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LETTER

Increased socially mediated plasticity in gene expression accompanies rapid adaptive evolution

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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, whereas 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.

Keywords

Adaptation, coevolution, genetic assimilation, genomic invasion, phenotypic plasticity, rapid evolution, social environment, *Teleogryllus oceanicus*, transcriptomics.

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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 & 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 capitalising 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 *c*. 20 generations, so dynamics of this system reflect the early stages of rapid adaptive evolution (Zuk *et al*. 2006). Flatwing males are protected from

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

would interfere with the silent treatment (flatwing males and females cannot sing but were also clipped to control for confounding effects due to cutting). One week later, cricket tissues were dissected and stored in RNALater at −20 °C.

RNA extractions, library preparation and sequencing

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

RNA-seq data analysis

Data analysis was conducted following the same pipeline as described in Pascoal *et al.* (2016a). Briefly, CASAVA version 1.8.2 (Illumina), Cutadapt version 1.2.1 (Martin 2011) and Sickle version 1.200 with a minimum window quality score of 20 were used for initial 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 25 bp (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 & Dewey 2011): reads were mapped to the *de novo* transcriptome assembly using BOWTIE 2 (Langmead & Salzberg 2012), and expected raw read counts for downstream differential expression (DE) analysis were generated using the mapping BAM (Binary Alignment/Map) 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 generalised 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 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 adjusted P-value < 5% were defined as significantly differentially expressed (Benjamini & 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

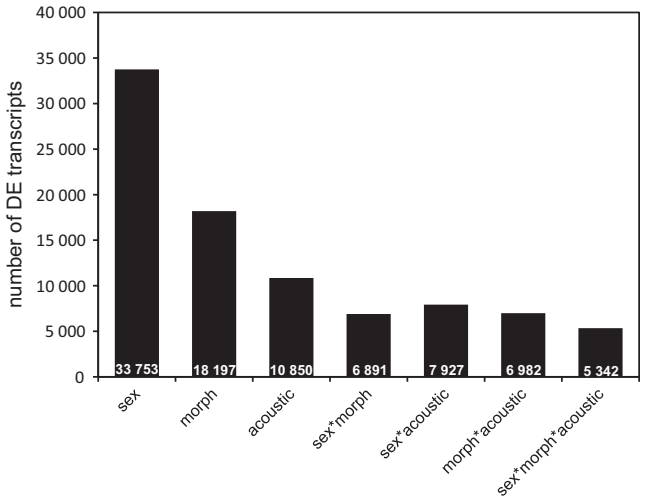


Figure 1 Differential transcript expression in cricket neural tissue. Expression differences were inferred using generalised linear models (GLMs). The bars show numbers (given in white text) of transcripts that were DE between sexes, between wing morphs and between acoustic treatments. Interaction terms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

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: (1) gene annotations of interest, (2) transcripts that were simultaneously DE in different contrasts (referred as overlap probes), (3) 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 hybridisation 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 hybridisation, 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, normalisation 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 normalisation (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 categorisation 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 & 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: 7927 transcripts showed different responses across acoustic treatments in males vs. females (sex*acoustic treatment interaction), and 6,982 transcripts showed different responses across acoustic treatments in flatwing vs. 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 vs. 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 across social environments, but little to negligible socially mediated plasticity in normal-wing crickets. Gene expression also responded

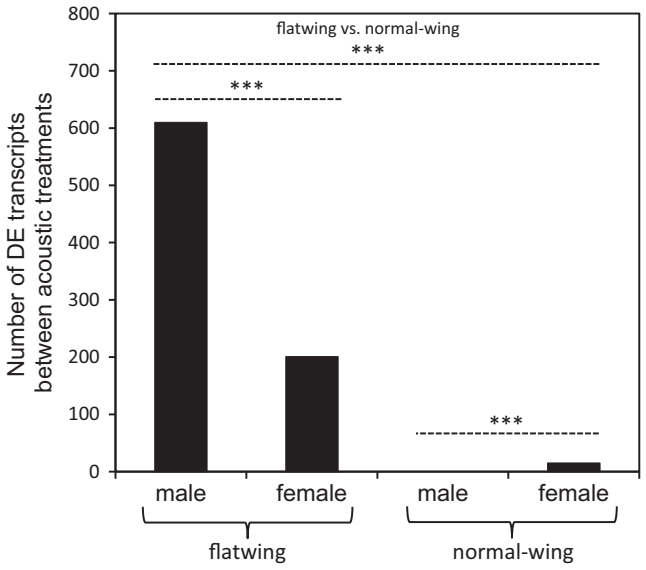


Figure 2 Socially mediated plasticity in gene expression is constitutively different between morph genotypes. The number of differentially expressed transcripts in the brains of adult crickets that had been reared in song vs. silence is indicated for each morph and sex. Differential expression was separately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

differently to the social environment in male vs. female neural tissue: there was no overlap of DE transcripts between the sexes. The lack of overlap is in agreement with the finding above that a significant number of transcripts show sexually dimorphic responses to the acoustic environment. While flatwing genotypes showed greater plasticity than normal-wing genotypes ($\chi^2 = 767.30$, d.f. = 1, $P < 0.001$), flatwing males showed greater transcriptome sensitivity to the acoustic environment than flatwing females ($\chi^2 = 206.32$, d.f. = 1, $P < 0.001$). The pattern of sex differences was reversed in normal-wing crickets, although this is based on a very small number of DE transcripts recovered in the normal-wing comparison ($n = 15$ in normal-wing females, vs. zero in normal-wing males) ($\chi^2 = 15.00$, d.f. = 1, $P = 0.001$).

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

A final set of analyses tested how morph genotype, acoustic treatment effects and their interaction impacted the transcriptomes of each sex separately. These broadly supported our previous findings, and indicated that although both sexes show expression 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 5547 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 vs. 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

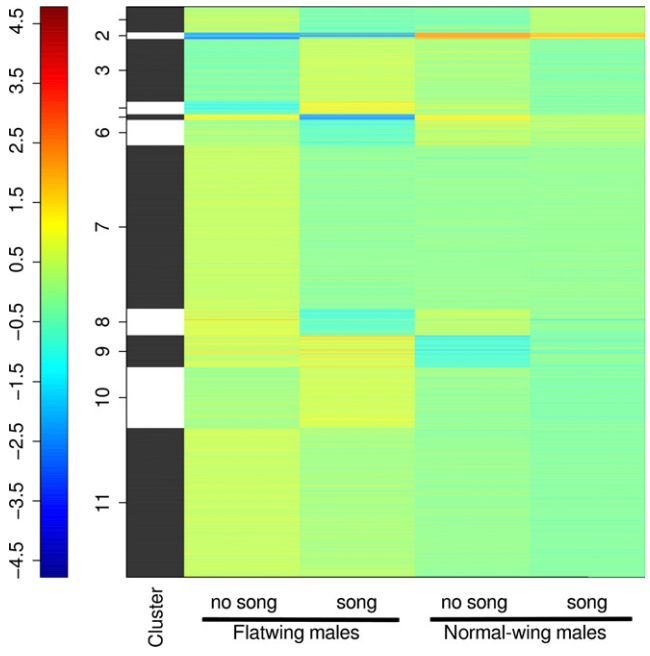


Figure 3 Comparison of socially mediated gene expression in flatwing vs. normal-wing males. Transcripts whose expression was significant in the morph*acoustic interaction of the male-specific expression analysis are depicted. The significance of the interaction term indicated that the two morph genotypes 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 upregulation, and smaller values (blue) indicating down regulation. For each gene, data from all samples are zero-centred to facilitate visual interpretation.

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

Transcriptome feminisation and sex differences in plasticity

The nearly 7000 transcripts identified as significant in the overall sex*morph interaction (Fig. 1) suggested that brain transcriptomes showed different levels of sex-biased expression in the two morphs. A comparison of differential expression between flatwing males vs. all females, and between normal-wing males vs. all females, revealed that there were fewer sex differences in flatwing male brain transcriptomes compared to normal-wing male brain transcriptomes (Fig. 4a) ($\chi^2 = 2011.79$, d.f. = 1, $P < 0.001$). Flatwing males thus had more female-like patterns of neural gene expression. We used multidimensional scaling (MDS) to plot similarities among samples in expression measured across all transcripts (Fig. 4b). The first and second dimensions separated the sexes and morph genotypes respectively. As with the previous analysis, flatwing male brain transcriptomes appeared more female-like than those of normal-wing males, but this feminisation was most prominent in flatwing males that had been reared in silence (Fig. 4b). Thus, flatwing males not only showed the greatest degree of transcriptome plasticity in response to acoustic signals in their environment, but their exposure to song appeared to mitigate female-like patterns of gene expression in the 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

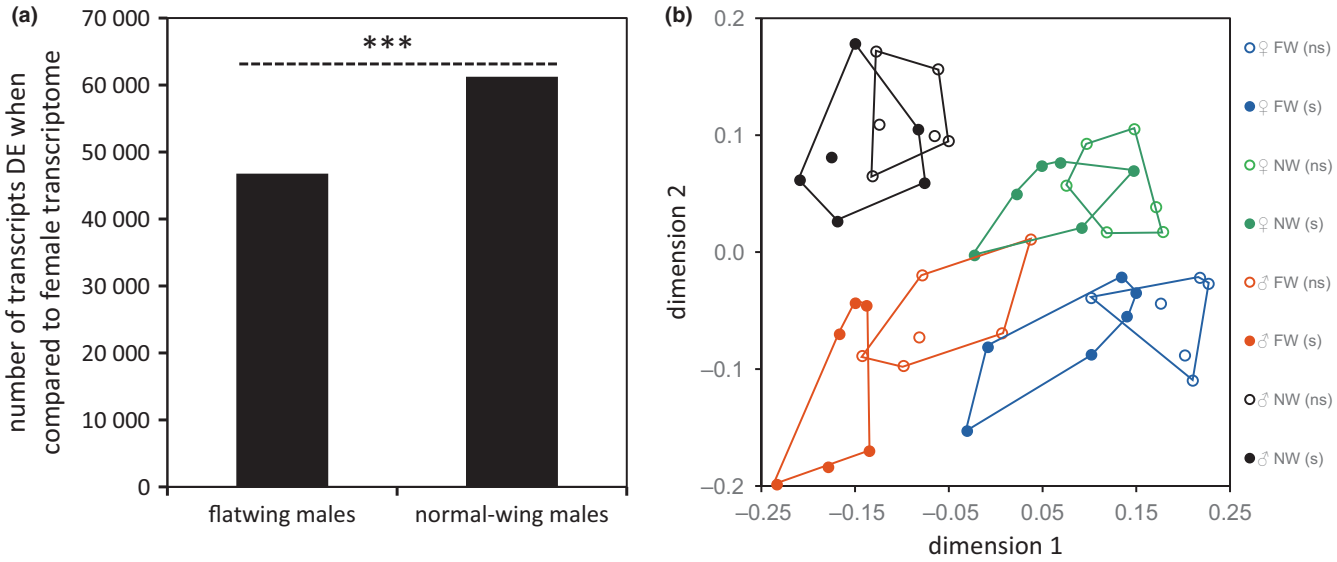


Figure 4 Neural transcriptomes are feminised in flatwing males. (a) Number of transcripts differentially expressed between flatwing males vs. all females and between normal-wing males vs. all females. Greater similarity between flatwing males and females than between normal-wing males and females indicates transcriptional feminisation of flatwing male neural tissue; asterisks indicate a significant difference ($\chi^2 = 2011.79$, d.f. = 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.

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, 2003, 2005; DeWitt & Scheiner 2004; Wund 2012; Zuk *et al*. 2014). These predictions have received mixed empirical support. Comparative work has linked diversity with patterns of ancestral plasticity in spadefoot toad species (Gomez-Mestre & Buchholz 2006), and patterns of plasticity have been found to recapitulate macroevolutionary patterns of trait divergence in *Polypterus*, the ray-finned fishes (Standen *et al*. 2014). Despite the intense interest and focus this topic has received, however, plasticity is often treated as a static property, rather than an evolvable quantity. For example the idea that pre-existing phenotypic plasticity acts as a pre-adaptation is appealing, and has received support in the cricket system we used here (Bailey *et al*. 2008; Tinghitella *et al*. 2009; Zuk *et al*. 2014), yet we still do not understand how plasticity interacts with traits under selection throughout the ongoing process of adaptive evolution. Our findings in *Teleogryllus oceanicus* reveal a genetic association between a rapidly evolving genotype and plasticity in neural gene expression supporting the view that plasticity itself is subject to evolutionary forces, and, in particular, can increase during the early stages of adaptive evolution in line with theoretical predictions (West-Eberhard 2005; Garland & Kelly 2006; Lande 2009). Box 1 provide a graphical description and explanation of this process.

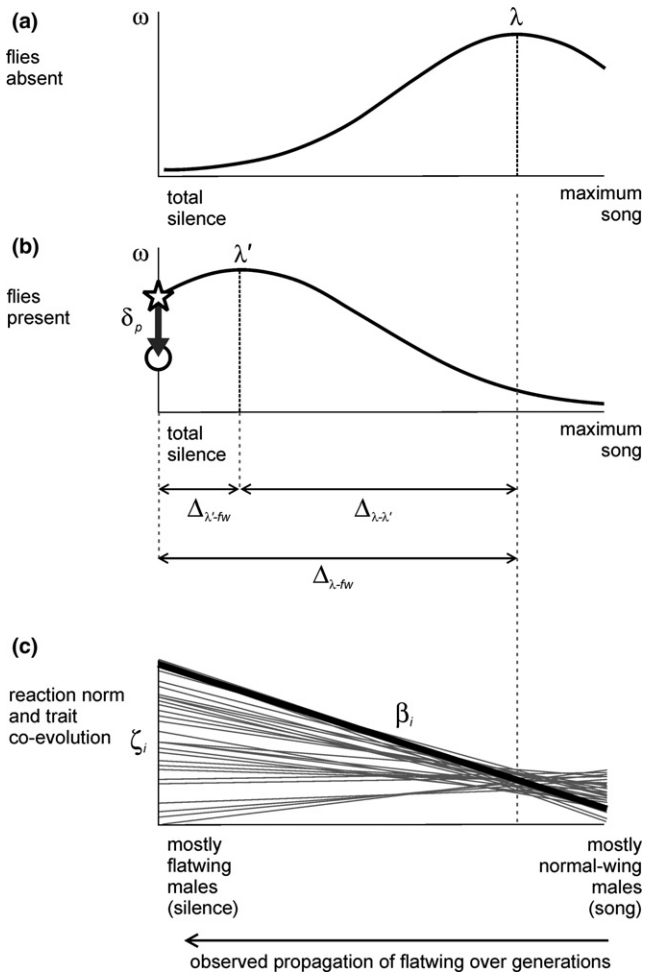
Prior work has revealed acoustically mediated plasticity in a broad spectrum of traits related to mating and reproduction in *T. oceanicus* from the island of Kauai, where alleles causing the erasure of sound-producing structures on male wings have rapidly spread, almost always in a manner that would be expected to increase fitness in a silent environment dominated by silent flatwing males (Zuk *et al*. 2006, 2014; Pascoal *et al*. 2014). The constitutive difference in acoustically mediated plastic gene expression in *T. oceanicus* crickets carrying flatwing vs. normal-wing genotypes is consistent with the rapid evolution of increased plasticity in neural gene expression in flatwing genotypes – 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 remains 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 (*x*-axis on MDS plot in Fig. 4b) in a male-biased direction.

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

We would not have expected a difference in plastic responses of flatwing and normal-wing genotypes if the average genotype in the population had been subjected to similar selection favouring the rapid evolution of socially mediated plasticity. It appears that the initial spread of flatwing was facilitated by pre-existing plasticity, followed by further differential selection on plasticity in flatwing vs. normal-wing genotypes. It is important to note that pre-existing genotypic variation in plasticity is necessary for plasticity to subsequently evolve: the existence of reaction norm variation prior to dramatic

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



[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.

[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 $\Delta_{\lambda\text{-}\lambda'}$, 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, 1998; Rotenberry *et al*. 1996). 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, $\Delta_{\lambda\text{-}fw}$, yet when flies are present it clearly confers an advantage despite having 'overshot' the optimal phenotype, $\Delta_{\lambda'\text{-}fw}$. 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.

[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 & 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 *c*. 15 years ago, thus the rapid spread of flatwings represents the earliest phase of this process (Zuk *et al*. 2006). Figure based on Lande (2009) (Fig. 1).

environmental change favouring increased plasticity is a key assumption of the Lande (2009) model. There is evidence for such reaction norm variation in *T. oceanicus* (Bailey & Zuk 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 vs. 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 canalised 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 & 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 occur on the order of hundreds to thousands of generations, not dozens (Box 1) (Lande 2009). Similar feedback effects are pervasive in evolving systems (Crespi 2004), and the relationship between flatwing and transcriptome plasticity in *T. oceanicus* demonstrates how the general impact of phenotypic plasticity on evolutionary change in other systems is likely to be inextricably linked to its own coevolution with traits under selection.

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AUTHORSHIP

NWB, Sonia P, MGR and MZ conceived and designed the experiments and wrote the paper. Sonia P and NR performed the experiments. NWB, XL, Sonia P, Steve P and YF analysed the data. NWB and NR contributed reagents/materials/analysis/tools.

DATA ACCESSIBILITY STATEMENT

Sequence data has been deposited in the NCBI BioProject database (accession number: PRJNA344019), and will be made available after a standard embargo period.

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