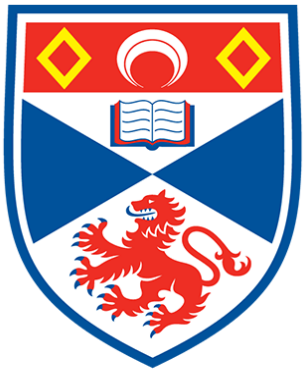
Evolutionary dynamics promoting and accompanying rapid adaptive trait loss

Jack G. Rayner



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Abstract

Understanding the conditions which promote adaptation is a key goal of evolutionary biology, and a pressing issue across fields of biology. Addressing this involves investigating not just the genetic and developmental mechanisms through which adaptive phenotypes arise, but also the environmental and ecological conditions which promote their spread. A major challenge in addressing these aims is that contemporary examples of rapid adaptive evolution are difficult to study, owing to the difficulty of identifying traits under selection during the early stages of adaptation. In this thesis, I use a Hawaiian field cricket system which provides a useful exception; males of the species *Teleogryllus oceanicus* ordinarily sing to attract females, however adaptive male-silencing ('flatwing') phenotypes have recently emerged and spread on at least three islands, under selection against male song exerted by a parasitoid fly, *Ormia ochracea*, which is attracted to singing males. Prior work indicates at least two of these flatwing phenotypes, from islands of Kauai and Oahu, have evolved independently under this shared selection pressure. This example of rapid, convergent evolution provides an opportunity to identify conditions which have promoted and resulted from rapid adaptation in wild populations evolving under extreme selection pressure. I investigate features which have contributed to the ability of these populations to rapidly, and repeatedly, adapt under strong selection against male song. The results indicate convergent sexual trait loss has been promoted by sex-biased development pathways maintained by sexually antagonistic selection; that pleiotropic, or associated, effects of adaptive mutation(s) in both sexes have played an important role in their spread; that adaptive male song-loss phenotypes have evolved repeatedly, above and beyond flatwing morphology; and that silent males nevertheless invest as much energy in practicing wing movement patterns associated with song and, despite reduced sexual dimorphism, are just as likely to be involved in aggressive intrasexual contests.

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Co-authorship statement

Chapter 1 – This introductory chapter was written with input from Nathan Bailey (NWB).

Chapter 2 – This chapter presents the results of a collaboration with Sonia Pascoal and NWB. Sonia Pascoal collected the original tissue samples and performed RNA extractions, and library preparation and RNA sequencing was performed by the Centre for Genomic Research at the University of Liverpool. The remainder of the work was performed by me with input from NWB.

Chapter 3 – I collected tissue samples and performed RNA extractions, and library preparation and RNA sequencing was performed by the Centre for Genomic Research at the University of Liverpool. The remainder of the work was performed by me with input by from NWB.

Chapter 4 – The work in this chapter was performed by me, with input from NWB.

Chapter 5 – The work in this chapter was performed by me, with input from NWB, who also helped design and perform field playback experiments. The microCT scan presented in Fig 5.1 was performed by Sarah Aldridge and Fernando Montealegre-Z.

Chapter 6 – This chapter presents the results of collaboration with Will Schneider (WTS) and NWB. WTS and I designed experiments, and WTS wrote and implemented the video processing scripts used to track behavioural wing movements, and validate their results. The remainder of the work was performed by me with input from NWB.

Chapter 7 – This discussion chapter was written with input from NWB.

Appendix 1 – This manuscript is authored by Sonia Pascoal, Judith Riise, Xiao Zhang et al., written by NWB. I performed transcriptomic analysis.

Table of contents

List of Figures

List of Tables

1. Introduction

1.1 What limits the rate of adaptive evolution?

A central goal in the study of evolutionary biology is to understand features of populations' biology which allow them to adapt to environmental and ecological change. Various genetic and developmental constraints are known to impede adaptive evolution, having received substantial consideration from evolutionary biologists laying the groundwork of the modern synthesis (Darwin 1859; Wright 1922; Fisher 1930; Dobzhansky 1937): these include limited standing genetic variation and the paucity, and pleiotropic effects of, adaptive *de novo* mutations (Barton & Turelli 2003; Nei 2005; Orr 2000). Despite these various constraints, it has been shown that populations nevertheless frequently adapt rapidly over contemporary timescales (Stockwell et al. 2003; Losos 2014). Moreover, similarly adaptive phenotypes often arise independently across populations and lineages evolving under shared selection pressures (Alves et al. 2019; Sackton et al. 2019; Therkildsen et al. 2019). Understanding how and under what conditions populations are able to rapidly and repeatedly adapt to extreme selection pressures therefore provides a compelling topic for study in evolutionary biology, with a range of important applications across related fields such as conservation biology and medicine (Prentis et al. 2008; Davies & Davies 2010; Sarrazin & Lecomte 2016).

Genetic adaptation requires either that there is sufficient standing variation for selection to act upon, or that newly beneficial mutations are able to arise and spread. The relative contribution of these two processes to adaptation across various scenarios is debated, but it is often thought standing variation will be of particular importance in the early stages of rapid adaptation (Hermisson & Pennings 2005; Lai et al. 2019). However, evolutionary forces such as genetic drift and stabilising or purifying selection are expected to erode standing genetic variation (Barton & Turelli 2003), thereby impeding rapid adaptive

evolution. Where there is not sufficient standing genetic variation for adaptation to occur through changes in frequencies of existing alleles, the capacity for adaptive evolution relies on beneficial mutations arising *de novo*. The rate of adaptation is therefore linked with mutation rate across species from the same lineage (Rousselle et al. 2019), but mutations are most often deleterious (Nei 2005) and so mutation rates are constrained by negative selection (Desai & Fisher 2007), imposing a further impediment to adaptation. Reconciling the anticipated erosion of genetic variation, and the paucity of novel adaptive mutations, with the observed ability of populations to rapidly and repeatedly adapt to changes in ecology or environment has presented a persistent challenge in evolutionary research (Hunt et al. 2007; Roff & Mousseau 1987; Stockwell et al. 2003).

These fundamental genetic constraints on adaptation are themselves shaped by features of the environment in which populations have evolved. Selectable genetic variation is theoretically related to factors of population size, fluctuating selection pressures associated with environmental stochasticity and frequency-dependent selection, heterozygote advantage, and admixture between genetically distinct populations (Barton & Turelli 2003; Charlesworth 2006). Small, fragmented populations with little or no migration between them are likely to suffer depleted genetic variation, while populations evolving under balancing selective forces such as negative frequency-dependent selection and fluctuating environmental pressures will likely harbour greater genetic variation. Larger populations are likely to produce a greater number of beneficial mutations, though their initial spread will be influenced by the fitness of the individual in which they arise (Charlesworth et al. 1993; Sniegowski et al. 1997). This linked selection, or genetic hitchhiking, is one further avenue through which standing genetic variation might be reduced in contemporary populations under selection. For example, in the case of a 'hard sweep' where a single adaptive mutation confers an extreme fitness advantage and quickly spreads to fixation, this will purge ancestral variation in nearby genomic regions

(Hermisson & Pennings 2005). This contrasts with soft selective sweeps, in which multiple adaptive mutations may arise, spread, and recombine, retaining greater levels of ancestral variation (Messer & Petrov 2013). The capacity for populations to generate and maintain genetic diversity is therefore strongly influenced by features of past selection and demography, and is likely to be substantially diminished in small, isolated populations, and those which have been exposed to extreme selection pressures.

1.2 Pleiotropy and evolvability

Another widely anticipated constraint upon adaptive evolution arises from genetic correlation between traits (Lande & Arnold 1983). The likelihood of adaptive change occurring in association with a given genetic variant is theoretically influenced by its pleiotropy, which is expected to limit the rate of evolution by reducing the likelihood of adaptive changes in value for one trait effecting a net fitness increase, due to non-adaptive 'off-target' effects (Fisher 1930; Orr 2000). For example, Chevillon *et al.* (1997) showed that in the mosquito *Culex pipiens* three insecticide resistance-associated alleles, each with an independent origin, all have negative pleiotropic effects upon other traits such as longevity, but nevertheless spread under extreme selection pressure.

The related 'cost of complexity' hypothesis states that more complex organisms should be evolutionarily constrained due to the greater number of interacting genes, i.e. greater extent of pleiotropy (Orr 2000). This constraint might be mitigated by genomic modularity (Welch & Waxman 2003): modules represent groups of 'integrated', tightly associated traits, while modules themselves are much less intrinsically associated with one another (Klingenberg 2008). Mutations in modular networks would therefore primarily affect a subset of integrated, related traits rather than the sum, reducing the likelihood of pervasive

negative pleiotropic fitness effects (Needham 1933; Wagner & Altenberg 1996; Welch & Waxman 2003). Consistent patterns of genetic or developmental integration of related traits are apparent in studies across morphological features and taxa (Cheverud et al. 1997; Klingenberg & Zaklan 2000; Klingenberg et al. 2001; Klingenberg et al. 2003).

Genomic modularity and related considerations have informed development of 'evolvability' frameworks, describing the likelihood of heritable and thus selectable, adaptive variants arising for a given trait, without widespread deleterious effects (Kirschner & Gerhart 1998). The latter feature is regulated through factors such as integration of related traits, the former by mutability of genomic regions. Once a controversial idea, primarily of interest to the field of 'evo-devo', there is growing evidence of differences in trait-specific evolvability, and heightened evolvability has been implicated in classic examples of adaptive evolution. A landmark recent study demonstrated that enhanced mutability of the pelvic enhancer region of the *Pitx1* gene in sticklebacks, caused by 'fragile' regions of repetitive thymine-guanine content in associated regulatory DNA sequences, is implicated in the repeated convergent and putatively adaptive loss of pelvic hind fins in freshwater populations (Xie et al. 2019). Similarly, Brimacombe *et al.* (2019) recently found evidence that genetically underpinned changes in chromatin composition which induce chromosome missegregation, and thereby aneuploidy, positively influence the ability of the yeast *Candida albicans* to evolve under environmental stress.

Whether features which influence a trait's mutability can feasibly be the product of selection is a topic of persistent debate (Kirschner & Gerhart 1998; Pigliucci 2008). The evolution of increased mutator rates in *Escherichia coli* (Zeyl & Bell 1997; Colegrave 2002; Peabody et al. 2017) is widely considered to represent one such example of selection for evolvability in novel environments (Pigliucci, 2008). Selection for genetically determined 'evolvability' has also been reported in *E. coli*, based on the finding that less fit clones are

able to evolutionarily out-compete those better adapted to the initial environment (Woods et al. 2011). However, the applicability of these second-order dynamics of selection to non-clonal populations, and to sexually reproducing Eukaryotes in particular, is unclear. Yet, sexual reproduction may itself provide an example of an evolved capacity to adapt under selection. Populations of dioecious fresh-water snails *Potamopyrgus antipodarum* exposed to greater rates of infection by trematode parasites exhibit a greater proportion of sexually reproducing individuals (Lively 1987), apparently due to selection for rare resistant phenotypes which arise with greater frequency in sexually reproducing populations (Dybdahl & Lively 2006).

An important tenet of the evo-devo framework is that phenotypic evolution occurs largely through changes to gene regulation, rather than 'structural' protein coding sequences, and that modularity and non-lethality of these changes are associated with changes to cis-regulatory regions (Monod and Jacob, 1961). These changes are expected to affect respective genes' regulation, and thus phenotype, with fewer widespread deleterious effects than would be expected from structural changes to coding sequences (Wray 2007). Though this hypothesis is difficult to test explicitly (but see Sackton *et al.*, 2019), and was met with early opposition (Hoekstra & Coyne 2007), the pervasiveness of cis-regulatory changes in studies of causal mutations underlying selected (be it natural or artificial) phenotypes is difficult to ignore (Chan et al. 2010; Mou et al. 2011; Feng et al. 2014; Guo et al. 2016; Kim et al. 2019; Sackton et al. 2019). Cis-regulatory changes and genomic or developmental integration, i.e. the parcellation of related traits, could therefore readily complement one another in promoting coordinated adaptive evolution of functionally related traits.

1.3 Adaptation over contemporary timescales

Despite the clear and numerous constraints upon the rate of adaptation, a key insight from research over the last century is that patterns of evolutionary change are not restricted to geological timescales, but can in fact be observed in real time over few generations (Stockwell et al. 2003; Losos 2014; Marchini et al. 2014). A famous example is that of the evolutionary response to coal pollution of populations of the peppered month, *Biston betularia*, in which the common pale form was rapidly supplanted by a darker, melanic form which benefited from lower predation rates owing to its reduced conspicuousness to predators (Cook 2003). In this case, the adaptive mutant phenotype was produced by the insertion of a transposable element into an untranscribed region of the gene 'cortex' (van't Hof et al. 2016). This capacity for rapid adaptation is also readily demonstrated in experimental evolution studies such as the 'Longshanks' experiment, where researchers observed an increase in tibia length of approximately 13% over 20 generations of selective breeding in mice (Castro et al. 2019). Such examples of rapid adaptation have generated particular interest in recent decades, given the unprecedented rate and extent of environmental change to which many wild populations are being subjected, largely through anthropogenic influence, and to which they must presumably adapt to survive (Stockwell et al. 2003). For example, Conover and Munch (2002) reported dramatic changes in body length of silverside fish (*Menidia menidia*) across replicate lines after just six generations of selective harvesting for size, with clear relevance to the impacts of anthropogenic fishing.

Genetic adaptation is not the only mechanism by which populations are able to respond to changes in selection. Just as individual reproductive fitness hinges on surviving to reproductive age (i.e. viability selection), genetic adaptation depends on populations persisting for long enough under selection for adaptive genetic combinations to arise and/or spread, and adaptive phenotypic plasticity is widely considered to play an important role in

this context (Ghalambor et al. 2007; Lande 2009). Some have gone further to argue plastic responses to selection will often precede genetic adaptation by producing beneficial phenotypes that will subsequently be genetically 'accommodated' (West-Eberhard 2005), for example through mutations which allow initially plastic adaptive phenotypes to be reached more efficiently (Baldwin 1896). In *Daphnia melanica*, production of melanic pigment is plastically down-regulated under low levels of UV radiation, reducing conspicuousness to predators, and these changes appear to have been genetically accommodated, as inferred from the loss of plasticity in populations evolving under consistently low UV and high predation rates (Scoville & Pfrender 2010). Experiments with *Arabidopsis thaliana* exposed to a range of environmental stressors show a plastically induced heightened recombination rate, which persists over generations and is heritable from both sexes (Molinier et al. 2006). Many adaptive plastic responses are nevertheless likely to depend largely on underlying genetic variation (Bradshaw 1965), so will be similarly constrained by features such as small population size, and low levels of gene flow. Additionally, while adaptive plastic responses to selection might act as an important buffer, benefitting fitness and permitting populations to persist in the short-term, they might also impede genetic adaptation by weakening selection pressures to which the individual is subject (Ghalambor et al. 2007).

1.4 How repeatable is adaptive evolution?

One way in which genetic and developmental constraints upon evolution might be investigated is to consider the repeatability of adaptation at these respective levels. Gould (1990) pondered how things would differ if one were able to 'replay the tape of life', arguing evolutionary outcomes are largely contingent on specific events, so would show limited similarity between lineages with dissimilar ancestral environments irrespective of their

current selection pressures. This question is especially pertinent to the study of convergent evolution, whereby phenotypic similarity is independently derived across multiple lineages (Losos 2011). Given an 'adaptive landscape' for a given trait, a single prominent peak would indicate that selection should lead populations to converge accordingly (Wright 1932). However, if the landscape is 'rugged' with multiple peaks, selection will be expected to produce different evolutionary 'solutions' under differing starting conditions (Blount et al. 2018), and evolutionary trajectories may differ between populations facing the same selection pressure. This conceptualisation is a simplification, however, and the likelihood of a given evolutionary trajectory will also depend on properties such as the specific developmental processes involved, i.e. the relationship between genotype and phenotype (Maynard Smith et al. 1985; Wagner & Altenberg 1996; Hall 2003).

Extended experimental evolution studies, such as the Long-Term Evolution Experiment (LTEE) in *Escherichia coli* (Lenski et al. 2002) in which 12 initially identical populations have been evolving in a glucose-limited medium to which they are poorly adapted since 1988, provide an excellent opportunity to test Gould's view that evolutionary outcomes are highly contingent on small differences between populations in their 'histories'. These studies have tended to document highly parallel increases in fitness across populations (Blount et al. 2018). For example, in the LTEE, after 50,000 generations derived populations had on average approximately 1.7 times the fitness of the ancestral population, growing 70% faster. Moreover, many of the genetic changes which have taken place show similarity between populations, with changes to protein coding sequences disproportionately affecting the same genes or genomic regions (Lenski 2017). There are nevertheless also important differences between derived populations owing to apparent evolutionary contingencies which are consistent with Gould's conception. Just one population evolved the ability to exploit citrate, an additional carbon source present in the growth medium, and subsequent 'replay'

experiments performed by re-evolving populations archived at various timepoints indicate that the evolution of this citrate-using variant benefited from a 'potentiating' mutation which arose by some 20,000 generations (Blount et al. 2008). This mutation might not have conferred any immediate fitness benefit, but is thought to have facilitated the subsequent evolution of citrate-use and concomitant fitness increase in a later generation.

In contrast with experimental evolution experiments, addressing the repeatability or contingency of adaptive evolution in wild populations is complicated by differences between populations in standing genetic variation, and the specific environmental factors they experience (Blount et al. 2018), begging the question of what qualifies as 'contingency'. Convergent evolution is nevertheless pervasive throughout nature, and studies of underlying genetic changes can help elucidate whether phenotypic convergence is recapitulated at the genetic level – through targeting the same alleles, genes, or gene pathways (Sackton & Clark 2019). These studies frequently find that convergent evolution is associated with genetic mutations within, or in the region of, the same few key genes. For example, mutations in the *Mc1r* gene are persistently associated with adaptive colour polymorphisms in mammals and birds (Theron et al. 2001; Mundy et al. 2004; Eizirik et al. 2003; Hoekstra et al. 2006). In another example, Alves et al. (2019) showed that wild rabbit populations from three countries which have suffered myxomatosis endemics – the United Kingdom, France and Australia – have independently evolved resistance through highly similar changes in allelic frequencies of the same genes, each benefitting from standing variation which preceded their intercontinental dispersal. This pattern, of convergent evolution being associated with shared selection pressures acting upon existing standing variation, is another emerging theme in studies of genetic changes underlying phenotypic convergence (Sackton & Clark 2019).

Convergent evolution may be brought about through changes at a number of levels in the developmental hierarchy bridging genome and phenotype. Phenotypic convergence might

be underpinned by identical changes in allelic frequencies a single gene; this might be common where populations share ancestral standing genetic variants (Alves et al. 2019), or where adaptive variants are introgressed from another population or closely related species through hybridisation (The Heliconius Genome Consortium, 2012). Alternatively, parallel changes in allele frequencies might occur in the event that identical adaptive mutations arise and spread. While presumably rare, this emergence and spread of identical genetic variants might be promoted in cases where particular genetic mutations are more likely to occur than others (Xie et al. 2019). Different mutations in nearby genomic regions may also have similar phenotypic effects, for example if they disrupt the function of the same regulatory elements, proteins, or downstream signalling pathways (Therkildsen et al. 2019). Next, different mutations in non-overlapping or distant genomic regions might affect expression of the same key genes (Warner et al. 2019), or components of the same gene expression networks (Parker et al. 2015), with shared effects upon downstream biological processes and phenotypes. Each of these are routes through which phenotypically similar patterns of convergent adaptation might occur, however their respective prevalence and the degree to which they account for phylogenetic patterns of pervasive phenotypic convergence is unclear (Stern 2013).

Losos (2011) detailed a number of ways in which convergent adaption might be wrongly inferred, or conversely overlooked, particularly in comparative phylogenetic studies which constitute the bulk of the empirical literature on the topic. Convergent adaptation might be wrongly inferred if populations have phenotypically converged for reasons other than adaptation under shared selection pressures (Gould & Vrba 1982); for example, through processes of genetic drift, or shared biases in the production of phenotypic variation (Maynard Smith et al. 1985). Moreover, phenotypes shared between populations might in fact represent correlated responses to selection on a trait not directly under selection (Lande &

Arnold 1983), potentially leading to the misidentification of convergent evolution on this associated trait.

In contrast, a scenario identified by Losos (2011) in which convergent adaptation would not be identified in comparative phylogenetic studies is where a variety of phenotypic 'solutions' exist to a given selection pressure, as in the divergent physiological modifications by which subterranean rodents have evolved to dig burrows (Stein 2000). Whether this constitutes convergent evolution in the strictest sense is a subject for debate, however other studies more clearly demonstrate that convergent evolution can occur through not just differing, but phenotypically divergent, morphological changes. Threespine sticklebacks, *Gasterosteus aculeatus,* have evolved morphologically divergent but ecologically and functionally similar adaptive specialisation to dietary differences following colonisation of benthic freshwater habitat, and this pattern of convergent but morphologically varied adaptation has not only benefited fitness of the derived populations but also generated phenotypic diversity (Mcgee & Wainwright 2013). Thus, functionally convergent but morphologically varied convergent adaptations might be one mechanism through which phenotypic variation is produced, potentially contributing to macroevolutionary processes such as population divergence and speciation (Bailey et al. 2019). However, if functional convergence is frequently overlooked in favour of morphological convergence, as may well be the case in phylogenetic studies, it is clear how this would bias understanding of the relationship between phenotypic and genetic convergence, and of convergent evolution more broadly.

1.5 What is the role of sexual dimorphism in evolution?

In sexually dimorphic organisms, a very large proportion of observable intraspecific phenotypic variation is associated with differences between sexes in pre- and post-translational processing and expression of shared genes (Mank 2017). This is most clearly demonstrated by sexually dimorphic species in which sex is determined by environmental variation, e.g. mediated by temperature as in many reptile species (Ciofi & Swingland 1997), or by differences in ploidy of shared chromosomes (e.g. XO/ZO sex determination; Bachtrog et al. 2014), but where all genes are shared. Some of these differences in processing or expression are involved in the development of sexually dimorphic reproductive organs, such as testes and ovaries (Dean & Mank 2016), however others relate to tissues not directly involved in reproduction, and are generally thought to represent contrasting selection pressures to which sexes are subject (Lande 1980).

Sex differences in gene expression and phenotype are both shaped by, and contribute to, evolutionary dynamics. Patterns of sex-biased gene expression appear to be strongly affected by mating system, apparently due to differences in the strength of sexual selection on males, though treatment-associated changes are not always consistent in direction between studies (Hollis et al. 2014; Veltsos et al. 2017), nor are sex differences necessarily consistent through ontogeny (Perry et al. 2014). Differences in the extent of sex-biased gene expression also contribute substantially to phenotypic variation within each sex, underlying alternative reproductive strategies (Pointer et al. 2013; Stuglik et al. 2014) and contributing to variance in fitness between individuals of the same sex (Dean et al., 2018). These differences thereby play an important contributing role in evolutionary dynamics, by producing both inter- and intra-sexual phenotypic differences.

Of particular interest with respect to the role of sex differences in evolution is sexually antagonistic selection, i.e. differences in selection pressures acting upon males and

females. These differences result in conflict over allelic identity or expression of shared genes, termed intralocus sexual conflict (Bonduriansky and Chenoweth, 2009); such intragenomic conflicts themselves represent a constraint upon the ability of evolution to achieve optimal phenotypic solutions. Sexually antagonistic intralocus genomic conflict is widely thought to be mitigated by differences in expression of shared genes via their sex-specific regulation, leading to phenotypic sex differences despite shared genes (Mank 2017), and which are often regulated by relatively few key genes. One family of genes with a taxonomically widespread, conserved role in regulating sexual dimorphism is that of the doublesex/*mab-3* related transcription factors (DMRT), all of which share a conserved DNA-binding domain and regulate sexual dimorphism through a variety of mechanisms across insects, mammals and nematodes (Kopp 2012). Sexually dimorphic patterns of phenotypic variation represent one of the clearest examples of integration of ecologically related traits – those which consistently differ between males and females in a coordinated manner – and the genetic and developmental modularity of these integrated traits is clearly defined (Bachtrog et al. 2014).

While it is clear that the evolution of sexually dimorphic phenotypes and divergent life histories will impose selection pressures of their own, e.g. through associated sexual conflict over optimal dietary composition (Rapkin et al. 2016), they may also contribute to a population's ability to evolve under selection. The importance of sexual reproduction in the production and maintenance of genetic variation is widely appreciated (Lively 1987; Zeyl & Bell 1997; Brimacombe et al. 2019). What is perhaps less often considered is that disruptive selection associated with sexually antagonistic selection pressures will also generate and maintain genetic variation (Lonn et al. 2017; Wright et al. 2018) which, in addition to novel variants produced by sexual recombination (Peabody et al. 2017), might provide an important substrate for evolution in sexually reproducing species (Fierst 2011). While sexually

antagonistic selection and associated intragenomic conflict will therefore reduce the absolute fitness values of either sex, it might also increase the ability of each to respond to changes in selection pressures by maintaining genetic variation associated with key ecologically important traits; similarly male-beneficial and female-beneficial variants will each be selected for and against.

1.6 Hawaiian field crickets: a model system for studying rapid adaptation

The oceanic field cricket *Teleogryllus oceanicus* exists in small, fragmented populations across the Hawaiian archipelago, having originally spread from Polynesian populations (Tinghitella & Zuk 2009; Tinghitella et al. 2011). At some point in the 20th century, a Tachinid fly, *Ormia ochracea*, was introduced to the Hawaiian islands from the mainland USA. The fly is a parasitoid of grylline crickets: gravid females are attracted to the songs of male crickets and deposit larvae on or near to singing males. These larvae latch onto and burrow into their host – be they male or female – and internally devour the cricket before emerging, killing them. *O. ochracea* do not occur in *T. oceanicus*' ancestral range across Australia and Polynesia, and so the introduction of this fatal parasitoid fly exposed them to an extreme and novel selection pressure. (Zuk et al. 1993)

In 2003, Robin Tinghitella, a researcher studying populations of *T. oceanicus* evolving under parasitism from *O. ochracea* on the island of Kauai, located an unusual looking individual. The cricket was missing an ovipositor, a female-specific trait required for the deposition of eggs into substrate, indicating it was a male. However, the cricket's wings also lacked visually distinctive male-specific sound-producing structures which are required to produce song (Fig 1.1), a secondary sexual trait which attracts females and thus creates mating opportunities. The cricket was confirmed as male, and Zuk et al. (2006) observed that

this male wing phenotype ('flatwing') spread to near-fixation in the population within just a few generations. The reason for this rapid spread was readily apparent: male crickets without sound-producing structures cannot sing, so do not attract the parasitoid fly: dissections revealed flatwing males were, like females, considerably less likely to harbour parasitic larvae compared with normal-wing males which retain the ability to sing. (Zuk et al. 2006)

Approximately three years after the discovery of the flatwing male phenotype in the Kauai population, a superficially similar phenotype was observed to emerge and rapidly spread on a second island, Oahu (Zuk et al. 2006). As in Kauai, males exhibiting the phenotype were unable to sing, so could not produce the signal which attracts the parasitoid fly, and the flatwing phenotype spread rapidly. In the years since their discovery, flatwing phenotypes appear to have spread to fixation in at least one population on each of Kauai and Oahu (Chapter Five; Rayner *et al.*, 2019), and similar flatwing phenotypes have also spread on two further islands of Hawaii and Molokai (Pascoal et al. 2014; Tinghitella et al. 2018). The process of adaptation has therefore been extremely rapid. Stockwell et al. (2003) define 'contemporary evolution' as observable phenotypic change over fewer than one-hundred years. In the case of *T. oceanicus*, flatwing phenotypes were observed to emerge and spread to near-fixation in fewer than ten (between 30 and 40 generations), despite small population sizes. This rapid change had dramatic knock-on consequences for parasitised populations, drastically altering their social environment through the elimination of important male inter- and intrasexual acoustic cues (Bailey et al. 2010; Logue et al. 2010; Pascoal et al. 2018).

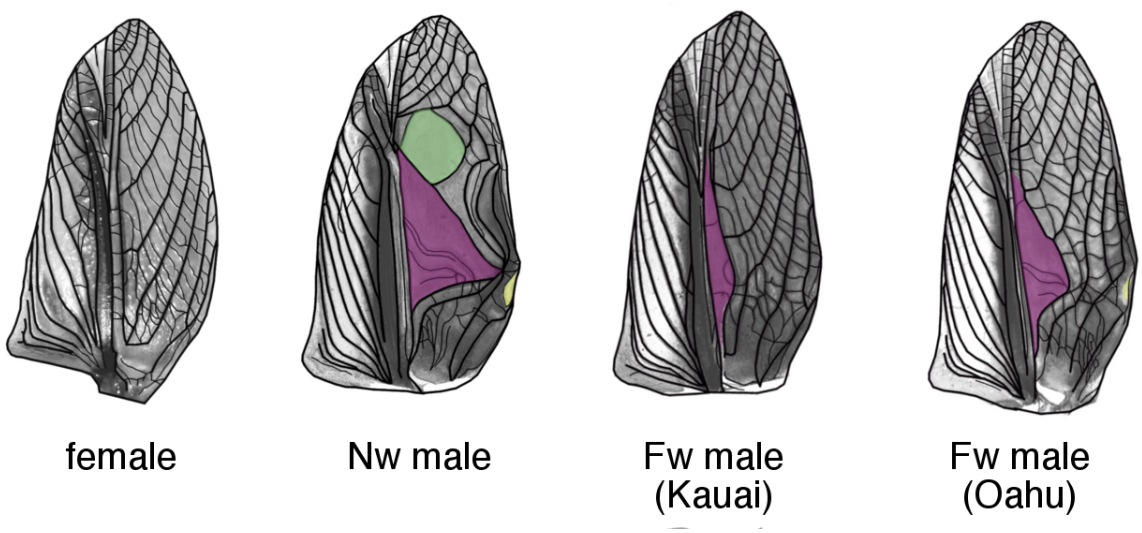

Figure 1.1 Diagrams of female and male forewing phenotypes
Traced micrographs showing forewing venation patterns (adapted from Pascoal et al. 2014) of a female and normal-wing (Nw) male and flatwing (Fw) males from Kauai and Oahu, with sound-producing structures highlighted (purple, harp; green, mirror; yellow, plectrum).

Flatwing phenotypes on Kauai and Oahu are heritable, and segregate in the manner of a single X-linked locus (Tinghitella 2008; Pascoal et al. 2014) – *T. oceanicus* follow an XO system of sex-determination, so the genetic variants (*flatwing* [*Fw*], or *normal-wing* [*Nw*]) are carried in single-copy in males, whereas females can be homo- or hetereozygous. Comparison of SNPs which segregate between male wing morphs has shown the vast majority of *flatwing*-associated SNPs on each island are non-overlapping, and those which do overlap show allelic reversal between islands, suggesting the two phenotypes have arisen independently and represent an example of rapid adaptive convergent evolution in the wild (Pascoal et al. 2014). Flatwing phenotypes have since been observed on islands of Hawaii and Molokai, however it is not known whether these phenotypes represent independent evolutionary origins, or have introgressed from other populations (Pascoal et al. 2014; Tinghitella et al. 2018).

The primary role of male field cricket song is to attract female mates. Males typically produce two types of song which are attractive to females. Calling song, consisting of longer

length ‘chirps’ and shorter, paired ‘pulses’ (Fig. 1.2A), enables female phonotaxis, drawing sexually receptive females within earshot towards the calling male (Alexander 1962). Crucially, calling song can also attract other males which pursue satellite mating tactics to intercept and attempt to mate with approaching females. Once physical contact has been made with the female, the singing male will begin to produce courtship song, distinguished from calling song by an especially energetically costly ‘trill’ feature (Hack, 1998; Fig 1.2B), and the features of which are under strong sexual selection (Rebar et al. 2009). Besides these two primary roles in attracting female mates, a distinctive third form of male song (‘aggressive song’) is characterised by repetitive, monotonous chirps, and functions as an aggressive display signal in male-male interactions (Logue et al. 2010).

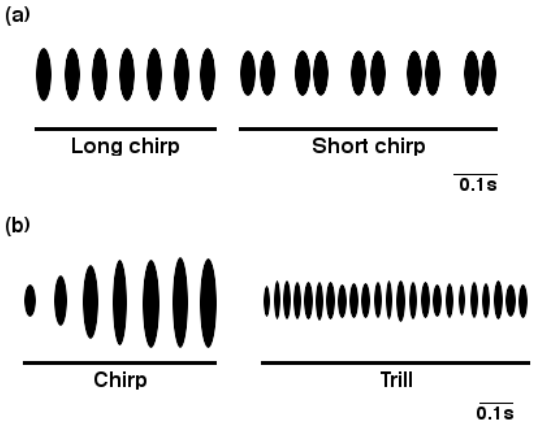

Figure 1.2 Calling and courtship song waveforms
Typical features of two forms of song produced by *T. oceanicus* males to attract and court females: (a) long-range calling song, (b) short-range courtship song. Figure adapted from Zuk, Rebar and Scott (2008).

In the presence of calling normal-wing males, flatwings are able to achieve matings by adopting satellite mating tactics and intercepting phonotactic females (Zuk et al. 2006). However, in the years following the emergence of flatwings on Kauai and Oahu, the respective phenotypes appear to have reached fixation in at least two separate populations, rendering them completely silent (Chapter Five; Rayner *et al.*, 2019). Flatwing males in these

populations must achieve matings, despite their inability to produce important sexually selected acoustic signals. *T. oceanicus* are active between the hours following sunset and preceding sunrise, under little-to-no light (Zuk et al. 1993); it is therefore unclear how individuals are able to locate one-another in the absence of acoustic or visual stimuli, but behavioural studies have demonstrated males and females raised in environments without song show increased phonotaxis (Bailey et al. 2010), and males from the Kauai population also show increased locomotion (Balenger & Zuk 2015), each of which might improve their chances of contacting conspecifics. This sensitivity to the acoustic environment appears to be a plastic response shaped by evolution (Bailey & Zuk 2012; Pascoal et al. 2018); individuals in less densely populated regions will be less exposed to acoustic signals, and will therefore benefit from heightened acoustic responsiveness and increased locomotion. It appears, therefore, that phenotypic plasticity, on the part of both silent males and receptive females, has played an important capacitating role in the rapid spread of flatwing phenotypes.

1.7 Using Hawaiian field crickets to test factors that promote and constrain adaptation

A substantial body of research has documented the spread of flatwing phenotypes in populations on multiple islands of Kauai, Oahu and Hawaii (Zuk et al. 2006; Pascoal et al. 2014; Zuk et al. 2018), the behavioural dynamics which have accompanied and perhaps facilitated their spread (Bailey et al. 2010; Balenger & Zuk 2015; Pascoal et al. 2018), and also begun to characterise their genetic underpinnings (Pascoal *et al.*, 2014, 2016, Appendix 1). This system provides an opportunity to address several outstanding questions with respect to the genetic and behavioural circumstances surrounding rapid adaptation. Following the view that pervasive pleiotropic effects of otherwise adaptive genetic variants should inhibit

their spread, the extent of off-target effects of mutations underlying flatwing phenotypes is of particular interest. For example, my analysis examining effects of the Kauai *Fw* genotype upon gene expression in developing embryos, presented in Appendix 1, revealed pervasive effects on gene expression across the full range of sex chromosomes and autosomes, and further work demonstrates it also feminises male cuticular hydrocarbon profiles (Appendix 1; Fig. 1.3). This suggests it may well have widespread phenotypic effects, but that any negative fitness consequences are outweighed by the adaptive loss of song (Chevillon et al. 1997), perhaps due in part to plastic or genetic accommodation of deleterious phenotypes.

Pleiotropic effects of the *flatwing* genotype might be expected not just in males, but in females too; what effects, if any, do the underlying genotypes have upon females, in which wing morphology is unaffected? The Kauai *Fw* genotype is known to have some effect upon female neural transcriptomes, but whether these changes affect females at the phenotypic level is unclear (Pascoal et al. 2018). Females carry two copies of the underlying genotype, while males carry just one, so any effects upon female phenotype and thus fitness will be evolutionary important owing to females' greater genetic contribution to the next generation (Rice, 1986). Additionally, the fact that *flatwing* genotypes underlie the loss of a male secondary sexual trait which is not expressed in females, but for which they share with males all the necessary genes, suggests that they might affect levels of sexual antagonism at the loci involved. Previous research supports the view that secondary sexual traits are associated with intralocus sexual conflict (Joag et al. 2016), even when the traits are themselves sex-limited in their expression (Harano et al. 2010). Patterns of sexually antagonistic selection are frequently considered in the evolution of secondary sexual traits (Lande, 1980; Rice, 1986), and expected to be attenuated, for example, through subsequent evolution of modifier genes in the sex to which they confer no fitness benefit. However, the idea that intralocus sexual conflict could play an important role in their eventual – and surprisingly common (Wiens

2001; Kraaijeveld 2014) – loss does not appear to have been addressed. Potentially important pleiotropic effects of the Kauai *flatwing* genotype are evaluated in Chapter Two by testing associated changes in gene expression across multiple tissues in each sex, and the effects of these changes upon patterns of sex-biased gene expression are used to evaluate whether intralocus sexual conflict might have played a facilitating role in the phenotype's rapid spread.

Understanding the causative genetic variants underlying adaptive evolution in the wild is crucial to understanding how, and under what circumstances, populations can rapidly adaptive to strong selective pressures. The genetic modifications underlying convergently evolved flatwing phenotypes remain unclear (Pascoal et al. 2014; Pascoal et al. 2016; Appendix 1). Addressing these underpinnings will greatly contribute to understanding how the underlying mutations were able to independently emerge and spread on at least two islands, despite small and fragmented populations. A recently produced map of quantitative trait loci statistically associated with the Kauai *Fw* genotype constitutes nearly a third of the length of the X-chromosome, likely due to a large number of hitchhiking genes following a recent selective sweep (Appendix 1). Complementary efforts in determining important genes within this region might involve testing for differences in gene expression associated with divergent wing phenotypes and cross-referencing with quantitative trait loci to identify candidates (Wainberg *et al.*, 2019). Moreover, differences in expression of genes between male wing morphs and sexes could be compared, to examine whether rapid adaptation benefitted from genetic and phenotypic variation associated with sexual dimorphism and, by extension, sexually antagonistic selection pressures (Fierst 2011; Wright et al. 2018). Whether convergently evolved flatwing phenotypes affect similar gene expression pathways, particularly those involved in regulating sexual dimorphism, forms the focus of Chapter Three. Given their female-like wing vein morphology, it is plausible flatwing males exhibit

feminised patterns of wing development. If flatwing males are less sexually dimorphic at the level of gene expression (Pascoal et al. 2018), this could also explain associated phenotypes such as their feminised cuticular hydrocarbon profiles (Appendix 1).

Sexual dimorphism is widely expected to play an important role in regulating male-male conflict and same-sex sexual behaviour (Steiner et al. 2005; Dukas 2010). Reduced sexual dimorphism might therefore impact not just inter-sexual, but also intra-sexual, interactions, for example by reducing levels of aggression flatwing males receive from conspecifics, if they are less readily distinguished from females (Norman et al. 1999), and this could have important implications for understanding the rapid spread of the phenotypes. The idea that flatwing males might in some circumstances benefit from feminised appearance and inability to produce characteristic male song through being mistaken for females is addressed in Chapter Four, by comparing the incidence of same-sex sexual behaviour across normal-wing, mixed, and flatwing pairs of males.

Patterns of rapid adaptation are frequently mirrored across populations through selection on shared ancestral variants, introgression between populations, or independent emergence of similarly adaptive phenotypes (Sackton & Clark 2019). Given flatwing phenotypes' clear fitness benefits in the context of natural selection conferred by the parasitoid fly, it is unclear why their distribution throughout the Hawaiian archipelago is highly heterogeneous (Fig. 1.3). The parasitoid fly is observed at the sites of all study populations of *T. oceanicus*, yet the proportion of silent flawing males varies between ~5% in a study population on the island of Hawaii, to 100% in at least one population on each of Kauai and Oahu (Zuk et al. 2018). Moreover, in a second high-density population on Hawaii, flatwing males are not observed at all, begging the question of how this population has persisted in the face of extreme selection against song. One possibility is that populations in which flatwing phenotypes have either not emerged or have not spread to an appreciable

degree have adapted under selection by *O. ochracea* through different means; for example through reduced calling effort, or alternative routes to silence. However, there is not yet strong evidence for these predictions. Chapter Five describes field observations and experiments which reveal many of the males in populations in which flatwing phenotypes have not spread to a high degree are nevertheless incapable of producing acoustic signals at ordinary levels, due to changes in wing shape and morphology, and are therefore protected against parasitism from the fly.

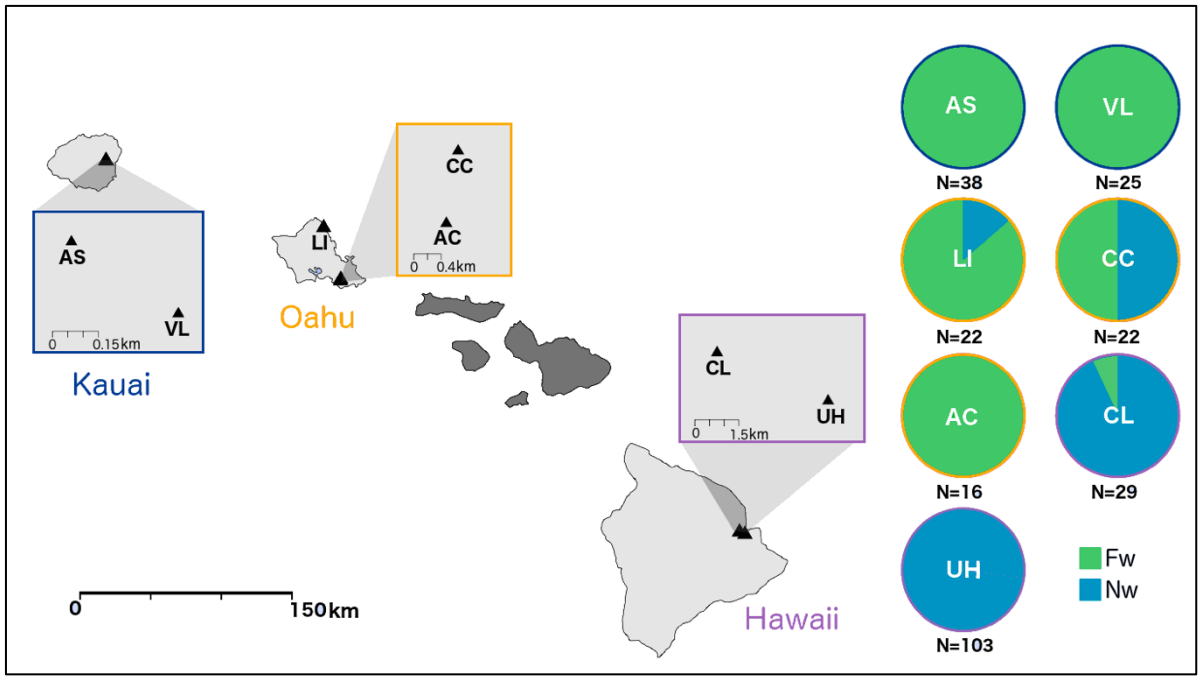

Figure 1.3 Distributions of normal-wing and flatwing phenotypes
Geographic distributions of parasitized populations of *T. oceanicus* and proportions of males showing flatwing and and normal-wing phenotypes from 2018 surveys. Two-letter codes correspond to site IDs. Figure adapted from Chapter 5.

Also surprising is the persistence of calling behaviour in silent populations. Flatwing males continue to practice the energetically costly patterns wing movement which in normal-wing males are associated with song, despite their inability to produce song at any appreciable amplitude (Schneider et al. 2018), and associated fitness costs (Hunt et al. 2004). One

possibility is that, while flatwing males still attempt to sing, they have evolved to invest less energy in doing so, due to the lack of fitness benefits in the context of sexual selection. Such evolutionary accommodation could play an important role in facilitating the spread of *de novo* mutations, and reduction in male calling behaviour among silent populations could provide important insight into evolutionary dynamics underlying the elaboration, maintenance and loss of sexually selected traits. Behaviour is often considered to play a key role in the early stages of adaptation (Wong & Candolin 2015; Bailey et al. 2018), and Chapter Six tests the hypothesis that silent males and/or silent populations should have evolved to invest less energy in calling effort.

2. Evolved loss of a male sexual trait demasculinises female gene expression*†

The loss of sexual ornaments is observed across taxa, and pleiotropic effects of such losses provide an opportunity to gain insight into underlying dynamics of sex-biased gene expression and intralocus sexual conflict (IASC). We investigated this in Hawaiian *T. oceanicus*, in which an X-linked genotype (*flatwing*) feminises males' wings and eliminates their ability to produce sexually selected songs. We profiled adult gene expression across somatic and reproductive tissues of both sexes. Despite the feminising effect of *flatwing* on male wings, we found no evidence of feminised gene expression in males. Instead, female transcriptomes were more strongly affected by *flatwing* than males', and exhibited demasculinised gene expression. These findings are consistent with a relaxation of IASC constraining female gene expression through loss of a male sexual ornament. In a follow-up experiment we found reduced testes mass in flatwing males, whereas female carriers showed no reduction in egg production. In contrast, female carriers exhibited greater measures of body condition. Our results support the view that sex-limited phenotypic expression offers only partial resolution to intralocus sexual conflict, owing to pleiotropic effects of the loci involved. Benefits conferred by release from intralocus conflict could help explain widespread loss of sexual ornaments across taxa.

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† Trimmed RNA-seq reads are available at the European Nucleotide Archive (PRJEB27211)

2.1 Introduction

Sex-biased gene expression produces striking phenotypic differences in species where the sexes share a substantial portion, if not all, of the same genome (Connallon & Knowles 2005; Ellegren & Parsch 2007; Bachtrog et al. 2014; Mank 2017). Such evolved differences between sexes in gene regulation play an important role in attenuating intralocus sexual conflict (IASC), which arises when sexes are under contrasting selection pressures at shared loci, by achieving phenotypic dimorphism (Lande 1980; Pizzari & Snook 2003; Bonduriansky & Chenoweth 2009; Harrison et al. 2015). However, it is increasingly recognised that resolution of such conflict is not necessarily complete (Rice & Chippindale 2001; Cox & Calsbeek 2009; Connallon et al. 2010; Berger et al. 2014), and that IASC can persist even when genes and phenotypes have evolved under contrasting selection pressures to exhibit sex-biased or even sex-limited expression (Harano et al. 2010; Cheng & Kirkpatrick 2016). One of the reasons for this is pleiotropy exerted by loci involved in the conflict upon other traits which are not directly under selection (Fig. 2.1). Sexual trait loci can thus exert spillover effects across sexes and tissues. For example, the enlarged mandibles of male broad-horned flour beetles *Gnatocerus cornutus* are genetically associated with reduced female lifetime fecundity (Harano et al. 2010) despite their male-limited expression, illustrating incomplete resolution of associated IASC.

As well as its role in regulating differences between sexes, recent studies have demonstrated that varying degrees of sex-biased gene expression are associated with intra-sexual phenotypic variance, often with fitness-associated effects (Dean et al. 2018). Pointer et al. (2013) found subordinate males of the wild turkey *Meleagris gallopavo* exhibit feminised patterns of gene expression relative to more ornamented dominant males. Similarly, in the bulb mite *Rhizoglyphus robini*, 'fighter' male morphs show exaggerated transcriptional sexual dimorphism compared with unarmoured 'scrambler' males (Stuglik et al. 2014), and

are associated with increased IASC at the population level (Joag et al. 2016; Plesnar-Bielak et al. 2014). An assumption of sexual selection models is that elaborated, dimorphic sexual traits should eventually be checked by countervailing natural selection (Fisher 1915; Lande 1981; Kirkpatrick 1982), but evidence for the involvement of sex-biased pathways of gene expression in naturally-selected adaptations is limited, and the consequences for IASC after sexual trait reduction or loss are of particular interest.

To explore these consequences, we examined the effects of sexual trait loss on patterns of sex-biased gene expression in Hawaiian populations of *T. oceanicus*. Adaptive, obligate silence is caused by mutation(s) that cause males to develop female-like wing venation, erasing sound-producing structures and protecting them against fatal parasitism. The flatwing phenotype segregates as a single-locus variant (the *flatwing* genotype) on the X chromosome (sex determination is XX/XO; males and females share all genes), though the exact nature of the mutation(s) is not known (Appendix 1). Although it is transmitted on the X, *flatwing*'s effects upon wing phenotype appear to be male-limited; female carriers show no readily detectable wing differences. There is evidence for widespread pleiotropic effects of *flatwing* in both sexes (Pascoal et al. 2016; Pascoal et al. 2018), and males carrying the genotype exhibit more female-like cuticular hydrocarbons (Appendix 1), in addition to their feminised wing membranes. We profiled gene expression from a range of non-wing, somatic and gonad tissues of adults from lines that were pure-breeding for *flatwing* or *normal-wing* genotypes. Our aims were to test the role of sex-biased genes in evolved song loss, and explore the latter's consequences for IASC.

If *flatwing* widely impacts sex-biased pathways of gene expression, we anticipated one of two patterns among affected loci. Given its feminising effect in male wing tissues and upon male cuticular hydrocarbons, *flatwing* might be associated with a general increase in female-biased gene expression, demasculinising female carriers and feminising male carriers

(Hypothesis 1 in Fig. 2.1) (Plesnar-Bielak et al. 2014; Hollis et al. 2014). An alternative, but non-mutually exclusive, scenario is that the loss of the male sexual trait relaxes pleiotropic IASC-associated constraints upon female gene expression, in which case we anticipated up-regulation of female-biased (or down-regulation of male-biased) gene expression (demasculinisation) predominantly affecting females (Hypothesis 2 in Fig. 2.1). The results of this study will inform debate regarding the roles of pleiotropy and intralocus sexual conflict in evolutionary dynamics of rapid adaptation and the widely observed loss of sexual traits (Wiens 2001).

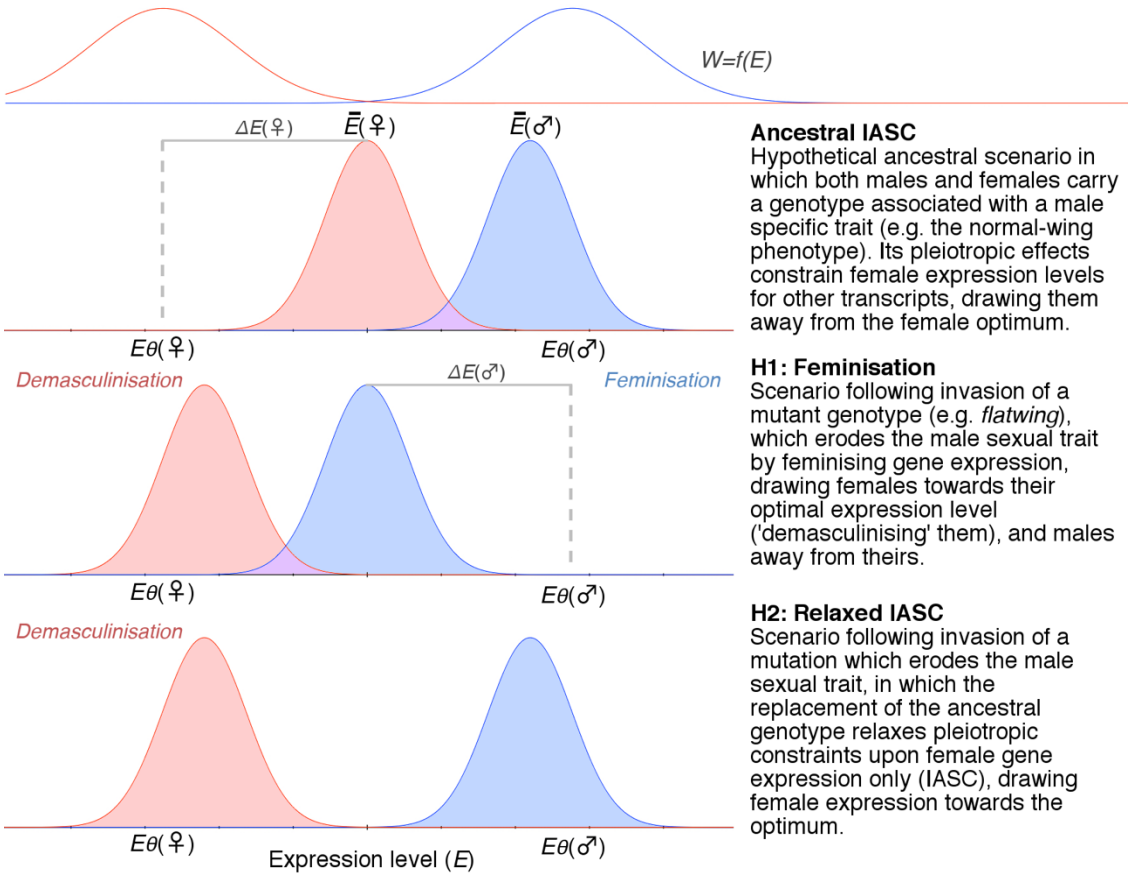

Figure 2.1 Hypothetical effects of male sexual trait loss on IASC
The schematic shows expression levels (E) and fitness (W) for a transcript assumed to be pleiotropically influenced by a sexual trait locus, thus contributing to incompletely resolved IASC. Expression optima (E_θ) and observed average expression values ($\bar{E}$) differ between the sexes, and shaded curves illustrate frequency distributions for sex-specific expression. Within each sex, fitness is a function of expression level, maximized at the optimum (top red and blue lines indicating hypothetical stabilizing fitness functions for females and males,

respectively). Thus, ΔE describes displacement from the optimum level of expression for each sex. The descriptors 'feminisation' and 'demasculinisation' refer to the identity of the individual under consideration: females whose gene expression shifts away from the male optimum are demasculinised, whereas males whose gene expression shifts in the same direction are feminized.

2.2 Methods

2.2.1 Cricket rearing and production of lines

Pure-breeding *normal-wing* (*Nw*) and *flatwing* (*Fw*) lines from which RNA samples were collected were derived from individuals caught in Kauai in 2012 following the procedures detailed in Pascoal et al. (2016), and thereafter maintained under common garden conditions at 25 °C on a 12h:12h light:dark cycle in an incubator with calling males present. All crickets were reared under common-garden conditions, exposed to acoustic signals of other crickets in the environment. Note that *Fw* and *Nw* lines have not been found to differ in development time (Rayner & Bailey, unpublished data).

2.2.2 Collection and sequencing of RNA samples

We collected tissue samples from virgin adults (ca. 3 months from egg stage) from replicate lines breeding pure with respect to each morph genotype (homozygous *flatwing* '*Fw*' or homozygous *normal-wing* '*Nw*'). RNA was extracted from three tissues (neural, thoracic, and gonad of a single male and a single female from each of 6 lines (N=3 lines of each morph), for a total of 36 samples from 12 individuals. Multiple lines were included in each group to account for between-line variance and to enable detection of expression differences attributable to morph genotype. Females were homozygous diploid for the respective genotype while males were hemizygous (XX/XO).

In sampling tissues, head and body tissues were separated and the digestive tract discarded, and tissue samples stored in RNAlater at -20 °C. Immediately prior to RNA

extraction, neural tissue was dissected from the head capsule, and thoracic muscle and testes or ovaries were dissected from the body cavity. Trizol RNA extractions were performed following Pascoal et al. (2018a). Quantity and integrity of RNA samples were assessed using Qubit broad range (Invitrogen) and Agilent Bioanalyzer assays, respectively. Total RNA was depleted with RiboZero and used in the ScriptSeq protocol (Epicentre), following manufacturer instructions. Samples were sequenced on an Illumina HiSeq 2000 with version 3 chemistry, generating 2×100 bp paired end reads. CASAVA v1.8.2 (Illumina) was used for basecalling and de-multiplexing of indexed reads. Adapter sequences were trimmed from fastq files using Cutadapt v1.2.1 (Martin 2011) and low quality bases were removed using Sickle v1.200 with a minimum window quality score of 20.

2.2.3 Sampling, sequencing and differential expression analysis

Paired-end reads of all 36 samples were generated on an Illumina HiSeq 2000, and a *de novo* transcriptome was assembled from trimmed reads of all samples in Trinity using *in silico* normalisation (Grabherr et al. 2013). Similar transcripts were then clustered in CD-hit-est (Li & Godzik 2006), and lowly expressed transcripts (those not expressed at >1 counts per million in at least 3 samples) and transcripts without an open reading frame of >100 amino acids were filtered from the transcriptome. Reads were aligned to the transcriptome using Bowtie2 (Langmead & Salzberg 2012) with strand-specific settings, and quantified in RSEM (Li & Dewey 2011). Differential expression (DE) analyses were performed in *edgeR* (Robinson et al. 2010) at the level of Trinity 'genes'; henceforth referred to as 'transcripts' in acknowledgement that not all Trinity-identified genes passing filtering will represent genes in the strictest sense. Because our analysis was at the gene level, isoform variants should not contribute to the patterns of DE we observe. Clustering of similar genes by CD-hit-est was used to further ensure isoform variants were not represented as multiple genes, and we used

the results of BUSCO analysis of conserved genes (Simão et al. 2015) to validate that our transcriptome was not highly duplicated. Separate models were constructed for somatic (neural, thoracic muscle) and gonad tissues, to examine effects of sex and morph, with significance testing performed using likelihood ratio tests. To restrict our analyses to transcripts showing strong evidence of DE, we adopted a conservative significance threshold of FDR<0.01 to consider a transcript significantly DE or sex-biased. We checked whether results qualitatively changed if we used another common approach of imposing a fold-change threshold of >2 for a transcript to be considered DE/sex-biased, with FDR <0.05 (e.g. Immonen et al. 2017), and found they did not (see Results).

Sequences of DE transcripts were entered as BLASTX queries against the NCBI non-redundant protein database, with an e-value threshold of 10^{-3} and a maximum of 20 hits. Mapping and annotation were performed in Blast2GO (Conesa et al. 2005) with default parameters. Functional enrichment of gene ontologies (GO) was assessed for all transcripts passing the expression filter against all Drosophila melanogaster proteins.

2.2.4 Differential Expression Analyses

Prior to constructing models, transcripts not expressed at a level above one count per million in a minimum of three samples were filtered from the dataset, as these were considered to have little empirical support and because their removal increased power to identify DE transcripts. After filtering, input counts were adjusted using trimmed mean of M-values (TMM) normalisation.

Separate models were initially constructed for somatic (including neural tissue and thoracic muscle from both sexes), and gonad (testes and ovaries) samples. Scaled normalisation procedures assume that no more than 50% of transcripts in a dataset are differentially expressed between any two groups of samples, which was likely to be violated

in a single model including both reproductive and somatic tissues. Importantly, however, the TMM method is relatively robust against violations of this assumption (Robinson & Oshlack 2010). For downstream investigation of sex-biased patterns of gene expression it was convenient to combine gonad tissues from both sexes in a single model, despite the expectation that a very large proportion of transcripts would be differentially expressed between the sexes. We tested the validity of results from this combined gonad model by also constructing and comparing separate models for testes and ovaries samples. For downstream analyses involving gonad tissues we used results from the model including gonad tissues from both sexes, as comparison of the identity of statistically DE transcripts indicated a high degree of overlap between separate and combined-sex models for ovaries (140 DE in ovaries model, 185 DE in combined sex model; 123 out of 140 (87.76%) DE transcripts shared between the two), while 16 transcripts were DE in testes samples in the separate sex model, versus 9 in the combined-sex model (6 out of 9 shared between the two).

Negative binomial generalised linear models (GLMs) were constructed in edgeR (Robinson et al. 2010), and tested using likelihood ratio tests. Once models had been constructed, pairwise contrasts were performed between groups of samples, as recommended by the authors of EdgeR for more complex experimental designs (Robinson et al. 2010), with a Benjamini and Hochberg-adjusted significance threshold of FDR<0.01. Contrasts were specified to examine the number of DE transcripts between morph genotypes for each of the tissues in each sex, as well as a sex comparison which was performed by contrasting average male and female expression values (i.e. samples for both morph genotypes were included for each sex). The approach of including both morph genotypes in sex comparisons was adopted to avoid statistical artefacts that could result from defining sex-biased transcripts using only normal-winged samples, i.e. using the same reference groups in both sex (*Nw* male vs *Nw* female) and morph (e.g. *Nw* male vs *Fw* male) comparisons (see: Mallard et al. 2018).

2.2.5 Gene expression feminisation, demasculinisation and tissue-specificity

We defined feminised and demasculinised expression, applied to males and females respectively, as up-regulation of female-biased transcripts (or down-regulation of male-biased transcripts) in males, and down-regulation of male-biased transcripts (up-regulation of female-biased transcripts) in females (Fig. 2.1). Thus, the terminology indicates the sex experiencing the effect. Identification of sex-biased genes was performed using differential expression analysis, averaging expression values across both morph genotypes in each sex; genes up-regulated at FDR<0.01 in males were considered male-biased, and genes up-regulated in females considered female-biased. To test for feminisation and demasculinisation, we took the subset of transcripts that were DE in both morph genotype and sex comparisons and compared the direction of change between the two for each tissue separately.

To understand whether changes in expression associated with morph genotype were correlated between sexes, we tested whether log-fold changes in expression for transcripts DE in one or both sexes were correlated between males and females. We also investigated the level of tissue-specificity of genotype-associated effects in each sex by comparing log-fold changes among all transcripts DE in either comparison (Dean & Mank 2016). To test whether sex-limited and tissue-specific transcripts were less likely to be DE between morph genotypes, which could support the involvement of pleiotropy affecting genes shared between sexes, we subset for each sex*tissue combination transcripts expressed at >1cpm in all 6 samples, and transcripts expressed at <1cpm in all 6 samples, then compared identity across tissues to define sets of sex-specific and tissue-specific transcripts.

2.2.6 Reproductive tissue and condition measures

We investigated whether sex-specific reproductive fitness measures differed between separate, recently outcrossed (see Supporting Information) pure-breeding *Nw* (*N*=4) and *Fw* (*N*=3) lines derived from the same base population. These lines were descended from the same lines from which RNA samples were collected, but had since been outcrossed by breeding *Nw* and *Fw lines* together to reduce effects of inbreeding. Pure-breeding *Nw* and *Fw* lines were subsequently re-established by mating heterozygous females with males of the desired morph, then backcrossing in the next generation and screening offspring to retain homozygous families. At 7 days post-adult eclosion, gonad characteristics were measured in virgin male (*N*=140; 18 to 21 per biological line) and female (*N*=145; 19 to 24 per biological line) crickets that had been reared at standard stock densities. As proximate measures of reproductive output, we obtained wet mass of dissected testes to the nearest mg, and for females counted the number and measured the total wet mass in mg of eggs contained within the ovaries. Next, scaled mass index (SMI) was calculated using the following equation following Peig & Green (2009), with pronotum length as the linear measurement.

Testes mass was analysed using a linear mixed model (LMM), while female total egg mass was analysed using a generalised linear mixed model (GLMM) with a negative binomial distribution. Total egg mass followed a negative binomial distribution owing to the Poisson distribution of egg numbers. Both models included predictor variables of morph genotype, log pronotum length, log somatic mass, and a random effect of biological line. We calculated somatic (i.e. not including gonad masses) scaled mass index (SMI) from pronotum length and somatic wet mass, often used as a proximate measure for individual body condition (Peig & Green 2009). Log-transformed SMI was analysed using an LMM with predictor variables of morph genotype, sex, an interaction between the two, and a random effect of biological line. Following the SMI comparison, contributions of differences in

pronotum length and somatic wet mass were investigated using LMMs with the same predictors and random effect. Mixed models were run in the R package *lme4* (Bates et al. 2016), with *MASS* used to fit the negative binomial GLMM. Significance of predictor terms was tested using Wald's χ^2.

2.3 Results

2.3.1 Morph genotype has larger effects on gene expression in females

Female transcriptomes were more strongly impacted by carrying the *flatwing* genotype than were males'. The unfiltered *T. oceanicus* transcriptome contained complete sequences for 90.6% conserved insect BUSCO genes, with low duplication rates (1.8% of complete genes; see Supporting Information), and 42,496 transcripts (Trinity-identified 'genes') passed filtering. Differential expression results are summarised in Table 2.1. In all tissues the number of DE transcripts (FDR<0.01) associated with morph genotype was greater among females than males, and female thoracic muscle and ovaries were particularly strongly affected (neural tissue: χ^2_1=11.571, P<0.001; thoracic muscle: χ^2_1=310.77, P<0.001; gonads: χ^2_1=159.67, P<0.001) (Fig. 2.2a). This interpretation remained unchanged if a fold-change of >2 and FDR <0.05 was instead adopted (greater DE in females: all P<0.001).

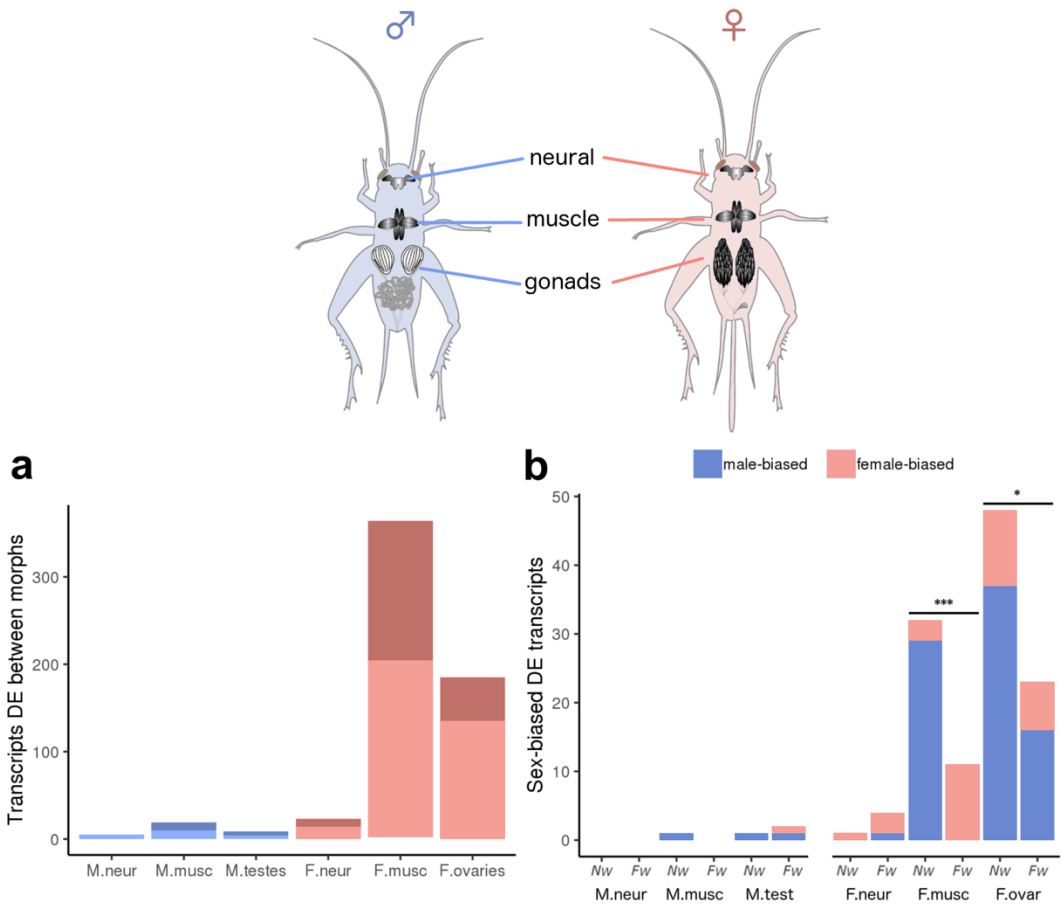

Figure 2.2 The *flatwing* genotype's effects on gene expression.
The top panel shows tissues sampled. **a)** Numbers of transcripts up-regulated in *Nw*-carrying crickets for males (light blue) and females (light red), versus up-regulated in *Fw*-carrying individuals of either sex (dark blue/red). **b)** Sex-biased genes that differed between female morph genotypes showed patterns of demasculinisation in *Fw* females. (Too few sex-biased genes were DE between male genotypes for statistical comparison.) Numbers of sex-biased transcripts up-regulated in each morph genotype with respect to the other are plotted, and colours show female-biased (red) vs. male-biased (blue) expression. Significance (*** $P<0.001$, * $P<0.05$) is shown for differences between genotypes in the number of transcripts showing masculinised/demasculinised expression. Significance was not tested for neural tissue, in which just 5 sex-biased transcripts were DE between genotypes.

Of 560 unique transcripts DE between genotypes in either sex, 296 (52.86%) had significant BLASTX hits. None of the annotated transcripts had obvious known functions or GO terms related to sexual dimorphism in insects. Overrepresented GO terms among transcripts up-regulated in each of the female genotypes are given in Table 2.2. Neither male morph showed significant overrepresentation for any GO categories.

2.3.2 Male trait loss is associated with demasculinised female gene expression

Fw females showed demasculinised gene expression compared with *Nw* females (Fig. 2.2b). Of the 119 sex-biased transcripts DE between female genotypes across all tissues, 87 (73.11%) showed expression patterns consistent with demasculinisation of *Fw* females (either female-biased transcripts up-regulated in *Fw* females or male-biased transcripts up-regulated in *Nw* females), compared with only 32 transcripts (26.89%) showing the reverse pattern (χ^2_1=25.420, P<0.001). The pattern of demasculinisation in *Fw* relative to *Nw* samples was consistent across female thoracic muscle and ovaries tissues (thoracic muscle: χ^2_1=31.837, P<0.001; ovaries: χ^2_1=4.070, P=0.044), but numbers were too low for quantitative comparison in neural tissues. Interpretation of demasculinised expression remained unchanged under fold-change >2 and FDR <0.05 criteria (neural tissue: too few for comparison; thoracic muscle: χ^2_1=57.791, P<0.001; ovaries: χ^2_1=5.921, P=0.015).

2.3.3 Magnitude of DE associated with male trait loss across sexes and tissues

For transcripts DE between genotypes in one or both sexes, changes in gene expression were positively correlated between sexes in neural (Spearman's rank: r=0.920, N=26, P<0.001) and gonad (r=0.203, N=193, P=0.005) tissues, but not in thoracic muscle (r=0.046, N=378, P=0.372). Across the 19 transcripts showing concordant and significant DE in males and females, after relaxing the significance threshold to FDR<0.05 to increase sample size, there was no indication that females showed greater log-fold changes; male genotypes tended to exhibit greater differences (male $\log_2$-fold change – female $\log_2$-fold change: average = 0.386, P=0.123). Changes in expression associated with the *Fw* genotype were concordant in pairwise comparisons across tissues within each of the sexes (Spearman's rank: all r>0.465,

P<0.01), suggesting a relatively high degree of pleiotropy. Interpretations above were unchanged under fold-change >2 and FDR <0.05 criteria.

Transcripts showing sex-limited expression did not show substantial DE between genotypes. In ovaries, the female tissue which showed the greatest degree of sex-limited expression, sex-limited transcripts (expressed >1cpm in all ovaries samples and <1cpm in all testes samples) tended to be underrepresented among those DE between morph genotypes (11 of 185 DE transcripts sex-limited, vs 1,782 of the 17,254 transcripts >1cpm in all 6 samples; χ^2_1=3.350, *P*=0.067). No sex-limited transcripts were DE between morph genotypes in testes, or neural and thoracic muscle tissues of either sex.

Among transcripts showing tissue-specific expression within each sex (e.g. expressed at >1cpm in all female neural samples but <1cpm in all female thoracic muscle and ovaries samples) fewer than expected were DE between morph genotypes in ovaries (7/178 DE transcripts showed tissue-specific expression, versus 1,576/17,254 of those expressed at >1cpm in all 6 samples; χ^2_1=5.161, *P*=0.023). No tissue-specific transcripts were DE between genotypes in any of the other tissues; including testes, despite the large number of tissue-specific transcripts (0/9 versus 6,658/20,998). In somatic tissues, tissue-specific transcripts were less likely to show sex-bias than were non- tissue-specific transcripts also expressed at >1cpm in all 6 samples for the respective tissue (χ^2: *P*<0.001 in both tissues and sexes), but this pattern was reversed in ovaries, where tissue-specific transcripts were more likely to show sex-bias (χ^2=26.763, *P*<0.001). There was no difference in testes samples (χ^2=0.300, *P*=0.584).

2.3.4 Sex and morph variation in reproductive tissues and condition

Adult *Nw* males grew larger testes (LMM: χ^2_1=8.800, *P*=0.003; Fig. 2.3A), but there was no difference in the mass of eggs produced by females of either genotype (GLMM: χ^2_1=0.011,

P=0.916; Fig. 2.3B) (Table 2.3). Nevertheless, *Fw* females achieved better condition. Their SMI was greater than that of *Nw* females, but a significant sex × morph interaction (LMM: χ^2_1=14.006, *P*<0.001) indicated there was no similar effect observed in males (Fig. 2.3C, Table 2.3). Thus, *Fw* lines showed greater divergence in SMI between sexes, and this effect appeared largely related to changes in mass. (Table 2.3,2.4)

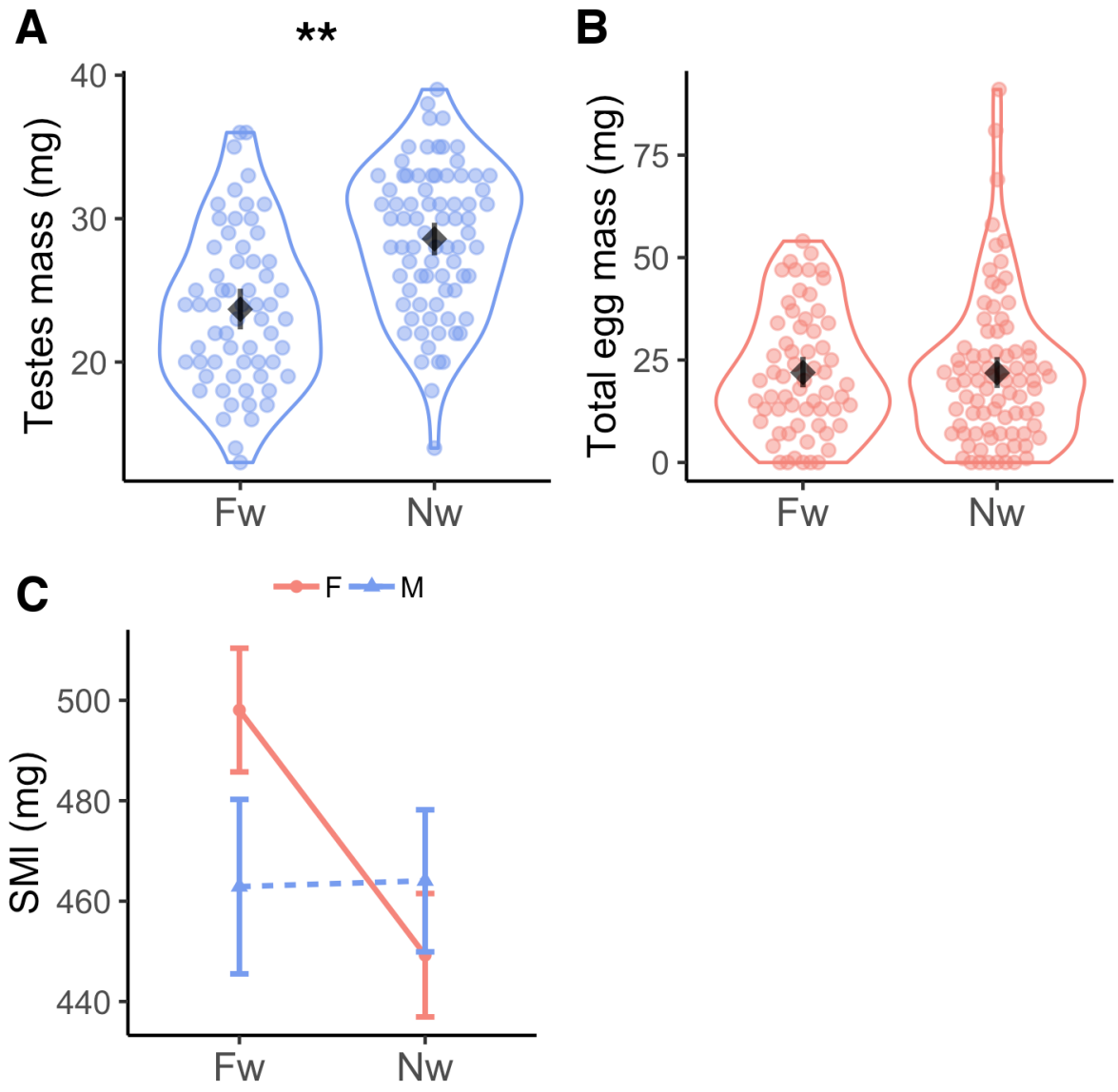

Figure 2.3 Sex-specific differences in fitness-associated phenotypes between genotypes **a**) Male testes mass, and **b**) female total egg mass, at 7 days post-eclosion. Black points illustrate means and 95% non-parametric confidence intervals, and ** indicates a significant difference at P<0.01 (see Table 2.3). **c**) *Fw* females showed increased SMI compared to *Nw* females, but SMI did not differ between male genotypes. Points illustrate means, error bars ±

2 standard errors. Points in a) and b) are scattered along the X-axis for purposes of visualisation only, with solid outlines representing density distributions.

2.4 Discussion

Influential models of sexual selection and sexual conflict predict that sex differences in gene expression underlying sexually selected traits arise due to IASC (Bonduriansky & Chenoweth 2009). However, such resolution of IASC is often expected to be incomplete, and costly elaboration of sexual traits should eventually be checked by natural selection (Fisher 1915; Lande 1981; Kirkpatrick 1982). Surprisingly, we found that the naturally-selected, genetic loss of a male sexual signal in crickets, via feminisation of male wing structures, affected gene expression more strongly in adult females than in males. There was no evidence of feminisation detectable in adult *flatwing* males, though this does not preclude such a role during earlier stages of development (e.g. Perry et al. 2014), which is hinted at by their reduced testes mass, and feminised CHCs (Appendix 1). In contrast, gene expression was demasculinised in female carriers of the *flatwing* genotype, which also showed increased body condition. These results best support our predictions under a scenario of relaxed IASC following male sexual trait loss (Fig. 2.1)

Sex-biased gene expression is likely to be associated with underlying IASC at loci where selection pressures differ between males and females (Mank 2017; Pizzari & Snook 2003), and sexual ornaments provide a clear example of a trait with contrasting fitness optima between sexes (Harano et al. 2010). The association between sexually selected traits and sexual conflict has frequently been inferred by comparing laboratory lines reared under contrasting selection regimes (Plesnar-Bielak et al. 2014; Hollis et al. 2014; Rice 1996; Holland & Rice 1999; Crudgington et al. 2005). In *T. oceanicus*, our results raise the intriguing possibility that relaxed IASC among females accompanied evolutionary loss of a

male sexual trait in the wild. Relaxation of IASC-associate constraints on expression of genes or traits in females could occur more widely than is generally considered, given repeated secondary losses of sexually-selected male traits across taxonomic groups (Wiens 2001; Porter & Crandall 2003; Morris et al. 2005; Ptacek et al. 2011), and could even facilitate these losses given the arms race-like dynamics with which IASC is frequently associated (Pennell et al. 2016).

Recent evidence suggests increased sexually dimorphic gene expression is associated with increased fitness (Dean et al. 2018). Following the results of the differential gene expression analyses, we therefore antipicated males and females from *flatwing* lines would show contrasting fitness effects of the mutant genotype, with females benefitting from demasculinised gene expression and males showing no variation. Flatwing males exhibited reduced testes mass, consistent with a previous report (Bailey et al. 2010) and contrary to our expectations, but females carrying the *flatwing* genotype did not differ in reproductive output. Instead, they exhibited increased SMI, a proximate measure of body condition, whereas *flatwing* males showed no such increase. While we are cautious about making direct inference about fitness effects of SMI, evidence of IASC over body size in species as diverse as humans (Stulp et al. 2012) and Indian meal moths *Plodia interpunctella* (Lewis et al. 2011), illustrates that males and females are frequently subject to contrasting optima for mass and structural size. In *T. oceanicus*, structural body size is likely to have an important influence on male mating success through male-male competition and female choice, while females less subject to pressures of sexual selection may benefit from maximising energy reserves (Whitman 2008). Phenotypic evidence suggests, therefore, that flatwing males are disadvantaged above and beyond their inability to signal, whereas female *flatwing* carriers are not strongly disadvantaged, and may actually benefit, potentially as a result of relaxed IASC.

While demasculinised gene expression and increased body condition in *flatwing*-carrying females support a hypothesis of relaxed IASC following male sexual trait loss, several caveats are worth considering. For example, demasculinised expression does not itself illustrate female benefit, though this interpretation is supported by the increased body condition observed, which may or may not be directly related to demasculinised gene expression, and by others' findings of an association between greater sex-biased gene expression and fitness-associated traits (Dean et al. 2018). Additionally, while our focus was on sex-biased transcripts, genotype also affected many transcripts in both sexes which did not show sex-bias. It is difficult to make inferences about the importance of these changes, or relate them to phenotypic traits, however it would affect interpretation of female benefit from carrying the *Fw* genotype if changes to non- sex-biased transcripts had contrasting fitness-associated effects (Chevillon et al. 1997). Finally, we examined differences between pure-breeding lines derived from a single wild population, but interpretation of our results would benefit from future work testing patterns of sex-specific selection across lines derived from wild populations with contrasting proportions of flatwing/normal-wing male phenotypes, to assess whether this influences IASC at the population level (Joag et al. 2016; Perry et al. 2017).

Comparing gene expression profiles across tissues within each sex revealed a strong pattern for transcripts differentially expressed between morphs in one tissue to show evidence of concordant differences in others. A lack of tissue specificity is often used as a proxy measure for pleiotropy (i.e. more pleiotropic loci are likely to be less tissue-specific) (Dean & Mank 2016), and extensive pleiotropy is widely expected to constrain the rate of evolution due to the reduced likelihood of a net increase in fitness (Orr 2000). We found that very few transcripts showing tissue-specific or sex-limited expression differed in expression between genotypes. This supports the view that changes we observe to be associated with carrying

flatwing are primarily among transcripts that have detectable levels of expression in both sexes, across tissues, and represent spillover effects of the *flatwing* locus in non-wing tissues. As well as showing *flatwing* has pervasive pleiotropic effects across multiple tissues, these results are consistent with the idea that the adaptive benefit of the flatwing phenotype in males outweighs costs associated with pleiotropic effects in non-focal tissues. Given the observed demasculinisation of female transcriptomes, and evidence for increased female body condition, our results also raise the intriguing prospect that positive pleiotropic effects of *flatwing* on females through relaxed IASC could actually have facilitated its rapid spread.

Our results are consistent with theoretical expectations for relaxed genomic conflict following reduction of sexual selection (Cox & Calsbeek 2009). The relaxation of genomic conflict may be an underappreciated yet capacitating feature of the widely-observed loss of sexual ornaments, for which the genetic and evolutionary mechanisms are not well understood (Wiens 2001). It is generally expected that the maintenance of sexually ornaments will be associated with IASC, and also acted against to varying degrees by natural selection. In *T. oceanicus*, the evolutionary loss of a male-specific sexual ornament may reduce IASC-associated constraints upon female gene expression, supporting the view that sex-biased gene expression only partially resolves underlying forces of intralocus sexual conflict even when phenotypes are sex-limited in their expression (Connallon et al. 2010; Harano et al. 2010). More generally, IASC may be an underappreciated driver during the evolutionary reduction or loss of secondary sexual traits.

Table 2.1. Numbers of DE genes for contrasts examining sex-biased expression and morph genotype in each tissue and sex.

Tissue	**DE_Down[2]**	**DE_Up[2]**	**DE_Sum[2]**
Sex (M)[1]			
Neural	379	152	631
Muscle	726	492	1218
Gonads	9030	11267	20297
Male genotype (*Nw*)			
Neural	0	5	5
Muscle	9	10	19
Testes	5	4	9
Male total	**14**	**19**	**33**
Female genotype (*Nw*)			
Neural	9	14	23
Muscle	160	204	364
Ovaries	50	135	185
Female total	**219**	**353**	**572**

[1] Reference group for each contrast is given in parentheses: M=males, Nw=normal-wing
[2] All DE inferred using FDR<0.01

Table 2.2 Significantly enriched GO categories for each of the female genotypes. Male genotypes showed no significant GO enrichment.

Genotype	GO Name	GO Category	FDR	Nr Test	Nr Reference	Non Annot Test	Non Annot Reference
Female.Fw	endomembrane system	CELLULAR_COMPONENT	1.82E-09	30	648	104	14195
Female.Fw	endoplasmic reticulum	CELLULAR_COMPONENT	1.96E-07	15	160	119	14683
Female.Fw	ncRNA metabolic process	BIOLOGICAL_PROCESS	0.005780891	9	118	125	14725
Female.Fw	tRNA aminoacylation for protein translation	BIOLOGICAL_PROCESS	0.0179214	5	33	129	14810
Female.Fw	cytoplasm	CELLULAR_COMPONENT	0.0179214	40	2291	94	12552
Female.Fw	ligase activity, forming aminoacyl-tRNA and related compounds	MOLECULAR_FUNCTION	0.0179214	5	32	129	14811
Female.Fw	ligase activity, forming carbon-oxygen bonds	MOLECULAR_FUNCTION	0.0179214	5	32	129	14811
Female.Fw	aminoacyl-tRNA ligase activity	MOLECULAR_FUNCTION	0.0179214	5	32	129	14811
Female.Fw	tRNA aminoacylation	BIOLOGICAL_PROCESS	0.0179214	5	33	129	14810
Female.Fw	amino acid activation	BIOLOGICAL_PROCESS	0.0179214	5	33	129	14810
Female.Fw	tRNA metabolic process	BIOLOGICAL_PROCESS	0.029074957	6	64	128	14779
Female.Fw	phenylalanyl-tRNA aminoacylation	BIOLOGICAL_PROCESS	0.041903784	2	0	132	14843
Female.Fw	cell	CELLULAR_COMPONENT	0.041903784	63	4622	71	10221
Female.Fw	phenylalanine-tRNA ligase activity	MOLECULAR_FUNCTION	0.041903784	2	0	132	14843
Female.Fw	endodermal digestive tract morphogenesis	BIOLOGICAL_PROCESS	0.041903784	2	0	132	14843
Female.Fw	cell part	CELLULAR_COMPONENT	0.041903784	63	4622	71	10221
Female.Fw	intracellular part	CELLULAR_COMPONENT	0.041903784	55	3827	79	11016
Female.Fw	UDP-glucose:glycoprotein glucosyltransferase activity	MOLECULAR_FUNCTION	0.041903784	2	0	132	14843
Female.Fw	cellular process	BIOLOGICAL_PROCESS	0.041903784	71	5412	63	9431
Female.Fw	intracellular	CELLULAR_COMPONENT	0.049092922	55	3861	79	10982
Female.Nw	muscle cell development	BIOLOGICAL_PROCESS	1.80E-12	15	87	91	14784
Female.Nw	striated muscle cell development	BIOLOGICAL_PROCESS	1.80E-12	15	86	91	14785
Female.Nw	myofibril assembly	BIOLOGICAL_PROCESS	8.63E-11	13	75	93	14796
Female.Nw	striated muscle cell differentiation	BIOLOGICAL_PROCESS	8.63E-11	15	121	91	14750
Female.Nw	muscle cell differentiation	BIOLOGICAL_PROCESS	1.25E-10	15	128	91	14743
Female.Nw	sarcomere	CELLULAR_COMPONENT	1.67E-09	12	77	94	14794

Female.Nw	supramolecular complex	CELLULAR_COMPONENT	1.67E-09	16	196	90	14675
Female.Nw	supramolecular polymer	CELLULAR_COMPONENT	1.67E-09	16	194	90	14677
Female.Nw	actomyosin structure organization	BIOLOGICAL_PROCESS	1.67E-09	13	103	93	14768
Female.Nw	myofibril	CELLULAR_COMPONENT	1.99E-09	12	83	94	14788
Female.Nw	contractile fiber part	CELLULAR_COMPONENT	1.99E-09	12	82	94	14789
Female.Nw	contractile fiber	CELLULAR_COMPONENT	1.99E-09	12	83	94	14788
Female.Nw	A band	CELLULAR_COMPONENT	1.23E-08	7	11	99	14860
Female.Nw	cellular component assembly involved in morphogenesis	BIOLOGICAL_PROCESS	1.23E-08	13	129	93	14742
Female.Nw	supramolecular fiber	CELLULAR_COMPONENT	1.23E-08	15	194	91	14677
Female.Nw	muscle structure development	BIOLOGICAL_PROCESS	9.56E-08	16	272	90	14599
Female.Nw	sarcomere organization	BIOLOGICAL_PROCESS	3.15E-07	9	55	97	14816
Female.Nw	skeletal myofibril assembly	BIOLOGICAL_PROCESS	4.20E-07	5	3	101	14868
Female.Nw	organelle assembly	BIOLOGICAL_PROCESS	1.34E-06	13	198	93	14673
Female.Nw	muscle thin filament assembly	BIOLOGICAL_PROCESS	1.60E-06	5	5	101	14866
Female.Nw	adult somatic muscle development	BIOLOGICAL_PROCESS	1.60E-06	6	14	100	14857
Female.Nw	skeletal muscle myosin thick filament assembly	BIOLOGICAL_PROCESS	4.36E-06	4	1	102	14870
Female.Nw	striated muscle myosin thick filament assembly	BIOLOGICAL_PROCESS	4.36E-06	4	1	102	14870
Female.Nw	supramolecular fiber organization	BIOLOGICAL_PROCESS	6.40E-06	13	232	93	14639
Female.Nw	muscle contraction	BIOLOGICAL_PROCESS	2.89E-05	6	26	100	14845
Female.Nw	actin cytoskeleton organization	BIOLOGICAL_PROCESS	4.46E-05	13	278	93	14593
Female.Nw	myosin filament assembly	BIOLOGICAL_PROCESS	5.12E-05	4	4	102	14867
Female.Nw	actin filament-based process	BIOLOGICAL_PROCESS	7.67E-05	13	294	93	14577
Female.Nw	muscle system process	BIOLOGICAL_PROCESS	8.63E-05	6	33	100	14838
Female.Nw	structural constituent of muscle	MOLECULAR_FUNCTION	1.10E-04	5	17	101	14854
Female.Nw	non-membrane-bounded organelle	CELLULAR_COMPONENT	5.72E-04	25	1251	81	13620
Female.Nw	intracellular non-membrane-bounded organelle	CELLULAR_COMPONENT	5.72E-04	25	1251	81	13620
Female.Nw	myosin filament organization	BIOLOGICAL_PROCESS	0.001041773	4	12	102	14859
Female.Nw	anatomical structure formation involved in morphogenesis	BIOLOGICAL_PROCESS	0.001205333	14	450	92	14421
Female.Nw	myosin complex	CELLULAR_COMPONENT	0.002402313	5	36	101	14835
Female.Nw	tissue development	BIOLOGICAL_PROCESS	0.003135179	21	1035	85	13836

Female.Nw	somatic muscle development	BIOLOGICAL_PROCESS	0.003667131	6	70	100	14801
Female.Nw	cytoskeleton organization	BIOLOGICAL_PROCESS	0.004851486	15	588	91	14283
Female.Nw	actin cytoskeleton	CELLULAR_COMPONENT	0.005463022	7	115	99	14756
Female.Nw	single-organism organelle organization	BIOLOGICAL_PROCESS	0.007631604	14	541	92	14330
Female.Nw	actin-dependent ATPase activity	MOLECULAR_FUNCTION	0.00827667	3	7	103	14864
Female.Nw	collagen trimer	CELLULAR_COMPONENT	0.010052005	2	0	104	14871
Female.Nw	Z disc	CELLULAR_COMPONENT	0.012809765	5	55	101	14816
Female.Nw	I band	CELLULAR_COMPONENT	0.013560177	5	56	101	14815
Female.Nw	flight behavior	BIOLOGICAL_PROCESS	0.019818808	4	31	102	14840
Female.Nw	flight	BIOLOGICAL_PROCESS	0.021919929	3	11	103	14860
Female.Nw	developmental process	BIOLOGICAL_PROCESS	0.026149506	38	3005	68	11866
Female.Nw	structural molecule activity	MOLECULAR_FUNCTION	0.028546329	9	270	97	14601
Female.Nw	cytoskeletal part	CELLULAR_COMPONENT	0.043255702	13	573	93	14298
Female.Nw	single-organism developmental process	BIOLOGICAL_PROCESS	0.044807148	37	2977	69	11894
Female.Nw	anatomical structure development	BIOLOGICAL_PROCESS	0.044807148	36	2864	70	12007

Table 2.3 Results from mixed models for proximate measures of reproductive output, body condition and body size.

	N		χ^2_1	*df*	P-value
ln testes mass (mg)[1]	139				
		Morph	8.800	1	**0.003**
		ln pronotum length	0.875	1	0.350
		ln somatic mass	33.841	1	**<0.001**
Egg mass (mg)[2]	145				
		Morph	0.011	1	0.916
		ln pronotum length	1.190	1	0.275
		ln somatic mass	2.688	1	0.101
ln somatic SMI (mg)[1]	284				
		Sex	14.071	1	**<0.001**
		Morph	5.095	1	**0.024**
		Sex × Morph	14.006	1	**<0.001**

All mixed models included a random effect of biological line, and pronotum length and somatic mass measures were standardized. ln indicates natural log.
[1] LMM
[2] Negative binomial GLMM

Table 2.4 Results from LMMs for measures of pronotum length and somatic mass.

	N		χ^2_1	*df*	**P-value**
Pronotum length (mm)	284				
		Sex	6.925	1	**0.008**
		Morph	1.109	1	0.292
		Sex × Morph	0.007	1	0.934
Somatic mass (mg)	284				
		Sex	5.521	1	**0.019**
		Morph	0.000	1	0.998
		Sex × Morph	6.493	1	**0.011**

All mixed models included a random effect of biological line.

3. Convergent adaptive trait loss is associated with parallel changes to transcriptomic sex differences

Sexes are often under contrasting selection pressures for shared traits, resulting in disruptive selection at associated loci. However, it is not well understood whether genetic variation associated with sexual dimorphism might also contribute to adaptation under natural selection. We investigated whether this process contributed to recurrent adaptive song loss in Hawaiian populations of *T. oceanicus*. Song loss on neighbouring islands occurred through genetic loss or reduction of sound-producing features on the male wing, resulting in female-like wing morphology. Using RNA-seq, we investigated whether, despite different underlying genetic mutations, 'flatwing' phenotypes arise through convergent disruption of transcriptomic sex differences which underlie sexual dimorphism in wings. Evidence for this prediction would suggest that independently evolved, adaptive *flatwing* genotypes have each targeted shared regulatory pathways involved in producing phenotypic sex differences. Our results show that the vast majority of changes in gene expression associated with genotypes underlying male song-loss are non-overlapping between island populations. However, sex-biased genes are highly represented among the few genes which do show parallel changes in expression, and include *doublesex* domains involved in insect sex-determination. Our results support the prediction that adaptive loss of male song occurred through convergent disruption of sex-specific wing development trajectories, and that genetic variation maintained by sexual dimorphism can be an important factor influencing the adaptive potential under natural selection.

3.1 Introduction

It is widely appreciated that evolutionary adaptation can occur and be observed over very few generations (Prentis, Wilson, Dormontt, Richardson, & Lowe, 2008; Losos, 2014). Standing genetic variation enhances the potential for this rapid adaptation (Hermisson & Pennings 2005; Lai et al. 2019; Alves et al. 2019), particularly given the very low frequency at which newly beneficial mutations are expected to arise (Nei 2005). In a seeming paradox, however, standing genetic variation should be eroded by evolutionary forces of genetic drift and stabilising or directional selection, particularly in small populations with low levels of gene flow (Barton & Turelli 2003). How small and fragmented populations subject to these constraints are able to adapt to changes in selection pressure is therefore a topic of enduring interest (Hunt et al. 2007).

Balancing selection is widely expected to play an important role in maintaining genetic variation. For example, sexually dimorphic traits maintained by sexually antagonistic selection pressures might play an important role in maintaining genetic variation affecting a range of ecologically important traits. Differences in gene expression between sexes underlie a considerable portion of the phenotypic diversity observed in populations (Pointer et al. 2013; Immonen et al. 2014; Mank 2017), accounting in large part for the sexual dimorphism which is ubiquitous across sexually reproducing organisms (Andersson 1994; Bachtrog et al. 2014), and helping mitigate underlying genomic conflict resulting from contrasting selection pressures acting on males and females (Bonduriansky & Chenoweth 2009; Mank 2017). This sexually antagonistic selection acting upon shared genes is associated with disruptive selection at the population level, creating and maintaining genetic diversity at loci involved in the conflict (Connallon & Clark 2014; Cheng & Kirkpatrick 2016; Lonn et al. 2017; Wright et al. 2018). Given the variety of phenotypic traits which exhibit sexual dimorphism, this balancing selection associated with sexually antagonistic selection is likely increase the

ability of either sex to rapidly adapt to changes in ecology or environment (Fierst 2011). Where genomes are shared, individuals of each sex must carry the genes which underlie both male and female-specific traits, and the expression of these genes across tissues will be regulated by existing sex determination pathways.

A factor which has been argued to be of key importance in promoting genetic adaptation is compartmentalisation, through which mutations have coordinated phenotypic effects restricted to some subset of related traits (Kirschner & Gerhart 1998; Welch & Waxman 2003). Sexual dimorphism is achieved largely through differences in gene expression (Perry et al. 2014; Mank 2017), often regulated in a coordinated manner by relatively few key genes (Bachtrog et al. 2014). For example, the doublesex/mab-3 related (*Dmrt*) family of transcription factors plays a key, conserved role in the determination of phenotypic sex differences across insects, nematodes and mammals (Kopp 2012) which are regulated via differences in expression or splicing between males and females (Price et al. 2015). Differences in the extent of sex-biased gene expression are also associated with phenotypic variation within each sex (Pointer et al. 2013; Stuglik et al. 2014), and carry adaptive significance (Dean et al. 2018). Thus, existing developmental pathways which regulate the production of sexually dimorphic phenotypes could provide a substrate for evolution by coordinated phenotypic change among ecologically related traits (Wagner & Altenberg 1996), such as morphology and diet, that can be effected by changes to just one or a few key regulatory genes (Kijimoto et al. 2012).

Whether, or to what degree, convergent phenotypic changes tend to be underpinned by the same sets of genes and pathways is not yet clear (Stern 2013; Warner et al. 2019). To test a role for phenotypic variation maintained by sexual dimorphism in adaptive evolution, we capitalised on the emergence and rapid spread of adaptive song-loss in Hawaiian populations of *T. oceanicus*. Adaptively silent flatwing males are unable to produce song

owing to female-like forewing vein morphology, and a key feature of interest is that multiple male-limited sound-producing structures are concomitantly reduced (Wagner & Altenberg 1996; Bailey et al. 2019) (Fig. 3.1). Although the ages of mutations underlying flatwing phenotypes are not known, both adaptive phenotypes appear to have emerged and spread recently in populations on multiple islands, and contemporarily, having been first observed in continuously-monitored populations from Kauai and Oahu within 3 years of one another (Zuk et al. 2006). Genomic analyses suggest the two silent male phenotypes have distinct underlying genetic architectures (Pascoal et al. 2014), indicative of rapid convergent evolution. Despite their different underlying architectures, flatwing phenotypes from both islands segregate as single X-linked Mendelian traits (Pascoal et al. 2014).

We tested whether flatwing and normal-wing genotypes from Kauai and Oahu show overlapping differences in expression affecting genes involved in regulating sexual dimorphism. Because flatwing male wings appear similar to those of females (Fig. 3.1), we anticipated any overlapping changes in gene expression would include genes involved in regulating phenotypic sex differences, which would support the idea rapid adaptation was facilitated by variation maintained by sexual dimorphism. Note that, given *T. oceanicus* have an XO sex determination system, with males carrying just one copy of the X-chromosome and females two, sexual dimorphism must result from differences in expression of shared genes related to differences in X-dosage. We predicted: 1) flatwing and normal-wing phenotypes would show partially overlapping differences in gene expression in the two island populations, despite their distinct genetic architectures; and, 2) among these shared changes in gene expression, sex-biased genes should be highly represented: more so in Kauai, where loss of male-specific sound-producing structures is more complete (Fig. 3.1). Evidence to support these predictions would indicate that rapid convergent adaptation was promoted by genetic and phenotypic variation maintained by sexually antagonistic selection pressures.

3.2 Methods

3.2.1 Terminology

We refer to the genotype underlying normal-wing morphology as *Nw*, and to those underlying flatwing morphology on Kauai and Oahu as Fw_K and Fw_O, respectively. Female wings appear the same irrespective of genotype, but we nevertheless indicate their genotype using the same terminology (i.e. ♀*Nw*, ♀Fw_K, or ♀Fw_O). Note that females used in experiments are all homozygous, and males hemizygous, for the respective genotype.

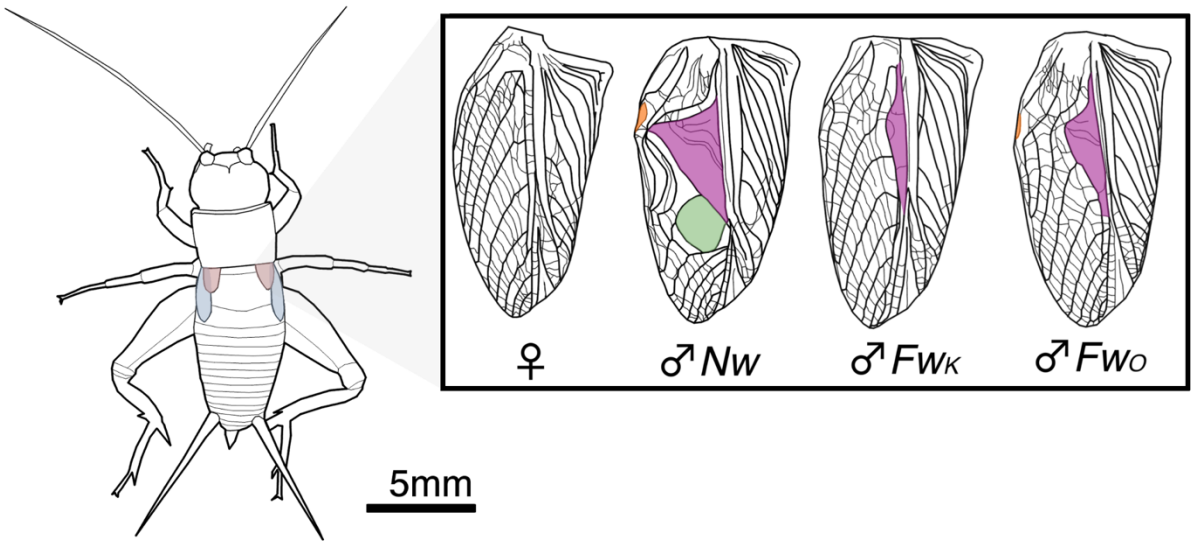

Figure 3.1 Male and female wing phenotypes
Developing forewings (red; hindwings are highlighted in blue) were sampled from second-penultimate instars. The inset shows adult wing vein phenotypes for a female (in which venation does not appear to differ between genotypes), a *Nw* male, and *Fw* males from Kauai (♂Fw_K) and Oahu (♂Fw_O), with sound-producing structures highlighted (green: mirror; pink: harp; orange: plectrum). Note the less complete reduction of sound-producing features in Oahu. Wing drawings are tracings of wing micrographs, adapted from Pascoal et al. (2014).

3.2.2 Rearing of experimental populations and biological lines

Laboratory populations were reared in a growth chamber at 25 C, under a 12-hour L:D cycle. Populations used in the experiment were derived from eggs laid by wild-caught females from populations of Wailua in Kauai and La'ie in Oahu in 2014, from which purebreeding *Nw* and *Fw* lines were produced by performing genetic crosses described in Pascoal et al. (2016); males and females from these lines are therefore purebreeding for the respective genotype, with males carrying one copy and females two. Kauai lines were recently outcrossed by mixing *Nw* and *Fw* lines, and purebreeding lines were reconstituted by performing genetic crosses two generations prior to the current experiment; otherwise Kauai and Oahu lines were reared and maintained under identical conditions, in 20L boxes with cardboard shelter, and food and water available *ad libitum*.

3.2.3 Sampling and extraction of RNA

We collected and sequenced 24 RNA samples, including *Nw* and *Fw* genotypes of both sexes from each island population, with 3 replicate purebreeding lines per group. For sampling, individuals were removed from stock boxes at second-penultimate instar (Fig. 3.1), when developing wings are first externalised, ca. 1.5 months after hatching. Wing differences between *Nw* and *Fw* genotype males arise early during their development, and become pronounced between second-penultimate and penultimate instar (Pascoal et al. 2016). Individuals were briefly anesthetised using CO_2, and the dorsal-right forewing bud (henceforth 'wingbud') removed using micro-dissection scissors then placed in RNAlater. Wingbuds from five individuals were pooled per sample, and samples were frozen at -20C after 24 hours at 4C.

RNA extractions were performed using a Trizol protocol. Extracted RNA purity was assessed using a NanoDrop ND-1000 spectrophotometer, and quality assessed using a Agilent

2100 Bioanalyzer. Following library preparation with RiboZero, samples were sequenced on an Illumina HiSeq 4000, generating 2×150 bp paired end reads. Due to logistical constraints, library preparation and sequencing were performed separately for samples from the two populations, but using identical protocols; we restrict differential expression analyses to within-population comparisons, so this should not affect our results. CASAVA v1.8.2 (Illumina) was used for basecalling and de-multiplexing of indexed reads. Adapter sequences were trimmed from fastq files using Cutadapt v1.2.1 (Martin 2011) and low quality bases were removed using Sickle v1.200 with a minimum window quality score of 20. Sequences with high similarity to Eukaryotic ribosomal RNAs were removed from the dataset using sortmeRNA (Kopylova et al. 2012).

3.2.4 Genome alignment and transcriptome assembly

Genome alignment, transcriptome assembly and quantification of expression were performed following Pertea et al. (2016). Reads were aligned to the *T. oceanicus* genome (v1, available from http://chirpbase.org/) using HiSat2 v2.1.0. A genome-guided transcriptome was then assembled from output files using StringTie v1.3.4, and gene expression values quantified for each of the samples. To retain in our transcriptome only genes with strong empirical support and which appear to be protein-coding, we filtered any without open reading frames of >100 amino acids, and which weren't expressed at >1 count per million in at least 3 samples from each population, as well as a previously sequenced Kauai wingbud RNA-seq dataset (see below).

3.2.5 Differential expression analysis

Gene counts produced by StringTie were prepared for input into edgeR v 3.20.9 (Robinson et al. 2010) using the *prepDE.py* script made available by the authors (Pertea et al. 2016). In edgeR, counts were normalised by trimmed means of M-values (TMM), after which a single

negative binomial GLM was fit incorporating all data, using per-gene normalised expression values as the response variable. Differential expression (DE) analyses were performed using likelihood ratio tests for pairwise comparisons between morph genotypes and sexes. P-values were FDR-adjusted using the Benjamini–Hochberg procedure, and genes were considered DE between groups if FDR values were < 0.05. This less stringent threshold was adopted due to relatively high variance within groups. Statistical analyses were performed within R v3.4.1 (R Core Team 2017).

As partial validation of our results, we compared the identity of genes DE between morph genotypes from Kauai with those found to be DE between male morphs in previously-sequenced samples from male Kauai wingbuds (see: Pascoal et al. 2016). The latter data were collected using methods considerably different to our own: individuals came from different biological lines, were not anaesthetised prior to sampling, there were fewer individuals per pooled sample and more samples per group (N=3 individuals per pool; N=6 samples per male morph). Samples were also sequenced on a different platform (Illumina HiSeq 2000). Thus, a high degree of overlap between datasets in genes identified as DE would be indicative of biologically meaningful and robust experimental procedures. We aligned these samples to the transcriptome and performed DE analysis following the same procedure as above.

To test hypotheses of overlapping changes in gene expression between Kauai and Oahu's respective *Nw* and *Fw* genotypes, we first subset genes which were significantly DE in one or both comparisons. We then used a chi-squared test to statistically compare the number of these DE genes for which log-fold changes were positively and negatively correlated between islands. Similarly, to test whether flatwing genotypes showed generally 'feminised' expression patterns, we subset genes which were DE between genotypes on each island, and which were also sex-biased, and compared the direction of change (in this case there were too few genes for statistical comparison).

3.2.6 Testing disproportionate involvement of the X chromosome

Because both flatwing phenotypes segregate as X-linked traits, and differences in expression of X-linked genes might play a particularly important role in producing sexual dimorphism, we tested for disproportionate differential expression of genes which are known to be located on the X chromosome, using the linkage map of Pascoal et al. (2018). To do so, we compared the proportion of X-linked genes passing filtering which showed differential expression between *Nw* and *Fw* genotypes, with that of autosomes. Additionally, we tested whether males and females showed differences in expression of X-linked relative to autosomal genes. Given the XO sex-determination system, males might exhibit complete or partial dosage compensation of X-linked genes, or females may exhibit deactivation of one of their X chromosomes, which could affect the number of genes identified as sex-biased. We calculated 'relative X expression' (RXE) as $\log_2(x) - \log_2(a)$, where x and a refer to X-linked and autosomal genes, respectively, and RXE values <0 indicate incomplete X up-regulation in males (or incomplete inactivation in females), while >=0 indicates full up-regulation (complete inactivation in females) (Duan et al. 2019). For comparison, we performed the same analyses above to test for sex differences in X-dosage in existing RNA-seq data for adult neural, thoracic and gonad tissues from males and females from Kauai (Chapter 2), for which alignment and quantification was re-performed (cf. Chapter 1) as above, using HISAT2, Stringtie and EdgeR. We also tested for disproportionate representation of X-linked genes among those DE between morph genotypes by comparing the proportion of DE genes which were X-linked with the proportion of genes in the linkage map which were X-linked, using a Chi-squared test.

3.2.7 Gene ontology and functional enrichment analyses

DE genes were entered as BLASTX queries against the NCBI non-redundant protein database, with an e-value threshold of 10^{-3} and a maximum of 20 hits. Mapping and annotation were performed in Blast2GO (Conesa et al. 2005) with default parameters. Functional enrichment of gene ontologies (GO) was assessed for all transcripts passing the expression filter against all *Drosophila melanogaster* proteins.

3.3 Results

After filtering, the genome-guided transcriptome assembly contained 30,299 unigenes (henceforth 'genes'). There was a proportionally high degree of overlap between genes found to be differentially expressed (DE) between Kauai male genotypes in the new and previously-collected datasets (10 of 30 shared; compared with 20 of 30,269 not), and their log-fold changes were positively correlated (rho=0.648, P=0.049), despite substantial differences in the methods used to collect each (see Methods). This consistency demonstrates our DE results are technically and biologically robust.

3.3.1 Gene expression changes strongly correlated between sexes

In each of the populations the effect upon gene expression of carrying the respective *flatwing* genotype (Fw_K or Fw_O) was highly correlated between males and females. About half the genes DE between male genotypes were also significantly DE between female genotypes in each population (Kauai: N=11 of 25 [44%]; Oahu: N=26 of 50 [52%]), and changes were strongly correlated across all genes showing DE in one or both sexes (Kauai: N=41, Spearman's rank rho=0.771, P<0.001; Oahu: N=90, rho=0.775, P<0.001). (Fig. 3.2A,B)

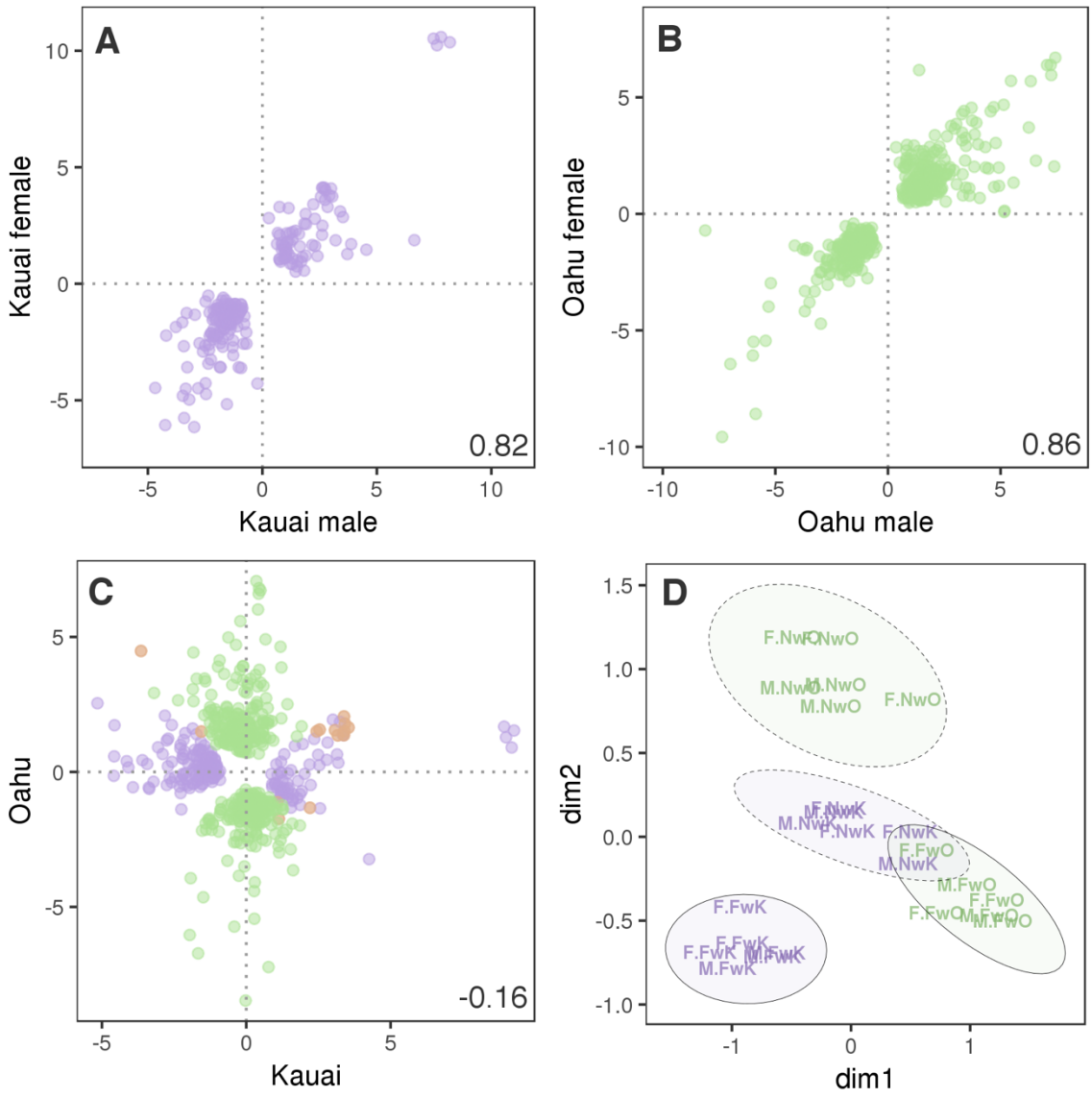

Figure 3.2 Correlated effects on gene expression between sexes but not islands
The effects of *Fw* genotypes on male and female transcriptomes were strongly correlated within each population for both (A) Kauai and (B) Oahu. (C) The correspondence of *Fw* effects in Kauai vs. Oahu (Fw_K and Fw_O), pooled across sexes; genes DE in both populations are highlighted in orange. In plots (A – C), points show differences in mean expression values ($\log_2$ counts per million) between *Nw* and respective *Fw* genotypes. Points > 0 and < 0 are *Nw*- and *Fw*-biased, respectively. Annotations show significant (P<0.001) Spearman's rank correlation coefficients. D) Multidimensional scaling plot showing differences along two major axes of variation (dim1 and dim2) between samples in expression of genes identified as significantly DE between genotypes in either population. Labels signify individual sex and genotype (F.FwO= female with Fw_O genotype, etc.), and ellipses show 95% confidence intervals assuming a multivariate t-distribution, for each genotype × island combination, again pooled across sexes. Ellipses are coloured by population, with solid/dotted outlines indicating the genotype underlies normal-wing/flatwing male phenotypes, respectively.

3.3.2 Largely discordant gene expression changes between populations

There was very little overlap in gene expression changes associated with Fw_K and Fw_O genotypes from the two respective populations. Because changes were strongly correlated between sexes in each population (Fig. 3.2A,B), we maximised our statistical power to identify shared differences between populations by pooling sexes for each genotype from both populations (Kauai: N=205 genes DE between genotypes; Oahu N=464). Just 15 (7.31%) of the 205 genes DE between Kauai genotypes were also DE between Oahu genotypes, and only 10 (4.88%) of these showed concordant changes; all showing concordant *Nw*-bias. Surprisingly, across genes DE between genotypes in either population, expression differences between genotypes in the other population were more likely to be in the opposite direction (concordant: N=230, non-concordant: N=409; X_1^2=50.142, P<0.001) (Fig. 3.2C). For these genes DE between *Nw* and respective *Fw* genotypes in either population, a multidimensional scaling plot clearly separated Fw_O and Fw_K genotypes along the first major axis of variation, along which *Nw* genotypes on the two islands showed no apparent differences. In contrast, the two *Fw* genotypes differed from *Nw* genotypes in the same direction along the second major axis of variation (Fig. 3.2D).

3.3.3 Sex-biased genes represented among those DE between genotypes

In contrast with the very small overlap in genotype-associated changes between populations, 13 of the 20 (65%, cf. 4.88% above) genes identified as sex-biased in Kauai were also sex-biased in Oahu, in all cases concordantly. Despite being few in number, genes which were sex-biased in either population were highly represented among those DE between genotypes in the two populations (Kauai: 3 of 20 sex-biased genes DE; Oahu: 7 of 30). Overall, 14 of the 654 (2.14%) of unique genes DE between genotypes in one or both populations reported sex-bias; contrasting with 23 sex-biased of the remaining 29,645 (0.078%) genes in the

transcriptome (X_1^2=206.71, P<0.001). Sex-biased genes were especially highly represented among genes DE in both populations (3/15 [20%]). There was, however, no clear evidence of feminised gene expression in flatwing genotypes; 2 of 3 concordantly DE between flatwings and females on Kauai, and 2 of 7 on Oahu, though our ability to make conclusions is precluded by the few genes involved in comparisons.

3.3.4 Down-regulation of doublesex across Fw *genotypes*

Despite the lack of feminised patterns of gene expression in *Fw* genotypes, 2 of the 10 genes showing concordant genotype-associated changes across both populations showed significant BLAST homology with doublesex (*dsx*) proteins: one of these contained the conserved DNA binding domain superfamily, the other the DNA dimerisation domain superfamily; these two domains are considered essential for *dsx* transcription factor activity (Price et al. 2015). Each showed strongly correlated down-regulation in *Fw* genotypes across sexes and populations, and differences between genotypes were stronger in Kauai (Fig. 3.4). We did not identify sex-specific *dsx* isoforms, which are often observed in insects; instead, expression patterns were consistent across isoforms. Both domains were also validated as DE in the previously collected Kauai wingbud data (unadjusted P < 5e-04) from Pascoal et al. (2016), and we found by cross-referencing with the QTL of Pascoal et al. (2018) that the DNA dimerization domain strongly co-localises with the Fw_K-associated region of the X-chromosome (Figs 3.3C, 3.4). The DNA-binding domain is not present in the linkage map.

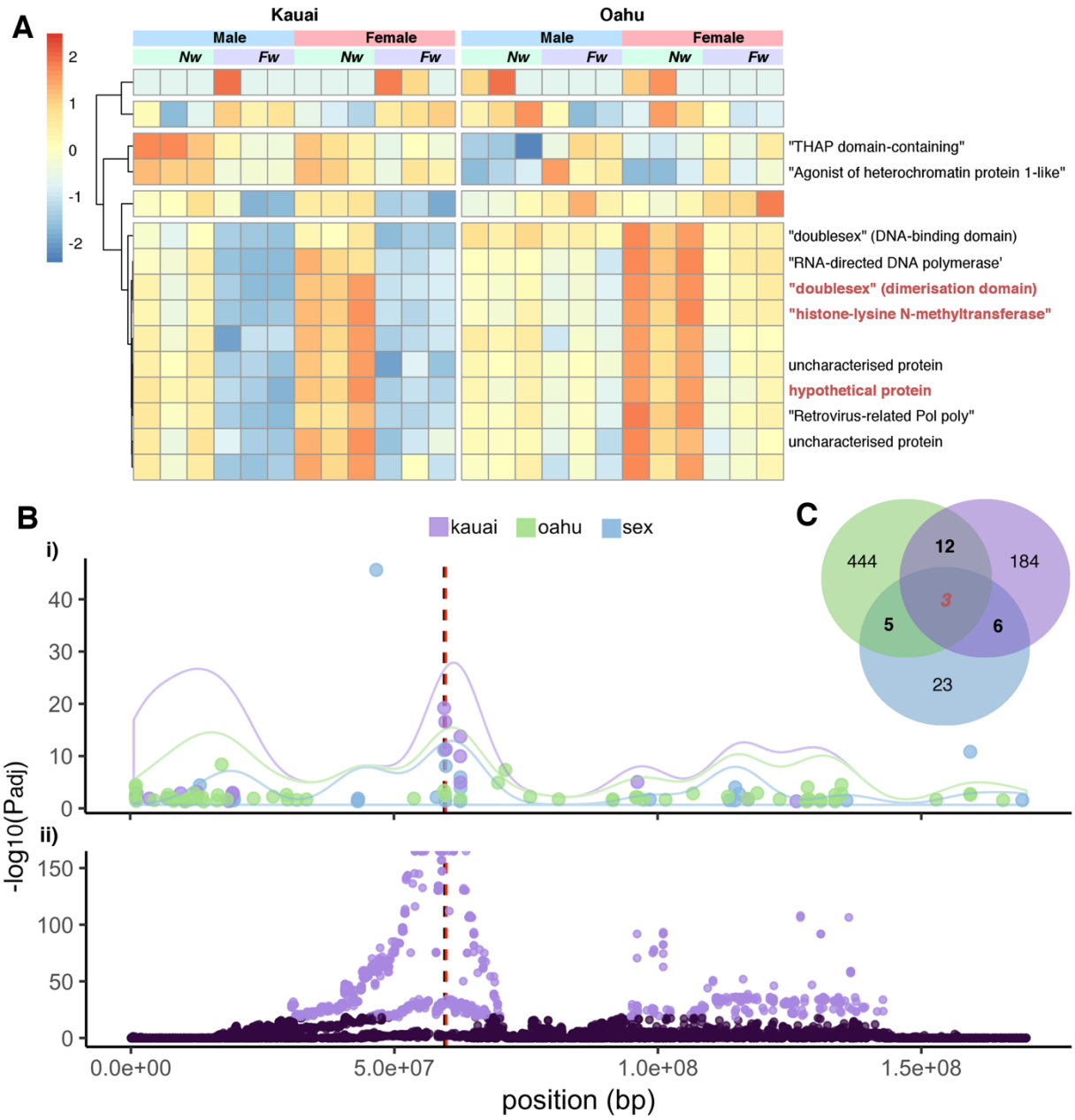

Figure 3.3 Genes and genomic regions of significance to flatwing morphology
A. Expression values in counts-per-million, scaled by row (i.e. gene), for all genes reported as DE between genotypes in both populations. Labels show BLASTX annotations; red are those also showing significant sex-bias. **Bi**. Positions of DE genes located on the X-chromosome. Lines show densities of DE genes for each contrast. **Bii**. Kauai flatwing-associated genomic QTL markers from Pascoal et al. (Appendix 1). Dotted lines show the physical position of the doublesex dimerization domain. Y-values in both Bi and Bii illustrate inverse $\log_{10}$ FDR (i.e., larger values on the Y-axis indicate greater significance in DE or QTL comparisons), and chromosomal positions on the x-axes are aligned. **C.** Venn diagram showing overlap in the identity of genes DE between Kauai (purple) and Oahu (green) genotypes, and those showing sex-biased expression (blue).

Of the other 8 DE genes showing correlated changes in both populations, 6 had BLASTX annotations; an RNA-directed DNA polymerase, a mariner *Mos1* transposase, a retrovirus-related *Pol* polyprotein, and 4 uncharacterised or hypothetical proteins. All of these that could be localised on the linkage map of Pascoal et al. were within the QTL region of the X-chromosome which is statistically associated with the Kauai *Fw* genotype. A homolog of *fem-1*, essential for sex-determination in *Caenorhabditis elegans* (Doniach & Hodgkin 1984), was up-regulated in males and females with *Fw*$_O$ genotypes, but showed no correlated change in the Kauai population.

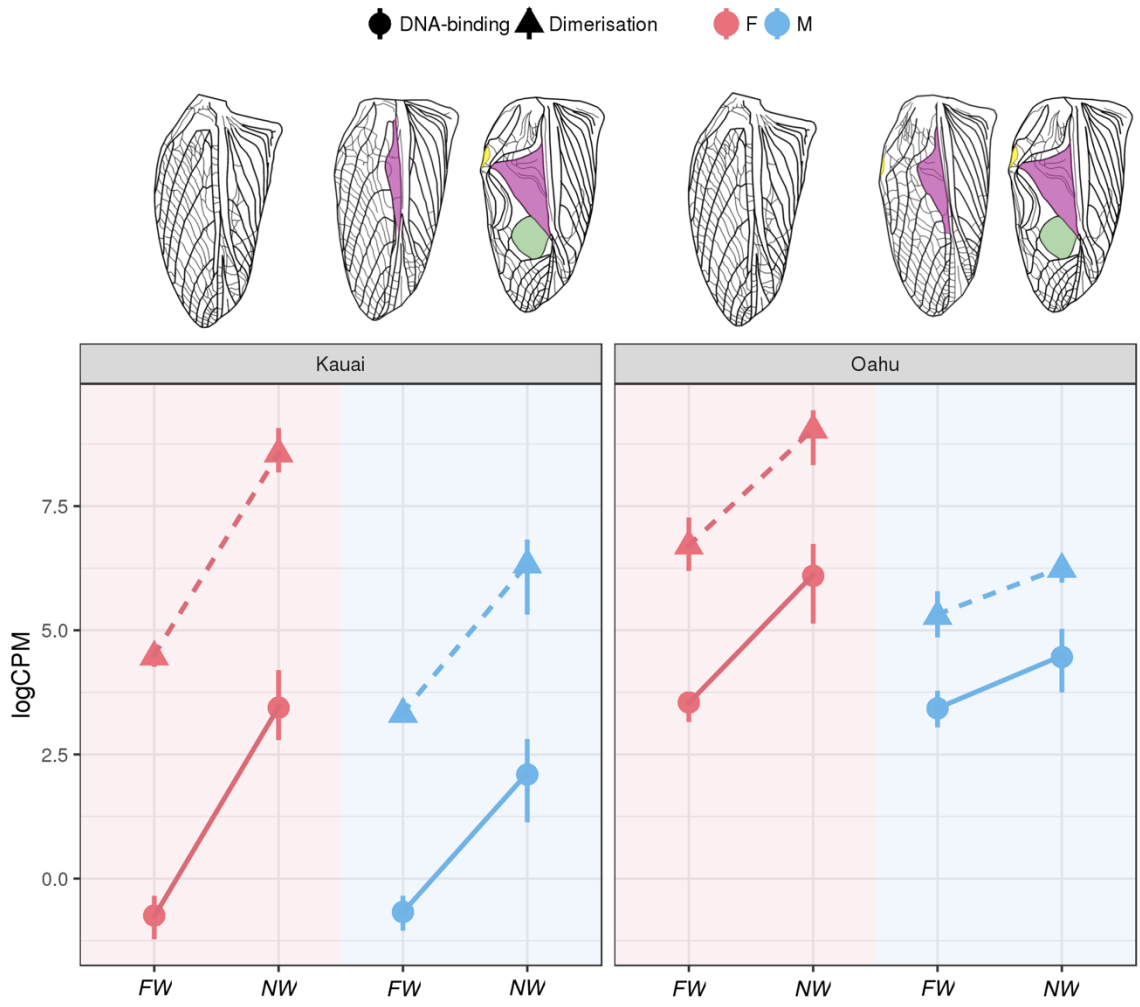

Figure 3.4 Coordinated down-regulation of doublesex domains
Loss of male song due to forewing feminisation is associated with down-regulation of *dsx* in developing wingbuds. Relative expression values (log_2 counts-per-million) are shown for the

two consistently DE *dsx* domains. Both are down-regulated, in Fw_K and Fw_O genotypes, and in both sexes. However, the extent of down-regulation is less in Fw_O, a genotype which is associated with less complete loss of sound-producing features on the wings of males which carry it (see wing diagrams, top). Points illustrate means, error bars the minimum and maximum values across replicates (N=3 per group of samples).

3.3.5 Functional enrichment, KEGG and GO analyses

KEGG analysis identified purine and thiamine metabolism pathways represented among genes DE between genotypes in both populations, and these were also the only pathways represented among sex-biased genes. Overall, 310 (45.99%) genes reported as DE between genotypes reported significant homology. Of the 30,299 total genes in the transcriptome, 12,182 (40.21%) reported significant BLASTX hits to proteins from *Drosophila melanogaster*. Only the set of genes up-regulated in Kauai normal-wing lines reported significant functional enrichment (FDR<0.05), for biological processes involved in cytokinesis, bearing similarity with previous results (Pascoal et al. 2018; Chapter 3).

3.3.6 No differences in dosage or disproportionate involvement of X-linked genes

There was no evidence of differences across sexes and genotypes of the two populations in summed expression of X-linked (N=6,091), relative to autosomal (N=13,224), genes (Kruskal-Wallis rank sum test: X^2_7=3.453, P=0.840). Relative expression of X-linked genes did not differ significantly between sexes despite differences in ploidy (Wilcoxon rank-sum test: P=0.143), indicative of X- dosage compensation in males or inactivation in females, and contrasting with adult tissues from Chapter 2, in which female relative expression of X-linked genes was consistently significantly greater than males (Wilcoxon rank-sum tests: all P<0.01).

There was also no evidence X-linked genes were overrepresented among those DE between developing wings of *Nw* and *Fw* genotypes (pooled across sexes) from either

population, compared with autosomal genes: 30 of the 116 DE (25.86%) genes present in the linkage map of Pascoal et al. (2018) were X-linked, versus 6,091 of the total 19,315 (31.54%) genes present in the linkage map (X^2=1.467, P=0.226).

3.4 Discussion

One way in which sexual reproduction might produce and maintain genetic variation, thereby contributing to populations' ability to adapt under selection, is through sexually antagonistic patterns of selection which are associated with balancing selection at the population level (Cheng & Kirkpatrick 2016; Wright et al. 2018). Such genetic variation produced and maintained at loci which underlie ecologically important, sexually dimorphic traits might therefore provide an important substrate for adaptive evolution. Our results show gene expression changes associated with adaptive silent phenotypes in *T. oceanicus* strongly involve genes with conserved roles in regulating phenotypic sex differences, supporting our prediction that variation associated with sexually dimorphic wing venation in *T. oceanicus* promoted their loss in flatwing males.

Most of the differences between genotypes underlying normal-wing and convergently evolved flatwing phenotypes were distinct between islands. Less than 5% of genes identified as DE between morph genotypes in either island population were the same and showed coordinated changes between convergently evolved flatwing phenotypes. In contrast, 65% of sex-biased genes were shared and showed correlated changes between island populations. Moreover, among the 95% of genes that did not show parallel changes in convergently evolved flatwing genotypes, the majority showed opposing patterns of expression changes. These results appear to support previous data indicating flatwing phenotypes evolved independently in the two populations. Pascoal et al. (2014) compared flatwing-associated

nucleotide polymorphisms from Kauai and Oahu, and found a considerable majority of differences between *Nw* and *Fw* genotypes in each island population were non-overlapping, while that those few that did overlap showed allelic reversal. The divergent changes in expression we observe in association with *Fw* genotypes underlying convergent flatwing phenotypes also supports growing evidence that convergent adaptation can provide a source of genetic and phenotypic variation between populations, which could promote future diversification (Mcgee & Wainwright 2013; Bailey et al. 2019).

Interestingly, however, there was a small but notable overlap in effects of the different genotypes upon gene expression, among which sex-biased genes were highly represented. Sex-biased genes were disproportionately affected by flatwing-associated genotypes, and among these was doublesex (*dsx*). Males and females carrying Fw_K and Fw_O were consistently associated with down-regulation of *dsx* domains. One of these, the dimerization domain, strongly co-localises with a QTL for the Kauai *Fw* genotype, and is downstream of a region with strongly reduced genetic diversity in flatwing males from Oahu, indicative of recent selection in this region (Xiao Zhang, unpublished data). *Dsx* was an early candidate for involvement in flatwing morphology (Pascoal et al. 2016) owing to its primary role in mediating sexual dimorphism in insects and related arthropods (Kunte et al. 2014; Price et al. 2015), particularly in sex-specific expression of sexual traits (Kijimoto et al. 2012). For example, in horned beetles *Onthophagus taurus*, males exhibit large exaggerated horns which function as a weapon in aggressive encounters, while females are typically hornless. However, down-regulation of *dsx* drastically reduces the size of males' horns while, counterintuitively, leading to their expression in ordinarily hornless females, illustrating its sex-specific effects in regulating phenotypic expression (Kijimoto et al. 2012). Our results suggest a similar pattern of sex-specific expression of male sound-producing wing structures in *T. oceanicus*, where multiple losses of the male sexual trait are associated with down-

regulation of *dsx*, the extent of which correlates with completeness of reduction of male sound-producing structures.

It is plausible that down-regulation of *dsx* and related pathways in developing tissues could also contribute to a range of 'feminised' phenotypes observed in adult males; including reduced reproductive tissue mass (Bailey et al. 2010; Rayner et al. 2019), and female-like cuticular hydrocarbon profiles (Appendix 1). It is nevertheless clear that the extent of 'feminisation' of flatwing males is substantially greater in the developing forewings, in which they strongly resemble females, compared with other sexually dimorphic phenotypes such as the gonads. Differences in expression of *dsx* might therefore be largely or partially restricted to developing forewings (Kunte et al. 2014). A recent study of *Drosophila melanogaster* demonstrated the extent of sexual dimorphism, in relation to differences in regulation of doublesex, is modified in a tissue-specific manner (Rice et al. 2019), reiterating the tissue-specific evolvability of gene expression networks underlying sexual dimorphism (Wagner & Altenberg 1996; Fierst 2011).

Transcriptional regulation of sex differences by *dsx* is frequently observed to involve sex-specific splicing, which we did not observe in *T. oceanicus*, however studies of arthropods and insects distantly related to model organisms such as *Drosophila melanogaster* have found that this is often not the case (Price et al. 2015; Ruiz et al. 2015). In these cases *dsx* appears to regulate sex differences largely via differences in expression, as are apparent in our data. In holometabolous insects, sexual differentiation involving sex-specific splicing of *dsx* is regulated by transformer (*tra*) (Kopp 2012), however no sequences in the *T. oceanicus* genome show significant homology with *tra*. Given that *Nw* females exhibit much greater expression of both *dsx* domains, compared with males, but their expression is strongly down-regulated in *Fw* females which show no apparent changes in wing morphology, it is clear that if *dsx* is responsible for effecting differences in wing venation it does so in a male-

limited manner. *Dsx* could play a primary role in mediating male-specific development (Kopp 2012) through sex-specific downstream effects; as in the male-specific effects of *distal-less* in regulating sexually dimorphic antennal shape in water striders *Rheumatobates rileyi*, despite expression in both sexes (Khila et al. 2012). Indeed, the strong correlation between gene expression effects of Fw_K and Fw_O between sexes, as well as the relatively few genes identified as showing sex-bias (20 in Kauai, 30 in Oahu, cf. substantial sex-bias in adult tissues (Chapter Two; Rayner, Pascoal, et al. 2019), suggests that many of the same genes must have sex-specific effects.

Our findings reveal the involvement of transcriptomic sex differences in rapid convergent loss of a male sexual trait and, specifically, show this loss is associated with down-regulation of a phylogenetically conserved sex-differentiating gene, doublesex, in developing wing tissues. By implicating parallel down-regulation of sex-determining pathways in the convergent adaptive loss of male song, these findings support our hypothesis for the role of variation maintained by sexually antagonistic selection in rapid adaptation in capacitating rapid adaptive loss of a male sexual trait. Our results are also consistent with a more general role for variation maintained within the genome by sexually antagonistic patterns of selection in providing a substrate for adaptive evolution (Fierst 2011). Additionally, our finding that convergently evolved mutant genotypes affect the expression of largely non-overlapping sets of genes, but do both affect the expression of a small, but perhaps key, subset of genes, contributes to understanding of how convergent evolution is able to occur through different genetic mutations by targeting the same developmental pathways (Stern 2013).

4. Same-sex sexual behaviour and the evolution of alternative male reproductive phenotypes*

Male same-sex sexual behaviour (SSB), where males court or attempt to mate with other males, is common among animal taxa. Recent studies have examined its fitness costs and benefits in attempts to understand its evolutionary maintenance, but the evolutionary consequences of SSB are less commonly considered. One potential impact of SSB might be to facilitate the evolution of traits associated with less sexually dimorphic males, such as alternative reproductive tactics, by diverting costly aggression from other males. To test this, we capitalized on the recent spread of a silent males in Hawaiian *T. oceanicus*, which are unable to produce characteristic male acoustic signals, benefit from satellite mating behaviour, and exhibit feminized appearance and cuticular hydrocarbon profiles. We tested the prediction that interactions involving these nonsignalling, less sexually dimorphic male morphs would show heightened rates of SSB, which could reduce the strength of male–male competition and permit greater access to females. We found no evidence that SSB was more common in trials involving silent males. Instead, SSB was predicted by courtship of females presented during a pretrial treatment. Our results provide evidence supporting the view that SSB represents a spillover of sexually selected courtship behaviour in a nonadaptive context, but do not support a strong role for SSB in the evolution of less ornamented males in this system.

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4.1 Introduction

Same-sex sexual behaviour (SSB), where individuals court or attempt to mate with members of the same sex, is taxonomically widespread (Bailey & Zuk 2009). Recent studies have tested various adaptive and nonadaptive explanations offered for the evolutionary origins and persistence of these behaviours. These have provided some support for nonadaptive hypotheses of SSB resulting from mistaken identity (Harari et al. 2000; Sales et al. 2018), with influences of social environment (Bailey & French 2012; Han & Brooks 2015; Han et al. 2016) and mating system (MacFarlane et al. 2007). However, SSB might also play important roles in mediating male competition (Lane et al. 2016; Kuriwada 2017) and increasing relative fitness under sexual selection of males that express it (McRobert & Tompkins 1988; Steiner et al. 2005; Preston-Mafham 2006; Bierbach et al. 2012). Despite these research efforts, little is known about the influence SSB might have upon evolutionary change of other traits (Bailey & Zuk 2009; Scharf & Martin 2013; Hoskins et al. 2015).

Often viewed as evolutionarily counterintuitive or costly (Maklakov & Bonduriansky 2009; Scharf & Martin 2013; Boutin et al. 2016), the prevalence of SSB across taxa nevertheless suggests it could exert a substantial influence on evolution, for example by affecting the social selection pressures individuals experience. One way in which it has been suggested to do so is by altering the fitness consequences of same-sex encounters (Lane et al. 2016). For example, same-sex female pairs of a female-biased population of Laysan albatross, *Phoebastria immutabilis*, exhibit cooperative breeding (Young et al. 2008), increasing their fitness and suggesting a role for SSB in facilitating the expression of alternative reproductive strategies (Young & VanderWerf 2014). In males, SSB is generally expected to reduce the strength of aggressive interaction (Peschke 1985; Preston-Mafham 2006; Bailey & Zuk 2009; Kuriwada 2017), although evidence for this is mixed (Ruther & Steiner 2008; Bailey & French 2012; Lane et al. 2016).

Perhaps the most intuitive evolutionary consequence that SSB could have, at least among invertebrates, arises from its well-supported link to 'mistaken identity' (Harari et al. 2000; Dukas 2010; Bailey & French 2012; Scharf & Martin 2013; Macchiano et al. 2018). In mating systems characterized by scramble competition, individuals that court or attempt to mate with a member of the same sex may do so because they have mistaken them for a member of the opposite sex. If mistaken identity is an important factor contributing to the incidence of male SSB, interactions involving less sexually dimorphic males should have a heightened likelihood of SSB (Preston-Mafham 2006; Steiner et al. 2005), conceivably to their benefit (Peschke 1985). For example, Norman et al. (1999) reported field-based observations that small, female-like males of the giant cuttlefish, *Sepia apama*, seem to avoid attack by mate-guarding males, while Dukas (2010) found immature male fruit flies, *Drosophila melanogaster*, are subject to heightened levels of SSB, apparently due to the ambiguity of their incompletely developed cuticular sex pheromones. These observations suggest an evolutionarily important role for SSB in facilitating the evolution of less sexually dimorphic males, through benefits arising from mistaken sex. Such benefits might consequently promote the evolution of alternative reproductive tactics, but this role for SSB in facilitating the spread of less sexually dimorphic males does not appear to have been evaluated.

We tested the prediction that interactions involving less sexually dimorphic males should show an increased incidence of SSB, by capitalizing on the recent evolutionary spread of adaptive, songless male morphs of Hawaiian *T. oceanicus*. Male calling and courtship songs are an important determinant of mating success in field crickets (Balakrishnan & Pollack 1996; Bailey & Zuk 2008; Rebar et al. 2009). However, flatwing males are rendered silent by genetically determined female-like wing morphology. Loss of song also has important consequences for male–male interactions. For example, aggressive song plays an

important role in agonistic contests (Logue et al. 2010). As well as feminized wing morphology, flatwing males have cuticular hydrocarbon profiles more similar to those of females, compared with more sexually dimorphic 'normal-wing' males (Appendix 1), and their neural transcriptomes are feminized (Pascoal et al. 2018). Importantly, flatwing males benefit from satellite mating strategies (Zuk et al. 2006; Zuk et al. 2018), and may thus profit from heightened levels of mistaken identity in male–male interactions. Increased incidence of SSB in interactions involving these less sexually dimorphic males could therefore have facilitated their recent and rapid evolution, by reducing the levels of aggression they experience, and enabling access to females.

To test these predictions, we conducted trials involving normal-wing and silent flatwing males, and a mixture of both, and recorded the incidence of SSB across treatments. We predicted that interactions involving less sexually dimorphic flatwing males would exhibit heightened levels of SSB, which could potentially benefit them and thereby have facilitated their rapid spread.

4.2 Methods

4.2.1 Stocks and rearing

Crickets used in experiments were taken from a mixed-morph laboratory stock population, derived from eggs laid by females from a population on Kauai in 2014 (Pascoal et al. 2016). The stock population has since been maintained at >100 individuals with approximately equal proportions of normal-winged (Nw) and flatwing (Fw) males. Populations were reared in 20-litre plastic containers, with Burgess Excel Junior and Dwarf rabbit pellets and water available ad libitum, at 25 ^{o}C under a 12:12 h photoreversed light:dark cycle.

Males were removed from the mixed-stock population as mature adults less than 4 weeks post-eclosion. To obtain a sufficient sample size, the stock population was sampled over four generations. The adult males were isolated in cylindrical clear plastic containers (65 mm diameter × 40 mm depth) for 3 days prior to trials, with cardboard shelter and food and water available ad libitum as above. On the second day of isolation, to enable their differentiation during trials, each individual's dorsal right wing was marked with one or two spots using a similar amount of white correction fluid (Tipp-Ex). Marking was performed on the day prior to males' use in trials to minimize the likelihood it would affect their behaviour.

4.2.2 Trials

Males of each wing morph were haphazardly assigned to one of three 'dyad' groups: normal-wing versus normal-wing (Nw.Nw), normal-wing versus flatwing (Nw.Fw) and flatwing versus flatwing (Fw.Fw). Trials and pretrial treatments were conducted in an incubator at 24 ^{o}C, under red light. Immediately prior to use in trials, each male was introduced to a 210 × 230 mm arena containing a female from the stock population of unknown age and mating status, and left to interact for 10 min. This pretrial exposure to females has been found to increase the incidence of SSB in subsequent male–male trials due to mistaken identity (Bailey & French 2012). As SSB is an infrequent behaviour, we performed the pretrial exposure to females to facilitate comparisons between dyads by increasing the incidence of SSB across trials. Presence/absence of wing movement patterns of male courtship song (flatwing males still perform wing movement patterns associated with the production of song, despite obligate silence; Schneider *et al.*, 2018) and female mounting was recorded over the course of the 10 min treatment. In field crickets, females must mount the male for mating to occur (Rebar et al. 2009), and male courtship is characterized by the production of distinctive courtship song (Balakrishnan & Pollack 1996). If the female mounted the male, the two were

gently separated using a paintbrush to prevent copulation (Bailey & French 2012). The same female was not used in multiple pretrial treatments.

After the pretrial treatment, the two males were removed from their respective arenas and gently placed at opposite ends of a third arena with the same dimensions. They were left to interact for 10 min, the duration of which was filmed using a Nikon D3300 digital camera, with no observers present. After trials, males were weighed to the nearest mg and their pronotum length recorded to the neared 0.01 mm. Equipment was cleaned with 80% ethanol between trials.

4.2.3 Scoring SSB and agonistic behaviours

Each film was studied by the same observer (J.G.R.) and the presence of SSB and agonistic behaviours recorded. Videos were scored without audio to avoid biasing measurements between normal-wing and flatwing males. The strength of agonistic contests was scored between 0 and 3 using a weighting adapted from Dixon and Cade (1986), frequently used in studies of field cricket interactions (Bailey & French 2012; Kuriwada 2017): no aggressive contests=0; antennal fencing=1; mandible engagement=2; flipping=3. Presence of SSB was recorded when one or both males produced wing movement patterns characteristic of courtship song in the vicinity of the other. Courtship song could be distinguished by distinctive wing movement patterns; it includes a long, constant-intensity trill, distinct from the short chirps of calling song and intense repetitive aggressive song in which the lateral magnitude of wing movements is much greater and is visually distinctive (Balakrishnan & Pollack 1996).

4.2.4 Statistical analyses

We first tested factors that might influence whether females mounted males in pretrial treatments using a generalized linear model (GLM) with binomial error distribution. The

response was whether females mounted the male. To examine whether the effect of male courtship upon female mounting differed between male wing morphs, we included in the full model 'courted' (yes or no) and 'morph' (flatwing or normal-wing) as categorical factors, their interaction, and 'mass' and 'pronotum length' as covariates. We also used a binomial GLM to test whether, given their inability to produce song, flatwing males were any less likely to produce wing movements associated with courtship song in the pretrial exposure to females. Here the response was whether or not the focal male produced courtship song wing movements, with the same covariates and 'morph' modelled as a categorical factor.

We next examined factors influencing the likelihood of SSB during the subsequent male–male behavioural trials. We treated the expression of SSB observed in each male–male dyad, irrespective of which cricket exhibited it, as a response in a binomial GLM. The unit of analysis in this initial test was therefore behaviour observed at the level of the dyad rather than the level of individual crickets (see below), which avoided pseudoreplication. Differences in mass and pronotum length for the two interacting males were included as covariates. Whether interacting males courted females in the pretrial treatment ('courtship') and whether they were mounted by females in the pretrial treatment ('mounted') were both modelled as categorical factors: because each male–male trial involved two males, these variables had three factor levels (i.e. neither male expressed or experienced the behaviour, only one did or both males did).

We performed a post hoc analysis to distinguish whether a given focal male's tendency to express SSB was affected by his own prior experience with females, his interacting male partner's prior experience or both. To do this, we randomly selected one male from each dyad. Using this randomly selected focal male's expression of SSB as a response, we ran a GLM with binomial distribution to examine the effects of pretrial experiences (male courtship and female mounting) of the focal male and his interacting

partner. The model also included predictor terms of focal and interacting male morph, mass and pronotum length. The process of randomly selecting focal and interacting males for the above GLM was repeated 10 000 times to avoid random sampling bias, discarding results from models that produced convergence errors. Distributions of coefficients and significance of predictors describing pretrial experiences of focal versus interacting males across all model runs were then compared, allowing us to evaluate whether SSB displayed by focal males was more strongly predicted by their own previous experience or by the previous experience of their interacting partner.

All GLMs also included 'generation' as a categorical predictor variable, specified as a fixed rather than random effect because it only had four levels, to account for any differences between cohorts. The strength of agonistic contests could not easily be transformed to approximate a normal distribution, so we used nonparametric Kruskal–Wallis and Wilcoxon rank sum tests to evaluate whether the strength of aggressive contests differed between trials in which SSB was or was not observed, or across dyads. Analyses were performed in R v3.4.4 (R Core Team, 2018). Binomial GLMs were checked for overdispersion and significance testing was performed using chi-square tests, with type II and III sum of squares for models with and without interaction terms, respectively.

4.2.5 Ethical note

We followed the ASAB/ABS Guidelines for the treatment of animals in behavioural research and teaching. Individuals were marked using a noninvasive procedure, that is, with temporary correction fluid, which gradually wore off over approximately 7 days. Arenas were large enough for males to escape aggressive rivals. After use in experiments, crickets were returned to the original stock population, with food and water available ad libitum.

4.3 Results

A total of 98 trials, involving 196 males, were recorded. Of these, 27 involved two normal-winged males (Nw.Nw), 30 two flatwing males (Fw.Fw) and 41 one of each male wing morph (Nw.Fw). Of trials in which males interacted (*N*=89), 60 (67.42%) exhibited aggressive interactions, 23 (25.74%) exhibited SSB and 14 (15.73%) exhibited both aggressive interactions and SSB (Fig. 4.1).

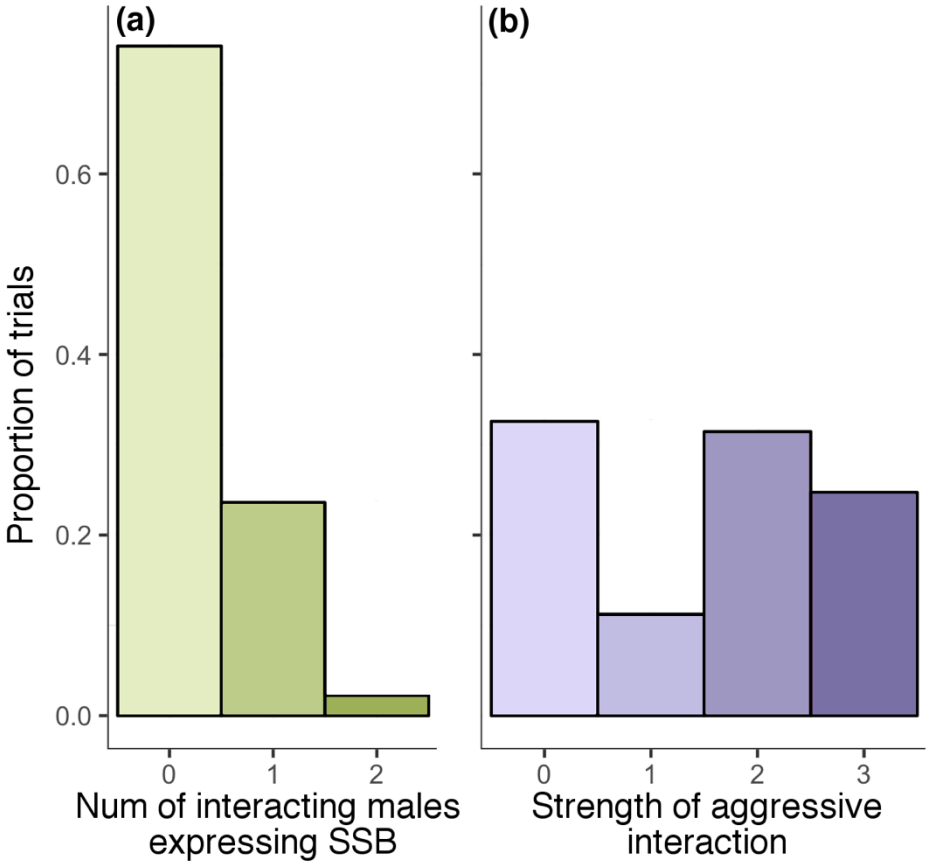


Figure 4.1 Proportions of trials showing SSB and aggressive interaction
(a) Proportions of trials in which neither, one or both interacting males expressed SSB. (b) Proportions of trials involving aggressive contests of varying strength (see Methods for criteria used to score aggressive contests).

4.3.1 Behaviour in pretrial treatment

Results for male courtship and female mounting behaviours during pretrial treatments are shown in Table 4.1. In the presence of a female, flatwing males were no less likely to attempt courtship song than normal-wing males, despite flatwing males' inability to generate an audible signal when making wing movements. Nevertheless, the effect of flatwing and normal-wing courtship efforts on female mounting differed significantly and in a predictable manner: flatwing males were less successful at eliciting female mounting behaviour if they tried to produce courtship song than were normal-wing males (Wilcoxon rank sum test: P=0.013). In cases where males did not attempt courtship, there was a nonsignificant trend for flatwing males to receive more mountings (Wilcoxon rank sum test: P=0.074). Attempting to court did nevertheless increase the likelihood of flatwing males being mounted (Fig. 4.2).

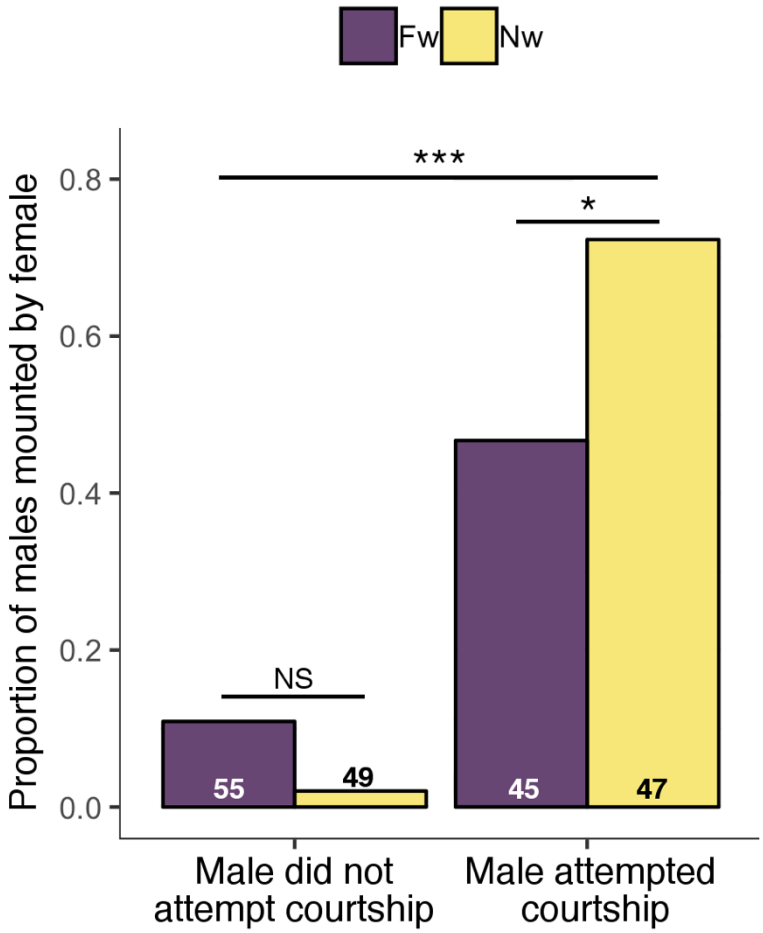


Figure 4.2 Effects of courtship on female mounting
The likelihood of females mounting males of each wing morph that did and not attempt courtship. Fw: silent flatwing males; Nw: singing normal-wing males. Numbers in/above bars indicate sample sizes. Asterisks indicate significance for 'courtship' in the overall GLM (top comparison) and 'morph' in post hoc tests within each courtship category (comparisons between Nw and Fw males): *P<0.05, ***P<0.001.

4.3.2 Rates of SSB

Results from the GLM for the incidence of SSB across trials are given in Table 4.2. The incidence of SSB was affected by the number of interacting males that had previously courted the female in the pretrial exposure: trials in which both males had courted females were on average 3.29 times more likely to exhibit SSB than those in which neither male had courted the female (Fig. 4.3). There was, however, little evidence for an effect of signalling ability or differences in size of males on the expression of SSB, with no indication that expression of

SSB differed between dyads with differing proportions of Nw and Fw males or that it was affected by differences in mass or pronotum length (Table 4.2).

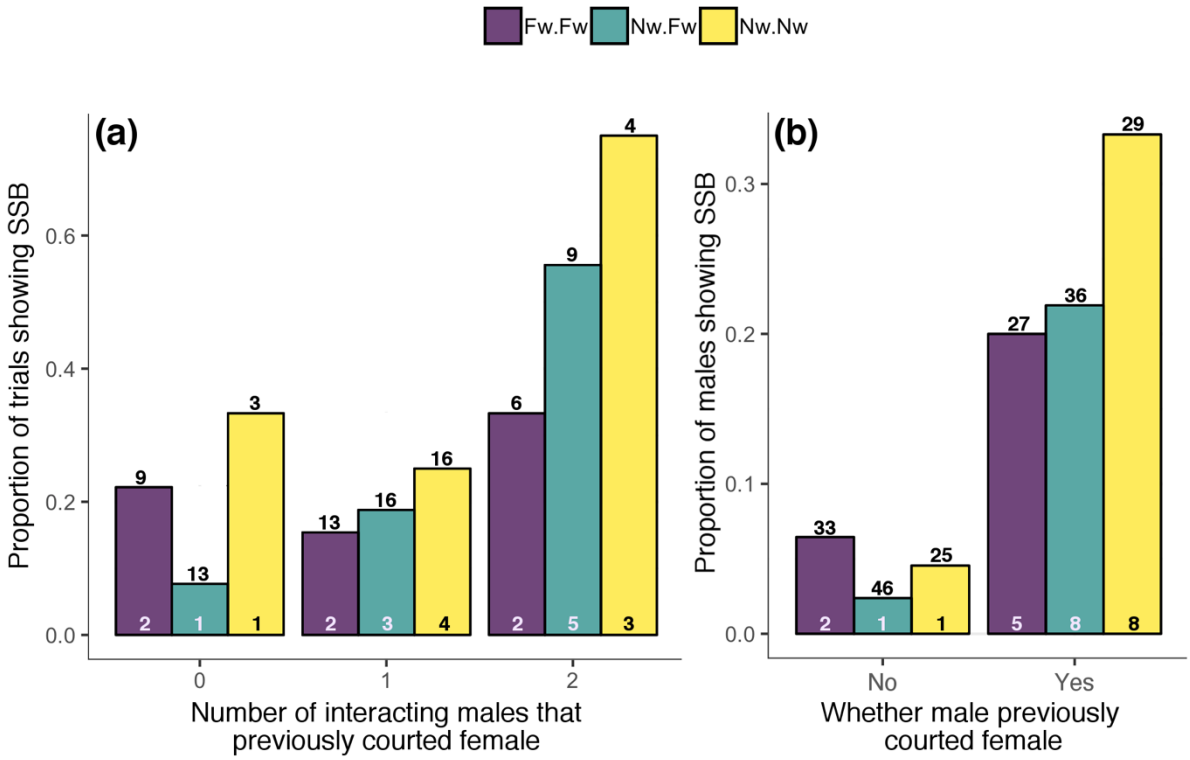


Figure 4.3 Effects of prior male courtship on expression of SSB
The relationship of male SSB to prior courtship of females across dyads with varying proportions of singing normal-wing (Nw) and silent flatwing (Fw) males. (a) Proportions of trials showing SSB, for each dyad group, in association with the number of males that previously courted a female. (b) Proportions of males from each dyad group that expressed SSB, in relation to whether they had previously courted a female. Numbers above bars show sample sizes, and numbers inside bars show the number of trials in which SSB was observed. Note differences in Y-axis limits between (a) and (b).

Follow-up analysis indicated that prior courtship by a focal male, rather than by their interacting male partner, increased the focal male's expression of SSB. Across 10 000 random subsets of single focal males selected from each dyad, prior courtship by the focal male was a significant positive predictor (P<0.05) of focal SSB in 5932 subsets, while prior courtship by the interacting male was a significant positive predictor in only 84. There was also little evidence that the interacting male having been mounted by the female in the pretrial

treatment had an effect on SSB (a significant positive predictor of focal SSB in 594 iterations), making it unlikely that focal SSB was positively influenced by residual female olfactory cues on the interacting male. (Supplementary Figure 4.1)

4.3.3 Rates of aggression

The strength of aggressive contests did not appear to differ between trials in which SSB was or was not observed (Wilcoxon rank sum test: W=785, P=0.803) or between dyads (Kruskal–Wallis rank sum test: χ^2_2=1.383, P=0.501). Similarly, the likelihood of an aggressive contest occurring did not appear to be associated with whether or not SSB occurred (W=803, P=0.443) or with the type of dyad (χ^2_2=0.679, P=0.712).

4.4 Discussion

There is an intuitive hypothetical mechanism linking mistaken identity, frequently associated with SSB, with the evolutionary spread and persistence of alternative reproductive tactics. A common assumption in systems where males adopt alternative mating tactics is that males that are less readily distinguished from females will benefit from reduced levels of male–male competition (Peschke 1985; Norman et al. 1999; Dukas 2010), enabling access to receptive females. SSB has been considered likely to reduce the strength of aggressive interactions that occur during such competition (Kuriwada 2017; Lane et al. 2016). The interaction of these two processes suggests a potential role for SSB in the evolutionary spread of less sexually dimorphic males that adopt alternative mating tactics. Despite these expectations, we found no evidence that a less sexually dimorphic, non-signalling male morph of field cricket, which benefits from satellite mating behaviours (Zuk et al. 2006), is more likely to express or be the recipient of SSB compared with more sexually dimorphic males. These results indicate that the rapid adaptive spread of silent, partially feminized male

crickets is unlikely to have been facilitated by flexible expression of SSB leading to a decrease in the fitness costs of aggressive contests. Instead, the best predictor of SSB was whether males courted females in pretrial treatments, a result that emphasizes the behaviour of the individual expressing SSB ('libido' sensu Logue *et al.*, 2009).

A male cricket's expression of SSB was predicted by his prior courtship behaviour but was not strongly affected by the phenotype or prior experiences of the male with whom he interacted. Whether dyads were all flatwing, all normal-wing or a mix had no apparent bearing on the likelihood that SSB would be expressed. These findings support the view that expression of SSB is influenced primarily by behaviour of the individual expressing it, rather than appearance or signalling of the male conspecific (Han et al. 2016), and is consistent with interpretations of SSB as a spillover of ordinary courtship behaviour into a nonadaptive context (Bailey & Zuk 2009; Logue et al. 2009), i.e. a behavioural syndrome (Sih et al. 2004; Boutin et al. 2016). Selection for male courtship behaviour is likely to be particularly strong in field crickets such as *T. oceanicus*, in which copulation can only occur if females mount males (Rebar et al. 2009), perhaps helping to explain the prevalence of SSB in this and related species (Bailey & French 2012; Kuriwada 2017; Boutin et al. 2016) due to fitness benefits of increased courtship behaviour (Logue et al. 2009).

We introduced each of the males used in the experiment to a female prior to male–male behavioural trials, which has been shown to increase the rate of SSB owing to mistaken identity (Bailey & French 2012). Flatwing males were no less likely to attempt courtship song during these pretrial treatments, despite being unable to produce song at an appreciable amplitude (Schneider et al. 2018). However, patterns of wing movement associated with the production of courtship song (whether silent in the case of flatwing males or audible in the case of normal-wing males) were not equally effective in inducing female mounting behaviour; not surprisingly, courtship song by normal-winged crickets has a stronger effect in

eliciting female mounting. This illustrates that flatwing males incur the substantial energetic costs associated with wing movement patterns that ordinarily generate song, despite their inability to sing (Hunt et al. 2004); courtship song is particularly costly, incurring twice the energetic expenditure of long-range advertisement song in the related field cricket *Acheta domesticus* (Hack 1998). Although being silent clearly had a negative impact on male courtship ability, courtship by flatwing males nevertheless had a positive effect on the likelihood of female mounting. This could be due to low levels of noise produced during stridulation (Tinghitella et al. 2018); however a more plausible explanation is that this increase is due to the involvement of non-acoustic courtship cues, such as posturing and time spent near the female, which were not recorded.

We did not find support for the prediction that less sexually dimorphic males of *T. oceanicus* receive, or benefit from, increased exposure to SSB, suggesting that SSB is unlikely to be a prominent mechanism of reducing male–male competition in this system. Nevertheless, observations from other species suggest this might be the case elsewhere (Mason & Crews 1985; Norman et al. 1999; Peschke 1985; Dukas 2010). Reduced sexual dimorphism, frequently referred to as 'female mimicry', is common among males of many species, and is thought to be an adaptive strategy that reduces the strength of intrasexual competition to which they are exposed, but whether a result of inconspicuousness, lack of perceived threat or mistaken sex is often unclear. For example, in the ruff, *Philomachus pugnax*, less sexually dimorphic 'faeder' males sneak matings in the vicinity of territorial, ornamented males. Observations suggest these 'female mimics' benefit from mistaken sex, and both express and receive SSB in interactions with aggressive territorial males (Jukema & Piersma 2006). In red-sided garter snakes, *Thamnophis sirtalis parietali*, and marine isopods, *Paracerceis sculpta*, less sexually dimorphic males benefit from production of female-like pheromones in the former, and female-like appearance in the latter, by avoiding male–male

competition and thereby gaining access to receptive females (Mason & Crews 1985; Shuster 1987)

In cases where less sexually dimorphic males that use alternative reproductive tactics benefit from reduced competition, they are often thought to do so by avoiding aggression from territorial males due to mistaken sex (Dominey 1980; Mason & Crews 1985). However, benefits of reduced investment in sexually dimorphic ornamentation could also derive from reduced conspicuousness to conspecific males and predators alike, and reallocation of nutritional and energetic resources (e.g. greater testes size in drab 'faeder' males of the ruff; Jukema and Piersma, 2006). Whether less sexually dimorphic males benefit from mistaken sex, providing a clear potential role for eliciting SSB as an adaptive strategy, or simply represent less conspicuous, unornamented males, is often unclear. Although we did not find evidence to support the hypothesis that SSB facilitated the spread of less sexually dimorphic male crickets, the potential for SSB to play a role in the spread of alternative reproductive tactics may be greater in cases where males actively 'mimic' female behaviours associated with courtship and reproduction (Arnold 1976; Thornhill 1979; Dominey 1980) (Arnold, 1976; Thornhill, 1979; Dominey, 1980).

Table 4.1. Results of binomial GLMs for male courting and female mounting behaviours in the pretrial treatment.

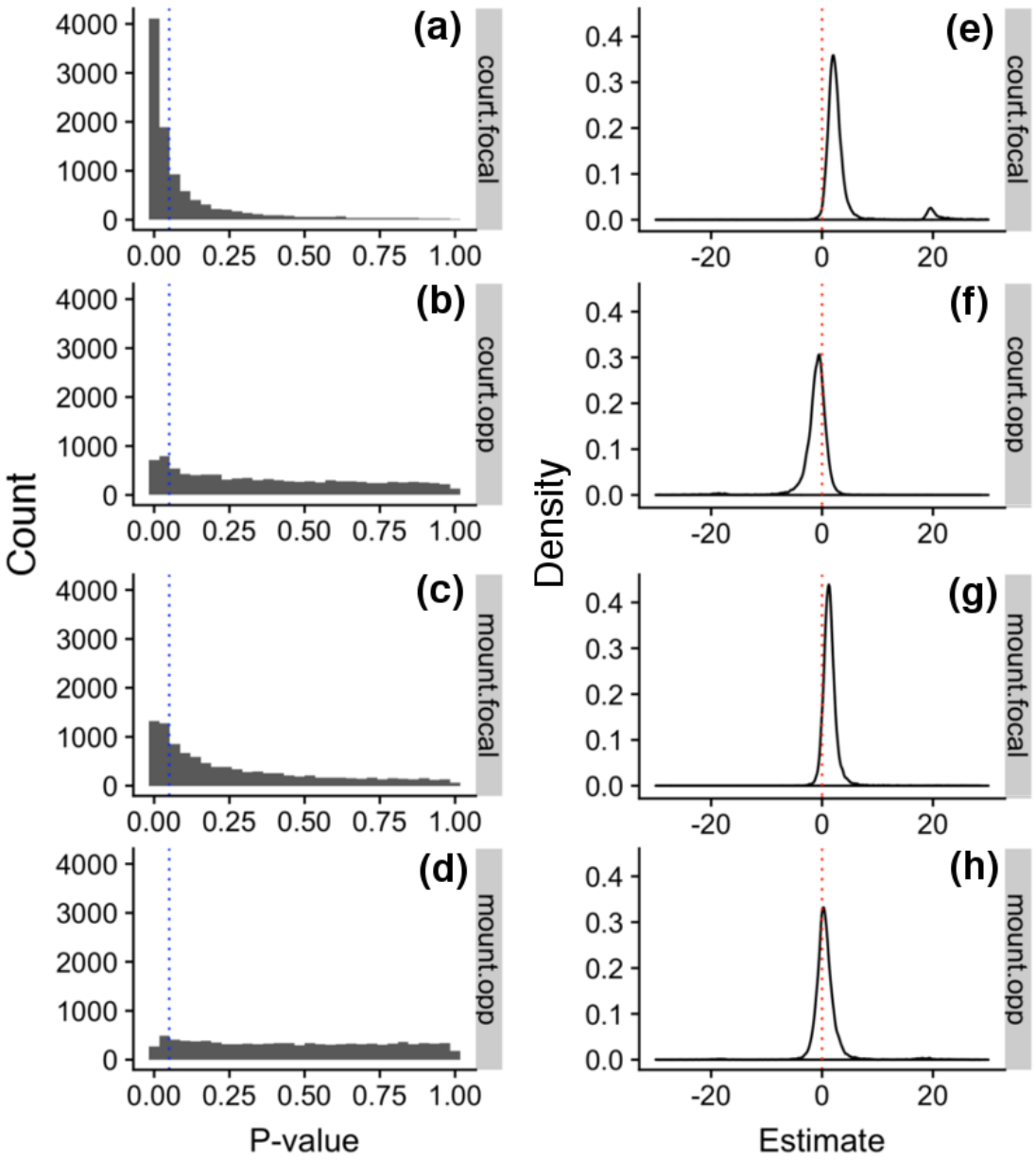
Response	R^2	Predictor	χ^2	*df*	*P*
Male courtship	0.052	Wing morph	0.379	1	0.538
		Mass	2.911	1	0.088
		Pronotum length	0.073	1	0.787
		Generation	3.755	3	0.289
Female mounting	0.394	Wing morph	4.593	1	**0.032**
		Courted	17.390	1	**<0.001**
		Mass	3.573	1	0.059
		Pronotum length	0.557	1	0.455
		Generation	0.960	3	0.811
		Morph:Courted	9.645	1	**0.002**

Significant (P<0.05) P values are highlighted in bold. Data are from 196 observations.

Table 4.2. Results of a binomial GLM for the incidence of SSB across trials.

Predictor	χ^2	*df*	*P*
Dyad	2.105	2	0.349
Proportion courted female	6.830	2	**0.033**
Proportion mounted by female	2.072	2	0.355
Mass difference	1.752	1	0.186
Pronotum difference	3.080	1	0.079
Generation	3.003	3	0.391

Significant ($P<0.05$) *P* values are highlighted in bold. Data are from 89 trials. The model had an R^2 of 0.236.



Supplementary Figure 4.1 Results from GLMs iterated across random subsets of males from each dyad.
(a-d) Histograms showing the distribution of *P* values, and (e-h) density plots showing the distribution of estimates of effect upon SSB by focal males, of predictor terms describing courtship behaviour performed by (a, e) focal males and (b, f) interacting male partners and female mounting elicited by (c, g) focal males and (d, h) interacting male partners, in the pretrial exposure to females. Dotted blue lines illustrate P=0.05 and dotted red lines illustrate an estimate of 0 (i.e. no effect upon expression of SSB in the focal individual). X-axes in plots of model coefficients have been truncated at ±30. Predictor terms were included in a GLM with a binomially distributed response variable of individual SSB, for randomly selected combinations of single males from 89 dyads. This process was repeated for 10 000 iterations.

5. Convergent song loss is morphologically varied and widespread*

Whether convergently evolved adaptive phenotypes reveal developmental and evolutionary constraints upon the direction of evolutionary change, or, in contrast, the ability of organisms to repeatedly find solutions to the same problem, is a topic of debate. While genomic lines of enquiry have found phenotypically similar adaptations are often underpinned by genetic changes to nearby genomic loci, broadly supporting the former view, convergent adaptation might be frequently overlooked where phenotypically distinct but functionally similar phenotypes independently arise and spread. It is well established that flatwing male morphs of *T. oceanicus* lost the ability to sing via genetically determined feminised wing venation which rapidly spread under selection from a parasitoid fly that locates them by their song. Here, we present evidence that males expressing previously unidentified and phenotypically dissimilar song-loss phenotypes are similarly unable to produce song at perceptible levels, and appear to be protected from the parasitoid fly. Our findings show that in at least two of the populations exhibiting surprisingly low proportions of flatwing males, a much larger proportion of males are unable to sing than was previously appreciated, and that silence has evolved on at least four occasions under the same selection pressure. These findings illustrate how multiple solutions to a selective pressure can result in the evolution of functionally convergent, but morphologically varied, adaptive phenotypes.

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* This chapter is a modified, extended version of an article published as: Rayner, Aldridge, et al. (2019) 'A silent orchestra: convergent song loss in Hawaiian crickets is repeated, morphologically varied, and widespread' in *Ecology*, vol. 100 (8).

5.1 Introduction

Convergent adaptive evolution has been considered variously as illustrative of the ability of organisms to repeatedly find solutions to the same selection pressure, and as evidence of constraints upon the evolution of new adaptive phenotypes (Gould & Lewontin 1979; Gould 1989; Losos 2011). This latter view is often thought to be supported by accumulating evidence indicating convergently evolved, phenotypically similar phenotypes are frequently underpinned by mutations or parallel changes in allelic frequencies at or near to the same, key loci (Alves et al. 2019; Sackton & Clark 2019). On the other hand, it is likely that established examples of convergent adaptation are subject to a degree of observer bias, being more readily recognised when phenotypic changes show greater similarity, and that convergent adaptation through phenotypically dissimilar means is frequently overlooked in wild populations (Losos 2011).

Functionally convergent phenotypes which are adaptive in the same ecological or physiological context, but which are reached through disparate phenotypic means, might provide important insight into the potential for evolution to find multiple solutions to same problem. For example, sticklebacks have adapted to parallel changes in diet following repeated colonisation of freshwater benthic habitats through morphologically divergent phenotypic changes, in each case promoting adaptive suction-based feeding strategies, and with the added effect of increasing the phenotypic variation between populations (Mcgee & Wainwright 2013). Additionally, fossorial rodent species exhibit various adaptive changes in morphology which render them suited to burrowing, but which show little phenotypic similarity, or involve different limbs entirely (Stein 2000; Losos 2011). In these scenarios, convergent adaptation through disparate phenotypic changes can be considered to demonstrate unambiguously that adaptive evolution has repeatedly occurred under shared selection pressures, not confined to the same genes, gene networks or even morphological

traits. This phenomenon of 'functional convergence' might, however, be frequently overlooked in studies that do not take into consideration important ecological contexts and selection pressures, instead relying largely on morphological similarity between populations or species.

The loss of male song in *T. oceanicus* subject to fatal parasitism by *O. ochracea* is a textbook example of rapid adaptive evolution (Dugatkin 2008). Song loss is caused by genetic mutations that greatly reduce or eliminate sound-producing structures by superficially feminising male wing venation ('flatwing', Fw; Fig. 5.1A), and which have independently arisen on at least two islands. Surprisingly, given that the parasitoid fly is observed at locations of all known *T. oceanicus* populations across the Hawaiian archipelago, the distribution of flatwing phenotypes across populations is highly heterogeneous (Zuk et al. 2018). Here, I present data which show a large proportion of males in populations within which flatwing phenotypes have not arisen or spread to predominate nevertheless express altered wing morphologically, which reduces their ability to sing and protects them against parasitism from the fly. These observations may help explain the heterogeneous spread of flatwing phenotypes across populations, and illustrate the multiple means through which *T. oceanicus* have lost the ability to sing under shared selection pressure from the acoustically-orienting parasitoid fly.

5.2 Methods and Results

5.2.1 Fieldwork observations

On visits to parasitized cricket populations in 2017 and 2018, we discovered two wing phenotypes – 'small-wing' (Sw: Fig. 5.1B) and 'curly-wing' (Cw: Fig. 5.1B) – which have not previously been described, and which differ phenotypically from normal-wing and

flatwing phenotypes. These aberrant wing phenotypes were discovered while performing annual transect surveys in parasitized populations of *T. oceanicus* to record numbers of normal-wing and flatwing males (Fig. 5.2A). We first identified curly-wing morphology in the 'CC' population (Fig. 5.2A) in 2017, and performed follow-up lab and field work in 2018, during which we identified the small-wing phenotype. Thus, curly-wing phenotypes constitute the primary focus of this section, but the results are likely to be similarly applicable to small-wing morphology (see 5.2.3).

The curly-wing phenotype we observed has not been previously described in crickets, so we named it for its similarity with the *Drosophila* wing mutation described nearly a century ago by Ward (1923). In lab populations reared from eggs of ca. 30 wild-caught females, curly-wing morphology persisted across five generations at similar proportions (~50%), strongly suggesting a heritable basis. The trait is observable immediately upon adult eclosion, and other lab populations reared in the same growth chamber do not express it. Males of *T. oceanicus* produce song through rhythmic movements of their forewings, leading sound-producing structures on either forewing to engage and produce resonating frequencies (Pfau & Koch 1994). We anticipated that curly-wing morphology would prohibit the engagement of the plectrum and stridulatory file, reducing the ability of males expressing this phenotype to produce song and protecting them against parasitism by *O. ochracea* (Zuk et al. 2006).

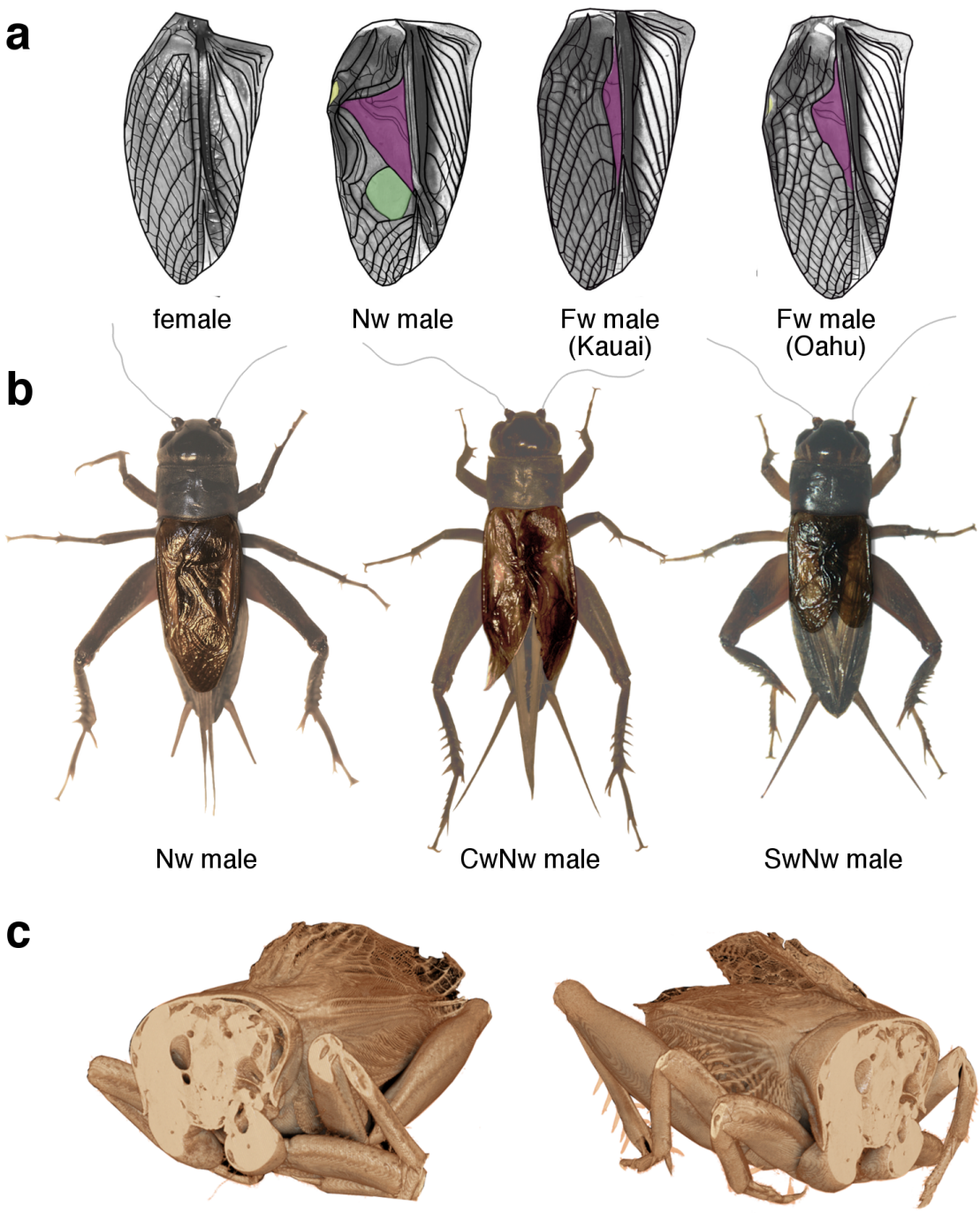


Figure 5.1 Alternative male-silencing wing morphs of Hawaiian *T. oceanicus*.
(A) Venation variants: traced micrographs showing forewing venation patterns (adapted from Pascoal et al. 2014) of a female and Nw male, and Fw males from the different islands, with sound-producing structures highlighted (purple='harp', green='mirror', yellow='plectrum'). (**B**) Newly described shape and size variants: typical Nw male (left) alongside CwNw male and SwNw male; note that both forewings and hindwings are reduced. (**C**) Micro-CT scans of a CwFw male with forewings in resting position and head and thorax omitted, showing how marginal wing surfaces 'peel up' and preclude physical engagement during wing movement.

5.2.2 Curly-wing recordings and playback trials

We found strong support for our prediction that curly-wing morphology protects calling males from parasitoid attack relative to typical Nw males (Fig. 5.2B,C). First, we found that males with Nw venation, but exhibiting curly-wing morphology like that shown in Fig. 5.1C, do not sing as loudly as typical Nw males (Wilcox rank sum test: N=15, P<0.001; Fig 5.2B; comparing songs measured in the lab using a CEM DT-805 sound level meter 5 cm from test subjects). Flatwing males also attempt to sing (Schneider et al. 2018), but the amplitude of acoustic stimuli produced during wing movement did not differ between CwNw and Fw males (N=13, P=1.000). Like Fw males, CwNw males produced variable, but lower-amplitude, peak frequencies (Fig. 5.2B).

To test whether CwNw males were protected from the parasitoid fly, we performed playback trials at the CC site using looped calling songs recorded in the lab at 25±1°C from 4 Nw and 4 CwNw males. Songs were played on SanDisk Mp3 players through Sony SRS-m30 speakers underneath fly traps (modified 1.5L plastic bottles with the funnel-end inverted), broadcast at their originally-recorded volumes. Since Cw males were only found in populations that also contained calling Nw males (Fig. 5.2A), we designed playbacks to mimic natural conditions by placing three traps 11m apart in a triangle: one typical Nw song, one CwNw song, and a third without playback as a negative control. Trials lasted 5 minutes and were performed in dry weather between sunset (~6.10pm) and 8.30pm when the fly is active (Beckers & Wagner 2012). All pairings of typical Nw and CwNw song models were repeatedly tested over 4 nights and rotated among speakers between trials. Like the negative controls, CwNw songs never resulted in a fly entering the trap, whereas typical Nw songs attracted flies in 28.13% of trials (paired Wilcoxon signed rank test: N=64, P<0.001). (Fig. 5.2C)

5.2.3 Small-wing observations

In the same field season, when surveying a different parasitised population of Hawaiian *T. oceanicus* ('UH' in Fig. 2A) in which less than 5% of males exhibit flatwing morphology , we noted a substantial proportion of males (N=28, 27.18%) with unusually small, but normally-veined forewings ('SwNw', Fig. 5.1B). We temporarily removed 12 SwNw males from the field and measured courtship song that they produced when exposed to females (mean = 61.83 dB ± 2.99 SE, see supplementary videos). Two of the 12 produced acoustic stimuli below the recordable atmospheric noise level of ca. 45 dB, so we conservatively dummy-coded these in analyses as producing song at 45 dB. One of the 12 had forewings of differing lengths and sang at up to 80 dB, towards the lower end of the normal range (Balakrishnan & Pollack 1996), but this was the exception. The other 11 produced acoustic signals at substantially lower than normal levels.

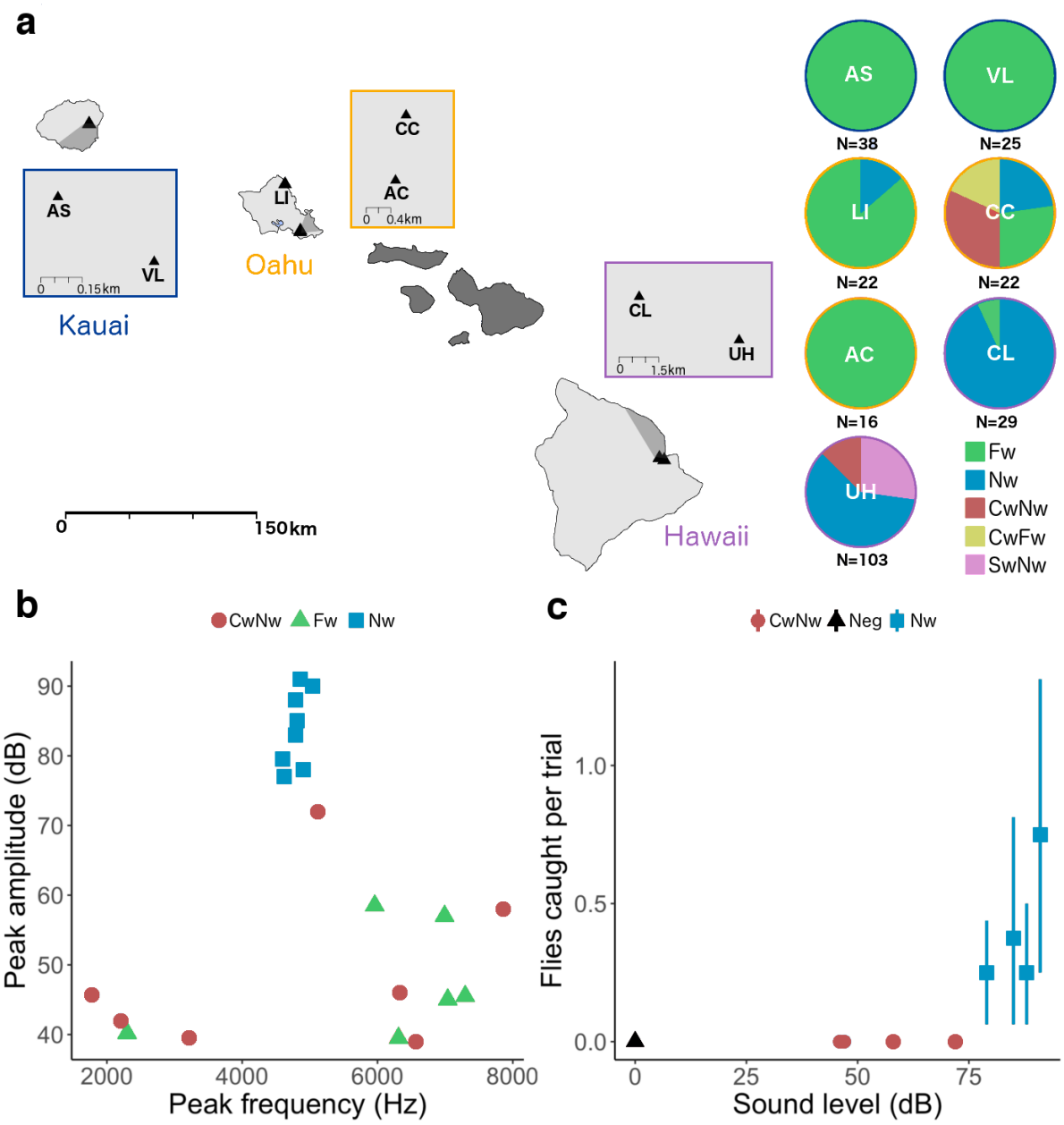


Figure 5.2 Distributions, song features and fly attack rates
A. Distributions of parasitized populations of *T. oceanicus* and proportions of males showing typical Fw and Nw morphology plus newly-identified CwNw, CwFw and SwNw phenotypes from 2018 surveys. Two-letter codes correspond to site IDs. **B.** Differences in calling song properties for Nw, Fw and CwNw males recorded using a Sennheiser ME66 microphone 5 cm from subjects in the lab. **C.** Flies attracted to CwNw, negative control and Nw playbacks in the field: points illustrate means and bars are 95% non-parametric confidence intervals.

5.3 Discussion

Whether convergent adaptive evolution demonstrates the surprisingly ability of populations to repeatedly adapt under shared selections, or, instead, the presence of developmental constraints upon the route that adaptive evolution may take is much debated (Gould & Lewontin 1979; Losos 2011). Our observations, alongside prior work (Zuk et al. 2006; Pascoal et al. 2014), reveal that silent phenotypes have independently arisen in Hawaiian field crickets predated by an acoustically-orienting parasitoid on at least four occasions. While convergent flatwing phenotypes from Kauai and Oahu populations share superficial phenotypic similarity (Pascoal et al. 2014), the two newly observed phenotypes reported here are morphologically distinct from flatwing phenotypes and from each other, illustrating that functionally convergent adaptation (i.e. loss of male song) can occur through a variety of phenotypic changes. This range of phenotypic variants, all of which appear to have spread under selection against male song, demonstrate convergent adaptation through morphologically dissimilar means. The finding that these silent male phenotypes coexist in multiple populations is also consistent with expectations of soft selective sweeps, in which multiple adaptive variants arise and spread under shared selection pressures (Messer & Petrov 2013); likely better preserving ancestral genetic variation in populations in which multiple adaptive variants have emerged.

We observed that small-wing morphology not only affected crickets' forewings, which males use to produce song, but also the hindwings, which both sexes use for flight (Fig. 5.1B). Brachyptery is commonly observed in the hindwings of related species (though not, to our knowledge, in *T. oceanicus*) and is highly heritable in related species (Roff 1994). An important distinction is that brachypterous forms of field crickets such as *Gryllus firmus* gain a fitness advantage by divesting energy from maintaining large hindwings and flight muscle, while boosting their attractiveness to females through increased calling effort using

the forewings (Crnokrak & Roff 1998). In *T. oceanicus*, small-wing males are unable to produce ordinary calling or courtship song, owing to their reduced forewings, and so would gain no such benefit. Intriguingly, however, small-wing males may still derive benefit from reduced investment in wing growth and associated wing musculature. Dealation – the active removal of wings – is known to occur across a variety of insect taxa, and in Orthopterans is known to enhance the rate of egg production in females (Tanaka 1976; Roff 1984). Even more striking, the experimental removal of wings in two species that do not practice dealation, *Gryllus firmus* and *T. oceanicus*, also increases the rate of egg production, seemingly related to histolysis of associated wing muscles (Roff 1989). Future work could investigate whether small-wing males and females similarly benefit from resource re-allocation associated with their drastically reduced wings.

The initial discovery of flatwing stimulated research into behavioural and physiological consequences of trait loss and rapid evolution, and a population of *T. oceanicus* was recently described on Molokai in which flatwing males produce severely attenuated, broad-band acoustic stimuli (Tinghitella et al. 2018). Our identification of additional protective, reduced-song wing morphs raises many questions. The emergence of alternative adaptive phenotypes may have inhibited the spread of flatwing males and could account for their variable proportions observed in different populations (Zuk et al. 2018). Do flatwing, curly-wing and small-wing males differ in attractiveness to females, and does one phenotype have an advantage over others? All phenotypes appear capable of co-expression in the same male, and females also express curly-wing and small-wing, so it will be important to dissect their genetic architecture. The repeated appearance of adaptive phenotypes through different underlying genetic changes is consistent with expectations of a soft selective sweep (Messer & Petrov 2013), so tracking their relative success in populations where multiple adaptive

phenotypes have appeared could provide important insight into their relative costs and fitness benefits.

The recurrent adaptive loss of song across small, fragmented populations of Hawaiian field crickets illustrates the multiple, morphologically varied routes by which this male trait can be functionally lost. Our observations are consistent with recent evidence for high evolvability of trait loss under negative selection (Xie et al. 2019), a phenomenon widely observed among costly sexually selected traits (Wiens 2001), and which may play an important role in rapid adaptation of populations to novel environments or selection pressures. While functionally convergent adaptive phenotypes have been observed across a range of phenotypic gains such as novel feeding and burrowing morphologies (Losos 2011; Mcgee & Wainwright 2013), it is plausible that this functionally convergent adaptation is particularly likely to occur under selection against traits, given the multitude of means by which an ecological trait might reasonably lost or reduced.

6. Persistence of behavioural singing effort in silent cricket populations*

Evolutionary loss of sexual signals is widespread. Examining the impact on behaviours associated with such signals can provide insight into factors promoting or inhibiting trait loss. We tested whether a costly behavioural component of a sexual trait, male calling effort, has been evolutionary reduced in populations of silent Hawaiian field crickets (*Teleogryllus oceanicus*). Sexual advertisement song requires energetically costly wing movements, but silent flatwing males have genetically feminised wings that preclude song and protect against a lethal, eavesdropping parasitoid. Flatwing males express wing motor patterns associated with singing, but in contrast to normal-wing males, sustained periods of wing movement cannot confer sexual selection benefits and should be subject to strong negative selection. We developed a novel, automated technique to quantify how long males spend expressing wing movements associated with song. We compared calling effort among populations of Hawaiian crickets with different proportions of silent males, and between male morphs. Contrary to expectation, silent populations invested as much in calling effort as non-silent populations. Flatwing and normal-wing males did not differ in calling effort. The lack of evolved reduction in behaviour following morphological change in silent Hawaiian crickets illustrates how behaviour might sometimes impede, rather than facilitate, evolutionary trait loss.

* This chapter is currently under review: Rayner, JG, Schneider, WT, & Bailey, NW (in review) 'Can behaviour impede evolution? Persistence of singing effort after morphological song loss in crickets'.

6.1 Introduction

A common intuition is that traits which no longer serve an adaptive function should be evolutionarily lost (Porter & Crandall 2003). The secondary loss and vestigialisation of morphological traits is well-documented (Fong et al. 1995), but whether behaviours associated with such traits are also evolutionarily lost, for example through the accumulation of neutral mutations (Carson et al. 1982; Wilkens 1988), is less well understood. Alternatively, behaviours could remain expressed and therefore available for evolutionary co-option (Moczek 2008). Secondary sexual traits provide an excellent opportunity to test this, because they frequently involve display behaviours such as limb, appendage or other bodily movements that work in tandem with specialised morphological features to produce a conspicuous signal. The reduction and loss of sexual signals under natural selection is theoretically predicted (Fisher 1930; Lande 1981) and widely observed (Wiens 2001; Kraaijeveld 2014). Here we use Hawaiian populations of field cricket *T. oceanicus* that have recently lost the ability to sing due to a morphological change (Zuk et al. 2006), yet retain central pattern generators that produce the behavioural component of song (Schneider et al. 2018), to test whether selection has reduced the amount of effort males expend on non-functional signalling behaviour.

Male crickets produce song via rhythmic movement of their forewings, causing sound-producing wing structures to resonate (Pfau & Koch 1994). Females strongly prefer males that sing more (Cade & Cade 1992; Crnokrak & Roff 1995; Holzer et al. 2003; Hunt et al. 2005; Drayton et al. 2010), but calling behaviour incurs substantial energy expenditure (Prestwich & Walker 1981; Hoback & Wagner 2008; Hack 1998) and is condition-dependent (Holzer et al. 2003; Hunt et al. 2004; Judge et al. 2008; Houslay et al. 2017). In *T. oceanicus*, males from populations on multiple Hawaiian islands have lost the ability to produce acoustic signals under selection from an acoustically-orienting parasitoid fly, *Ormia ochracea* (Zuk et

al. 2006). In the best characterised example, silence is caused by the loss of sound-producing structures on the wings (the 'flatwing' phenotype). The timeline of flatwing's appearance and spread has been well-documented in several populations on different islands: Kauai (first observed in 2003), Oahu (2005), and Hawaii (2010) (Pascoal et al. 2014). In both Kauai and Oahu, flatwing segregates as a single-locus trait underpinned by mutation(s) on the X-chromosome (Zuk et al. 2006; Pascoal et al. 2014). Silent males are strongly disadvantaged in the context of sexual selection (Tanner et al. 2019; Rayner & Bailey 2019), but nevertheless spread rapidly under selection from the fly. Flatwing males are capable of expressing the precise patterns of rhythmic forewing movement that produce advertisement song in normal-wing males (Schneider et al. 2018). Given its energetic costs, behavioural calling effort – the amount of time spent producing wing movements associated with calling song – should be selected against.

We developed a novel, automated video analysis technique to assay calling effort of males from populations with consistent but contrasting proportions of normal-wing and flatwing male phenotypes (Zuk et al. 2018). In at least one population of *T. oceanicus* on each of Kauai and Oahu, 100% of males are now silent (Chapter Five). We predicted these silent populations would show reduced calling effort compared with populations where more normal-wing males are found. We also estimated male condition and measured testes mass to evaluate whether calling effort is associated with proxy measures of male quality. Finally, we tested whether, within a population, flatwing males show lower calling effort than normal-wing males. Evidence consistent with our predictions of reduced calling effort in silent populations would support the idea that behaviour played an important role in the rapid spread of a mutation causing adaptive silence in Hawaiian crickets. No difference in calling effort among populations or between morphs, however, would suggest that selection has not

reduced this non-adaptive trait, and its persistence could diminish the overall advantages of flatwing and enable co-option for other functions (Porter & Crandall 2003).

6.2 Methods

6.2.1 Sampling and rearing of test populations

We sampled sites on Kauai (K_{VL}: 100% flatwing), Hawaii (H_{CL}: 90-100% normal-wing), and two nearby (~1km apart) sites on Oahu with contrasting proportions of silent and non-silent males (O_{AC}: 100% flatwing; O_{CC}: ~50% flatwing) in 2017 (Fig. 6.1A) (Chapter Five). Our estimation that ~50% of O_{CC} males would be able to sing owing to normal-wing venation was revised down to ~25%, following the identification of an additional silencing phenotype in this population ('curly-wing'; Chapter 5). No curly-wing males were used in the current experiment. Assuming ca. 4 generations per year, flatwing males had been present in the Kauai and Oahu populations for approximately 56 and 48 generations, respectively, at the time of sampling; well within the ability of populations to exhibit adaptive evolution (Fricke & Arnqvist 2007; Marchini et al. 2014; Szűcs et al. 2017; Foucault et al. 2018). Flatwing males have been observed in Hawaii since 2010 (approx. 28 generations before sampling), but at consistently low proportions (<10%) (Pascoal et al. 2014; Zuk et al. 2018; Chapter Five).

Offspring from approximately 30 wild-caught females and 30 wild-caught males were reared and maintained in an incubator at 25C, on a 12:12 LD cycle. Lab stocks were maintained at >150 individuals for two generations prior to testing to minimise field-based maternal effects, with food (Burgess Excel Junior and Dwarf rabbit pellets) and water *ad libitum*. Penultimate instar males from F_2 lab populations were isolated in clear plastic

containers (65mm diameter × 40mm depth) until use in trials between 7 and 11 days post-adult eclosion.

Upon adult eclosion, the plectrum of the right wing, which is necessary to produce song, was surgically removed from normal-wing males (Fig. 6.1B). Flatwing males were sham-operated. All males were therefore silent to avoid confounds that could arise if males receive auditory feedback from their own song or sing in response to others (Jang 2011). Calling effort estimates thus reflect the constitutive tendency of males to produce wing movements associated with advertisement songs, rather than social feedback. Experimental males were tested once between 7 and 11 days post-adult eclosion; previous studies have found cricket calling behaviour is repeatable across adult ages (Kolluru 1999; Bertram et al. 2011), and is heritable (Hedrick 2002; Webb & Roff 1992; Gray & Cade 1999). Experimental males were weighted to the nearest 0.001 g and pronotum lengths measured to the nearest 0.01 mm. Scaled mass index (SMI) was calculated as a proxy of body condition (Peig & Green 2009), with pronotum length as the linear measurement.

6.2.2 Trials and video processing

On the day before trials, a small reflective tag (3-4mg) was attached near the distal end of males' dorsal-right forewing using a small amount of superglue (Loctite, Germany) (Fig. 6.1B). After a ca. 12-hour recovery period, we filmed two-hour calling effort trials using a Nikon D3300 digital camera, recording under dim red light. Trials began 15 minutes after onset of the dark cycle to coincide with peak calling activity in wild Hawaiian populations (Kolluru 1999). During trials, males occupied 55 × 43 × 35mm compartments within a larger box containing 20 such compartments, visually isolated from one another but visible to the camera, which recorded the red light reflected from each cricket's wing tag. Between 12 and 16 crickets were filmed in each trial. Crickets were allowed 15 minutes to acclimate prior to

the start of the trial. After trials, crickets were massed to the nearest mg and the length of their pronota measured to the nearest .01mm. Males were euthanised by freezing at -20C, with the exception of those needed for maintaining lab stocks (*N*=24). Frozen males were dissected and their testes massed to the nearest mg.

Automated analysis was performed in MATLAB using custom scripts to quantify the duration of wing movement bouts associated with calling song for each cricket (Supporting Information). Briefly, image brightness and contrast were adjusted and the background removed so that only reflective wing tags were visible. Centre coordinates for each tag were recorded so distances moved between frames could be calculated. Differences between X-coordinates were used to determine whether each distance was positive or negative. Distances were converted into the time/frequency domain using a continuous wavelet transform (CWT) between 1 to 20 Hz. For wing movements to qualify as singing, four criteria needed to be met: (1) mean power between 10-16 Hz (13 Hz being the observed frequency of *T. oceanicus* wing movements during calling song, based on Schneider et al. (2018)) exceed a threshold of 0.7 of the CWT output, (2) 90% of the power in the CWT output was between 10-16 Hz, (3) peak frequency power was between 9 and 17 Hz, and at this peak the CWT output power was greater than 1.5, (4) the duration of singing exceeded 0.6 seconds. Episodes of singing that restarted within 10 seconds were recorded as a single bout.

The novel methodology employed for assaying calling effort by video tracking was validated using separate trials of audibly-singing crickets (N = 22, 9 hours 34 minutes of video) to ensure accurate detection of singing. Spectrograms of the audio from these trial videos were manually assessed to determine whether a singing bout (defined as successive chirps and trills) occurred. This information was then cross-referenced with the results of the automated detection algorithm. All occurrences of singing bouts were successfully detected by the video tracking approach, though cross-referencing did report a low rate of false

positives. During the 573.85 minutes of video, the code detected a total of 49.65 minutes of singing. Of this, 48.64 minutes was corroborated, in that it lined up with manually scored singing, whereas 1.02 minutes (ca. 2%) was incorrect and was caused by unusual movements in the video.

6.2.3 Statistical analysis

Statistical analysis was performed in R v.3.4.4. Calling effort (i.e. time spent singing, (Hunt et al. 2004; Judge et al. 2008; Houslay et al. 2017)), was normalised by log-transformation (log_2[time singing+1]). Variation in calling effort was analysed using a linear mixed model (LMM), with *population* modelled as a categorical factor and *trial ID* as a random effect to account for batch effects. *Age* in days post-eclosion, *total mass*, and *SMI* were included as covariates. Morph variation within mixed populations was analysed in the same way, but with *morph* replacing *population* as the categorical variable. Visualisation indicated a non-linear effect of SMI, so it was included as both a linear and quadratic predictor using the R function *poly* to account for covariance.

6.3 Results

Calling effort was recorded for 342 males (H_{CL}=95 [90 normal-wing, 5 flatwing]; O_{CC}=82 [45 normal-wing, 37 flatwing]; O_{AC}=85 flatwing, and K_{VL}=80 flatwing). Consistent with our expectations based on field observations, O_{AC} and K_{VL} populations reared in the lab from eggs collected from wild-caught females contained only silent flatwing males, supporting the observation that normal-wing males are absent in these populations (Tinghitella et al. 2018; Zuk et al. 2018; Rayner, Aldridge, et al. 2019).

There was no evidence males from silent populations spent less time calling (Table 6.1; Fig 6.1C). Similarly, there was no evidence in the O_{CC} population, in which normal-wing

and silent flatwing morphs co-occur, of any reduced investment in song by flatwing males (Table 6.2); there was a similar pattern in the H_{CL} population, though with too few flatwing males for statistical comparison (Fig. 1D). Time spent calling scaled positively with condition, but this relationship appeared to tail off at the highest measures of condition (Fig. 6.S1). Mass was negatively associated with time spent calling but this was only significant when SMI was included in the model. Overall, the LMM accounted for little of the total variance in calling effort (R^2=0.083). Including testes mass in the model as an additional covariate (reducing *N* from 342 to 318) alongside somatic measures of mass and SMI produced similar results, and testes mass did not predict time spent calling (Table 6.3).

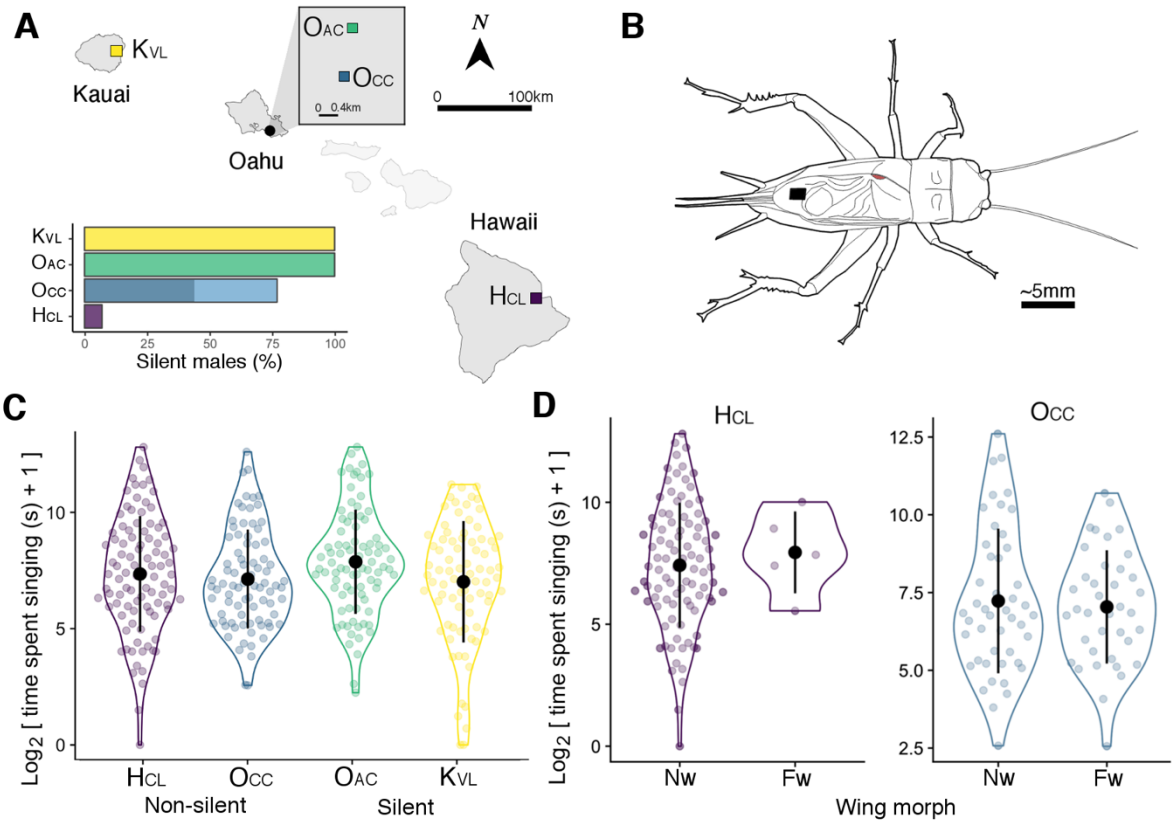


Figure 6.1 No reduction in calling effort among silent populations
A) Map of the Hawaiian islands, with locations of the study populations. The inset graph shows proportions of silent males in respective wild populations (see Chapter Five): the segmented bar for O_{CC} illustrates that ~33% of the silent males (the lighter portion) are silent

due to curly-wing rather than flatwing morphology. **B)** Diagram of an adult normal-wing male with a reflective tag (black) applied to its dorsal-right forewing. The plectrum, which was surgically removed or sham-operated in all test males, is highlighted in red. **C)** Calling effort (time spent singing) for males from each population. **D**) Time spent singing by Nw and Fw males in O_{CC} and H_{CL} populations in which they co-occur. In C) and D), points are jittered along the X-axis, and black points and error bars represent means and standard deviations. Points in C) and D) are scattered along the X-axis for purposes of visualisation only, with solid outlines representing density distributions.

6.4 Discussion

Sexually selected traits are often opposed by countervailing natural selection (Fisher 1930; Zahavi 1975; Lande 1981; Andersson 1986), which can lead to their evolutionary reduction or loss (Wiens 2001; Kraaijeveld 2014). Understanding the consequences for behaviours previously associated with the signal can provide information about factors that may facilitate (if behaviour is rapidly lost) or stymie (if costly behaviour persists) such trait losses. We found no evidence of reduced investment in energetically costly calling effort among male *T. oceanicus* in which wing movements do not produce song. Neither silent populations, nor silent males in mixed populations, showed any indication of reduced calling effort. This costly calling effort will deplete energy reserves and involve resource allocation tradeoffs (Hack 1998; Hoback & Wagner 2008), reducing the relative fitness advantages of silent males, and its persistence appears inconsistent with the view that behaviour generally facilitates the early stages of adaptive evolution (Wong & Candolin 2015; Bailey et al. 2018). Here, persistent signalling behaviour imposes a double cost on flatwing males: not only can they not produce signals, they still expend considerable effort trying to do so.

Evolved loss of song in Hawaiian populations of *T. oceanicus* has been well-documented, and has occurred convergently through a variety of morphological means (Zuk et al. 2006; Pascoal et al. 2014; Chapter Five). Nevertheless, in all cases, males which cannot produce song at ordinary levels persist in attempting to sing (Schneider et al. 2018; Chapter

Five; Rayner, Aldridge, et al. 2019). It is unsurprising that silent flatwing males from mixed-morph populations exhibit the same calling effort as normal-wing males: to evolve a lower investment in song effort would require that mutations underlying reduced investment are or have become genetically linked with the *flatwing* genotype on the X-chromosome, which is perhaps unlikely. However, it is counter-intuitive that a seemingly optimal solution to selection against song by the parasitoid fly – for males to evolve lower calling effort – has not evolved in predominantly silent populations. Shifts in diurnal patterns of calling in response to the fly have previously been documented in this system which suggests a capacity for such behavioural evolution (Zuk et al. 1993), so it remains an open question why morphological losses of sexual signalling in this system appear to be the more successful adaptations.

The persistence of costly calling behaviours in silent males and in silent populations of *T. oceanicus* is not without parallel in other species, and has important implications for the evolvability of the same, or novel, phenotypes. For example, populations of rattlesnakes, such as *Crotalus catalinensis* and *C. ruber lorenzoensis* in the gulf of California, express only vestigial rattles and are incapable of producing a warning signal, but nevertheless silently 'rattle' their tails when disturbed by humans (Johnson 1972; Shaw 1964; Radcliffe & Maslin 1975). Along similar lines, several species of the *adiastola* group of Hawaiian *Drosophila* exhibit a courtship ritual in which they raise and vibrate their abdomen, yet males of only one species express long clavate hairs which sweep the female's head as this occurs. The elaboration of the hairs is suggested to have evolved following the courtship behaviour, rather than having been lost in related lineages (Carson 1978). Note, however, that in Hawaiian Drosophila, abdomen vibration is likely to function as a courtship signal even in the absence of the elaborated hairs, and it is feasible also that tail display behaviours in rattlesnakes could retain functionality as a visual signal following the vestigialisation of the rattle appendage. It is feasible that the wing movements associated with song could be similarly co-opted for

non-acoustic courtship displays, or otherwise lead to the regain of song in Hawaiian field crickets if selection against song is relaxed (Tinghitella et al. 2018). Gray et al. (2018) demonstrated the feasibility of such a scenario by inducing the expression of calling song in a cricket species, *Gryllus ovisopis*, in which it has been evolutionarily lost.

Our results suggest an interesting counterpoint to the widely supposed role of behaviour in the early stages of rapid adaptation (Wong & Candolin 2015; Bailey et al. 2018); constraints associated with less evolutionarily responsive phenotypes might reduce the benefit of novel adaptive variants. In the case of song-loss in Hawaiian *T. oceanicus* the costs of this evolutionary lag are evidently marginal compared with the overwhelming benefits of evading parasitism, but under different conditions, similar costs could inhibit or even preclude populations from rapidly adapting to changes in their environment, and the maintenance of 'vestigial behaviours' could play an important role in eventual re-emergence of previously lost traits over longer-term evolutionary timescales (Tinghitella et al. 2018; Bailey et al. 2019).

6.5 Tables

Table 6.1. The results of an LMM (N=342, R^2=0.083) for total time spent singing (log_2-transformed), with a random effect of trial ID.

Predictor	***df***	***X²***	***P***
Population	3	3.108	0.375
Age	1	0.311	0.577
Mass	1	4.901	**0.027**
SMI*	2	16.055	**<0.001**

Significant P-values (<0.05) are highlighted in bold.
* SMI is included in the model as an orthogonal polynomial with 2 degrees to account for non-linear effects

Table 6.2 The results of an LMM (N=82, R^2=0.045) for time spent singing (log2-transformed) for males from the OCC population, with a random effect of trial ID.

Predictor	***df***	***X²***	***P***
Wing morph	1	0.268	0.605
Mass	1	0.008	0.927
SMI*	2	2.846	0.241

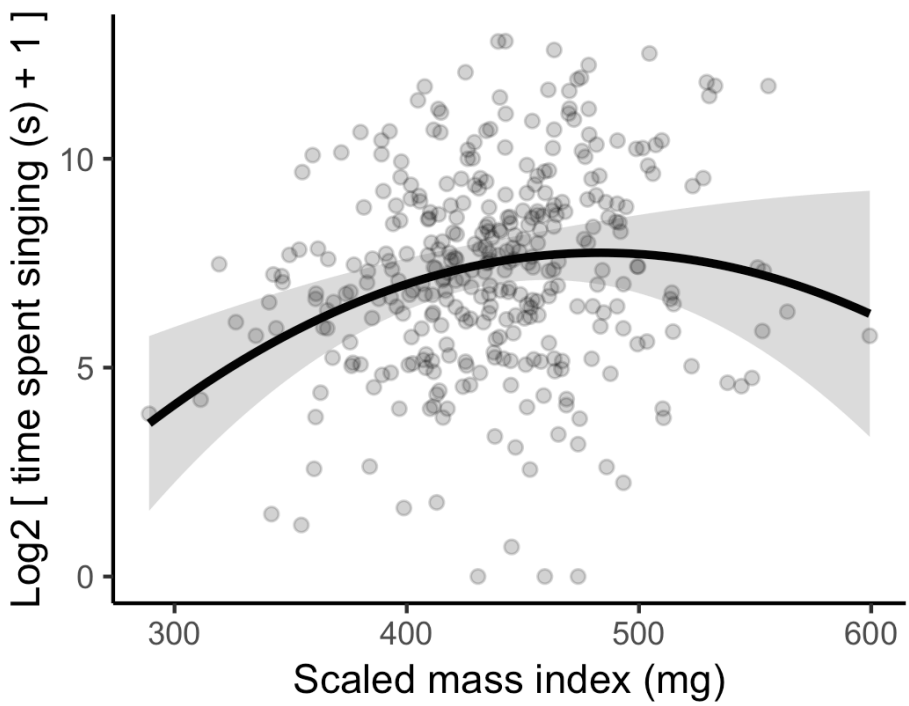
* Somatic SMI is included in the model as an orthogonal polynomial with 2 degrees, to account for non-linear effects

Table 6.3. The results of an LMM (N=318, R^2=0.075) for total time spent singing (log_2-transformed), with a random effect of trial ID, including testes mass as an additional predictor variable. Significant P-values (<0.05) are highlighted in bold.

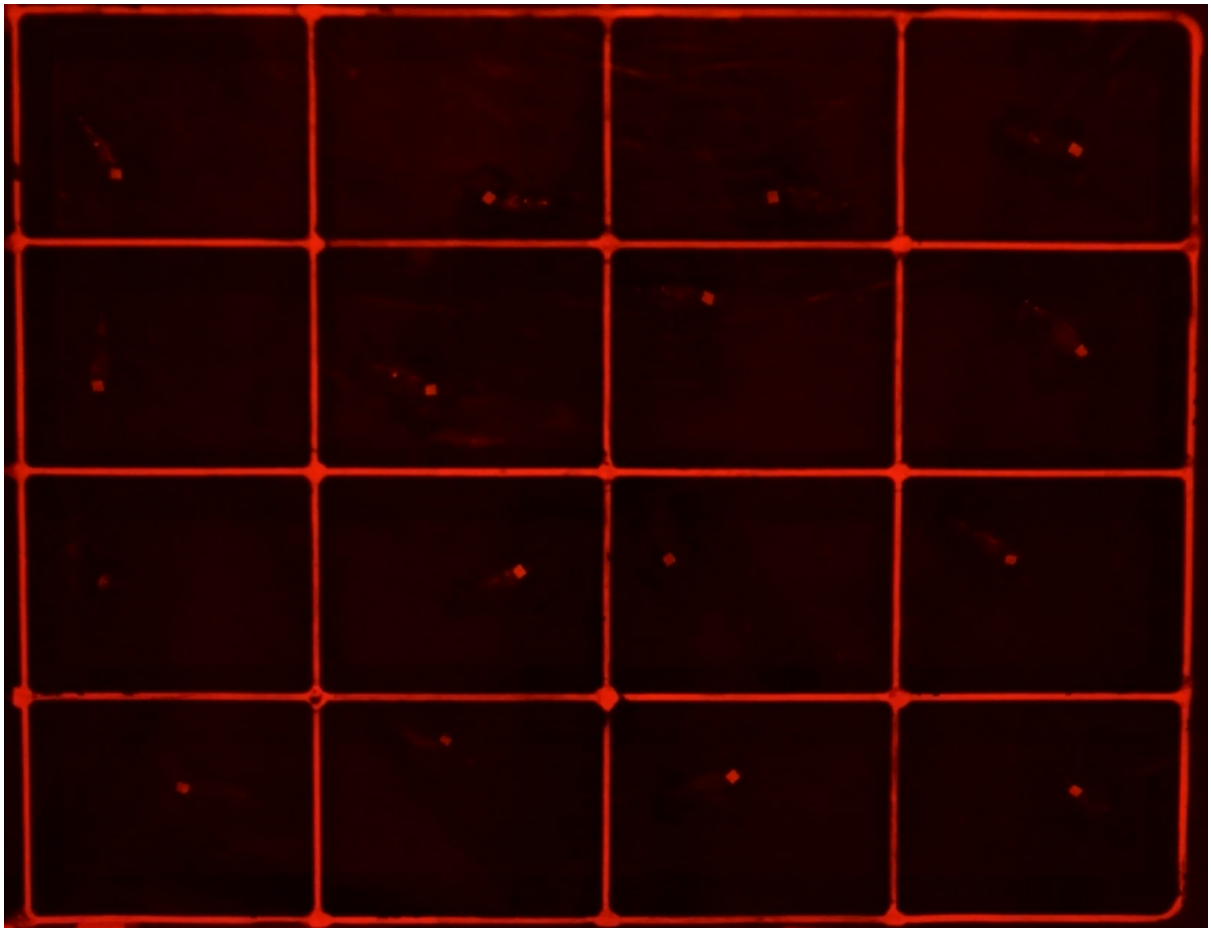
Predictor	***df***	***X²***	***P***
Population	3	3.799	0.284
Age	1	0.054	0.816
Somatic mass	1	1.646	0.199
Somatic SMI*	2	13.094	**0.001**
Testes mass	1	1.789	0.181

Significant P-values (<0.05) are highlighted in bold.
* Somatic SMI is included in the model as an orthogonal polynomial with 2 degrees to account for non-linear effects

6.6 Supporting Figures



Supplementary Figure 6.1 Relationship between SMI and calling effort
The non-linear relationship between scaled mass index and log_2-transformed calling effort across all populations. The line shows predicted values for the SMI term from the linear mixed model in Table 6.1, and with 95% confidence intervals.



Supplementary Figure 6.2 Arrangement of experimental males
Screenshot from a trial video, showing 16 isolated male crickets under a low level of red light, with their reflective tags visible.

7. General Discussion

Understanding the factors which promote rapid adaptive evolution in the face of the various ecological and genetic features which act to constrain it is central to the study of evolutionary biology, and has motivated a great deal of research. In this thesis I have used the loss of male song in Hawaiian populations of *T. oceanicus* as a system in which to test factors that have promoted and resulted from recurrent and rapid adaptive evolution across small, diffuse and fragmented wild populations evolving under extreme selection from the acoustically-orienting parasitoid fly, *O. ochracea*. Alongside prior work, the results have shown that song loss has evolved repeatedly, and through a variety of independent means, but with potentially key unifying characteristics which provide insight into the conditions which promote rapid adaptation. For example, the evolution of genetically distinct flatwing phenotypes appears to have been facilitated by variation underlying sexual dimorphism (Chapter Three), and pleiotropic or otherwise associated effects on non-focal phenotypes of the underlying mutations in each sex are likely to have played an important role in promoting or constraining their spread (Chapter Two). Additionally, all characterised adaptive variants involve the loss of male song rather than alternative adaptive strategies, such as reduced investment in song (Chapters Five and Six), suggesting morphological – rather than behavioural – trait losses may lend themselves particularly to rapid adaptation, given the multitude of ways in which a trait may be functionally lost.

In contrast, and despite expectations that phenotypic plasticity will play a key role in the early stages of adaptation (West-Eberhard 2005), the results reported here do not add to existing evidence of behaviour, plasticity or alterations to the male social environment having played an important capacitating role in the spread of adaptive song-loss variants. All males which cannot sing (due to obligate, or experimentally manipulated, silence) continue to

expend considerable energy trying to do so (Chapter Five), as do populations which have evolved in silence for upwards of 50 generations, despite the readily apparent fitness advantages of divesting energy from the costly behaviour in the absence of benefits in female mate choice (Chapter Six). Additionally, there was no evidence that the loss of the male sexual trait, or associated feminised appearance and cuticular pheromones, had any mitigating effect with respect to reducing the aggressiveness of male agonistic encounters (Chapter Four). While there are various potential explanations for these null findings, the results of this thesis hint at a primary importance of genetic and developmental factors, such as balancing selection and pleiotropy, in promoting the rapid adaptive spread of songless male phenotypes. While an important role for associated phenotypic plasticity and evolutionary accommodation in this system is supported by prior work (Zuk et al. 2006; Bailey et al. 2010; Bailey & Zuk 2012; Balenger & Zuk 2015), this role might be secondary to that of more immediate genetic and developmental constraints in determining the ability of small and fragmented populations to evolve under strong selection.

7.1 Standing genetic variation and rapid adaptation

A primary constraint acting upon the evolution of novel adaptive phenotypes is the amount of standing genetic variation in the genome (Barton & Turelli 2003). This existing variation is widely expected to play a primary role in determining the ability of small or medium-sized populations to adapt to rapid changes in ecology (Hermisson & Pennings 2005), as *de novo* adaptive mutations may be vanishingly rare (Lai et al. 2019). At least in Kauai and Oahu, adaptive flatwing phenotypes are underpinned by separate mutations which appear to have arisen and spread under selection from the parasitoid fly (Zuk et al. 2006). Evidence from the differential expression study in Chapter Three indicates each of these adaptive mutations

interact with and disrupt existing patterns of sex-biased gene expression in the affected developing wing tissues. This suggests that evolution of newly adaptive mutant phenotypes nevertheless benefitted from the existence of variation underlying sexual dimorphism; specifically, variation associated with sex-specific developmental trajectories (i.e. genes expressed differently between sexes) which are regulated by differences in expression of key modifier genes, and which are shared across populations (Alves et al. 2019; Lai et al. 2019). This would in turn indicate that two fundamental substrates for genetic adaptation – genomic variation and mutation – interacted synergistically in bringing about the emergence and spread of flatwing phenotypes. Such a pattern of *de novo* mutations, i.e. those underlying flatwing phenotypes in Kauai and Oahu, interacting with standing variation, i.e. variation underlying sexually dimorphic wing venation patterns, to produce adaptive change is consistent with the idea that existing, evolutionarily shaped patterns of developmental regulation play an important role influencing genomes' evolvability (Wagner & Altenberg 1996; Kirschner & Gerhart 1998).

In contrast, the results of Chapters Five and Six illustrate that while song-loss phenotypes have repeatedly emerged, males carrying each of these phenotypes still invest heavily in trying to sing, and the energy invested in attempting to produce song does not differ between silent and non-silent populations. The fitness benefits of investing less energy in costly calling effort following the erosion of sound-producing features are clear, and a more intuitive and advantageous route of adaptation under selection from the parasitoid fly might have been to simply stop exhibiting this costly behaviour, or to modulate the timing and level of singing behaviour to avoid the fly (Zuk et al. 1993). The persistence of calling behaviour in silent and non-silent populations alike therefore hints at constraints upon the adaptive modulation of calling behaviour, and a plausible explanation for this is depleted standing genetic variation in associated regions as a result of many thousands of generations

of selection for substantial investment in male song (Wagner & Reiser 2000; Barton & Turelli 2003). Thus, while standing variation associated with sexually antagonistic selection pressures, and thus sexual dimorphism, might have capacitated the multiple emergence of adaptive songless male phenotypes, modulation of calling behaviour might have been impeded by the purging of variation in associated genomic regions under strong sexual selection for calling effort (Wagner & Reiser 2000).

The finding that multiple adaptive songless phenotypes have apparently emerged and spread under selection from the parasitoid fly raises an intriguing question for future research. Soft selective sweeps, in which multiple adaptive variants arise and spread under shared selection pressure, are expected to less drastically reduce standing genetic variation compared with hard selective sweeps, in which a single adaptive variant arises in a single ancestor (Hermisson & Pennings 2005). If so, populations in which combinations of flatwing, curly-wing, and/or small-wing males co-exist might be expected to exhibit greater levels of genetic variation – and thus effective population size – compared with those in which flatwing (or other adaptive songless variant) phenotypes emerged and spread to fixation. These dynamics might therefore increase the probability of evolutionary rescue, and promote evolvability in future environments (Wilson et al. 2017).

7.2 Phenotypic plasticity and compensatory adaptation

Phenotypic plasticity, of which behavioural flexibility is one form, is expected by many to play an important role in the early stages of adaptation (West-Eberhard 2003; Ghalambor et al. 2007; Wong & Candolin 2015; Bailey et al. 2018). Consistent with this, the spread of flatwing males appears to have benefitted from pre-existing flexibility in female mate choice (Zuk et al. 2006; Bailey et al. 2008), and plastic responses to the acoustic environment

(Bailey et al. 2010; Balenger & Zuk 2015; Pascoal et al. 2018); the latter presumably representing an ancestral form of phenotypic plasticity in response to changes in population density (Tinghitella & Zuk 2009).

Besides this behavioural flexibility in the context of the level of acoustic stimulation, I found little evidence of phenotypic plasticity or behavioural flexibility having played further important roles in the adaptive emergence and spread of song-loss phenotypes. The loss of song does not appear to influence the incidence of same-sex sexual behaviour via mistaken identity, which might have reduced the levels of male-male aggressiveness to which they are subject. This is perhaps surprising, as a large body of literature supports the view that less sexually dimorphic males, such as juveniles (Dukas 2010), and those which pursue alternative reproductive tactics (Mason & Crews 1985; Norman et al. 1999), tend to be exposed to lower levels of intra-specific aggression via increased likelihood of conspecific males expressing same-sex sexual behaviour. In fact, a previous study of *T. oceanicus* found that silent males actually experience greater levels of aggression, apparently owing to their inability to produce the victory display of aggressive song (Logue et al. 2010).

Additionally, there was no evidence silent flatwing males from mixed-morph populations, or experimentally silenced males, showed any behavioural flexibility in terms of refraining from expending substantial energetic resources in trying to sing, despite the obvious benefits of refraining from doing so. Much discussion of the important role played by plasticity, and particularly of behavioural flexibility, in the early stages of adaptive evolution places emphasis on the consequences of initially adaptive changes in individual behaviour following a change in environment or ecology (Wong & Candolin 2015). These initially adaptive changes might represent ancestral reaction norms selected for in the presence of fluctuating ecological parameters (Chevin & Lande 2015), or could be produced by chance (Moczek 2008); for example as a result of stress-induced variation some of which

will be in an adaptive direction (Lande 2009). However, plasticity or behavioural flexibility is likely to be eroded for traits which are under strong, persistent directional or stabilising selection (Lande 2009), such as those under sexual selection.

7.3 The repeatability of adaptive evolution

Genomic approaches to studying convergent evolution have provided insight into the genetic changes which underlie morphologically similar but evolutionarily independent changes in phenotype under shared selection pressures (Sackton & Clark 2019). While the insights from this research into the features of genetics and development that underlie the multiple emergence and spread of similar phenotypes are undeniable, it is also likely that this avenue of research biases understanding of the routes by which populations independently adapt to common selection pressures (Losos 2011). Recent work has shown that convergent adaptation can occur through contrasting morphological changes (Mcgee & Wainwright 2013), and the results of Chapter Five showing that adaptive silence has arisen through at least three distinct mechanisms (feminised wing veins, altered 3D wing morphology, and reduced wing size) demonstrate this potentially key but underappreciated feature of convergent adaptation. While the genomic changes underlying these divergent phenotypes have not yet been characterised, it is beyond reasonable doubt, given the divergent morphological changes with which they are associated, that they are underpinned by different causative genetic changes.

Research into convergent patterns of phenotypic evolution, particularly those relying largely on comparative phylogenetic analyses, might frequently overlook important patterns of parallel adaptation under similar selection pressures through divergent means. Moreover, if patterns of convergent evolution are considered to provide insight into the repeatability of adaptive evolution (Blount et al. 2018), or the genetic and developmental constraints to which

it is subject (Losos 2011), then it is especially important that the phenomenon of functionally convergent, but morphologically variable, evolution is not overlooked. It is nevertheless apparent from the results of Chapter Three that morphologically similar flatwing phenotypes are associated with overlapping changes in expression of potentially key genes, consistent with growing evidence that convergent evolution through phenotypically similar changes frequently occurs via changes at nearby genomic regions (Sackton & Clark 2019).

7.4 The role of sexual dimorphism in rapid adaptation

Two potentially key features that might have contributed to the rapid evolutionary dynamics with respect to flatwing phenotypes are 1) that flatwing male wings lack male-specific sound-producing structures, rendering them female-like in appearance, and 2) that the XO system of sex-determination requires that males and females share all genes – though females are diploid and male haploid for the X-chromosome on which *flatwing* genotypes reside. Combined, these two features suggest gene expression variation associated with sexual dimorphism might have played an important capacitating role in flatwing's emergence – supported by results from Chapter Three – and that pleiotropic or otherwise genetically linked effects of the *flatwing* genotypes in females could have played an important role in promoting or constraining their spread.

Although gene expression data from Chapter Three show down-regulated expression of phylogenetically conserved sex-determining doublesex transcripts in wings of males and females carrying *flatwing* genotypes, there was no evidence of pervasive 'feminisation' of male gene expression in the wings or across adult non-wing tissues (Chapter Two). There nevertheless remains a strong indication that flatwing males exhibit reduced sexual dimorphism: apart from the female-like wing membranes, they also exhibit feminised

cuticular pheromones (Appendix 1) and reduced testes mass (Chapter Two; Bailey et al. 2010) in Kauai males. It is possible feminisation of gene expression occurs at an earlier stage of development, or that phenotypic feminisation occurs through some other means, such as changes to hormonal regulation which could result from differences in the expression of genes such as *dsx* during development (Li et al. 2018).

There was, however, evidence in the results of Chapter Two that the Kauai *flatwing* genotype affects patterns of sex-biased gene expression in a manner consistent with 'demasculinisation' of these females, i.e. down-regulation of male-biased genes and/or up-regulation of female-biased genes. We also found females homozygous for the Kauai *flatwing* genotype showed greater measures of body condition. Together, these results could indicate a reduction in negative, sexually antagonistic pleiotropy affecting females via the emergence of a genotype which erodes the male-limited sexual trait. An important consequence of these results is the implication that females might benefit from carrying the adaptive *flatwing* genotype, which could therefore have facilitated its rapid spread, particularly owing to females' greater genetic contribution with respect to X-linked genes (Rice 1986). In contrast, negative pleiotropic effects of the underlying mutation in males which are potentially consistent with a shift in the expression of sexual dimorphism in females' favour – i.e. reduced expression of male-specific traits – are likely to have been outweighed by the extreme immediate benefits of song loss in the context of natural selection conferred by the parasitoid fly.

While the results of Chapter Two replicate an earlier study in finding flatwing males from Kauai exhibit reduced testes mass (Bailey et al. 2010), which might be expected to reduce their competitive ability in the context of post-copulatory sexual selection, a recent study found counter-intuitive evidence flatwing males from Kauai actually sire more offspring per successful mating event, suggesting they might carry a reproductive advantage

(Heinen-Kay et al. 2019). However, there was no overall indication in that study that flatwing males exhibit greater reproductive success once zero-counts (i.e. mating events that did not produce offspring) were accounted for. Moreover, while the results of Chapter Two revealed no difference in female fecundity associated with the Kauai *flatwing* genotype, Heinen-Kay et al. (2019) find that these females experience more instances of mating failure than do females homozygous for the normal-wing genotype. While these results are not directly contradictory, these potential inconsistencies suggest further, clearly outlined hypothesis-driven research into pleiotropic effects of flatwing genotypes across populations would be beneficial.

7.5 Future research

The results of this thesis provide several avenues for future research, which will offer insight into the evolutionary dynamics of the flatwing system and some of the conditions which might promote rapid adaptation more generally. The causative genetic mutations underlying flatwing phenotypes remain largely unclear, though recent work has narrowed down genomic regions of interest (Chapter Three; Appendix 1; Xiao Zhang unpublished data), and the newly adaptive variants documented in Chapter Five are also yet to be characterised at the genetic level. For example, it is not known whether mutations underlying flatwing morphology are located in intronic or exonic regions, which could contribute to ongoing debate regarding the importance of cis-regulatory and structural genetic changes (Wray 2007; Hoekstra & Coyne 2007; Sackton et al. 2019). Continuing advances in sequencing technology mean that identifying these genetic changes is increasingly feasible, and could conceivably also provide an approximate date for the emergence of the respective mutations (van't Hof et al. 2016). While it appears that flatwing phenotypes arose under selection from the parasitoid fly (Zuk

et al. 2006), it is also plausible that the respective underlying genotypes had persisted at low levels in the population or nearby populations but only spread to appreciable frequencies under this introduced selection pressure. Addressing whether mutations underlying flatwing phenotypes arose prior to or following the introduction of the parasitoid fly is complicated by the lack of an established introduction date for *O. ochracea* (Zuk et al. 1993), but could involve evaluating rates of recombination and mutation in the regions of associated QTL regions. For example, this approach has suggested the melanic form of the peppered moth first emerged and subsequently persisted at low levels in the decades prior to its adaptive proliferation as a result of industrial pollution, in the region of the year 1819 (van't Hof et al. 2016). Similarly, the suggestion in Chapter Five that functionally convergent curly-wing and small-wing phenotypes arose under the same direct selection pressure conferred by the parasitoid fly would benefit from further examination.

The results of Chapter Three provide an important candidate pathway for future research into the causative genetic basis of convergently evolved flatwing phenotypes from Kauai and Oahu. Both phenotypes were found to be associated with down-regulation of doublesex (*dsx*), a gene with a key conserved role in the regulation and expression of sexual dimorphism in insects (Kijimoto et al. 2012; Kopp 2012; Kunte et al. 2014; Price et al. 2015; Ruiz et al. 2015). Moreover, the genomic position of *dsx* on the X-chromosome places it at the centre of the recently produced QTL for the Kauai *flatwing* genotype (Appendix 1), and recent work has revealed similar results in association with Oahu and Hawaii *flatwing* genotypes (Xiao Zhang, unpublished data). Accumulated transcriptomic and genomic evidence for the involvement of *dsx* in the production of flatwing phenotypes, as well as the intuitive mechanism by which down-regulation of sex differences might lead to the loss of the male sexual trait, therefore provides a clear avenue for future research. RNAi knockdown of *dsx* transcripts in normal-wing lines could be used to functionally validate the importance

of associated sex-determination pathways (Kijimoto et al. 2012). If knockdown of *dsx* transcripts induces a flatwing-like phenotype this would strongly support a causative role for this gene, also corroborating the interpretation that genetic variation associated with sexual dimorphism played an important capacitating role in the repeated emergence of flatwing phenotypes. Efforts to accurately quantify expression of doublesex and reference genes through RT-qPCR are ongoing, representing a further step in corroborating the expression patterns observed in Chapter Three, and which will be vital for validating successful *dsx* knockdown.

Still less is known with respect to curly-wing and small-wing phenotypes. Initial research into understanding their genetic basis will involve performing crosses between laboratory strains to understand whether, like flatwing phenotypes, they segregate as single locus traits, or are instead polygenic, and to what extent. This will also reveal whether the phenotypes show sex-linkage, as in the case of flatwing (Tinghitella 2008; Pascoal et al. 2014), and whether curly-wing phenotypes identified on multiple islands share a genetic basis, or are similarly convergently evolved (Pascoal et al. 2014). Further research into these alternative song-loss phenotypes will involve studying their frequencies over time, especially in populations where they co-occur with flatwing phenotypes. The conditions following a soft-selective sweep, in which multiple adaptive variants co-occur (Hermisson & Pennings 2005), provide an intriguing and rare opportunity to investigate the relative costs and benefits of each of the alternative variants that might influence their subsequent success.

In spite of its overarching importance to this well-studied system, remarkably little is known about the ecology of the parasitoid fly, *Ormia ochracea*, which targets *T. oceanicus*. An important step in understanding more about the host-parasite interaction, and the conditions under which song-loss variants have repeatedly evolved, will be to quantify the population density of *O. ochracea* across various study sites. This is of particular interest

given the multiple adaptive silent male phenotypes that have been identified, and how the proportion of silent males varies across populations. Addressing this gap in understanding represents the focus of an upcoming research project, in which I will be involved as a post-doc, titled "How Repeatable is Adaptive Evolution? Testing What Promotes Rapid Adaptation in a Replicated Natural System". Utilising flytraps similar to those used to corroborate the fitness benefit of curly-wing males in Chapter Five, we will play back standardised song recordings from normal-wing males across multiple habitats which harbour populations of *T. oceanicus*. The number of flies captured per unit of time will be used as indicative of the relative density of parasitoid flies targeting males in that population. Combined with mating crosses and genetic analyses designed to elucidate the genomic underpinning of short-wing and curly-wing phenotypes, these data will inform understanding of the interplay between forces of mutation, migration and selection in wild populations. For example: do proportions of silent males (flatwing or otherwise) vary across populations in a predictable manner, based on the relative density of *O. ochracea*? And do mutations underlying adaptive changes in wing morphology disproportionately target nearby loci, i.e. 'hotspots' of genomic adaptation, such as regions involved in wing development and morphology (Sackton et al. 2019)?

A recent study by Tanner et al. (2019) quantified the fitness costs experienced by flatwing males in the context of sexual selection, by contrasting the proportion of normal-wing male offspring in female clutches with that of normal-wing males in the population, and reported a strong overrepresentation of normal-wing male phenotypes consistent with strong female discrimination against silent males. The persistence of silent male morphs in the face of these fitness costs therefore indicates that selection against male song in the context of natural selection must also remain very strong. If these two selection pressures are approximately equal to one another, this could indicate that the proportion of silent males in a

population might eventually reach a predictable evolutionarily stable state, though likely complicated in populations where there are multiple adaptive variants. Outcomes from these evolutionary interactions between alternative adaptive phenotypes will help to inform debate about what factors promote and constrain their spread. For example; are less pleiotropic, or more complete, routes to the loss of male song favoured by selection? Do flatwing males suffer a cost of feminised wing venation through reduced sexual dimorphism in other tissues, such as via reduced testes mass (Chapter Two; Bailey et al. 2010) and feminised cuticular pheromones, whereas males that cannott sing due to reduced forewings and hindwings benefit from histolysis of wing muscle (Roff 1989)?

7.6 Conclusions

Adaptive songless flatwing phenotypes appear to have benefitted from genetic variation underlying sexually dimorphic phenotypes, which are associated with sexually antagonistic selection. Flatwing males from Kauai are phenotypically feminised, and demasculinised patterns of gene expression and increased body condition suggest females may have benefitted from the loss of the male sexual trait via relaxation of sexual conflict over shared genes. Despite this phenotypic feminisation, flatwing males do not appear any more likely to be mistaken for females by conspecific males, nor are they less frequently involved in aggressive male-male contests. While flatwing phenotypes seem to have benefitted from the maintenance of genetic variation associated with sexually dimorphic wing phenotypes, additional silent wing phenotypes have evolved which do not morphologically resemble those observed in males or females of other populations. Together, these newly identified male-silencing ‘small-wing’ and ‘curly-wing’ phenotypes suggest that functional traits such as the ability to produce song can be lost in a variety of ways, and demonstrate that convergent

evolution does not rely upon parallel changes in allelic frequencies, or highly similar morphological changes. Finally, there was no evidence that silent male morphs or silent populations divest energy from singing, signifying constraints upon the capacity for adaptive phenotypic accommodation of novel song-loss phenotypes, nor do these less sexually dimorphic silent males benefit from reduced intrasexual competition. Altogether, the results support the view that genetic constraints such as limited standing genetic variation and extensive pleiotropy impede adaptive evolution, but illustrate a number of ways in which these constraints can be partially overcome, such as through balancing selection and reduced sexual conflict.

8. References

Alexander, R.D., 1962. Evolutionary change in cricket acoustical communication. *Evolution*, 16, pp.443–467.

Alves, J.M. et al., 2019. Parallel adaptation of rabbit populations to myxoma virus. *Science*, 363(6433), pp.1319–1326.

Andersson, M., 1986. Evolution of condition-dependent sex ornaments and mating preferences: sexual selection based on viability differences. *Evolution*, 40(4), pp.804–816.

Arnold, S.J., 1976. Sexual behavior, sexual interference and sexual defense in the salamanders Ambystoma maculatum, Ambystoma tigrinum and Plethodon jordani. *Zeitschrift für Tierpsychologie*, 42(3), pp.247–300.

Bachtrog, D. et al., 2014. Sex determination: why so many ways of doing it? *PLoS Biology*, 12(7), pp.1–13.

Bailey, N.W. & French, N., 2012. Same-sex sexual behaviour and mistaken identity in male field crickets, Teleogryllus oceanicus. *Animal Behaviour*, 84(4), pp.1031–1038.

Bailey, N.W., Gray, B. & Zuk, M., 2010. Acoustic experience shapes alternative mating tactics and reproductive investment in male field crickets. *Current Biology*, 20(9), pp.845–849.

Bailey, N.W., Marie-Orleach, L. & Moore, A.J., 2018. Indirect genetic effects in behavioral ecology: Does behavior play a special role in evolution? *Behavioral Ecology*, 29(1), pp.1–11.

Bailey, N.W., McNabb, J.R. & Zuk, M., 2008. Preexisting behavior facilitated the loss of a sexual signal in the field cricket Teleogryllus oceanicus. *Behavioral Ecology*, 19(1), pp.202–207.

Bailey, N.W., Pascoal, S. & Montealegre-Z, F., 2019. Testing the role of trait reversal in evolutionary diversification using song loss in wild crickets. *Proceedings of the National Academy of Sciences*, 116(18), pp.8941–8949.

Bailey, N.W. & Zuk, M., 2008. Acoustic experience shapes female mate choice in field crickets. *Proceedings of the Royal Society B: Biological Sciences*, 275(1651).

Bailey, N.W. & Zuk, M., 2009. Same-sex sexual behavior and evolution. *Trends in Ecology & Evolution*, 24(8), pp.439–446.

Bailey, N.W. & Zuk, M., 2012. Socially flexible female choice differs among populations of the Pacific field cricket: geographical variation in the interaction coefficient psi ( ). *Proceedings of the Royal Society B: Biological Sciences*, 279(May), pp.3589–3596.

Balakrishnan, R. & Pollack, G.S., 1996. Recognition of courtship song in the field cricket, Teleogryllus oceaniens. *Animal Behaviour*, 51(353–366).

Baldwin, J.M., 1896. A new factor in evolution. *The American Naturalist*, 30(354), pp.441–451.

Balenger, S.L. & Zuk, M., 2015. Roaming romeos: male crickets evolving in silence show increased locomotor behaviours. *Animal Behaviour*, 101, pp.213–219.

Barton, N.H. & Turelli, M., 2003. Evolutionary quantitative genetics: how little do we know? *Annual Review of Genetics*, 23, pp.337–370.

Bates, D., Maechler Martin & Walker, S., 2016. Package "lme4." *CRAN Repository*, p.113.

Berger, D. et al., 2014. Multivariate intralocus sexual conflict in seed beetles. *Evolution*, 68(12), pp.3457–3469.

Bertram, S.M. et al., 2011. Variation in cricket acoustic mate attraction signalling explained by body morphology and metabolic differences. *Animal Behaviour*, 82(6), pp.1255–1261.

Bierbach, D. et al., 2012. Homosexual behaviour increases male attractiveness to females.

Biology Letters, 9(1), p.20121038.

Blount, Z.D., Borland, C.Z. & Lenski, R.E., 2008. Historical contingency and the evolution of a key innovation in an experimental population of Escherichia coli. *Proceedings of the National Academy of Sciences*, 105(23), pp.7899–7906.

Blount, Z.D., Lenski, R.E. & Losos, J.B., 2018. Contingency and determinism in evolution: Replaying life's tape. *Science*, 362(6415).

Bonduriansky, R. & Chenoweth, S.F., 2009. Intralocus sexual conflict. *Trends in Ecology & Evolution*, 24(5), pp.280–288.

Boutin, S.R.T. et al., 2016. Same-sex sexual behaviour in crickets: Understanding the paradox. *Animal Behaviour*, 114, pp.101–110.

Bradshaw, A.D., 1965. Evolutionary significance of phenotypic plasticity in plants. *Advances in Genetics*, 13(C), pp.115–155.

Brimacombe, C.A. et al., 2019. A natural histone H2A variant lacking the Bub1 phosphorylation site and regulated depletion of centromeric histone CENP-A foster evolvability in Candida albicans. *PLOS Biology*, 17(6), p.e3000331.

Cade, W.H. & Cade, E.S., 1992. Male mating success, calling and searching behaviour at high and low densities in the field cricket, Gryllus integer. *Animal Behaviour*, 43(1), pp.49–56.

Carson, H.L., 1978. Speciation and sexual selection in Hawaiian Drosophila. In P. F. Brussard, ed. *Ecological Genetics: The Interface*. Berlin: Springer-Verlag, pp. 93–107.

Carson, H.L., Chang, L.S. & Lyttle, T.W., 1982. Decay of female sexual behavior under parthenogenesis. *Science*, 218(4567), pp.68–70.

Castro, J.P. et al., 2019. An integrative genomic analysis of the Longshanks selection experiment for longer limbs in mice. *eLife*, 8, p.e42014.

Chan, Y.F. et al., 2010. Adaptive evolution of pelvic reduction in sticklebacks by recurrent

deletion of a pitxl enhancer. *Science*, 327(5963), pp.302–305.

Charlesworth, B., Morgan, M.T. & Charlesworth, D., 1993. The effect of deleterious mutations on neutral molecular variation. *Genetics*, 134(4), pp.1289–1303.

Charlesworth, D., 2006. Balancing selection and its effects on sequences in nearby genome regions. *PLoS Genetics*, 2(4), p.e64.

Cheng, C. & Kirkpatrick, M., 2016. Sex-specific selection and sex-biased gene expression in humans and flies. *PLoS Genetics*, 12(9).

Cheverud, J.M., Routman, E.J. & Irschick, D.J., 1997. Pleiotropic effects of individual gene loci on mandibular morphology. *Evolution*, 51(6), pp.2006–2016.

Chevillon, C. et al., 1997. Pleiotropy of adaptive changes in populations: comparisons among insecticide resistance genes in Culex pipiens. *Genetical Research*, 70(3), pp.195–203.

Chevin, L.M. & Lande, R., 2015. Evolution of environmental cues for phenotypic plasticity. *Evolution*, 69(10), pp.2767–2775.

Ciofi, C. & Swingland, I.R., 1997. Environmental sex determination in reptiles. *Applied Animal Behaviour Science*, 51, pp.251–265.

Colegrave, N., 2002. Sex releases the speed limit on evolution. *Nature*, 420, pp.646–666.

Conesa, A. et al., 2005. Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics*, 21(18), pp.3674–3676.

Connallon, T. & Clark, A.G., 2014. Balancing selection in species with separate sexes: Insights from fisher's geometric model. *Genetics*, 197(3), pp.991–1006.

Connallon, T., Cox, R.M. & Calsbeek, R., 2010. Fitness consequences of sex-specific selection. *Evolution*, 64(6), pp.1671–1682.

Connallon, T. & Knowles, L.L., 2005. Intergenomic conflict revealed by patterns of sex-biased gene expression. *Trends in Genetics*, 21(9), pp.495–499.

Conover, D.O. & Munch, S.B., 2002. Sustaining fisheries yields over evolutionary time

scales. *Science*, 297(5578), pp.94–96.

Cook, L.M., 2003. The rise and fall of the carbonaria form of the peppered moth. *Quarterly Review of Biology*, 78(4), pp.399–417.

Cox, R.M. & Calsbeek, R., 2009. Sexually antagonistic selection, sexual dimorphism, and the resolution of intralocus sexual conflict. *The American Naturalist*, 173(2), pp.176–187.

Crnokrak, P. & Roff, D.A., 1995. Fitness differences associated with calling behaviour in the two wing morphs of male sand crickets, Gryllus firmus. *Animal Behaviour*, 50(6), pp.1475–1481.

Crnokrak, P. & Roff, D.A., 1998. The genetic basis of the trade-off between calling and wing morph in males of the cricket gryllus firmus. *Evolution*, 52(4), pp.1111–1118.

Crudgington, H.S. et al., 2005. Experimental removal and elevation of sexual selection: does sexual selection generate manipulative males and resistant females? *The American Naturalist*, 165, pp.s72-87.

Darwin, C., 1859. *On the origin of species by means of natural selection*, London: John Murray.

Davies, J. & Davies, D., 2010. Origins and evolution of antibiotic resistance. *Microbiologia and Molecular Biology Reviews*, 74(3), pp.417–433.

Dean, R. et al., 2018. Masculinization of gene expression is associated with male quality in Drosophila melanogaster. *Evolution*, 72(12), pp.2736–2748.

Dean, R. & Mank, J.E., 2016. Tissue specificity and sex-specific regulatory variation permit the evolution of sex-biased gene expression. *The American Naturalist*, 188(3), pp.E74–E84.

Desai, M.M. & Fisher, D.S., 2007. Beneficial mutation-selection balance and the effect of linkage on positive selection. *Genetics*, 176(3), pp.1759–1798.

Dixon, K.A. & Cade, W.H., 1986. Some factors influencing male-male aggression in the field

cricket Gryllus integer (time of day, age, weight and sexual maturity). *Animal Behaviour*, 34(2), pp.340–346.

Dobzhansky, T., 1937. *Genetics and the origin of species* 3rd ed., New York: Columbia University Press.

Dominey, W.J., 1980. Female mimicry in male bluegill sunfish - A genetic polymorphism? *Nature*, 284, pp.546–548.

Doniach, T. & Hodgkin, J., 1984. A sex-determining gene, fem-1, required for both male and hermaphrodite development in Caenorhabditis elegans. *Developmental Biology*, 106(1), pp.223–235.

Drayton, J.M. et al., 2010. Inbreeding and advertisement calling in the cricket Teleogryllus commodus: Laboratory and field experiments. *Evolution*, 64(10), pp.3069–3083.

Dugatkin, L.A., 2008. *Principles of animal behavior* 2nd ed., New York City: W.W. Norton and Co.

Dukas, R., 2010. Causes and consequences of male-male courtship in fruit flies. *Animal Behaviour*, 80(5), pp.913–919.

Dybdahl, M.F. & Lively, C.M., 2006. Host-parasite coevolution: evidence for rare advantage and time-lagged selection in a natural population. *Evolution*, 52(4), pp.1056–1066.

Eizirik, E. et al., 2003. Molecular genetics and evolution of melanism in the cat family. *Current Biology*, 13(5), pp.448–453.

Ellegren, H. & Parsch, J., 2007. The evolution of sex-biased genes and sex-biased gene expression. *Nature Reviews Genetics*, 8(9), pp.689–698.

Feng, C. et al., 2014. A cis-regulatory mutation of pdss2 causes silky-feather in chickens. *PLoS Genetics*, 10(8), p.e1004576.

Fierst, J.L., 2011. Sexual dimorphism increases evolvability in a genetic regulatory network. *Evolutionary Biology*, 38(1), pp.52–67.

Fisher, R.A., 1915. The evolution of sexual preference. *The Eugenics review*, 7(3), pp.184–192.

Fisher, R.A., 1930. *The genetical theory of natural selection*, Oxford, England: Clarendon Press.

Fong, D.W., Kane, T.C. & Culver, D.C., 1995. Vestigialization and loss of nonfunctional characters. *Annual Review of Ecology and Systematics*, 26, pp.249–268.

Foucault, Q. et al., 2018. Rapid adaptation to high temperatures in Chironomus riparius. *Ecology and Evolution*, 8(24), pp.12780–12789.

Fricke, C. & Arnqvist, G., 2007. Rapid adaptation to a novel host in a seed beetle (Callosobruchus maculatus): The role of sexual selection. *Evolution*, 61(2), pp.440–454.

Ghalambor, C.K. et al., 2007. Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Functional Ecology*, 21(3), pp.394–407.

Gould, J.S., 1989. *Wonderful life: The Burgess shale and the nature of history*, New York: W.W. Norton.

Gould, S.J. & Lewontin, R.C., 1979. The spandrels of San Marco and the Panglossian paradigm: a critique of the adaptationist programme. *Proceedings of the Royal Society of London - Biological Sciences*, 205(1161), pp.581–598.

Gould, S.J. & Vrba, E.S., 1982. Exaptation—a missing term in the science of form. *Paleobiology*, 8(1), pp.4–15.

Gray, D.A. et al., 2018. Induced expression of a vestigial sexual signal. *Biology Letters*, 14(5).

Gray, D.A. & Cade, W.H., 1999. Quantitative genetics of sexual selection in the field cricket, Gryllus integer. *Evolution*, 53(3), pp.848–854.

Guo, Y. et al., 2016. A complex structural variation on chromosome 27 leads to the ectopic

expression of hoxb8 and the muffs and beard phenotype in chickens. *PLoS Genetics*, 12(6), p.e1006071.

Hack, M.A., 1998. The energetics of male mating strategies in field crickets (Orthoptera: Gryllinae: Gryllidae). *Journal of Insect Behavior*, 11(6), pp.853–867.

Hall, B.K., 2003. Evo-Devo: Evolutionary developmental mechanisms. *International Journal of Developmental Biology*, 47(7), pp.491–495.

Han, C.S. & Brooks, R.C., 2015. Same-sex sexual behaviour as a by-product of reproductive strategy under male-male scramble competition. *Animal Behaviour*, 108, pp.193–197.

Han, C.S., Santostefano, F. & Dingemanse, N.J., 2016. Do social partners affect same-sex sexual behaviour in male water striders? *Animal Behaviour*, 116, pp.53–59.

Harano, T. et al., 2010. Intralocus sexual conflict unresolved by sex-limited trait expression. *Current Biology*, 20(22), pp.2036–2039.

Harari, A.R., Brockmann, H.J. & Landolt, P.J., 2000. Intrasexual mounting in the beetle Diaprepes abbreviatus (L.). *Proceedings of the Royal Society B: Biological Sciences*, 267(1457), pp.2071–2079.

Harrison, P.W. et al., 2015. Sexual selection drives evolution and rapid turnover of male gene expression. *Proceedings of the National Academy of Sciences*, 112(14), pp.4393–4398.

Hedrick, A. V., 2002. Female choice and the heritability of attractive male traits: an empirical study. *The American Naturalist*, 132(2), pp.267–276.

Heinen-Kay, J.L., Urquhart, E.M. & Zuk, M., 2019. Obligately silent males sire more offspring than singers in a rapidly evolving cricket population. *Biology Letters*, 15(7).

Hermisson, J. & Pennings, P.S., 2005. Soft sweeps: Molecular population genetics of adaptation from standing genetic variation. *Genetics*, 169(4), pp.2335–2352.

Hoback, W.W. & Wagner, W., 2008. The energetic cost of calling in the variable field cricket, Gryllus lineaticeps. *Physiological Entomology*, 22(3), pp.286–290.

Hoekstra, H.E. et al., 2006. A single amino acid mutation contributes to adaptive beach mouse color pattern. *Science*, 313(5783), pp.101–104.

Hoekstra, H.E. & Coyne, J.A., 2007. The locus of evolution: Evo devo and the genetics of adaptation. *Evolution*, 61(5), pp.995–1016.

Holland, B. & Rice, W.R., 1999. Experimental removal of sexual selection reverses intersexual antagonistic coevolution and removes a reproductive load. *Proceedings of the National Academy of Sciences*, 96(9), pp.5083–5088.

Hollis, B. et al., 2014. Evolution under monogamy feminizes gene expression in Drosophila melanogaster. *Nature Communications*, 5.

Holzer, B., Jacot, A. & Brinkhof, M.W.G., 2003. Condition-dependent signaling affects male sexual attractiveness in field crickets, Gryllus campestris. *Behavioral Ecology*, 14(3), pp.353–359.

Hoskins, J.L., Ritchie, M.G. & Bailey, N.W., 2015. A test of genetic models for the evolutionary maintenance of same-sex sexual behaviour. *Proceedings of the Royal Society B: Biological Sciences*, 282(1809), p.20150429.

Houslay, T.M. et al., 2017. Mating opportunities and energetic constraints drive variation in age-dependent sexual signalling. *Functional Ecology*, 31(3), pp.728–741.

Hunt, J. et al., 2004. High-quality male field crickets invest heavily in sexual display but die young. *Nature*, 432(7020), pp.1024–1027.

Hunt, J. et al., 2007. Reconciling strong stabilizing selection with the maintenance of genetic variation in a natural population of black field crickets (Teleogryllus commodus). *Genetics*, 177(2), pp.875–880.

Hunt, J., Brooks, R. & Jennions, M.D., 2005. Female mate choice as a condition-dependent life-history trait. *The American Naturalist*, 166(1), pp.79–92.

Immonen, E., Snook, R.R. & Ritchie, M.G., 2014. Mating system variation drives rapid

evolution of the female transcriptome in Drosophila pseudoobscura. *Ecology and Evolution*, 4(11), pp.2186–2201.

Jang, Y., 2011. Male responses to conspecific advertisement signals in the field cricket Gryllus rubens (Orthoptera: Gryllidae). *PLoS ONE*, 6(1), p.e16063.

Joag, R. et al., 2016. Transcriptomics of intralocus sexual conflict: Gene expression patterns in females change in response to selection onamale secondary sexual trait in the bulb mite. *Genome Biology and Evolution*, 8(8), pp.2351–2357.

Johnson, W.W., 1972. *Baja California: The American Wilderness*, New York: Time-Life Books.

Judge, K.A., Ting, J.J. & Gwynne, D.T., 2008. Condition dependence of male life span and calling effort in a field cricket. *Evolution*, 62(4), pp.868–878.

Jukema, J. & Piersma, T., 2006. Permanent female mimics in a lekking shorebird. *Biology Letters*, 2(2), pp.161–164.

Khila, A., Abouheif, E. & Rowe, L., 2012. Function, developmental genetics, and fitness consequences of a sexually antagonistic trait. *Science*, 336(6081), pp.585–589.

Kijimoto, T., Moczek, A.P. & Andrews, J., 2012. Diversification of doublesex function underlies morph-, sex-, and species-specific development of beetle horns. *Proceedings of the National Academy of Sciences*, 109(50), pp.20526–20531.

Kim, K.-W. et al., 2019. Genetics and evidence for balancing selection of a sex-linked colour polymorphism in a songbird. *Nature Communications*, 10(1), p.1852.

Kirkpatrick, M., 1982. Sexual selection and the evolution of female choice. *Evolution*, 36(1), pp.1–12.

Kirschner, M. & Gerhart, J., 1998. Evolvability. *PNAS*, 95(15), pp.8420–8427.

Klingenberg, C.P. et al., 2001. Inferring developmental modularity from morphological integration: Analysis of individual variation and asymmetry in bumblebee wings.

American Naturalist.

Klingenberg, C.P., 2008. Morphological integration and developmental modularity. *Annual Review of Ecology, Evolution, and Systematics*, 39, pp.115–132.

Klingenberg, C.P., Mebus, K. & Auffray, J.C., 2003. Developmental integration in a complex morphological structure: How distinct are the modules in the mouse mandible? *Evolution and Development*, 5(5), pp.522–531.

Klingenberg, C.P. & Zaklan, S.D., 2000. Morphological integration between developmental compartments in the Drosophila wing. *Evolution*, 64(4), pp.1273–1285.

Kolluru, G.R., 1999. Variation and repeatability of calling behavior in crickets subject to a phonotactic parasitoid fly. *Journal of Insect Behavior*, 12(5), pp.611–626.

Kopp, A., 2012. Dmrt genes in the development and evolution of sexual dimorphism. *Trends in Genetics*, 28(4), pp.175–184.

Kopylova, E., Noé, L. & Touzet, H., 2012. SortMeRNA: Fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. *Bioinformatics*, 28(24), pp.3211–3217.

Kraaijeveld, K., 2014. Reversible Trait Loss: The Genetic Architecture of Female Ornaments. *Annual Review of Ecology, Evolution, and Systematics*, 45, pp.159–177.

Kunte, K. et al., 2014. doublesex is a mimicry supergene. *Nature*, 507(7491), pp.229–232.

Kuriwada, T., 2017. Male–male courtship behaviour, not relatedness, affects the intensity of contest competition in the field cricket. *Animal Behaviour*, 126, pp.217–220.

Lai, Y.T. et al., 2019. Standing genetic variation as the predominant source for adaptation of a songbird. *Proceedings of the National Academy of Sciences of the United States of America*, 116(6), pp.2152–2157.

Lande, R., 2009. Adaptation to an extraordinary environment by evolution of phenotypic plasticity and genetic assimilation. *Journal of Evolutionary Biology*, 22(7), pp.1435–1446.

Lande, R., 1981. Models of speciation by sexual selection on polygenic traits. *Proceedings of the National Academy of Sciences*, 78(6), pp.3721–3725.

Lande, R., 1980. Sexual dimorphism, sexual selection, and adaptation in polygenic characters. *Evolution*, 34(2), pp.292–305.

Lande, R. & Arnold, S.J., 1983. The measurement of selection on correlated characters. *Evolution*, 37(6), pp.1210–1226.

Lane, S.M. et al., 2016. Same-sex sexual behaviour as a dominance display. *Animal Behaviour*, 114, pp.113–118.

Lenski, R.E., 2017. Convergence and divergence in a long-term experiment with bacteria. *The American Naturalist*, 190, pp.S57–S68.

Lenski, R.E. et al., 2002. Long-term experimental evolution in escherichia coli. I. Adaptation and divergence during 2,000 generations. *The American Naturalist*, 138(6), pp.1315–1341.

Lewis, Z., Wedell, N. & Hunt, J., 2011. Evidence for strong intralocus sexual conflict in the indian meal moth, plodia interpunctella. *Evolution*, 65(7), pp.2085–2097.

Li, S. et al., 2018. Identification and characterization of a doublesex gene which regulates the expression of insulin-like androgenic gland hormone in Fenneropenaeus chinensis. *Gene*, 649, pp.1–7.

Li, W. & Godzik, A., 2006. Cd-hit: A fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics*, 22(13), pp.1658–1659.

Lively, C.M., 1987. Evidence from a New Zealand snail for the maintenance of sex by parasitism. *Nature*, 328, pp.519–521.

Logue, D.M. et al., 2009. A behavioral syndrome linking courtship behavior toward males and females predicts reproductive success from a single mating in the hissing cockroach, Gromphadorhina portentosa. *Behavioral Ecology*, 20(4), pp.781–788.

Logue, D.M. et al., 2010. Does signalling mitigate the cost of agonistic interactions? A test in a cricket that has lost its song. *Proceeding of The Royal Society B: Biological Sciences*, 277, pp.2571–2575.

Lonn, E. et al., 2017. Balancing selection maintains polymorphisms at neurogenetic loci in field experiments. *Proceedings of the National Academy of Sciences*, 114(14), pp.3690–3695.

Losos, J.B., 2011. Convergence, adaptation, and constraint. *Evolution*, 65(7), pp.1827–1840.

Losos, J.B., 2014. What darwin got wrong. *Chronicle of Higher Education*, pp.B13–B15.

Macchiano, A., Razik, I. & Sagot, M., 2018. Same-sex courtship behaviors in male-biased populations: evidence for the mistaken identity hypothesis. *Acta Ethologica*, 21(3), pp.147–151.

MacFarlane, G.R. et al., 2007. Same-sex sexual behavior in birds: Expression is related to social mating system and state of development at hatching. *Behavioral Ecology*, 18(1), pp.21–33.

Maklakov, A.A. & Bonduriansky, R., 2009. Sex differences in survival costs of homosexual and heterosexual interactions: evidence from a fly and a beetle. *Animal Behaviour*, 77(6), pp.1375–1379.

Mank, J.E., 2017. The transcriptional architecture of phenotypic dimorphism. *Nature Ecology and Evolution*, 1(1).

Marchini, M. et al., 2014. Impacts of genetic correlation on the independent evolution of body mass and skeletal size in mammals. *BMC Evolutionary Biology*, 14, p.258.

Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal*, 17(1), p.10.

Mason, R.T. & Crews, D., 1985. Female mimicry in garter snakes. *Nature*, 316(6023), pp.59–60.

Maynard Smith, J. et al., 1985. Developmental constraints and evolution. *The Quarterly review of biology*, 60(3), pp.265–287.

Mcgee, M.D. & Wainwright, P.C., 2013. Convergent evolution as a generator of phenotypic diversity in threespine stickleback. *Evolution*, 67(4), pp.1204–1208.

McRobert, S.P. & Tompkins, L., 1988. Two consequences of homosexual courtship performed by drosophila melanogaster and Drosophila affinis males. *Evolution*, 42(5), pp.1093–1097.

Messer, P.W. & Petrov, D.A., 2013. Population genomics of rapid adaptation by soft selective sweeps. *Trends in Ecology and Evolution*, 28(11), pp.659–669.

Moczek, A.P., 2008. On the origins of novelty in development and evolution. *BioEssays*, 30(5), pp.432–447.

Molinier, J. et al., 2006. Transgeneration memory of stress in plants. *Nature*, 442(7106), pp.1046–1049.

Monod, J. & Jacob, F., 1961. General conclusions: teleonomic mechanisms in cellular metabolism, growth, and differentiation. *Cold Spring Harbor Symposia on Quantitative Biology*, pp.389–401.

Morris, M.R. et al., 2005. The role of sexual selection in the loss of sexually selected traits in the swordtail fish Xiphophorus continens. *Animal Behaviour*, 69(6), pp.1415–1424.

Mou, C. et al., 2011. Cryptic patterning of avian skin confers a developmental facility for loss of neck feathering. *PLoS Biology*, 9(3), p.e1001028.

Mundy, N.I. et al., 2004. Conserved genetic basis of a quantitative plumage trait involved in mate choice. *Science*, 303(5665), pp.1870–1873.

Needham, J., 1933. On the dissociability of the fundamental processes in ontogenesis. *Biological Reviews*, 8(2), pp.180–223.

Nei, M., 2005. Selectionism and neutralism in molecular evolution. *Molecular Biology and*

Evolution, 22(12), pp.2318–2342.

Norman, M.D., Finn, J. & Tregenza, T., 1999. Female impersonation as an alternative reproductive strategy in giant cuttlefish. *Proceedings of the Royal Society B: Biological Sciences*, 266(1426), p.1347.

Orr, H.A., 2000. Adaptation and the cost of complexity. *Evolution*, 54(1), pp.13–20. Available at: http://doi.wiley.com/10.1111/j.0014-3820.2000.tb00002.x.

Parker, D.J. et al., 2015. How consistent are the transcriptome changes associated with cold acclimation in two species of the Drosophila virilis group? *Heredity*, 115, pp.13–21.

Pascoal, S. et al., 2018. Increased socially mediated plasticity in gene expression accompanies rapid adaptive evolution. *Ecology Letters*, 21(4), pp.546–556.

Pascoal, S. et al., 2014. Rapid convergent evolution in wild crickets. *Current Biology*, 24(12), pp.1369–1374.

Pascoal, S. et al., 2016. Rapid evolution and gene expression: a rapidly evolving Mendelian trait that silences field crickets has widespread effects on mRNA and protein expression. *Journal of Evolutionary Biology*, 29(6), pp.1234–1246.

Peabody, G.L.V., Li, H. & Kao, K.C., 2017. Sexual recombination and increased mutation rate expedite evolution of Escherichia coli in varied fitness landscapes. *Nature Communications*, 8, p.2112.

Peig, J. & Green, A.J., 2009. New perspectives for estimating body condition from mass/length data: The scaled mass index as an alternative method. *Oikos*, 118(12), pp.1883–1891.

Pennell, T.M. et al., 2016. Contrasting effects of intralocus sexual conflict on sexually antagonistic coevolution. *Proceedings of the National Academy of Sciences of the United States of America*, 113(8), pp.E978–E986.

Perry, J.C., Garroway, C.J. & Rowe, L., 2017. The role of ecology, neutral processes and

antagonistic coevolution in an apparent sexual arms race. *Ecology Letters*, 20(9), pp.1107–1117.

Perry, J.C., Harrison, P.W. & Mank, J.E., 2014. The ontogeny and evolution of sex-biased gene expression in drosophila melanogaster. *Molecular Biology and Evolution*, 31(5), pp.1206–1219.

Pertea, M. et al., 2016. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. *Nature Protocols*, 11, pp.1650–1667.

Peschke, K., 1985. Immature males of Aleochara curtula avoid intrasexual aggressions by producing the female sex pheromone. *Naturwissenschaften*, 72(5), pp.274–275.

Pfau & Koch, 1994. The functional morphology of singing in the cricket. *The Journal of experimental biology*, 195(1), pp.147–167.

Pigliucci, M., 2008. Is evolvability evolvable? *Nature Reviews Genetics*, 9(1), pp.75–82.

Pizzari, T. & Snook, R.R., 2003. Perspective: Sexual conflict and sexual selection: Chasing away paradigm shifts. *Evolution*, 57(6), pp.1223–1236.

Plesnar-Bielak, A. et al., 2014. Selection for alternative male reproductive tactics alters intralocus sexual conflict. *Evolution*, 68(7), pp.2137–2144.

Pointer, M.A. et al., 2013. Masculinization of gene expression is associated with exaggeration of male sexual dimorphism. *PLoS Genetics*, 9(8).

Porter, M.L. & Crandall, K.A., 2003. Lost along the way: The significance of evolution in reverse. *Trends in Ecology & Evolution*, 18(10), pp.541–547.

Prentis, P.J. et al., 2008. Adaptive evolution in invasive species. *Trends in Plant Science*, 13(6), pp.288–294.

Preston-Mafham, K., 2006. Post-mounting courtship and the neutralizing of male competitors through “homosexual” mountings in the fly Hydromyza livens F. (Diptera: Scatophagidae). *Journal of Natural History*, 40(1–2), pp.101–105.

Prestwich, K.N. & Walker, T.J., 1981. Energetics of singing in crickets: Effect of temperature in three trilling species (Orthoptera: Gryllidae). *Journal of Comparative Physiology*, 143(2), pp.199–212.

Price, D.C., Egizi, A. & Fonseca, D.M., 2015. The ubiquity and ancestry of insect doublesex. *Scientific Reports*, 5, p.13068.

Ptacek, M.B. et al., 2011. Phylogenetic Evidence for the Gain and Loss of a Sexually Selected Trait in Sailfin Mollies. *ISRN Zoology*, 2011, p.251925.

R Core Team, 2017. *R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.*

R Core Team, 2018. *R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.*

Radcliffe, C.W. & Maslin, T.P., 1975. A new subspecies of the red rattlesnake, Crotalus ruber, from San Lorenzo Sur Island, Baja California Norte, Mexico. *Copeia*, 1975(3), pp.490–493.

Rapkin, J. et al., 2016. Macronutrient intake regulates sexual conflict in decorated crickets. *Journal of Evolutionary Biology*, 29(2), pp.395–406.

Rayner, J.G., Aldridge, S., et al., 2019. A silent orchestra: convergent song loss in Hawaiian crickets is repeated, morphologically varied, and widespread. *Ecology*, 100(8), p.e02694.

Rayner, J.G. & Bailey, N.W., 2019. Testing the role of same-sex sexual behaviour in the evolution of alternative male reproductive phenotypes. *Animal Behaviour*, 157.

Rayner, J.G., Pascoal, S. & Bailey, N.W., 2019. Release from intralocus sexual conflict? Evolved loss of a male sexual trait demasculinizes female gene expression. *Proceedings of the Royal Society B: Biological Sciences*, 286(1901).

Rebar, D., Bailey, N.W. & Zuk, M., 2009. Courtship song's role during female mate choice in

the field cricket Teleogryllus oceanicus. *Behavioral Ecology*, 20(6), pp.1307–1314.

Rice, G.R. et al., 2019. Modular tissue-specific regulation of doublesex underpins sexually dimorphic development in Drosophila. *Development*, 146, p.dev178285.

Rice, W.R., 1986. Sex chromosomes and the evolution of sexual dimorphism. *Evolution*, 38(4), pp.735–742.

Rice, W.R., 1996. Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. *Nature*, 381(6579), pp.232–234.

Rice, W.R. & Chippindale, A.K., 2001. Intersexual ontogenetic conflict. *Journal of Evolutionary Biology*, 14(5), pp.685–693.

Robinson, M.D., McCarthy, D.J. & Smyth, G.K., 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, 26(1), pp.139–140.

Roff, D.A., 1989. Exaptation and the evolution of dealation in insects. *Journal of Evolutionary Biology*, 2(2), pp.109–123.

Roff, D.A., 1984. The cost of being able to fly: a study of wing polymorphism in two species of crickets. *Oecologia*, 63(1), pp.30–37.

Roff, D.A., 1994. Why is there so much genetic variation for wing dimorphism? *Researches on Population Ecology*, 36, p.145.

Roff, D.A. & Mousseau, T.A., 1987. Quantitative genetics and fitness: Lessons from drosophila. *Heredity*, 58, pp.103–118.

Rousselle, M. et al., 2019. Is adaptation limited by mutation? A timescale-dependent effect of genetic diversity on the adaptive substitution rate in animals. *bioRxiv*. Available at: https://doi.org/10.1101/643619.

Ruiz, M.F. et al., 2015. An unusual role for doublesex in sex determination in the dipteran Sciara. *Genetics*, 200(4), pp.1181–1199.

Ruther, J. & Steiner, S., 2008. Costs of female odour in males of the parasitic wasp Lariophagus distinguendus (Hymenoptera: Pteromalidae). *Naturwissenschaften*, 95(6), pp.547–552.

Sackton, T.B. et al., 2019. Convergent regulatory evolution and loss of flight in paleognathous birds. *Science*, 364, pp.74–78.

Sackton, T.B. & Clark, N., 2019. Convergent evolution in the genomics era: new insights and directions. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 374(1777), p.20190102.

Sales, K. et al., 2018. Experimental evolution with an insect model reveals that male homosexual behaviour occurs due to inaccurate mate choice. *Animal Behaviour*, 139, pp.51–59.

Sarrazin, F. & Lecomte, J., 2016. Evolution in the Anthropocene. *Science*, 351(6276), pp.922–923.

Scharf, I. & Martin, O.Y., 2013. Same-sex sexual behavior in insects and arachnids: Prevalence, causes, and consequences. *Behavioral Ecology and Sociobiology*, 67(11), pp.1719–1730.

Schneider, W.T. et al., 2018. Vestigial singing behaviour persists after the evolutionary loss of song in crickets. *Biology Letters*, 14(2), p.20170654.

Scoville, A.G. & Pfrender, M.E., 2010. Phenotypic plasticity facilitates recurrent rapid adaptation to introduced predators. *Proceedings of the National Academy of Sciences*, 107(9), pp.4260–4263.

Shaw, C.E., 1964. A snake easily ruffled but unrattled. *Zoonoz*, 37, pp.1–7.

Shuster, S.M., 1987. Alternative reproductive behaviors: three discrete male morphs in paracerceis sculpta, an intertidal isopod from the northern gulf of california. *Journal of Crustacean Biology*, 7(2), pp.318–327.

Sih, A., Bell, A. & Johnson, J.C., 2004. Behavioral syndromes: An ecological and evolutionary overview. *Trends in Ecology and Evolution*, 19(7), pp.372–378.

Sniegowski, P.D., Gerrish, P.J. & Lenski, R.E., 1997. Evolution of high mutation rates in experimental populations of E. coli. *Nature*, 387(6634), pp.703–705.

Stein, B.E., 2000. Morphology of subterranean rodents. In J. L. Patton & G. N. Cameron, eds. *Life underground: the biology of subterranean rodents*. Chicago, IL: The University of Chicago Press, pp. 19–61.

Steiner, S., Steidle, J.L.M. & Ruther, J., 2005. Female sex pheromone in immature insect males - A case of pre-emergence chemical mimicry? *Behavioral Ecology and Sociobiology*, 58(2), pp.111–120.

Stern, D.L., 2013. The genetic causes of convergent evolution. *Nature Reviews Genetics*, 14, pp.751–764.

Stockwell, C.A., Hendry, A.P. & Kinnison, M.T., 2003. Contemporary evolution meets conservation biology. *Trends in Ecology and Evolution*, 18(2), pp.94–101.

Stuglik, M.T. et al., 2014. Alternative reproductive tactics and sex-biased gene expression: The study of the bulb mite transcriptome. *Ecology and Evolution*, 4(5), pp.623–632.

Stulp, G. et al., 2012. Intralocus sexual conflict over human height. *Biology Letters*, 8(6), pp.976–978.

Szűcs, M. et al., 2017. Rapid adaptive evolution in novel environments acts as an architect of population range expansion. *Proceedings of the National Academy of Sciences*, 114(51), pp.13501–13506.

Tanaka, S., 1976. Wing polymorphism, egg production, and adult longevity in Pteronemobius taprobanensis Walker (Orthoptera: Gryllidae). *Kontyû*, 44(3), pp.327–333.

Tanner, J.C., Swanger, E. & Zuk, M., 2019. Sexual signal loss in field crickets maintained despite strong sexual selection favoring singing males. *Evolution*, 73(7), pp.1482–1489.

The Heliconius Genome Consortium et al., 2012. Butterfly genome reveals promiscuous exchange of mimicry adaptations among species. *Nature*, 487, pp.94–98.

Therkildsen, N.O. et al., 2019. Contrasting genomic shifts underlie parallel phenotypic evolution in response to fishing. *Science*, 365(6452), pp.487–490.

Theron, E. et al., 2001. The molecular basis of an avian plumage polymorphism in the wild: A melanocortin-1-receptor point mutation is perfectly associated with the melanic plumage morph of the bananaquit, Coereba flaveola. *Current Biology*, 11(8), pp.550–557.

Thornhill, R., 1979. Adaptive female-mimicking behavior in a scorpionfly. *Science*, 205(4404), pp.412–414.

Tinghitella, R.M. et al., 2011. Island hopping introduces Polynesian field crickets to novel environments, genetic bottlenecks and rapid evolution. *Journal of Evolutionary Biology*, 24(6), pp.1199–1211.

Tinghitella, R.M. et al., 2018. Purring crickets: the evolution of a novel sexual signal. *The American Naturalist*, 192(6), pp.773–782.

Tinghitella, R.M., 2008. Rapid evolutionary change in a sexual signal: genetic control of the mutation “flatwing” that renders male field crickets (Teleogryllus oceanicus) mute. *Heredity*, 100(3), pp.261–267.

Tinghitella, R.M. & Zuk, M., 2009. Asymmetric mating preferences accommodated the rapid evolutionary loss of a sexual signal. *Evolution*, 63(8), pp.2087–2098.

van’t Hof, A.E. et al., 2016. The industrial melanism mutation in British peppered moths is a transposable element. *Nature*, 534, pp.102–105.

Veltsos, P. et al., 2017. Mating system manipulation and the evolution of sex-biased gene expression in Drosophila. *Nature Communications*, 8(1).

Wagner, G.P. & Altenberg, L., 1996. Complex adaptations and the evolution of evolvability.

Evolution, 50(3), pp.967–976.

Wagner, W.E. & Reiser, M.G., 2000. The importance of calling song and courtship song in female mate choice in the variable field cricket. *Animal Behaviour*, 59(6), pp.1219–1226.

Wainberg, M. et al., 2019. Opportunities and challenges for transcriptome-wide association studies. *Nature Genetics*, 51(592–599).

Ward, L., 1923. The genetics of curly wing in drosophila. Another case of balanced lethal factors. *Genetics*, 8(3), pp.276–300.

Warner, M.R. et al., 2019. Convergent eusocial evolution is based on a shared reproductive groundplan plus lineage-specific plastic genes. *Nature Communications*, 10(1), p.2651.

Webb, K.L. & Roff, D.A., 1992. The quantitative genetics of sound production in Gryllus firmus. *Animal Behaviour*, 44(5), pp.823–832.

Welch, J.J. & Waxman, D., 2003. Modularity and the cost of complexity. *Evolution*, 57(8), pp.1723–1734.

West-Eberhard, M.J., 2003. Developmental plasticity and evolution. *Nature*, 424, p.794.

West-Eberhard, M.J., 2005. Developmental plasticity and the origin of species differences. *Proceedings of the National Academy of Sciences*, 102(2), pp.6543–6549.

Whitman, D.W., 2008. The significance of body size in the Orthoptera: a review. *Journal of Orthoptera Research*, 17(2), pp.117–134.

Wiens, J.J., 2001. Widespread loss of sexually selected traits: How the peacock lost its spots. *Trends in Ecology and Evolution*, 16(9), pp.517–523.

Wilkens, H., 1988. Evolution and genetics of epigean and cave Astyanax fasciatus (Characidae, Pisces): support for the neutral mutation theory. In M. K. Hecht & B. Wallace, eds. *Evolutionary biology*. Boston, MA: Springer.

Wilson, B.A., Pennings, P.S. & Petrov, D.A., 2017. Soft selective sweeps in evolutionary

rescue. *Genetics*, 205(4), pp.1573–1586.

Wong, B.B.M. & Candolin, U., 2015. Behavioral responses to changing environments. *Behavioral Ecology*, 26(3), pp.665–673.

Woods, R.J. et al., 2011. Second-order selection for evolvability in a large Escherichia coli population. *Science*, 331(6023), pp.1433–1436.

Wray, G.A., 2007. The evolutionary significance of cis-regulatory mutations. *Nature reviews. Genetics*, 8(3), pp.206–216.

Wright, A.E. et al., 2018. Sex-biased gene expression resolves sexual conflict through the evolution of sex-specific genetic architecture. *Evolution Letters*, 2(2), pp.52–61.

Wright, S., 1922. Coefficients of inbreeding and relationship. *The American Naturalist*, 56(645), pp.330–338.

Wright, S., 1932. The roles of mutation, inbreeding, crossbreeding and selection in evolution. In *Proc of the 6th International Congress of Genetics*.

Xie, K.T. et al., 2019. DNA fragility in the parallel evolution of pelvic reduction in stickleback fish. *Science*, 363(6422), pp.81–84.

Young, L.C. & VanderWerf, E.A., 2014. Adaptive value of same-sex pairing in Laysan albatross. *Proceedings of the Royal Society B: Biological Sciences*, 281(1775).

Young, L.C., Zaun, B.J. & VanderWerf, E.A., 2008. Successful same-sex pairing in Laysan albatross. *Biology Letters*, 4(4), pp.323–325.

Zahavi, A., 1975. Mate selection-A selection for a handicap. *Journal of Theoretical Biology*, 53(1), pp.205–214.

Zeyl, C. & Bell, G., 1997. The advantage of sex in evolving yeast populations. *Nature*, 388(6641), pp.454–468.

Zuk, M. et al., 2018. Sexual signal loss: The link between behaviour and rapid evolutionary dynamics in a field cricket. *Journal of Animal Ecology*, 87(3), pp.623–633.

Zuk, M., Rebar, D. & Scott, S.P., 2008. Courtship song is more variable than calling song in the field cricket Teleogryllus oceanicus. *Animal Behaviour*, 76(3), pp.1065–1071.

Zuk, M., Rotenberry, J.T. & Tinghitella, R.M., 2006. Silent night: adaptive disappearance of a sexual signal in a parasitized population of field crickets. *Biology letters*, 2(4), pp.521–524.

Zuk, M., Simmons, L. & Cupp, L., 1993. Calling characteristics of parasitized and unparasitized populations of the field cricket Teleogryllus oceanicus. *Behavioral Ecology and Sociobiology*, 33(5), pp.339–343.

9. Appendix 1: Field cricket genome reveals footprint of recent adaptation in the wild

The Genomic Footprint of Recent, Abrupt Adaptation in the Wild

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Evolutionary adaptation is generally thought to occur through incremental mutational steps, but large mutational leaps can occur during its early stages. These are challenging to study in nature due to the difficulty of observing new genetic variants as they arise and spread, but characterising their genomic dynamics is important for understanding factors favouring rapid adaptation. Here, we report genomic consequences of recent, adaptive song loss in a Hawaiian population of field crickets (*Teleogryllus oceanicus*). A discrete genetic variant, *flatwing*, appeared and spread approximately 15 years ago. *Flatwing* erases sound-producing veins on male wings. These silent flatwing males are protected from a lethal, eavesdropping parasitoid fly. We sequenced, assembled and annotated the cricket genome, produced a linkage map, and identified a *flatwing* quantitative trait locus (QTL) covering a large region of the X chromosome. Gene expression profiling showed that *flatwing* is associated with extensive genome-wide effects on embryonic gene expression. We found that flatwing male crickets express feminised chemical pheromones. This male feminising

effect, on a different sexual signalling modality, is genetically associated with the *flatwing* genotype. Our findings suggest that the early stages of evolutionary adaptation to extreme pressures can be accompanied by greater genomic and phenotypic disruption than previously appreciated, and highlight how suites of traits that characterise alternative sexual polymorphisms might arise through pleiotropy or genomic hitchiking.

KEY WORDS: Adaptation, Feminisation, Genomics, Rapid Evolution, Sexual Signalling, Trait Loss

Impact Summary

What are the genomic consequences of extremely rapid evolution in the wild? The adaptive evolutionary loss of male song in Hawaiian field crickets (*Teleogryllus oceanicus*) protects silent "flatwing" males from a lethal eavesdropping parasitoid fly, and invasion and spread of genetic variants causing silence was observed to occur over approximately 20 generations in a population on the island of Kauai and now appears to be fixed. To investigate the genomic and phenotypic consequences of this abrupt bout of adaptation, we first sequenced, assembled and annotated the cricket genome – the first annotated reference genome for a field cricket. To provide a genomic resource for future work in crickets and allied taxa, we created a new, open-access genome browser and database for crickets and katydids (www.chirpbase.org) and curated our data and scripts in it. Using RAD-seq, we then constructed a high-density linkage map for the species and found that the variant or variants causing flatwing are localised to a large region of the X chromosome, consistent with widespread genomic hitchiking. We performed gene expression analysis of embryonic crickets and found that flatwing is genetically associated with genome-wide regulatory disruption during development. We quantified variation in another sexual signal, chemical pheromones, and discovered that flatwing is also strongly genetically associated with male pheromone feminisation. Our findings illustrate how strong, widespread genetic and phenotypic effects can accompany the rapid emergence and spread of adaptive variants during the very earliest stages of rapid adaptation, and demonstrate how suites of traits that characterise alternative sexual polymorphisms might arise through pleiotropy or genomic hitchhiking following such genomic alteration.

Introduction

Empirical studies have struggled to characterise genomic dynamics of the earliest stages of evolutionary adaptation in natural system, because it is difficult to detect new genetic variants at the moment they first appear and then spread in wild populations. However, understanding genomic causes and consequences of new adaptive mutations can help to identify and test factors that facilitate or inhibit rapid adaptation. For example, R. A. Fisher (Fisher 1930; Orr 2005) developed a 'geometric' model that predicts adaptation should occur via mutations of small effect size, with impacts narrowly limited to a small number of phenotypic traits (Bank et al. 2014). Later refinements to models of adaptation became more permissive of larger effect mutations, particularly during the earliest stages of adaptation under extreme selection (Kimura 1983; Orr 1998). However, questions remain about the extent to which novel adaptive variants of large effect are genetically associated with changes to other traits, altered gene expression, and potential loss of homeostasis, for example through pleiotropy or genomic hitchhiking, (Nadeau et al. 2003). Here, we identified and characterised the genomic signature of very recent sexual signal loss in Hawaiian field crickets, *Teleogryllus oceanicus*, and tested the associated genetic consequences of this rapid adaptation for a different sexual signal, chemical pheromones.

Male crickets sing to attract and court females and to fight with rivals, but approximately 16 years ago, silent *T. oceanicus* males were detected in populations on the Hawaiian archipelago (Zuk et al. 2006; Zuk et al. 2018) (Fig. 1A). They spread rapidly. First observed in 2003 in a population on Kauai, where they were previously not observed, silent male crickets rapidly spread in fewer than 20 generations (with 3-4 generations per year) to near-fixation under selection imposed by a lethal parasitoid fly, *Ormia ochracea* (Fig. 1B) (Zuk et al. 2006). Female flies acoustically locate male crickets by eavesdropping on their songs, but silent flatwing males have feminised wings lacking structures used to produce sound and are thus protected (Fig. 1C). The genetic mutation(s) underlying the flatwing phenotype have been shown previously using standard genetic crosses to follow discrete segregation patterns. Sex determination is XX/XO (female/male), and flatwing's sex-linked, male limited expression indicates it is a variant, or cluster of closely linked variants, that segregate in the manner of a single-locus located on the X chromosome (Tinghitella 2008; Pascoal et al. 2014). The morph has been observed emerging in parasitized populations on other Hawaiian islands, and in at least one case appears to be caused by distinct genetic mechanisms (Tinghitella 2008; Zuk et al. 2018). The genetic loss of male song in the Kauai population is a canonical example of rapid evolution in the wild (Dugatkin 2008), and all males in this population now appear to be flatwing (Rayner et al. 2019a). Nevertheless, the continued existence of the population indicates that silent males still find mates and must compensate for their inability to sing. The selective environment promoting the rapid spread of flatwing crickets is understood, but the genomic causes and consequences of this rapid evolutionary event remain open questions. Flatwing males have distinctly

feminised wings and cannot produce sexual signals critical for reproductive fitness: how did such a spectacularly disruptive phenotypic change invade the genome of crickets so quickly?

Materials and Methods

CRICKET REARING AND MAINTENANCE

Laboratory stocks of *Teleogryllus oceanicus* were originally derived from the population in which the flatwing phenotype was first observed on Kauai (Zuk et al. 2006), and a population near Daintree, Australia (Pascoal et al. 2016b) which contains no flatwing crickets. Stocks were maintained in 16 L plastic containers containing cardboard egg cartons for shelter. All crickets were reared in a single, temperature-controlled chamber a 25 °C, on a 12:12 light:dark cycle. They were maintained regularly and fed *ad libitum* with Excel Junior and Dwarf rabbit pellets (Burgess) and provided water in a moist cotton pad that also served as oviposition substrate.

GENOME SEQUENCING

Three Illumina sequencing libraries (paired-end TruSeq libraries with insert sizes of 180, 300 and 600 bp) were prepared at Edinburgh Genomics using gDNA extracted using a DNeasy Blood & Tissue kit (Qiagen) from the head capsule and muscle tissue of a single *T. oceanicus* female sourced from the Kauai stock population. DNA was quality-checked using Nanodrop and Qubit. We supplemented reads from the above libraries with additional sequences from two TruSeq Nano Pippin selected libraries with insert sizes of 350 bp and 550 bp, one 8 kb Nextera gel-plus mate-pair library, and 1 PacBio library. For these four libraries, gDNA from a separate, single female cricket from the same laboratory population was extracted using a high molecular weight Genera Puregene Cell Kit (Qiagen). The TruSeq libraries were sequenced on 5 lanes of an Illumina HiSeq 2000 v3 to yield 100 bp paired-end reads. NanoPippin libraries and the Nextera mate-pair library were sequenced on 2 Illumina HiSeq 2500 lanes to yield 250 bp paired-end reads. The PacBio library was constructed by purifying the extraction with 1x AMPure beads (Agencourt). DNA quality was checked using Nanodrop and Qubit. Average DNA size and degradation was assessed using a high sensitivity genomic kit on a fragment analyzer. Size-selected and non-size-selected libraries were made by shearing gDNA using g-TUBEs (Covaris). Size selection was performed using the BluePippin DNA Size Selection System with 0.75% cassettes and cutoffs between 7 and 20 kb. Preparation of both libraries then proceeded using the same protocol. We treated DNA for 15 min at 37 °C with Exonuclease V11. DNA ends were repaired by incubating for 20 min at 37 °C with Pacific Biosciences damage repair mix. Samples were then incubated with end repair mix for 5 min at 25 °C followed by washing with 0.5x AMPure and 70% ethanol. DNA adapters were ligated overnight at 25 °C. Incubation at 65 °C for 10 min was used to terminate ligation reactions, then samples were treated with exonuclease for 1 hr at 37 °C. We purified the SMRTbell library using 0.5x AMPure beads and

checked quality and quantity using Qubit. Average fragment size was quantified using a fragment analyser. For sequencing, primers were annealed to the SMRTbell library at values determined using PacBio's Binding Calculator. A complex was formed using DNA polymerase (P6/C4 chemistry), bound to MagBeads, and then used to set up 43 SMRT cells for sequencing to achieve 10X coverage. Sequencing was performed using 240 min movie times.

GENOME ASSEMBLY

Raw reads from all Illumina libraries were trimmed using cutadapt v1.8.3 (Martin 2011) to remove adapters, primers and poor quality bases, and then error-corrected using BLESS v1p02 (Heo et al. 2014). PacBio reads <1,000 bp were discarded. The original fragment length of the 350 bp library was shorter than the sequenced paired read length of 500bp, so reads from this library were merged using Vsearch v1.10.1 (Rognes et al. 2016). Platanus v1.2.4 (Kajitani et al. 2014) was used to assemble error-corrected reads from all Illumina libraries except the mate-pair library; reads from the latter were added at the scaffolding stage. Next, we selected the contigs >1,000 bp and combined these with the PacBio data to generate a hybrid assembly using PBJelly v15.2.20 (English et al. 2012). Pilon v2.1 (Walker et al. 2014) was used to improve local base accuracy, and BUSCO v2.1 (Simao et al. 2015) was used to assess genome quality through gene completeness.

REPEAT ANNOTATION

We used *de novo* and homology-based approaches to identify repetitive regions. We first built a *de novo* repeat library using RepeatModeler v1.0.10 (Tarailo-Graovac and Chen 2009), with dependencies RECON v1.08 and RepeatScout v1.0.5 (Price et al. 2005). To scan and classify interspersed repeats and low complexity DNA sequences at the DNA level, we searched the cricket genome sequence against the Dfam consensus database (20170127) (Hubley et al. 2016), RepBase (20170127) (Bao et al. 2015), and the *de novo* repeat library using RMBlast v2.6.0+ (Boratyn et al. 2012) and RepeatMasker v4.0.7 (Smit et al. 2013-2015). Protein-level repeats were identified by searching against the TE Protein Database using RepeatProteinMask v4.0.7 (Smit et al. 2013-2015). Unclassified repetitive elements were further classified by TEclass v2.1.3 (Abrusan et al. 2009), a programme using a support vector machine learning algorithm. Tandem repeats were also identified in the cricket genome using Tandem Repeat Finder v4.09 (Benson 1999).

GENE PREDICTION

Before running gene prediction pipelines, repetitive regions identified above were masked using an in-house Perl script. We built a pipeline including *ab initio*, homology and transcriptome-based methods to predict protein-coding genes in the cricket genome (Fig. S1). For *ab initio* prediction, SNAP 2013-11-29 (Korf 2004), Glimmer-HMM v3.0.4 (Majoros et al. 2004), GENEID v1.3 (Blanco

et al. 2007), and BRAKER v2.0.4 (Hoff et al. 2016) were used to generate preliminary gene sets from the repeat-masked genome. Specifically, reads obtained from the *T. oceanicus* transcriptome were aligned against the repeat masked genome with TopHat2 v2.0.10 (Kim et al. 2013). SAMTOOLS v0.1.19 (Li et al. 2009) was used to sort and index the resulting Binary Alignment Map (BAM) format file. This BAM file was processed in BRAKER v2.0.4 (Hoff et al. 2016), which used transcriptome data to train GENEMARK-ET v4.33 (Lomsadze et al. 2014), generate initial gene structures, and then subsequently train AUGUSTUS v3.2.2 (Stanke et al. 2008) and finally integrate RNA-seq information into final gene predictions. For other *ab initio* gene prediction programmes, gene sets from *Locusta migratoria* (Wang et al. 2014), *Acyrthosipon pisum* (International Aphid Genomics Consortium 2010), and *Drosophila melanogaster* (Gramates et al. 2017) were used for model training. For homology-based prediction, we aligned protein sequences of five insect species (*L. migratoria* (Wang et al. 2014)*, Drosophila melanogaster, Anoplophora glabripennis* (McKenna et al. 2016)*, Nilaparvata lugens* (Xue et al. 2014)*,* and *Cimex lectularius* (Benoit et al. 2016)) to the repeat-masked cricket genome using TBLASTN v2.2.26 ($E < 10^{-5}$) (Boratyn et al. 2012). The boundaries of potential genes were further identified using BLAST2GENE v17 (Suyama et al. 2004). We then ran GENEWISE2 2-4-1 (Birney et al. 2004) to obtain accurate spliced alignments and generate a final, homology-based gene set. For prediction based on transcriptome data, a de novo *T. oceanicus* transcriptome assembly generated for a separate study (Rayner et al. 2019b) using Trinity v2.2.0 (Grabherr et al. 2011) was filtered based on gene expression level, and then passed to Program to Assemble Spliced Alignments (PASA v2.2.0) (Xu et al. 2006). PASA performed transcript alignments to the cricket genome, generated a new transcript assembly, and predicted gene structures. All *ab initio*, homology, and transcriptome-based gene sets were then combined into a weighted consensus set using EVidenceModeler (EVM r2012-06-25) (Haas et al. 2008). We removed genes likely to be spurious, those with low EVM support, partial genes with coding lengths shorter than 150 bp, and genes only supported by a minority (≤ 2) of *ab initio* methods (Yang et al. 2017). PASA was used to further update the filtered consensus gene set to produce a finalised official gene set. The completeness of this final gene set was assessed by both BUSCO v2.1 (using the arthropoda dataset) (Simao et al. 2015) and transcriptome data.

FUNCTIONAL ASSIGNMENT

Putative gene functions were assigned using InterPro (InterProScan 5) (Finn et al. 2017), SwissProt (Feb.2018) (Bairoch and Apweiler 2000), TrEMBL (Feb.2018) (Bairoch and Apweiler 2000) and RefSeq non-redundant (NR) protein (106,376,657 sequences) and Kyoto Encyclopedia of Genes and Genomes (KEGG) gene (family_eukaryotes) databases. Briefly, we first obtained protein sequences from the final gene set using EVM r2012-06-25 (Haas et al. 2008). Functional annotation and gene ontology (GO) terms were assigned to genes based on protein sequence, using InterProScan 5 (Jones

et al. 2014). These proteins were also blasted against SwissProt, TrEMBL and NR databases (BLASTP, $E < 10^{-5}$), and assigned their best hits as functional annotations. GO terms were assigned using GO annotations downloaded (26.03.2018) from the GO Consortium (Adams et al. 2000; The Gene Ontology Consortium 2017). BLAST2GO (unix_4_1_x54) (Gotz et al. 2008) was implemented to further assign unassigned genes using NCBI databases, and KEGG Orthology (KO) terms were assigned using BlastKOALA v2.1 (Kanehisa et al. 2016b).

GENOME ANCHORING

ALLMAPS v0.7.7 (Tang et al. 2015) was used to detect chimeric scaffolds, anchor the cricket genome to the linkage map (see below), and construct pseudo-molecules (reconstructed portions of chromosomal sequence). We first built a consensus genetic map based on male and female genetic distances obtained from linkage maps, in which equal weighting was applied for both sexes. Then, scaffolds for which more than four markers mapped to multiple linkage groups were designated as chimeric scaffolds, and split. After this correction was applied, scaffolds anchored to the linkage maps were oriented and ordered based on the consensus genetic map. We used a custom Perl script to order unanchored scaffolds according to their length, and liftOver (Mar.2018) (Kent et al. 2002) to convert genome coordinates based on anchoring results.

CHIRPBASE – A GENOME BROWSER AND DATABASE

We created ChirpBase, an open-access community genomics resource for singing insects such as field crickets and katydids. It can be accessed at www.chirpbase.org where users may view and download genomic data and scripts presented in this study in addition to uploading data. An index page links to an ensembl page, where assembly statistics can be visualised using a Challis plot or compared in tabular format. A plot illustrating codon usage is presented, and BUSCO scores can be visualised. Additional linking pages include a basic local alignment search tool (BLAST) page and a download page for accessing raw data. We used the GenomeHubs framework (Challis et al. 2017) to set up ChirpBase. The databased is hosted using a Linux container (LXC) on a remote computer, linked to a cluster via an intermediate import computer. A MySQL docker container was started in the LXC, where a database *ini* file resided to guide additions to the database. An Ensembl-easy mirror Docker container was run to import the database into the MySQL container, uploading data designated in the *ini* file from the LXC to the database. The MySQL container links to an Ensembl EasyMirror container, BLAST container, and a download container.

LINKAGE AND QTL MAPPING CROSSES

We constructed a linkage map for *T. oceanicus* crosses designed to maximise recombination on the X chromosome by retaining only families where *flatwing*-carrying and *normal-wing*-carrying X

chromosomes were present together in dams, as the X is only diploid in females (Fig. S2), combined with restriction-site associated DNA sequencing (RAD-seq) to identify markers. *Flatwing* segregates on the X chromosome (Tinghitella 2008; Pascoal et al. 2014), so mapping was performed with F_3 offspring to increase recombination on the X. We set up two parental mapping families by crossing a flatwing sire from the Kauai stock line with a virgin dam from the Daintree, Australia stock line. Daintree females were used to maximise our opportunity to genetically map segregating variation in other phenotypes. Female F_1 offspring from parental crosses were heterozygous for *flatwing*, enabling recombination on the X. Full-sib matings were then performed with F_1 males, all of which were normal-wing. The resulting F_2 female offspring were a mix of homozygous *normal-wing* genotypes on the X, or heterozygous with respect to wing morph. Recombination between *flatwing* and *normal-wing* genotypes was similarly possible in the heterozygous F_2 females, but their phenotype is not externally detectable. We then mated F_2 females with full-sib *flatwing* males from the same generation. Screening male morph types in the resulting F_3 offspring enabled us to identify F_2 crosses involving heterozygous females, as all male offspring of homozygous *normal-wing* females expressed normal-wing morphology. The crossing procedure resulted in 10 F_3 mapping families from the original two parental families, from which a total of 192 females, 113 normal-wing males, and 86 flatwing males were used for RAD-seq analysis (below).

MARKER IDENTIFICATION USING RAD-SEQ

RAD-seq was used to identify single nucleotide polymorphisms (SNPs) in F_3 offspring (n = 391), P_0 dams and sires (n = 4), and the F_2 sires and dams (n = 19) that were used to produce mapping individuals in the F_3 generation. For each individual, gDNA extraction and quality control was performed as described above prior to library preparation. gDNA was digested using SbfI (New England BioLabs). We barcoded individuals by ligating P1 adapters (8 nM), then sheared and size selected 300-700 bp fragments. After ligating P2 adapters to sheared ends, parents were sequenced to an average coverage of 120x and offspring to 30x on an Illumina HiSeq 2000.

LINKAGE MAP CONSTRUCTION

Reads from all paired end RAD libraries were demultiplexed by sample using process_radtags from Stacks v1.46 (Catchen et al. 2013), mapped against the reference genome assembly using BWA-MEM v0.7.15-r1140 (Li and Durbin 2009) and duplicates marked using PicardTools MarkDuplicates v2.9.2 (http://broadinstitute.github.io/picard). Variants were called using samtools mpileup (v1.3, parameters -d 2000 -t DP,DPR,DV,DP4,SP -Aef -gu) and bcftools call (version 1.3, parameters -vmO z -f GQ). The resulting variants were filtered using vcfutils.pl (included with bcftools) with minimum quality 50 and a minimum read depth of 150 (-Q 50 -d 150) to only retain high quality variants. The vcf format was converted to the required Lep-MAP2 input format using a custom script of the

RADmapper pipeline (RAD_vcf_to_lepmap_with_sexmarker_conversion.py, https://github.com/EdinburghGenomics/RADmapper). During this conversion samples that did not fit initial relatedness expectations (n = 8, using vcftools relatedness2 and visual inspection of a heatmap) and samples from family I (which lacked a genotyped father, n = 59) and P0 parents (n = 4) were excluded from linkage map creation. Putative X-linked markers (male_het <=1, female_het > 20, het_sire <=1) were converted to biallelic markers in the relevant male offspring and sires using a dummy allele (Table S1). The linkage map was then created using the following steps and parameters in Lep-MAP2 v0.2 (Rastas et al. 2015) (Filtering: dataTolerance 0.05 keepAlleles=1; SeparateChromosomes: losLimit=10 sizeLimit=10 informativeMask=3;JoinSingles: lodLimit=5;OrderMarkers: filterWindow=10 polishWindow=100; OrderMarkers evaluateOrder: filterWindow=10 polishWindow=100). The resulting linkage map files were merged with the marker and sample information using a custom script from the RADmapper pipeline (LG_to_marker.py).

QTL MAPPING

To identify the flatwing locus on the putative X chromosome (LG1), we performed ANOVA for each marker using the lm package in R (v3.1) and 178 male samples (105 normal-wing + 73 flatwing; as above excluding all grandparental, parental and female samples together with samples that clustered with the wrong family, had insufficient coverage to calculate relatedness or did not have cuticular hydrocarbon (CHC) data, see below). Individual p-values were corrected to account for multiple testing using Bonferroni correction and markers supported by a log-of-odds (LOD)10 cutoff were plotted. QTL for all 26 CHC peaks as well as the principle components from the CHC analysis were mapped to the linkage groups using mixed linear models in ASReml v4. Mapping used a GWAS-type approach, taking into account genetic relatedness between individuals (Calus 2010). The markers mapped to the autosomal linkage groups 2-19 were filtered to contain only bi-allelic SNP markers with a MAF <=0.01 and <5% missing samples per marker. Only male samples were selected (the same n = 178 as for mapping *flatwing* above), as our aim was to map male CHCs (not sex-related associations) on the putative X (LG1) and autosomes using principle components from the CHC analysis as well as individual compounds as traits. The remaining 21,047 markers were used to calculate pairwise genetic relatedness with the first normalisation (VanRaden 2008). The resulting inverse relatedness matrix was used as random effect in a model: CHC trait ~ mu marker r! Giv(animal). P-values for all markers were extracted from the results and corrected for multiple testing using Bonferroni correction. The same model was used to assess LG1 separately with the same set of samples, for which 6,537 markers were used after filtering.

PURE-BREEDING LINES AND EMBRYO SAMPLING FOR RNA-SEQ

Kauai lines homozygous for the *flatwing* and *normal-wing* genotypes were used for examining

differential gene expression. Their establishment has been described previously (Pascoal et al. 2016a). Briefly, one generation of crosses was performed, starting with the laboratory population derived from Kauai and crossing males of either wing phenotype to virgin females of unknown genotype. Because the phenotypic effects of *flatwing* are sex-limited, family-level screening of the resulting male offspring was performed to select homozygous *flatwing* and homozygous *normal-wing* lines, resulting in a final selection of three pure-breeding lines for each morph genotype. Developing embryos were sampled from eggs laid by females from each line. Females were maintained in laboratory culture as above, and their oviposition substrates were monitored. Eggs were removed from the substrate and immediately preserved in 500 µL of RNAlater (Qiagen) at the stage when eye pigmentation first develops, ca. 2 weeks after laying. This time point corresponds approximately to embryonic stage 13-14 in the related grylline species *Gryllus bimaculatus* (Donoughe and Extavour 2016). After removing the outer egg chorion, the thoracic segment of each nymph was microdissected. Nymphs cannot be sexed based on external morphology until a later stage of juvenile development, and as chromosomal sex determination is XX/XO, screening for sex-specific markers is not possible. To minimise potential variation in sex ratio of samples between lines, and ensure a sufficient volume of tissue to extract RNA, thoracic tissue from n = 8 nymphs was pooled for each replicate, and 6 biological replicates were produced for each morph type (2 per line).

RNA-SEQ AND GENE EXPRESSION PROFILING

Total RNA was extracted using the TRIzol plus RNA purification kit (Life Technologies) and DNAse treated using PureLink (Invitrogen). RNA was quantified and quality checked using Qubit assessment (Invitrogen) and Bioanalyser RNA Nano Chips (Agilent), respectively. To isolate mRNA we depleted samples with RiboZero. After verifying depletion, cDNA libraries were constructed using the ScriptSeq protocol (Epicentre) with AMPure XP beads for purification. Following barcoding and multiplexing, final quality was checked and qPCR performed using Illumina's Library Quantification Kit (Kapa). Sequencing was performed on an Illumina HiSeq 2000 v3, with 1% PhiX DNA spike-in controls to produce 100 base paired-end reads. CASAVA v1.8.2 was used to demultiplex reads and produce raw fastq files, which were then processed with Cutadapt v1.2.1 (Martin 2011) and Sickle v1.200 (Joshi and Fass 2011) to remove adaptor sequences and trim low-quality bases. Further quality assessment was performed in FastQC. Expression analysis of RNA-seq data was performed broadly following the protocol published by Pertea et al. (2016) (Pertea et al. 2016). Reads were aligned to the genome using HISAT2 v2.1.0 with strand-specific settings, and transcripts compiled for each sample in StringTie v1.3.4, using the gene annotation file as a reference, which were then merged across all samples to produce a single annotated reference transcriptome. Sample transcript abundances were estimated with the parameter -e specified to restrict abundance estimation to annotated transcripts. Differential expression analysis was performed at the gene level following normalisation of counts by

trimmed mean of M-values (TMM), using a generalised linear model (GLM) with negative binomial distribution and a single predictor variable of 'morph' in the edgeR v3.20.9 package (Robinson et al. 2010) in R v3.4.1. Only genes with an expression level greater than 1 count per million in at least 3 samples were included in the analysis. Significance-testing was performed using likelihood ratio tests, and genes were considered significantly differentially expressed between morph genotypes if false-discovery rate (FDR)-adjusted P-values were below a threshold of 0.05.

SCREENING FOR TOP CANDIDATE GENES ASSOCIATED WITH FLATWING

We adjusted *P*-values for significant marker associations in the flatwing QTL mapping procedure using Bonferroni correction with a cut-off of $P < 0.001$. Three sources of information were used to comprehensively and robustly detect a set of top candidate genes associated with the flatwing phenotype. We detected genes (i.e. any overlapping portion of a predicted gene sequence) located in 1 kb flanking regions of all significant QTL markers after FDR correction as above, and defined these as QTL-associated candidates. We then subsetted these genes to retain only those located in the 1 kb flanking regions of the most significant (top 1%) of all QTL markers, and defined these as Top 1%-associated candidates. We also obtained the flatwing-associated sequences from a previously published bulk segregant analysis (BSA) of Kauai flatwings (Pascoal et al. 2014), and defined the BSA reference sequences containing flatwing-associated SNPs as flatwing-associated BSA sequences. We mapped these BSA sequences to the *T. oceanicus* reference genome using BWA-MEM with default parameters (Li and Durbin 2009). Coordinates of mapped sequences were extracted from the resulting BAM files using SAMTOOLS (Li et al. 2009) and custom Perl scripts, and we only retained those sequences that were anchored to LG1. Genes within 1 kb of these retained sequences were defined as BSA-associated candidates. Finally, we extracted differentially expressed genes from the embryonic thoracic transcriptome analysis above, and defined these as DEG-associated candidates. To ensure a reliable final candidate gene set for flatwing, we only retained genes supported by at least two of these four gene sets. We used KEGG pathway mapping (colour pathway) to reconstruct pathways and obtain reference pathway IDs (Kanehisa et al. 2016a). To characterise significantly enriched GO terms and KEGG pathways in DEGs, we implemented the hypergeometric test in enrichment analyses. *P* values for each GO and KEGG map term were calculated and FDR-adjusted in R.

CUTICULAR HYDROCARBON EXTRACTION AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY

We extracted CHCs from 394 individuals from the F_3 mapping generation prior to extracting gDNA for RAD-seq. Extraction and analysis of CHCs followed previous methodology (Pascoal et al. 2016b), which is briefly described here. Subjects were flash-frozen for several minutes at -20 °C and then

thawed. They were individually placed into 4 mL borosilicate glass vials (QMX Laboratories) and immersed for 5 minutes in 4 mL of HPLC-grade hexane (Fisher Scientific), then removed from the vials and stored for later processing. We evaporated a 100 µL aliquot of each sample overnight in a 300 µL autosampler vial (Fisher Scientific). CHC extracts were transported to the University of Exeter for gas chromatography mass spectrometry (GC/MS) using an Agilent 7890 GC linked to an Agilent 5975B MS. Extracts were reconstituted in 100 µL of hexane with a 10 ppm pentadecane internal standard, and 2 µL of this was injected into the GC/MS using a CTC PAL autosampler at 5 °C. The carrier gas was helium and we used DB-WAX columns with a 30 m x 0.25 mm internal diameter and 0.25 µm film. Injection was performed in split-less mode. The column profile was optimised for separation of the CHC extract (Pascoal et al. 2016b) to start at 50 °C for 1 minute, followed by a temperature ramp of 20 °C per minute until finally holding at 250 °C for a total run time of 90 minutes. The inlet temperature was 250 °C and the MS transfer line was 230 °C. We recorded electron-impact mass spectra using a 70 eV ionization voltage at 230 °C, and a C_7-C_{40} alkane standard was run as a standard to enable the later calculation of peak retention indices.

QUANTIFICATION AND ANALYSIS OF CHC PROFILES

For each individual, we used MSD CHEMSTATION software (vE.02.00.493) to integrate the area under each of 26 CHC peaks (Table S2) following (Pascoal et al. 2016b). Peak abundances were standardized using the internal pentadecane standard and $\log_{10}$ transformed prior to analysis. After accounting for samples that failed during extraction or during the GC run (n = 10), labelling error (n = 1), and one normal-wing male CHC profile that was identified as an outlier and removed during analysis (Fig. S3), we analyzed a total of n = 86 flatwing males, n = 112 normal-wing males, and n = 185 females of unknown genotype. To test whether CHC profiles differed between males of either wing morph, we first performed dimension reduction using principal components analysis (PCA) on male data only. JMP Trial v14.1.0 (SAS Institute Inc.) was used to draw a 3D scatterplot of the first three PCs. To assess statistical significance, we performed a MANOVA using all principal components with eigenvalue > 1.00 (n = 6). This indicated a highly significant difference among male morphs which formed the basis of QTL mapping described above. To visualise the difference between flatwing and normal-wing male CHC profiles with respect to female CHC profiles, we performed a discriminant function analysis (DFA) for all samples and all 26 peaks. DFA highlights the maximal difference between pre-defined groups, with maximum group differences indicated by the first DF axis. Statistical analyses of CHC data were done in SPSS (v23).

Results and Discussion

SEQUENCING THE CRICKET GENOME AND MAPPING FLATWING

We studied the genomic signature of song loss in the Kauai population where flatwing crickets were

first discovered, and in which rapid spread has been most thoroughly documented (Zuk et al. 2006). Using females from laboratory stock, we sequenced the *T. oceanicus* genome and generated an assembly of 2.045 Gb consistent with flow cytometry size estimates (Tinghitella et al. 2018), with a scaffold N50 of 62.6 kb (Table S3). We established an F_3 mapping population using crosses designed to maximise recombination on the X chromosome (Fig. S2). Mapping offspring and parents were sequenced using RADseq, and a map was assembled containing 19 linkage groups. We identified linkage group 1 (LG1) as the X chromosome by applying coverage and heterozygosity filters and dummy coding putative X-markers prior to constructing the map. LG1 was the largest linkage group, with a female recombination length of 379 cM and a male length of 195 cM (Fig. S4). After resolving chimeric scaffolds (Table S4), 35.6% of the genome was anchored to a linkage map using a LOD5 cutoff (Fig. 2A and Table S5). *T. oceanicus* has a haploid chromosome number of (13+X), so the additional 5 linkage groups likely correspond to unjoined chromosomal segments.

We performed gene prediction and annotation using custom pipelines incorporating *ab initio*, homology, and transcriptome-based approaches (Fig. S1). Evidence from different gene prediction and annotation methods was weighted and filtered to predict a final, conservative set of 19,157 genes, 75% of which had functional annotation (Table S6 and Fig. S5). Gene density was assessed (Fig. 2A track i), and we tested whether the putative X linkage group showed a different distribution of repeat content relative to the other linkage groups, across eight common categories of repeats. It did not (Fig. 2A track iii, Table S7, Fig. S6). *T. oceanicus* gene features were compared to 10 other insect species (Table S8), and we contrasted transposable element classifications with three other recently published insect genomes (Table S9). The *T. oceanicus* genome and metadata associated with it are curated in ChirpBase (www.chirpbase.org), a GenomeHubs Ensembl genome browser (Challis et al. 2017) that we created as an openly available, community-based genomics resource for researchers working on singing insects.

Flatwing was definitively mapped to the putative X chromosome using markers supported by a LOD10 cutoff and a mixed model, ANOVA-based approach designed to control for uneven genomic relatedness caused by family structure in the mapping crosses (Fig. 2B; no other linkage group had markers showing associations exceeding the genome-wide significance threshold of $P < 0.001$). To cope with the particularly high marker association on the putative X chromosome caused by the discrete mode of inheritance of *flatwing* and the different effective population size of the X compared to autosomes, we identified the QTL using only the top 1% of markers after FDR correction, yielding a prominent peak occupying approximately one third of the X chromosome (Fig. 2C).

REGULATORY CONSEQUENCES ASSOCIATED WITH *FLATWING*

Flatwing morphology is observable in males during mid- to late-instar stages of juvenile development,

so we examined early embryonic gene expression differences associated with *flatwing*. Females carrying the genotype cannot be visually distinguished and embryos cannot be readily sexed, so we used replicate laboratory lines homozygous for *flatwing* or *normal-wing* genotypes to detect widespread differential gene expression in the developing thoraces of embryonic crickets. We found 830 genes differentially expressed (DE), 204 of which had a $\log_2$ fold-change > 1, and a predominant pattern of down-regulation in *flatwing* crickets (Table S10 and Fig. S7). DE genes associated with *flatwing* were widely distributed across linkage groups and unmapped scaffolds (Fig. 2A track iv).

These physically dispersed expression effects are consistent with a scenario in which *flatwing* acts as a master regulatory switch during early development, with a broad cascade of downstream effects. Pathways reconstructed using differential expression data are consistent with a master regulatory switch. For example adherens junction activity was enriched, which affects epithelial patterning during early development (Tables S11 and S12). Using a stringent and redundant approach combining information from gene sets identified in the QTL study, RNA-seq experiment and a previously-published bulked segregant analysis (Pascoal et al. 2014), we identified 51 annotated protein-coding genes located within LG1 as top flatwing-associated candidates (Table S13). Gene ontology (GO) enrichment analysis indicated that *positive regulation of developmental process* was overrepresented in this candidate gene set, with three genes in particular (*NBL1, GOGA4, UNC89*) known to play a fundamental role in the regulation of cell differentiation (Table S14). However, it is plausible that loci hitchhiking with the causal genetic variant(s) underlying the flatwing phenotype also have regulatory effects. Such joint effects could compound gene regulatory consequences of novel adaptive variants.

CANDIDATE GENE DISCOVERY

In most pterygote insects, wings are derived from imaginal discs formed during development by the invagination of embryonic ectoderm (Snodgrass 1993). Previous work mainly in *Drosophila melanogaster* has established that the developmental elaboration of wing venation patterns requires the involvement of numerous transcription factors and complex coordination across numerous signalling pathways (De Celis 2003). Here, we found that 7 of 51 flatwing associated candidate genes have reported involvement in wing development in *D. melanogaster*. For example, *Collier* encodes a transcription factor required for wing disc patterning (Vervoort et al. 1999), and *Myoglianin* expression is required for normal wing disc development (Hevia and de Celis 2013). *ROR1* encodes a transmembrane tyrosine-protein kinase receptor involved in phosphorylating MAP kinases (Bicocca et al. 2012), and reduction of MAPK activity through *ROR1* silencing can lead to a loss of wing venation phenotype (De Celis 2003). The protein krasavietz is encoded by *PKRA*, and establishes planar cell polarity in the wing (Carvajal-Gonzalez et al. 2016), disruption of which can lead to wing distortion (Adler 2012). Knockouts and mutants in *Pelle*, *Gcn5*, and *Plexin-A4* show wing shape and

venation alterations with features similar to flatwing (Carre et al. 2005; Wu et al. 2015; Okada et al. 2016).

GENETICALLY ASSOCIATED FEMINISATION OF MALE PHEROMONES

We tested the consequences of the rapid invasion of *flatwing* into the *T. oceanicus* genome for other relevant phenotypes by focusing on a distinct, close-range sexual signalling modality that operates alongside acoustic signalling in field crickets. Cuticular hydrocarbons (CHCs) are long-chain, waxy molecules expressed on insect cuticles. CHCs are thought to have evolved for dessication resistance, and they tend to be expressed as a bouquet of numerous individual hydrocarbon compounds. *T. oceanicus* CHCs are sexually dimorphic and function as sexual signals during male and female mate choice (Tregenza and Wedell 1997; Thomas and Simmons 2009, 2010), and they have been found to vary between flatwing and normal-wing male crickets (Simmons et al. 2014). We characterised the CHC profiles of F_3 mapping individuals, all of which were raised in a common garden environment, by extracting their CHCs and using gas chromatography – mass spectrometry (GCMS) to measure the abundance of 26 individual compounds (Fig. 3A) (Table S2). By performing dimension reduction using principal components (PC) analysis of the CHC profiles, we first established that, in our mapping population, males carrying *flatwing* showed noticeably different CHC profiles from *normal-wing* males (Fig. 3B) (multivariate analysis of variance on 6 principal components with eigenvalues > 1 describing male CHC blends: $F_{6,191} = 29.769$, $p < 0.001$) (Table S15).

QTL analysis was performed on the first six CHC PCs using the same set of male mapping individuals, to determine whether *flatwing*-associated variation in male CHC profiles mapped to identifiable genomic regions. The putative X chromosome, LG1, was of particular interest, because we hypothesized that the striking variation between CHC profiles of flatwing and normal-wing males could be due to pleiotropy or hitchhiking associated with *flatwing*. Genetic mapping of CHCs was performed blind to male morphotype. PC1, which explained over a third of the variance in male CHC profiles, mapped to a ca. 2.5 cM region strongly co-localised with *flatwing* (Fig. 3C). PCs 4 and 6 also had co-localizing peaks (Fig. S8). As dimension reduction for CHCs can obscure phenotypic patterns in the original individual chemical compounds, we mapped each of the 26 compounds separately. Of these, 9 showed significant peaks co-localising with *flatwing* (Fig. 3D). We recovered no autosomal QTL peaks for PCs 1-6, and only one QTL peak for one compound on one autosome (compound 11, 7-C31ene, on LG8). However, the latter peak was weakly supported, with only a single marker showing an association at FDR-corrected $p < 0.001$.

We interrogated genes on scaffolds under the CHC QTL peaks following a similar procedure used to produce the *flatwing* candidate gene set (Table S16). Of 55 protein-coding genes, a subset of 6 were implicated for every CHC trait with a significant QTL peak, and these 6 genes were also present in the *flatwing* candidate gene set. These are strong candidates for testing for any pleiotropic or linked

effects of evolved acoustic sexual signal loss on chemical sexual signals. Our final step was to explore the nature of the phenotypic shift in flatwing male CHC profiles. It is unknown how flatwing males' profiles compare to those of females (Simmons et al. 2014), but given the generally feminising effect of *flatwing* on male wing morphology, we predicted that flatwing males' CHC profiles would also be feminised. We compared them to the profiles of normal-wing males and females using discriminant function analysis on profiles from all three groups. Discriminant function 1 (eigenvalue = 2.526) explained 78.8 % of the variance, and indicated that flatwing male crickets' CHC profiles are strongly feminised (Fig. 3E). Their CHCs appear to be correspondingly less attractive to females (Gray et al. 2014).

CONCLUSIONS

Factors constraining rapid adaptation will be increasingly important to evaluate as natural populations are placed under pressure from climate change, anthropogenic disturbances, and the application of biological control agents (Tomasetto et al. 2017). The rapid emergence and spread of flatwing crickets on Kauai is a textbook example of rapid adaptation in the wild (Dugatkin 2008). Previous work on this population of crickets has found differences in the level of phenotypic plasticity, gene expression, and other reproductive characteristics such as male testis size between male *normal-wing* and *flatwing* genotypes (Bailey et al. 2010; Pascoal et al. 2016a; Pascoal et al. 2018), and our present findings reveal the genomic footprint of strong, associated effects on sexual signalling in an entirely different sensory channel. These consequences of rapid adaptive trait loss are early-acting, genome-wide, and impact a range of important fitness traits. The suite of characters affected in flatwing crickets is reminiscent of feminised alternative male morphs in ruff (*Calidris pugnax*) in which a supergene controls size, ornament and behavioural traits simultaneously (Kupper et al. 2016), and in feminised bulb mites (Joag et al. 2016). What is surprising is that an evolved loss of function could lead to such similarly wide-ranging phenotypic impacts so quickly, and yet still be adaptive. Examples of rapid adaptive evolution are well-known, from industrial melanism in Kettlewell's peppered moths (*Biston betuliaria*) (van't Hof et al. 2011), to insecticide resistance in mosquitoes (Ranson et al. 2002), but in general, adaptation has been thought to be mutation-limited with negative pleiotropic consequences ascribed a prominent impeding role (Barrett and Schluter 2008). Strikingly, at least three independent male song-loss variants in the Hawaiian cricket system have been recently described: a less-feminised version of flatwing on the island of Oahu (Pascoal et al. 2014), plus "curly-wing" and "short-wing" crickets on Oahu and the Big Island, respectively (Rayner et al. 2019a). All of these adaptations involve morphological disruption to forewings, and their proliferation under fly selection hints that episodes of rapid adaptive evolution might be more likely when adaptation can proceed via secondary trait loss rather than gain. Future work would benefit from investigating whether the indirect genomic consequences of adaptive trait-loss mutations are less detrimental than those of mutations underlying

trait gain. The genomic signature of recent, abrupt song loss in Hawaiian crickets uniquely illustrates how genetic variants exerting large effects and accompanying widespread, associated consequences on gene expression and other phenotypes can invade genomes in the wild. Our results raise the possibility that disruptive genomic consequences of new genetic variants might place fewer constraints on rapid adaptation than previously appreciated.

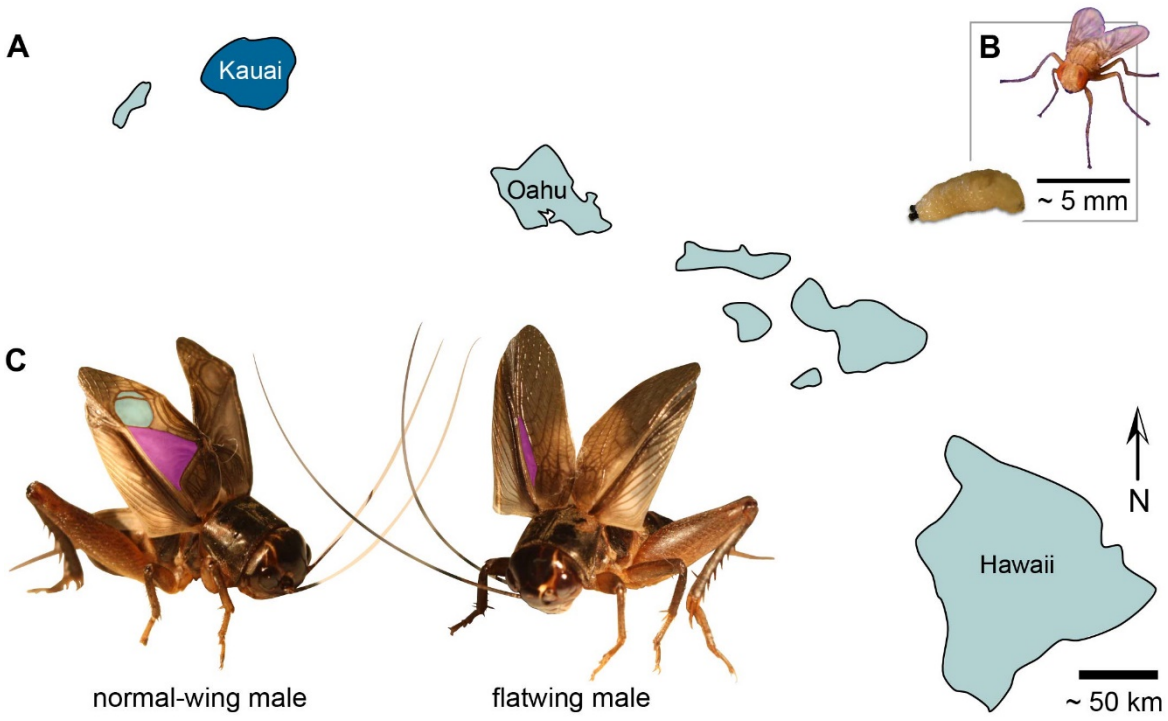

Figure 1. Evolutionary loss of song in Hawaiian crickets. (A) The field cricket *T. oceanicus* is thought to have migrated to the Hawaiian archipelago from other islands in Oceania, and is attacked by the fatal, acoustically-orienting parasitoid fly *Ormia ochracea* on Kauai, Oahu and Hawaii. We studied crickets from a population in Kauai, highlighted in dark blue, where parasitoid infestation rates have historically been highest. (B) Adult female fly and mature parasitoid larva. Gravid female flies locate hosts by eavesdropping on singing male crickets, then they eject larvae that burrow into the host and consume its viscera before emerging to pupate. Infestation is fatal, and the flies exert significant natural selection against male song. (C) Normal-wing males (left) of this field cricket species produce advertisement, courtship and aggressive songs by elevating and rubbing together forewings that bear specialised sound-producing venation. A toothed file on the right wing engages with a thickened ridge of tissue on the opposite, causing resonators to vibrate and produce sound. Two principal resonators are highlighted on this male's right forewing: the harp in purple and the mirror in turquoise. Flatwing males (right) have wings that are feminised and lack, or have severely reduced, resonators. They still make wing motions characteristic of singing despite the structural inability to produce sound (Schneider et al. 2018), but their silence protects them from the fly (Zuk et al. 2006). Currently, 100% of males from the population studied on Kauai exhibit flatwing morphology. (Photo credits: N.W. Bailey (cricket antennae drawn by hand))

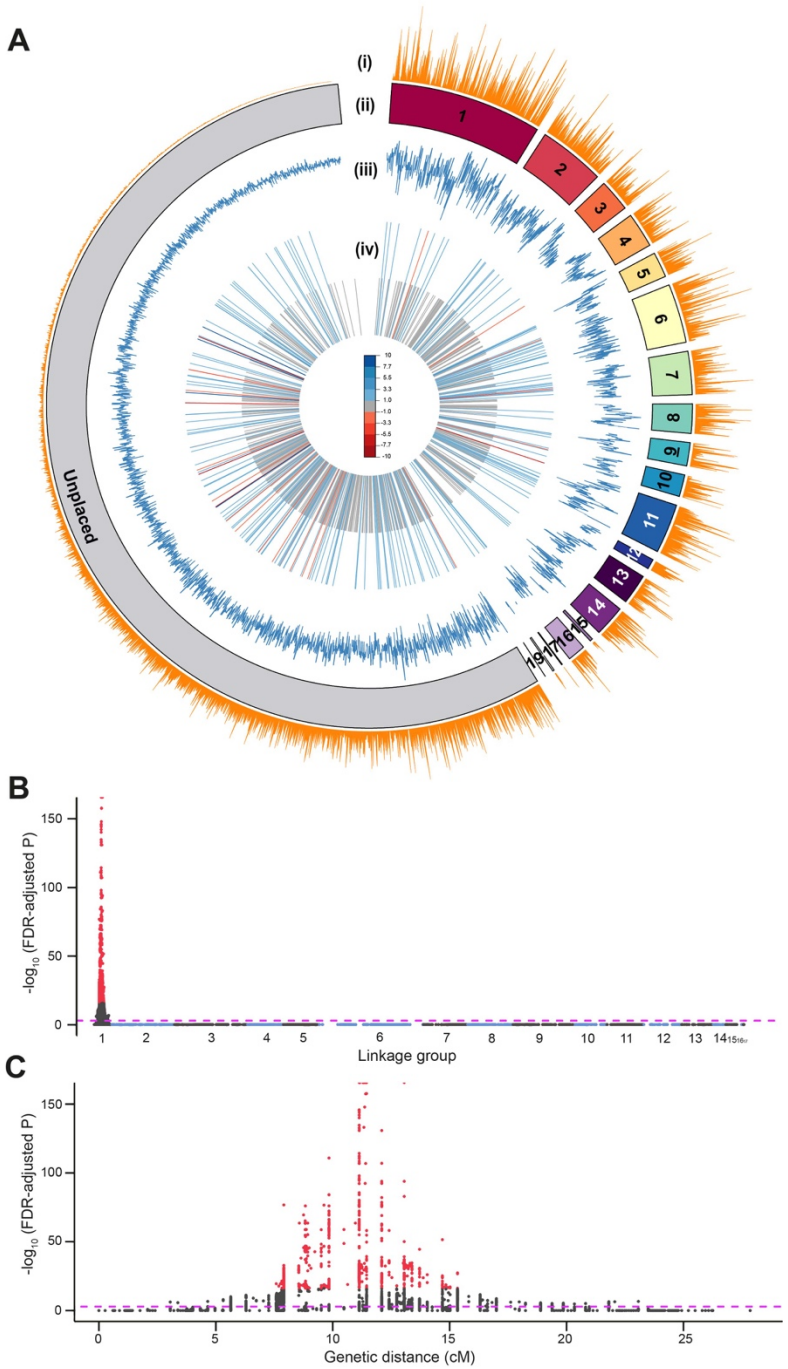

Figure 2. *Teleogryllus oceanicus* genome and regions associated with the flatwing phenotype. (A) Circos plot providing an overview of the genome. Linkage groups (LGs) upon which genome scaffolds were anchored are shown in different colours, with unplaced scaffolds in gray. LG1 was identified as the X chromosome based on heterozygosity and coverage filters (see Main Text). Tracks (i): gene density, (ii): linkage group pseudomolecules, (iii): transposable element density, (iv): genes differentially expressed (DE) in the thoracic tissues of embryos homozygous for *flatwing* vs. *normal-wing* genotypes. Longer bars are DE genes for which $\log_2$fold-change > 1 between genotypes, and short grey bars are all other DE genes. Colours indicate the magnitude of upregulation (red) versus downregulation (blue) in *flatwing* compared to *normal-wing* embryos. (B) Genome-wide Manhattan plot of the flatwing QTL. Alternating shades of grey and blue indicate different LGs. The horizontal dashed line indicates an FDR-corrected significance threshold of (P < 0.001), and the top 1% most

significant QTL markers are plotted in red. (C) Enlarged plot for LG1 (X chromosome) showing the flatwing-associated peak.

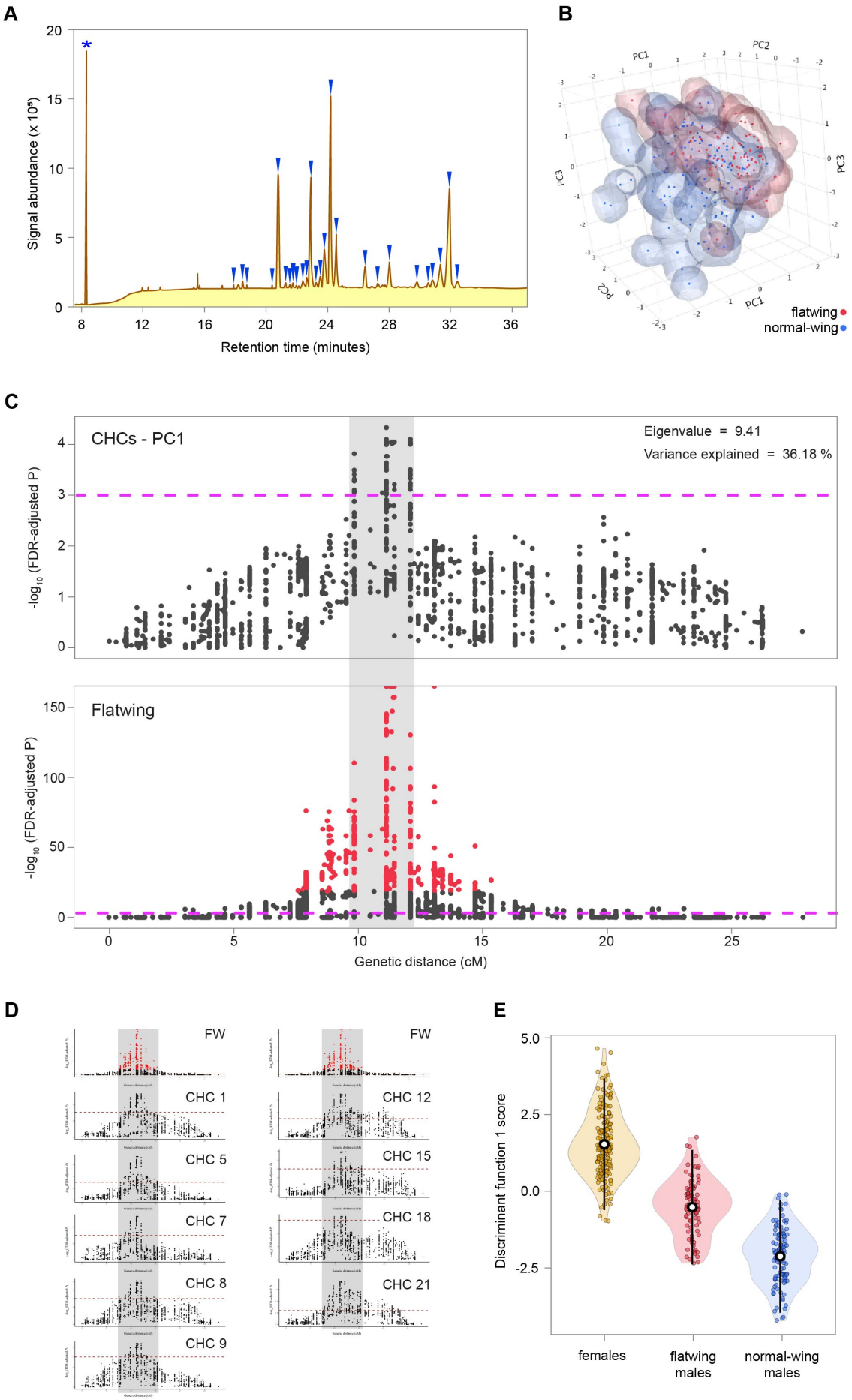
A
Signal abundance (x 10⁵)
Retention time (minutes)
B
PC1
PC2
PC3
flatwing
normal-wing
C
CHCs - PC1
Eigenvalue = 9.41
Variance explained = 36.18 %
-log10 (FDR-adjusted P)
Flatwing
Genetic distance (cM)
D
FW
CHC 1
CHC 5
CHC 7
CHC 8
CHC 9
CHC 12
CHC 15
CHC 18
CHC 21
E
Discriminant function 1 score
females
flatwing males
normal-wing males

Figure 3. Genetic colocalisation of the flatwing phenotype and male chemical pheromone feminisation. (A) Diagram of a *T. oceanicus* cuticular hydrocarbon (CHC) chromatogram, with the 26 measured peaks indicated by blue wedges. The asterisk indicates the internal standard (pentadecane). (B) Space-filling scatterplot of the first three principal components describing male CHC profiles, illustrating differences between flatwing and normal-wing males (variance explained for PC1: 35.18%, PC2: 10.14%, PC3: 9.58%). (C) Comparison of QTL on the putative X chromosome for CHCs (top; first principal component mapped) and flatwing (bottom, same as Fig. 2C). Grey shading indicates the extent (in cM) of the CHC peak, showing overlap with the flatwing QTL. Dashed lines indicate FDR-corrected significance of $p < 0.001$, red points the top 1% significant flatwing QTL markers. Note the different y-axis scales. (D) Univariate analyses revealed nine individual CHC components which also co-localised with flatwing. The original flatwing QTL is plotted at the top of each column. Grey shading spans the genetic region of co-localisation. Numbers refer to compounds indicated in *A*, and dashed lines indicate an FDR-corrected significance threshold of $p < 0.001$. (E) Discriminant function scores describing variation in CHC profiles among female, flatwing male and normal-wing male mapping individuals. Discriminant function 1 explained 78.8% of the variance in CHC profiles between groups. Means ± 2 s.d. are indicated by open black-and-white circles and lines, respectively. Points in E) are scattered along the X-axis for purposes of visualisation only, with solid outlines representing density distributions.

AUTHOR CONTRIBUTIONS

N.W.B. conceived and led the study. S.P., K.G., M.B., M.G.R. and N.W.B. designed experiments. S.P. led data collection, did genetic crosses and wet lab work. S.P., J.E.R., X.Z., T.C., E.L., X.L., J.H., J.G.R., B.L.S., U.T. and N.W.B. performed analyses. M.B., R.J.C., S.J., E.L., M.B. and N.W.B. designed ChirpBase. N.W.B. led manuscript writing. S.P., J.E.R., X.Z., E.L., M.B., M.G.R. and N.W.B contributed to writing.

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DATA ARCHIVING

Raw reads from Illumina and PacBio genome sequencing libraries have been deposited in the European Nucleotide Archive under acceession number PRJEB24786. Embryo RNAseq reads are available in the same archive (PRJEB27235) as are RADseq reads used in the linkage map and QTL analyses (PRJEB29921). CHC phenotype data and custom scripts are available online at http://chirpbase.org if not stated otherwise.

LITERATURE CITED

Abrusan, G., Grundmann, N., DeMester, L., and Makalowski, W. (2009). TEclass--a tool for automated classification of unknown eukaryotic transposable elements. *Bioinformatics* 25:1329-1330.

Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G.*, et al.* (2000). The genome sequence of Drosophila melanogaster. *Science* 287:2185-2195.

Adler, P. N. (2012). The frizzled/stan pathway and planar cell polarity in the Drosophila wing. *Curr Top Dev Biol* 101:1-31.

Bailey, N. W., Gray, B., and Zuk, M. (2010). Acoustic experience shapes alternative mating tactics and reproductive investment in male field crickets. *Curr Biol* 20:845-849.

Bairoch, A. and Apweiler, R. (2000). The SWISS-PROT protein sequence database and its supplement TrEMBL in 2000. *Nucleic Acids Res* 28:45-48.

Bank, C., Hietpas, R. T., Wong, A., Bolon, D. N., and Jensen, J. D. (2014). A bayesian MCMC approach to assess the complete distribution of fitness effects of new mutations: uncovering the potential for adaptive walks in challenging environments. *Genetics* 196:841-852.

Bao, W., Kojima, K. K., and Kohany, O. (2015). Repbase Update, a database of repetitive elements in eukaryotic genomes. *Mob DNA* 6:11.

Barrett, R. D. and Schluter, D. (2008). Adaptation from standing genetic variation. *Trends Ecol Evol* 23:38-44.

Benoit, J. B., Adelman, Z. N., Reinhardt, K., Dolan, A., Poelchau, M., Jennings, E. C.*, et al.* (2016). Unique features of a global human ectoparasite identified through sequencing of the bed bug genome. *Nat Commun* 7:10165.

Benson, G. (1999). Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acids Res* 27:573-580.

Bicocca, V. T., Chang, B. H., Masouleh, B. K., Muschen, M., Loriaux, M. M., Druker, B. J.*, et al.* (2012). Crosstalk between ROR1 and the Pre-B cell receptor promotes survival of t(1;19) acute lymphoblastic leukemia. *Cancer Cell* 22:656-667.

Birney, E., Clamp, M., and Durbin, R. (2004). GeneWise and Genomewise. *Genome Res* 14:988-995.

Blanco, E., Parra, G., and Guigo, R. (2007). Using geneid to identify genes. *Curr Protoc Bioinformatics* Chapter 4:Unit 4 3.

Boratyn, G. M., Schaffer, A. A., Agarwala, R., Altschul, S. F., Lipman, D. J., and Madden, T. L. (2012). Domain enhanced lookup time accelerated BLAST. *Biol Direct* 7:12.

Calus, M. P. (2010). Genomic breeding value prediction: methods and procedures. *Animal* 4:157-164.

Carre, C., Szymczak, D., Pidoux, J., and Antoniewski, C. (2005). The histone H3 acetylase dGcn5 is a key player in Drosophila melanogaster metamorphosis. *Mol Cell Biol* 25:8228-8238.

Carvajal-Gonzalez, J. M., Mulero-Navarro, S., Smith, M., and Mlodzik, M. (2016). A Novel Frizzled-Based Screening Tool Identifies Genetic Modifiers of Planar Cell Polarity in Drosophila Wings. *G3 (Bethesda)* 6:3963-3973.

Catchen, J., Hohenlohe, P. A., Bassham, S., Amores, A., and Cresko, W. A. (2013). Stacks: an analysis tool set for population genomics. *Mol Ecol* 22:3124-3140.

Challis, R. J., Kumar, S., Stevens, L., and Blaxter, M. (2017). GenomeHubs: simple containerized setup of a custom Ensembl database and web server for any species. *Database (Oxford)* 2017.

De Celis, J. F. (2003). Pattern formation in the Drosophila wing: The development of the veins. *Bioessays* 25:443-451.

Donoughe, S. and Extavour, C. G. (2016). Embryonic development of the cricket Gryllus bimaculatus. *Dev Biol* 411:140-156.

Dudchenko, O., Batra, S. S., Omer, A. D., Nyquist, S. K., Hoeger, M., Durand, N. C.*, et al.* (2017). De novo assembly of the Aedes aegypti genome using Hi-C yields chromosome-length scaffolds. *Science* 356:92-95.

Dugatkin, L. A. (2008). Principles of Animal Behavior. W. W. Norton & Company, New York.

English, A. C., Richards, S., Han, Y., Wang, M., Vee, V., Qu, J.*, et al.* (2012). Mind the gap: upgrading genomes with Pacific Biosciences RS long-read sequencing technology. *PLoS One* 7:e47768.

Finn, R. D., Attwood, T. K., Babbitt, P. C., Bateman, A., Bork, P., Bridge, A. J.*, et al.* (2017). InterPro in 2017-beyond protein family and domain annotations. *Nucleic Acids Res* 45:D190-D199.

Fisher, R. (1930). The Genetical Theory of Natural Selection. The Clarendon Press, Oxford.

Gotz, S., Garcia-Gomez, J. M., Terol, J., Williams, T. D., Nagaraj, S. H., Nueda, M. J.*, et al.* (2008). High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Res* 36:3420-3435.

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I.*, et al.* (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat Biotechnol* 29:644-652.

Gramates, L. S., Marygold, S. J., Santos, G. D., Urbano, J. M., Antonazzo, G., Matthews, B. B.*, et al.* (2017). FlyBase at 25: looking to the future. *Nucleic Acids Res* 45:D663-D671.

Gray, B., Bailey, N. W., Poon, M., and Zuk, M. (2014). Multimodal signal compensation: do field crickets shift sexual signal modality after the loss of acoustic communication? *Animal behaviour* 93:243-248.

Haas, B. J., Salzberg, S. L., Zhu, W., Pertea, M., Allen, J. E., Orvis, J.*, et al.* (2008). Automated eukaryotic gene structure annotation using EVidenceModeler and the Program to Assemble Spliced Alignments. *Genome Biol* 9:R7.

Heo, Y., Wu, X. L., Chen, D., Ma, J., and Hwu, W. M. (2014). BLESS: bloom filter-based error correction solution for high-throughput sequencing reads. *Bioinformatics* 30:1354-1362.

Hevia, C. F. and de Celis, J. F. (2013). Activation and function of TGFbeta signalling during Drosophila wing development and its interactions with the BMP pathway. *Dev Biol* 377:138-153.

Hoff, K. J., Lange, S., Lomsadze, A., Borodovsky, M., and Stanke, M. (2016). BRAKER1: Unsupervised RNA-Seq-Based Genome Annotation with GeneMark-ET and AUGUSTUS. *Bioinformatics* 32:767-769.

Hubley, R., Finn, R. D., Clements, J., Eddy, S. R., Jones, T. A., Bao, W.*, et al.* (2016). The Dfam database of repetitive DNA families. *Nucleic Acids Res* 44:D81-89.

International Aphid Genomics Consortium. (2010). Genome sequence of the pea aphid Acyrthosiphon pisum. *PLoS Biol* 8:e1000313.

Joag, R., Stuglik, M., Konczal, M., Plesnar-Bielak, A., Skrzynecka, A., Babik, W.*, et al.* (2016). Transcriptomics of Intralocus Sexual Conflict: Gene Expression Patterns in Females Change in Response to Selection on a Male Secondary Sexual Trait in the Bulb Mite. *Genome Biol Evol* 8:2351-2357.

Jones, P., Binns, D., Chang, H. Y., Fraser, M., Li, W., McAnulla, C.*, et al.* (2014). InterProScan 5: genome-scale protein function classification. *Bioinformatics* 30:1236-1240.

Joshi, N. A. and Fass, J. N. (2011). SICKLE: a sliding-window, adaptive, quality-based trimming tool for FastQ files [Software]. Available at: at https://github.com/najoshi/sickle.

Kajitani, R., Toshimoto, K., Noguchi, H., Toyoda, A., Ogura, Y., Okuno, M.*, et al.* (2014). Efficient de novo assembly of highly heterozygous genomes from whole-genome shotgun short reads. *Genome Res* 24:1384-1395.

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., and Tanabe, M. (2016a). KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Res* 44:D457-462.

Kanehisa, M., Sato, Y., and Morishima, K. (2016b). BlastKOALA and GhostKOALA: KEGG Tools for Functional Characterization of Genome and Metagenome Sequences. *J Mol Biol* 428:726-731.

Kent, W. J., Sugnet, C. W., Furey, T. S., Roskin, K. M., Pringle, T. H., Zahler, A. M.*, et al.* (2002). The human genome browser at UCSC. *Genome Res* 12:996-1006.

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., and Salzberg, S. L. (2013). TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol* 14:R36.

Kimura, M. (1983). The Neutral Theory of Molecular Evolution. Cambridge University Press, Cambridge.

Korf, I. (2004). Gene finding in novel genomes. *BMC Bioinformatics* 5:59.

Kupper, C., Stocks, M., Risse, J. E., Dos Remedios, N., Farrell, L. L., McRae, S. B.*, et al.* (2016). A supergene determines highly divergent male reproductive morphs in the ruff. *Nat Genet* 48:79-83.

Li, H. and Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 25:1754-1760.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N.*, et al.* (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25:2078-2079.

Li, S., Zhu, S., Jia, Q., Yuan, D., Ren, C., Li, K.*, et al.* (2018). The genomic and functional landscapes of developmental plasticity in the American cockroach. *Nat Commun* 9:1008.

Lomsadze, A., Burns, P. D., and Borodovsky, M. (2014). Integration of mapped RNA-Seq reads into automatic training of eukaryotic gene finding algorithm. *Nucleic Acids Res* 42:e119.

Majlat, P., Erdos, Z., and Takacs, J. (1974). Calculation and Application of Retention Indexes in Programmed-Temperature Gas-Chromatography. *J Chromatogr* 91:89-103.

Majoros, W. H., Pertea, M., and Salzberg, S. L. (2004). TigrScan and GlimmerHMM: two open source ab initio eukaryotic gene-finders. *Bioinformatics* 20:2878-2879.
Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. journal* 17:pp. 10-12.
McKenna, D. D., Scully, E. D., Pauchet, Y., Hoover, K., Kirsch, R., Geib, S. M.*, et al.* (2016). Genome of the Asian longhorned beetle (Anoplophora glabripennis), a globally significant invasive species, reveals key functional and evolutionary innovations at the beetle-plant interface. *Genome Biol* 17:227.
Miller, J. R., Koren, S., Dilley, K. A., Puri, V., Brown, D. M., Harkins, D. M.*, et al.* (2018). Analysis of the Aedes albopictus C6/36 genome provides insight into cell line utility for viral propagation. *GigaScience* 7:1-13.
Nadeau, J. H., Burrage, L. C., Restivo, J., Pao, Y. H., Churchill, G., and Hoit, B. D. (2003). Pleiotropy, homeostasis, and functional networks based on assays of cardiovascular traits in genetically randomized populations. *Genome Res* 13:2082-2091.
Okada, H., Ebhardt, H. A., Vonesch, S. C., Aebersold, R., and Hafen, E. (2016). Proteome-wide association studies identify biochemical modules associated with a wing-size phenotype in Drosophila melanogaster. *Nat Commun* 7:12649.
Orr, H. A. (1998). The Population Genetics of Adaptation: The Distribution of Factors Fixed during Adaptive Evolution. *Evolution* 52:935-949.
Orr, H. A. (2005). The genetic theory of adaptation: a brief history. *Nat Rev Genet* 6:119-127.
Pascoal, S., Cezard, T., Eik-Nes, A., Gharbi, K., Majewska, J., Payne, E.*, et al.* (2014). Rapid convergent evolution in wild crickets. *Curr Biol* 24:1369-1374.
Pascoal, S., Liu, X., Fang, Y., Paterson, S., Ritchie, M. G., Rockliffe, N.*, et al.* (2018). Increased socially mediated plasticity in gene expression accompanies rapid adaptive evolution. *Ecol Lett* 21:546-556.
Pascoal, S., Liu, X., Ly, T., Fang, Y., Rockliffe, N., Paterson, S.*, et al.* (2016a). Rapid evolution and gene expression: a rapidly evolving Mendelian trait that silences field crickets has widespread effects on mRNA and protein expression. *J Evol Biol* 29:1234-1246.
Pascoal, S., Mendrok, M., Mitchell, C., Wilson, A. J., Hunt, J., and Bailey, N. W. (2016b). Sexual selection and population divergence I: The influence of socially flexible cuticular hydrocarbon expression in male field crickets (Teleogryllus oceanicus). *Evolution* 70:82-97.
Pertea, M., Kim, D., Pertea, G. M., Leek, J. T., and Salzberg, S. L. (2016). Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. *Nat Protoc* 11:1650-1667.
Price, A. L., Jones, N. C., and Pevzner, P. A. (2005). De novo identification of repeat families in large genomes. *Bioinformatics* 21 Suppl 1:i351-358.
Ranson, H., Claudianos, C., Ortelli, F., Abgrall, C., Hemingway, J., Sharakhova, M. V.*, et al.* (2002). Evolution of supergene families associated with insecticide resistance. *Science* 298:179-181.
Rastas, P., Calboli, F. C., Guo, B., Shikano, T., and Merila, J. (2015). Construction of Ultradense Linkage Maps with Lep-MAP2: Stickleback F2 Recombinant Crosses as an Example. *Genome Biol Evol* 8:78-93.
Rayner, J. G., Aldridge, S., Montealegre, Z. F., and Bailey, N. W. (2019a). A silent orchestra: convergent song loss in Hawaiian crickets is repeated, morphologically varied, and widespread. *Ecology* 100:e02694.
Rayner, J. G., Pascoal, S., and Bailey, N. W. (2019b). Release from intralocus sexual conflict? Evolved loss of a male sexual trait demasculinizes female gene expression. *Proc Biol Sci* 286:20190497.
Robinson, M. D., McCarthy, D. J., and Smyth, G. K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26:139-140.
Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahe, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 4:e2584.

Rosenfeld, J. A., Reeves, D., Brugler, M. R., Narechania, A., Simon, S., Durrett, R.*, et al.* (2016). Genome assembly and geospatial phylogenomics of the bed bug Cimex lectularius. *Nat Commun* 7:10164.

Schneider, W. T., Rutz, C., Hedwig, B., and Bailey, N. W. (2018). Vestigial singing behaviour persists after the evolutionary loss of song in crickets. *Biol Lett* 14.

Simao, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V., and Zdobnov, E. M. (2015). BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics* 31:3210-3212.

Simmons, L. W., Thomas, M. L., Gray, B., and Zuk, M. (2014). Replicated evolutionary divergence in the cuticular hydrocarbon profile of male crickets associated with the loss of song in the Hawaiian archipelago. *J Evol Biol* 27:2249-2257.

Smit, A., Hubley, R., and Green, P. (2013-2015). RepeatMasker Open-4.0. Available at: http://www.repeatmasker.org.

Snodgrass, R. E. (1993). Principles of Insect Morphology. Cornell University Press, Ithaca.

Stanke, M., Diekhans, M., Baertsch, R., and Haussler, D. (2008). Using native and syntenically mapped cDNA alignments to improve de novo gene finding. *Bioinformatics* 24:637-644.

Suyama, M., Torrents, D., and Bork, P. (2004). BLAST2GENE: a comprehensive conversion of BLAST output into independent genes and gene fragments. *Bioinformatics* 20:1968-1970.

Tang, H., Zhang, X., Miao, C., Zhang, J., Ming, R., Schnable, J. C.*, et al.* (2015). ALLMAPS: robust scaffold ordering based on multiple maps. *Genome Biol* 16:3.

Tarailo-Graovac, M. and Chen, N. (2009). Using RepeatMasker to identify repetitive elements in genomic sequences. *Curr Protoc Bioinformatics* Chapter 4:Unit 4 10.

Terrapon, N., Li, C., Robertson, H. M., Ji, L., Meng, X., Booth, W.*, et al.* (2014). Molecular traces of alternative social organization in a termite genome. *Nat Commun* 5:3636.

The Gene Ontology Consortium. (2017). Expansion of the Gene Ontology knowledgebase and resources. *Nucleic Acids Res* 45:D331-D338.

Thomas, M. L. and Simmons, L. W. (2009). Sexual selection on cuticular hydrocarbons in the Australian field cricket, Teleogryllus oceanicus. *BMC Evol Biol* 9:162.

Thomas, M. L. and Simmons, L. W. (2010). Cuticular hydrocarbons influence female attractiveness to males in the Australian field cricket, Teleogryllus oceanicus. *J Evol Biol* 23:707-714.

Tinghitella, R. M. (2008). Rapid evolutionary change in a sexual signal: genetic control of the mutation 'flatwing' that renders male field crickets (Teleogryllus oceanicus) mute. *Heredity (Edinb)* 100:261-267.

Tinghitella, R. M., Broder, E. D., Gurule-Small, G. A., Hallagan, C. J., and Wilson, J. D. (2018). Purring Crickets: The Evolution of a Novel Sexual Signal. *Am Nat* 192:773-782.

Tomasetto, F., Tylianakis, J. M., Reale, M., Wratten, S., and Goldson, S. L. (2017). Intensified agriculture favors evolved resistance to biological control. *Proc Natl Acad Sci U S A* 114:3885-3890.

Tregenza, T. and Wedell, N. (1997). Definitive evidence for cuticular pheromones in a cricket. *Anim Behav* 54:979-984.

van't Hof, A. E., Edmonds, N., Dalikova, M., Marec, F., and Saccheri, I. J. (2011). Industrial melanism in British peppered moths has a singular and recent mutational origin. *Science* 332:958-960.

VanRaden, P. M. (2008). Efficient methods to compute genomic predictions. *J Dairy Sci* 91:4414-4423.

Vervoort, M., Crozatier, M., Valle, D., and Vincent, A. (1999). The COE transcription factor Collier is a mediator of short-range Hedgehog-induced patterning of the Drosophila wing. *Curr Biol* 9:632-639.

Walker, B. J., Abeel, T., Shea, T., Priest, M., Abouelliel, A., Sakthikumar, S.*, et al.* (2014). Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. *PLoS One* 9:e112963.

Wang, X., Fang, X., Yang, P., Jiang, X., Jiang, F., Zhao, D*., et al.* (2014). The locust genome provides insight into swarm formation and long-distance flight. *Nat Commun* 5:2957.
Wu, C., Chen, Y., Wang, F., Chen, C., Zhang, S., Li, C*., et al.* (2015). Pelle Modulates dFoxO-Mediated Cell Death in Drosophila. *Plos Genet* 11:e1005589.
Xu, Y., Wang, X., Yang, J., Vaynberg, J., and Qin, J. (2006). PASA--a program for automated protein NMR backbone signal assignment by pattern-filtering approach. *J Biomol NMR* 34:41-56.
Xue, J., Zhou, X., Zhang, C. X., Yu, L. L., Fan, H. W., Wang, Z*., et al.* (2014). Genomes of the rice pest brown planthopper and its endosymbionts reveal complex complementary contributions for host adaptation. *Genome Biol* 15:521.
Yang, Y., Wang, Y., Zhao, Y., Zhang, X., Li, R., Chen, L*., et al.* (2017). Draft genome of the Marco Polo Sheep (Ovis ammon polii). *GigaScience* 6:1-7.
Zuk, M., Bailey, N. W., Gray, B., and Rotenberry, J. T. (2018). Sexual signal loss: The link between behaviour and rapid evolutionary dynamics in a field cricket. *J Anim Ecol* 87:623-633.
Zuk, M., Rotenberry, J. T., and Tinghitella, R. M. (2006). Silent night: adaptive disappearance of a sexual signal in a parasitized population of field crickets. *Biol Lett* 2:521-524.