatwing males also have reduced investment in reproductive tissues (Bailey *et al*. 2010) and partially-feminised cuticular hydrocarbon profiles (unpublished data). The fitness decrement due to negative pleiotropy in flatwing males, δ_p, is indicated by the solid grey arrow, which shows how the potential maximum fitness benefits of flatwing (star) exceed the realised fitness benefits (circle). Plasticity to the changed signalling environment caused by the spread of silent flatwing males is known to enable males to mitigate consequences of obligate silence, reducing the fitness decrement δ_p associated with flatwing (Fig. 5B).

[C] Evolution of phenotypic plasticity during "extraordinary" environmental change caused by proliferation of silent flatwing males. Here, the y-axis represents a generic trait ζ_i that mitigates negative pleiotropic effects of flatwing by responding to the acoustic social environment—for example, the tendency of males to adopt satellite mating tactics. The x-axis now represents the proportion of flatwing males present in the population, which determines the amount of song present within the environment. Here, we consider the shift towards a silent social environment an "extraordinary" environmental change, cf. Lande (2009). An optimal reaction norm with slope β_i is indicated by the thick line, and selection

will favour individuals expressing phenotypes close to this line. If there is genetic variation for plasticity, for example as a result of past environmental stochasticity caused by demographic fluctuations or environmental signal interference (indicated by "silence" and "song" in parentheses on the x-axis), then reaction norms for individual genotypes are predicted to be distributed as indicated by the light grey lines, with little genetic variance available to selection under ordinary environmental circumstances that characterise populations rich in singing, normal-wing males, but with increasing exposure of cryptic genetic variation as the social environment shifts due to the proliferation of flatwing males (Gibson and Dworkin 2004). As the environment changes (following the lower arrow from right to left along the x-axis), phenotypes that mitigate negative effects of flatwing (i.e. reducing δ_p) will be positively selected, favouring reaction norms with increasingly large slopes β. Short-term reaction norm evolution over a timescale of tens to hundreds of generations is expected to be rapid, whereas a longer period of genetic assimilation is predicted to occur subsequently over many thousands of generations (Lande 2009). The evolution of flatwing crickets in Hawaii is very recent as they appear to have arisen approximately 15 years ago, thus the rapid spread of flatwings represents the earliest phase of this process (Zuk *et al.* 2006) (Fig. 5C). Figure based on Lande (2009) (Fig. 1).

Supplementary Material

Supplemental Tables

Table S1. Average calling song parameters (10 songs per individual) recorded from 24 normal-wing males from a laboratory Kauai stock population. Grand means used to construct song playbacks are indicated in bold on the bottom row. *T. oceanicus* song consists of a trill-like series of higher-amplitude pulses (commonly referred to as the "long chirp") followed by a series of lower-amplitude pulses typically clustered into pairs, the "short chirp" (Bailey and Macleod 2013). LC: long chirp; SC: short chirp; IPI: inter-pulse interval; ICI: inter-chirp interval.

ID	# long chirps	# short chirps	total duration	carrier freq.	LC total duration	LC pulse duration	LC pulse interval	LC - SC interval	SC chirp duration	SC pulse duration	SC IPI	SC ICI	SC total duration	inter-song interval
W1	4.9	6.5	1.207	5030.8	0.276	0.034	0.024	0.062	0.068	0.027	0.012	0.069	0.867	0.087
W2	6.6	7.2	1.563	4739.6	0.389	0.039	0.028	0.079	0.078	0.030	0.015	0.085	1.093	0.078
W3	4.1	4.1	0.737	5206.1	0.212	0.030	0.028	0.047	0.066	0.031	0.009	0.064	0.478	0.061
W4	6.0	4.1	0.936	4809.6	0.344	0.031	0.032	0.065	0.070	0.029	0.013	0.078	0.525	0.087
W5	5.6	6.0	1.210	4646.6	0.311	0.034	0.030	0.066	0.060	0.029	0.011	0.082	0.833	0.098
W6	5.9	6.8	1.289	5052.3	0.317	0.032	0.029	0.065	0.058	0.022	0.014	0.081	0.944	0.091
W7	4.7	4.4	0.925	4811.6	0.277	0.037	0.026	0.057	0.070	0.023	0.014	0.063	0.589	0.064
W11	5.5	6.0	1.281	4751.4	0.325	0.040	0.021	0.072	0.079	0.033	0.017	0.081	0.886	0.125
W12	5.9	5.6	1.170	4976.4	0.347	0.038	0.022	0.063	0.067	0.029	0.010	0.070	0.761	0.085
W13	5.8	7.0	1.462	4856.7	0.344	0.034	0.026	0.068	0.064	0.027	0.010	0.079	1.050	0.095
W14	6.5	8.8	1.908	5051.0	0.400	0.038	0.024	0.056	0.069	0.035	0.010	0.088	1.450	0.098
W15	6.4	8.0	1.500	4741.7	0.357	0.029	0.030	0.068	0.064	0.025	0.009	0.068	1.077	0.086
W16	5.2	4.1	0.899	5363.5	0.326	0.035	0.029	0.061	0.073	0.027	0.014	0.065	0.511	0.093
W17	5.2	5.0	0.988	4758.4	0.316	0.039	0.022	0.048	0.071	0.031	0.009	0.057	0.620	0.062
W18	5.7	3.6	0.888	4821.9	0.346	0.037	0.029	0.062	0.081	0.033	0.013	0.064	0.479	0.069
W19	5.1	5.7	1.234	4838.4	0.327	0.038	0.032	0.071	0.079	0.037	0.012	0.078	0.835	0.093
W20	7.3	8.5	1.883	4655.8	0.448	0.033	0.036	0.080	0.076	0.028	0.013	0.080	1.356	0.068
W21	7.9	6.4	1.418	5576.2	0.467	0.036	0.021	0.055	0.072	0.032	0.007	0.071	0.896	0.086
W22	6.2	6.8	1.312	4965.7	0.385	0.042	0.022	0.056	0.076	0.037	0.008	0.064	0.876	0.098
W23	5.8	6.1	1.328	5051.9	0.340	0.073	0.023	0.065	0.071	0.030	0.009	0.092	0.922	0.120
W24	7.6	5.2	1.157	5008.9	0.428	0.037	0.020	0.055	0.072	0.032	0.009	0.067	0.673	0.100
W26	5.3	5.9	1.208	4810.9	0.303	0.039	0.015	0.055	0.068	0.030	0.007	0.074	0.850	0.095
W27	5.1	8.6	1.583	4659.1	0.285	0.034	0.026	0.067	0.072	0.029	0.011	0.072	1.231	0.090
W28	5.8	6.8	1.464	4740.6	0.337	0.035	0.022	0.076	0.077	0.034	0.012	0.081	1.052	0.102
grand mean	**5.8**	**6.1**	**1.273**	**4913.5**	**0.342**	**0.037**	**0.026**	**0.063**	**0.071**	**0.03**	**0.011**	**0.074**	**0.869**	**0.089**

790 **Table S2.** Experimental design summary.

sex	morph genotype	acoustic treatment	incubator	biological replicates
males	normal-wing	song	A	3
			B	3
		no song	C	3
			D	3
	flatwing	song	A	3
			B	3
		no song	C	3
			D	3
females	normal-wing	song	A	3
			B	3
		no song	C	3
			D	3
	flatwing	song	A	3
			B	3
		no song	C	3
			D	3

791

Table S3. Summary of raw and trimmed sequence data. The proportion of trimmed reads that were unpaired after trimming was generally low (<1%), indicating that the data were of good quality.

Sample	Index	Pool	Untrimmed reads	Trimmed reads	R1/R2 pairs	R0 reads (%)[1]
Sample_65_Normal21_4	ATCACGAT-TCTTTCCC	m10	51213110	50388088	24948575	490938 (0.97)
Sample_66_Normal6_2	CGATGTAT-TCTTTCCC	m10	45735160	45209613	22,385,500	438,613 (0.97)
Sample_67_Normal8_3	TTAGGCAT-TCTTTCCC	m10	53038412	52423136	25,947,709	527,718 (1.01)
Sample_68_Normal6_20	TGACCAAT-TCTTTCCC	m10	62684826	61589560	30,521,397	546,766 (0.89)
Sample_69_Normal8_12	ACAGTGAT-TCTTTCCC	m10	51146242	50240263	24,869,694	500,875 (1.00)
Sample_70_Normal21_11	GCCAATAT-TCTTTCCC	m10	47476394	43246580	21,409,818	426,944 (0.99)
Sample_71_Normal6_14	CAGATCAT-TCTTTCCC	m10	55389280	53792333	26,621,039	550,255 (1.02)
Sample_72_Normal8_16	ACTTGAAT-TCTTTCCC	m10	63735242	62889776	31,101,990	685,796 (1.09)
Sample_73_Normal21_13	GATCAGAT-TCTTTCCC	m10	39795704	39292576	19,426,286	440,004 (1.12)
Sample_74_Normal6_8	TAGCTTAT-TCTTTCCC	m10	39213464	38706053	19,178,113	349,827 (0.90)
Sample_75_Normal8_5	GGCTACAT-TCTTTCCC	m10	40659330	40193367	19,878,742	435,883 (1.08)
Sample_76_Normal21_19	CTTGTAAT-TCTTTCCC	m10	52701328	51845955	25,671,123	503,709 (0.97)
Sample_77_FW11_3	AGTCAAAT-TCTTTCCC	m11	43404602	43103150	21,409,018	285,114 (0.66)
Sample_78_FW17_1	AGTTCCAT-TCTTTCCC	m11	33319756	33046763	16,392,856	261,051 (0.79)
Sample_79_FW23_1	ATGTCAAT-TCTTTCCC	m11	38747894	38381221	19,030,316	320,589 (0.84)
Sample_80_FW11_5	CCGTCCAT-TCTTTCCC	m11	43424404	43095693	21,415,093	265,507 (0.62)
Sample_81_FW17_5	GTAGAGAT-TCTTTCCC	m11	35454676	35145354	17,429,104	287,146 (0.82)
Sample_82_FW23_6	GTGAAAAT-TCTTTCCC	m11	35627960	35253705	17,471,064	311,577 (0.88)
Sample_83_FW11_9	GTGGCCAT-TCTTTCCC	m11	48753966	48021554	23,852,141	317,272 (0.66)
Sample_84_FW17_18	CGTACGAT-TCTTTCCC	m11	46751338	46357206	23,015,914	325,378 (0.70)
Sample_85_FW23_9	GAGTGGAT-TCTTTCCC	m11	39954704	39522528	19,618,474	285,580 (0.72)
Sample_86_FW11_15	CACCGGAT-TCTTTCCC	m11	43454276	43070149	21,375,323	319,503 (0.74)
Sample_87_FW17_16	CACGATAT-TCTTTCCC	m11	50661862	50097667	24,870,404	356,859 (0.71)
Sample_88_FW23_14	TCCCGAAT-TCTTTCCC	m11	47736978	47339870	23,497,415	345,040 (0.73)
Sample_89_Normal6_21	ATCACGAT-TCTTTCCC	m12	48,182,384	47,798,219	23,711,773	374,673 (0.78)
Sample_90_Normal8_1	CGATGTAT-TCTTTCCC	m12	53,957,708	53,488,753	26,530,364	428,025 (0.80)

Sample_91_Normal21_17	TTAGGCAT-TCTTTCCC	m12	45,211,642	44,737,241	22,165,232	406,777 (0.91)
Sample_92_Normal6_18	TGACCAAT-TCTTTCCC	m12	51,896,536	51,385,601	25,508,876	367,849 (0.72)
Sample_93_Normal8_5	ACAGTGAT-TCTTTCCC	m12	51,634,454	50,965,943	25,297,584	370,775 (0.73)
Sample_94_Normal21_18	GCCAATAT-TCTTTCCC	m12	42,417,674	41,797,660	20,735,523	326,614 (0.78)
Sample_95_Normal6_12	CAGATCAT-TCTTTCCC	m12	50,339,926	49,856,365	24,735,866	384,633 (0.77)
Sample_96_Normal8_11	ACTTGAAT-TCTTTCCC	m12	44,997,936	44,361,627	22,008,624	344,379 (0.78)
Sample_97_Normal21_10	GATCAGAT-TCTTTCCC	m12	38,004,450	37,638,634	18,675,819	286,996 (0.76)
Sample_98_Normal6_14	TAGCTTAT-TCTTTCCC	m12	37,369,320	36,638,383	18,167,764	302,855 (0.83)
Sample_99_Normal8_14	GGCTACAT-TCTTTCCC	m12	28,662,486	28,110,963	13,930,418	250,127 (0.89)
Sample_100_Normal21_13	CTTGTAAT-TCTTTCCC	m12	34,753,666	34,101,236	16,909,101	283,034 (0.83)
Sample_101_FW11_2	AGTCAAAT-TCTTTCCC	m13	41,281,256	40,713,985	20,234,480	245,025 (0.60)
Sample_102_FW17_1	AGTTCCAT-TCTTTCCC	m13	43,094,538	42,818,603	21,289,617	239,369 (0.56)
Sample_103_FW23_1	ATGTCAAT-TCTTTCCC	m13	39,692,492	39,412,319	19,581,222	249,875 (0.63)
Sample_104_FW11_6	CCGTCCAT-TCTTTCCC	m13	42,186,768	41,753,255	20,748,837	255,581 (0.61)
Sample_105_FW17_8	GTAGAGAT-TCTTTCCC	m13	41,076,258	40,742,216	20,252,790	236,636 (0.58)
Sample_106_FW23_7	GTGAAAAT-TCTTTCCC	m13	42,621,620	42,178,361	20,970,276	237,809 (0.56)
Sample_107_FW11_11	GTGGCCAT-TCTTTCCC	m13	38,435,250	38,101,992	18,909,722	282,548 (0.74)
Sample_108_FW17_17	CGTACGAT-TCTTTCCC	m13	32,553,664	32,319,194	16,046,684	225,826 (0.70)
Sample_109_FW23_11	GAGTGGAT-TCTTTCCC	m13	40,169,036	39,704,569	19,729,851	244,867 (0.62)
Sample_110_FW11_13	CACCGGAT-TCTTTCCC	m13	41,294,536	40,916,496	20,314,203	288,090 (0.70)
Sample_111_FW17_16	CACGATAT-TCTTTCCC	m13	43,717,190	43,338,556	21,519,773	299,010 (0.69)
Sample_112_FW23_13	TCCCGAAT-TCTTTCCC	m13	41,002,624	40,034,832	19,876,536	281,760 (0.70)

[1] % of all trimmed reads

Table S4. Assembly metrics for *de novo* transcriptome constructed using data from 48 sequencing libraries.

Assembled sequences	**Size range**	**Mean length**	**N50** [1]	**Number of sequences** **<500 bp**	**Number of sequences** **500 bp – 5 kb**	**Number of sequences** **5 kb – 10 kb**	**Number of sequences** **10 kb – 50 kb**	Trinity "genes" with a single "isoform"	Trinity "genes" with multiple "isoforms" (%) [2]
1,545,564	201-20,090	543	712	1,071,927	471,717	1,873	47	1,378,017	167,547 (13.34%)

[1] N50 = 50% of bases occur in sequences of this size or larger

[2] Calculated as the number of "Components and subcomponents" associated with >1 "seq" (% of total "Components and subcomponents"; n=1,256,357)

Table S5. Summary of trimmed sequence data mapping against *de novo* transcriptome assembly.

Sample	R1+R2 reads (trimmed)	R1+R2 reads mapped to the assembly	% R1+R2 reads mapped to the assembly
Sample_65_Normal21_4	49,897,150	38,105,620	76.37%
Sample_66_Normal6_2	44,771,000	34,452,978	76.95%
Sample_67_Normal8_3	51,895,418	39,197,006	75.53%
Sample_68_Normal6_20	61,042,794	47,335,750	77.55%
Sample_69_Normal8_12	49,739,388	37,080,698	74.55%
Sample_70_Normal21_11	42,819,636	32,965,310	76.99%
Sample_71_Normal6_14	53,242,078	40,366,564	75.82%
Sample_72_Normal8_16	62,203,980	47,127,042	75.76%
Sample_73_Normal21_13	38,852,572	28,302,580	72.85%
Sample_74_Normal6_8	38,356,226	29,574,182	77.10%
Sample_75_Normal8_5	39,757,484	30,169,664	75.88%
Sample_76_Normal21_19	51,342,246	38,445,788	74.88%
Sample_77_FW11_3	42,818,036	32,830,286	76.67%
Sample_78_FW17_1	32,785,712	24,668,246	75.24%
Sample_79_FW23_1	38,060,632	28,749,404	75.54%
Sample_80_FW11_5	42,830,186	33,162,330	77.43%
Sample_81_FW17_5	34,858,208	25,838,696	74.13%
Sample_82_FW23_6	34,942,128	26,527,616	75.92%
Sample_83_FW11_9	47,704,282	37,237,598	78.06%
Sample_84_FW17_18	46,031,828	35,899,416	77.99%
Sample_85_FW23_9	39,236,948	30,365,624	77.39%
Sample_86_FW11_15	42,750,646	33,422,012	78.18%
Sample_87_FW17_16	49,740,808	38,280,754	76.96%
Sample_88_FW23_14	46,994,830	36,001,654	76.61%
Sample_89_Normal6_21	47,423,546	37,130,662	78.30%
Sample_90_Normal8_1	53,060,728	41,761,678	78.71%
Sample_91_Normal21_17	44,330,464	34,546,178	77.93%
Sample_92_Normal6_18	51,017,752	39,657,376	77.73%
Sample_93_Normal8_5	50,595,168	39,503,388	78.08%
Sample_94_Normal21_18	41,471,046	32,403,190	78.13%
Sample_95_Normal6_12	49,471,732	38,614,454	78.05%
Sample_96_Normal8_11	44,017,248	34,804,912	79.07%
Sample_97_Normal21_10	37,351,638	29,378,420	78.65%
Sample_98_Normal6_14	36,335,528	28,426,270	78.23%
Sample_99_Normal8_14	27,860,836	21,636,444	77.66%
Sample_100_Normal21_13	33,818,202	26,369,794	77.98%
Sample_101_FW11_2	40,468,960	32,288,476	79.79%
Sample_102_FW17_1	42,579,234	33,197,186	77.97%
Sample_103_FW23_1	39,162,444	30,570,240	78.06%
Sample_104_FW11_6	41,497,674	32,508,230	78.34%
Sample_105_FW17_8	40,505,580	31,198,968	77.02%
Sample_106_FW23_7	41,940,552	32,857,162	78.34%

Sample_107_FW11_11	37,819,444	29,694,136	78.52%
Sample_108_FW17_17	32,093,368	24,838,774	77.40%
Sample_109_FW23_11	39,459,702	31,028,792	78.63%
Sample_110_FW11_13	40,628,406	32,040,942	78.86%
Sample_111_FW17_16	43,039,546	34,247,024	79.57%
Sample_112_FW23_13	39,753,072	31,194,008	78.47%

Table S6. Generalized linear models performed separately for each sex: summary of DE transcripts by contrast.

GLM by Sex	**Males**			**Females**		
Contrasts	**DE**	**DE_Up**	**DE_Down**	**DE**	**DE_Up**	**DE_Down**
morph (ref. FW)	16586	4737	11849	463	278	185
acoustic treatment (ref. No Song)	6225	4817	1438	3	1	2
morph x acoustic treatment	5547	4099	1488	2	0	2

Supplemental Figures and Results

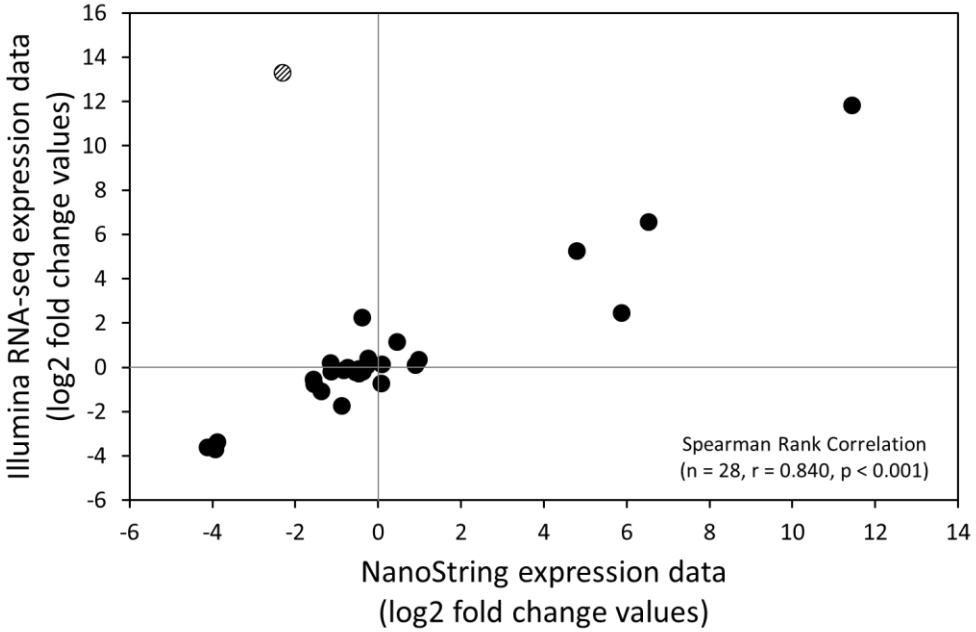


Figure S1. Comparison of RNA-seq and NanoString expression analyses. Log2 fold change values comparing expression between sexes are indicated for each technique. The three control transcripts that were not DE in the RNA-seq experiment are excluded from the plot, because they did not have associated log2 fold change values. The hatched datapoint represents a failed NanoString probe (see Fig. S2 for details). Results of a Spearman Rank correlation using the remaining data are shown inset. Paired samples t-tests were also performed for expression data from each technique by the three main contrasts, which similarly found no evidence for an overall difference in expression patterns quantified using the two techniques (Sex: n = 28, t = -1.150, p = 0.260 excluding one probe that malfunctioned; n = 29, t = -1.333, p = 0.193 if it is included. Morph: n = 29, t = 0.258, p = 0.798. Acoustic treatment: n = 29, t = -0.184, p = 0.855).

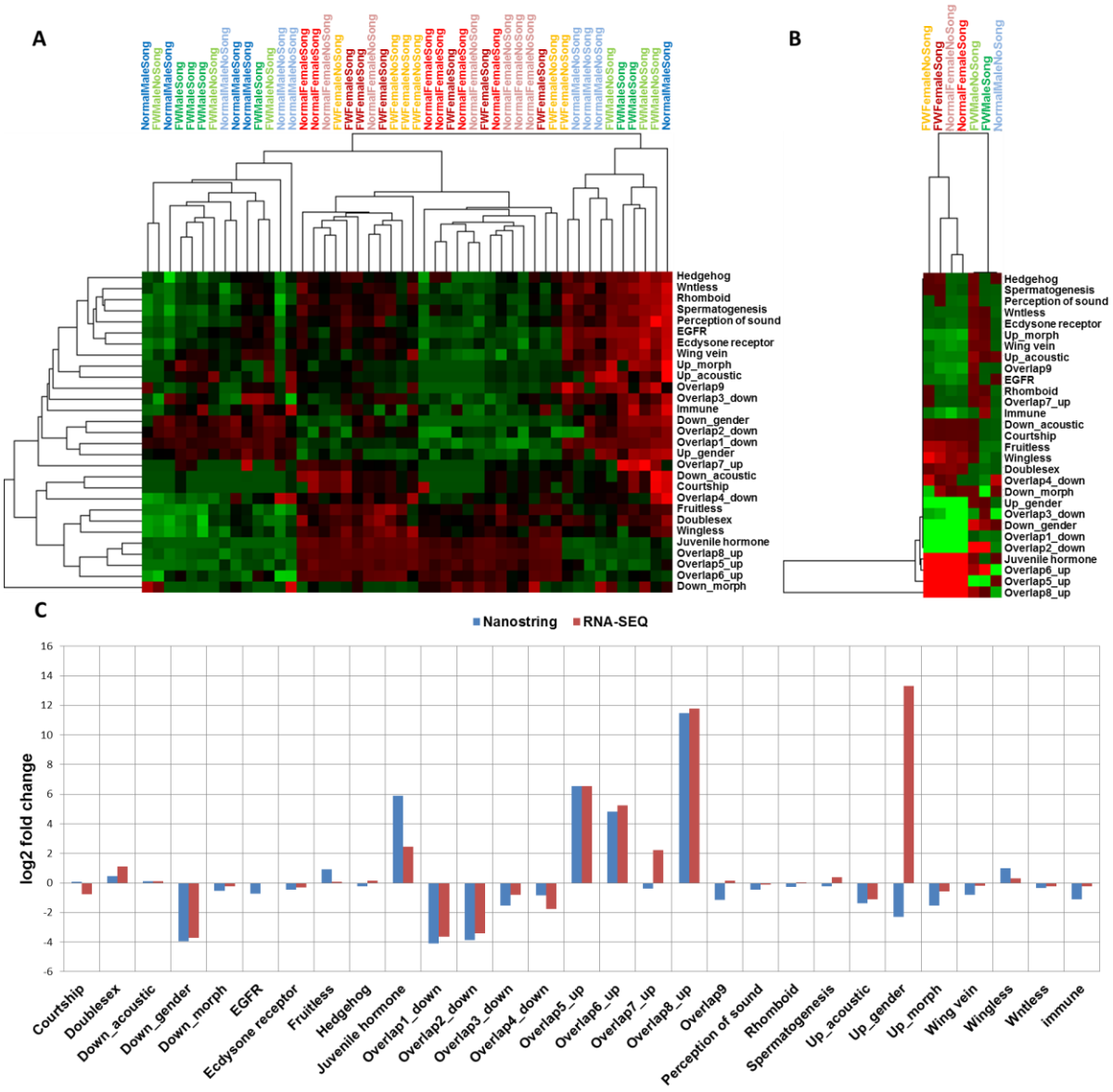


Figure S2. Nanostring validation data based on 32 probes. (A) Normalized Nanostring data and (B) fold change ratio agglomerative cluster heatmaps. Background subtraction was done using all the negative controls, normalization was obtained using the positive controls and 3 reference transcripts that were not DE in the RNA-seq experiment and fold change ratios were estimated using data partitioning with normal-wing male song treatment as baseline. (C) Fold change comparison between sexes for Nanostring (blue) and RNA-seq (red) datasets for the tested probes. Note that these exclude the three negative controls, as they lacked associated log2FC values. The data for the "Up_gender" probe corresponds to the outlier in Figure S1. The overall low number of read counts for this probe across samples indicated that it likely represented a failed Nanostring probe. Such failures may arise when the folding structure of a transcript prevents access to the probe during annealing.

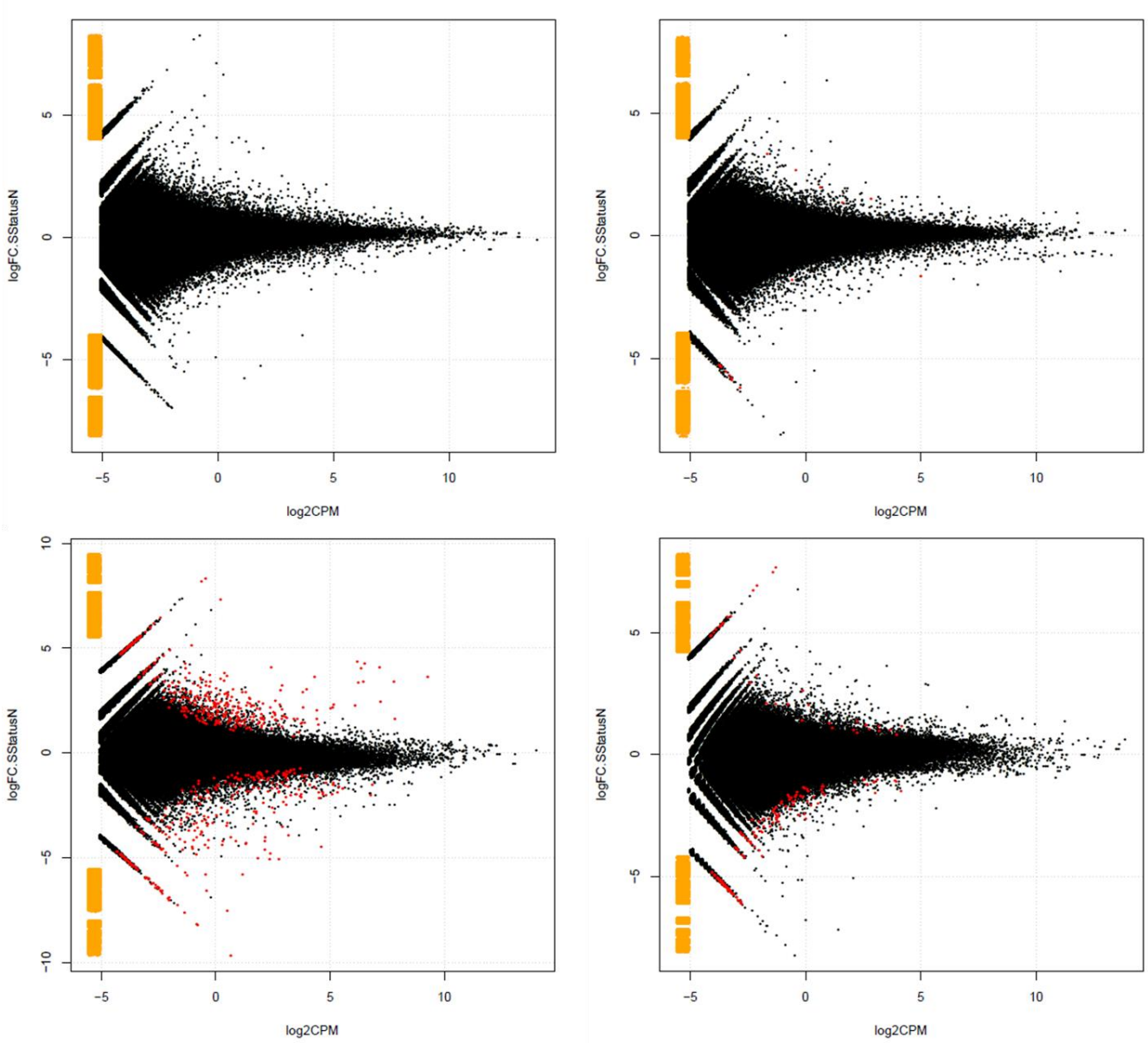
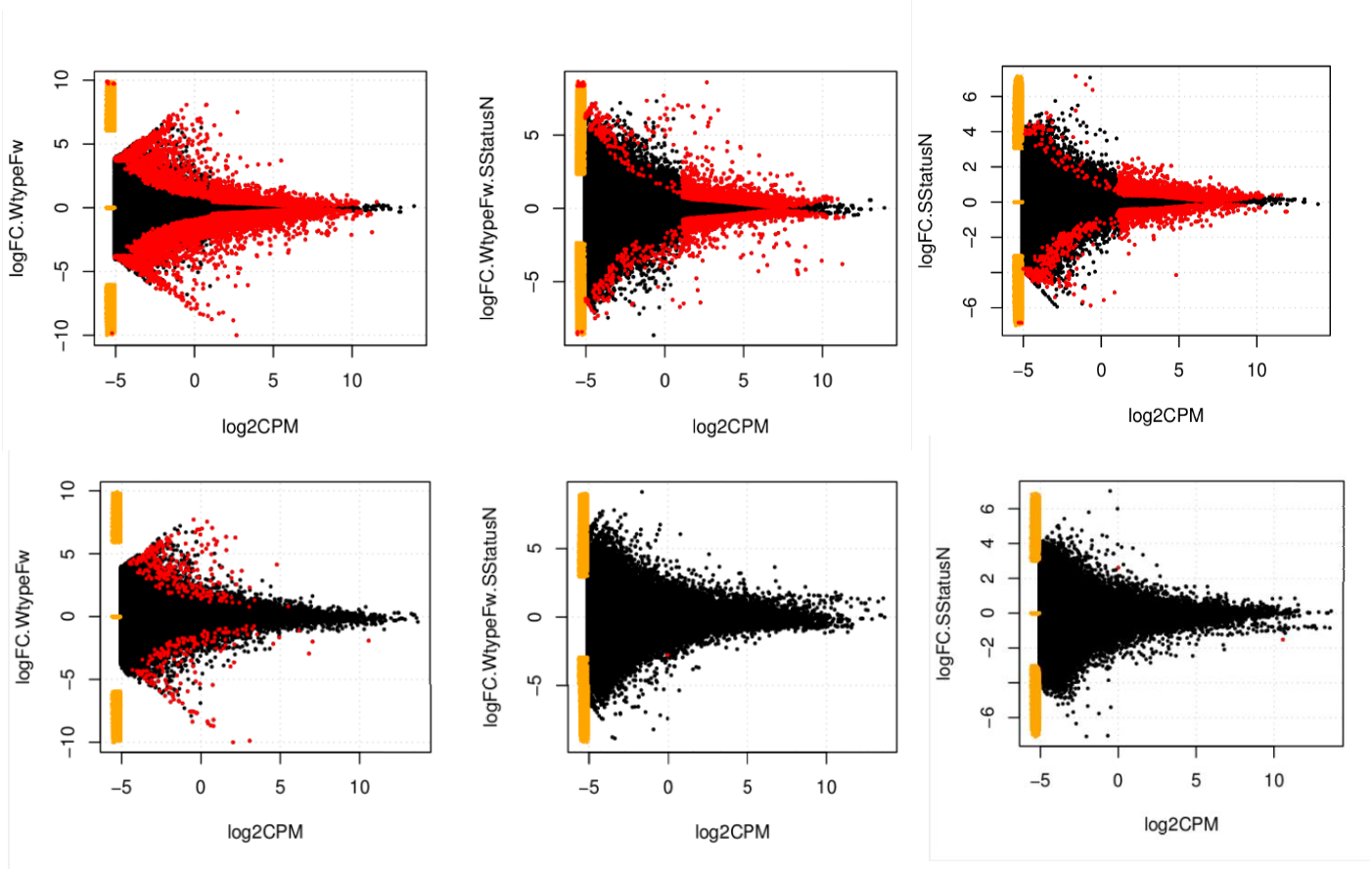


Figure S3. MA plots showing significantly, differentially-expressed transcripts between song versus no-song treatments (in red), separated by cricket type. Log2 fold change (log2FC) is plotted against log2 counts per million (log2CPM). Normal-wing males (top left), normal-wing females (top right), flatwing males (bottom left), and flatwing females (bottom right).



847

848 **Figure S4. MA plots showing transcripts that were significantly, differentially expressed in generalized linear models (GLMs) undertaken**
849 **separately for each sex.** The top row shows males, and the bottom row shows females. The contrast by morph genotype is shown in the left
850 column, the interaction between morph genotype and acoustic treatment is in the middle column, and the acoustic treatment contrast is on
851 the right. Low expression genes (log2CPM < -5) are colored in orange, and significant DE genes are colored in red. FC: fold change, CPM: counts
852 per million, Wtype: wing type (morph), SStatus: song status (acoustic treatment).

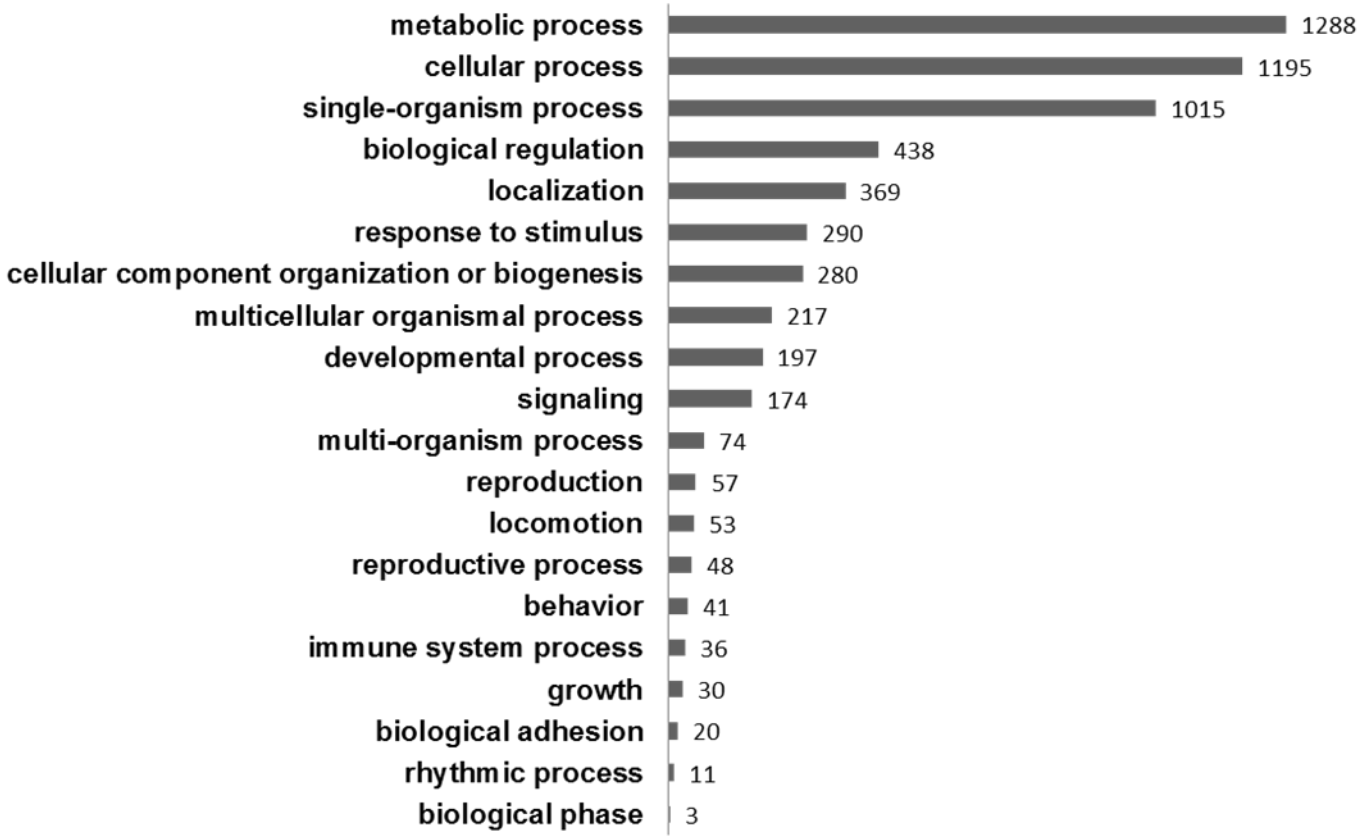


Figure S5. Gene ontology (GO) terms for the 5,547 transcripts that were significant in the morph*acoustic treatment interactions in male-only generalized linear models. Terms for Biological Process, Level 2 are shown, and thus describe functional annotation information for transcripts that showed different magnitudes or directions of plastic change in response to the acoustic environment in normal-wing versus flatwing males.

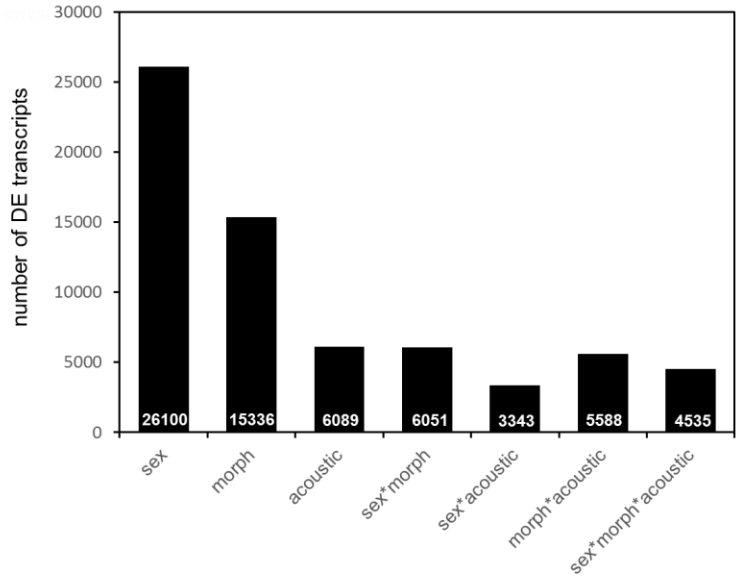


Figure S6. Differential transcript expression in the global GLM presented in Figure 1 of the main text, when a more stringent filter for lowly-expressed transcripts is applied. In this analysis, transcripts were removed if they were not expressed at a level of at least 1 count per million in a minimum of 3 samples. Fewer DE transcripts are recovered overall (n = 36,561 with more stringent filtering, versus n = 46,511), but the general pattern of DE genes represented across the GLM contrasts remains similar. The morph*acoustic interaction is of particular importance, because it indicates transcripts for which flatwing versus normal-wing genotypes of crickets respond differently to the social environment (i.e. divergent reaction norms). More stringent filtering did not decrease the relative fraction of transcripts that were significantly DE in this interaction category (15.28% of all DE transcripts with zero-count plus 1cpm filter, versus 15.01% of all DE transcripts with zero-count filter only).