Report

Current Biology

Natural variation at a single gene generates sexual antagonism across fitness components in *Drosophila*

Highlights

- *Drosophila* use cuticular hydrocarbons as sexual signals and to avoid abiotic stress
- A fatty-acyl CoA reductase, *DsFAR2-B*, produces polymorphic cuticular hydrocarbons
- The polymorphism is sexually antagonistic, harming males but benefiting females
- *DsFAR2-B* shows a population genetic signal of balancing selection in nature

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In brief

Rusuwa et al. study the diverse chemical sexual signals of *Drosophila*. In tropical Australia, a fatty-acyl CoA reductase gene *DsFAR2-B* creates polymorphic cuticular hydrocarbons with sexually antagonistic fitness effects. Population genomics shows balancing selection at *DsFAR2-B*, shedding light on how genetic variation is maintained in nature.

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Report

Natural variation at a single gene generates sexual antagonism across fitness components in *Drosophila*

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SUMMARY

Mutations with conflicting fitness effects in males and females accumulate in sexual populations, reducing their adaptive capacity.[1,2] Although quantitative genetic studies indicate that sexually antagonistic polymorphisms are common,[3–5] their molecular basis and population genetic properties remain poorly understood.[6,7] Here, we show in fruit flies how natural variation at a single gene generates sexual antagonism through phenotypic effects on cuticular hydrocarbon (CHC) traits that function as both mate signals and protectors against abiotic stress[8] across a latitudinal gradient. Tropical populations of *Drosophila serrata* have polymorphic CHCs producing sexual antagonism through opposing but sex-limited effects on these two fitness-related functions. We dissected this polymorphism to a single fatty-acyl CoA reductase gene, *DsFAR2-B*, that is expressed in oenocyte cells where CHCs are synthesized. RNAi-mediated disruption of the *DsFAR2-B* ortholog in *D. melanogaster* oenocytes affected CHCs in a similar way to that seen in *D. serrata*. Population genomic analysis revealed that balancing selection likely operates at the *DsFAR2-B* locus in the wild. Our study provides insights into the genetic basis of sexual antagonism in nature and connects sexually varying antagonistic selection on phenotypes with balancing selection on genotypes that maintains molecular variation.

RESULTS AND DISCUSSION

The contrasting means by which males and females maximize fitness favors the evolution of sexually dimorphic traits. Because the sexes share a genome, mutations usually affect traits in males and females similarly, which constrains the evolution of sexual dimorphism and produces sexually antagonistic effects on fitness.[1] Although quantitative genetic studies have revealed that sexually antagonistic genetic variation is common in sexual populations,[3,4,9–13] how sexual antagonism manifests at the genome level remains poorly understood.[6,14,15] Little is known of the selective contexts across which alleles produce sexually antagonistic fitness effects or the population genetic processes shaping the DNA sequences that encode them.[7] Required are integrative studies that can both characterize sexually antagonistic selection on shared traits and dissect the underlying sexually antagonistic loci.[7] However, such investigations remain rare, and those available have detected resolved, rather than ongoing, antagonism.[16–18]

Here, we focus on a shared trait that is subject to both natural and sexual selection in fruit flies: namely, cuticular hydrocarbons (CHCs). Many insects use CHCs as contact pheromones for mate choice[19,20] and also as waterproofing agents to prevent desiccation.[21,22] These dual functions simultaneously expose them to the combined evolutionary forces of natural and sexual selection, which can differ significantly between the sexes. CHCs are non-volatile long-chain hydrocarbons and exhibit parallel latitudinal clines across different continents in several fruit fly species, which is a strong indication that they experience spatially varying selection in nature.[23–26] Notably, in populations closer to the equator, the relative abundance of short-chain CHCs decreases while the abundance of long-chain CHCs tends to increase.[23,25] Experimental evolution[27,28] and association studies[25] implicate factors such as temperature and humidity as agents of spatially varying selection affecting these clines, which is consistent with the hypothesis that long-chain CHCs confer better protection against evaporative water loss than short-chain homologs.[29]

The CHC profiles of the Australian fly *Drosophila serrata* exhibit such a cline along the eastern Australian coastline. Importantly, this cline is genetically based and known to be shaped by natural selection (Figure 1A).[26] Northernmost populations express

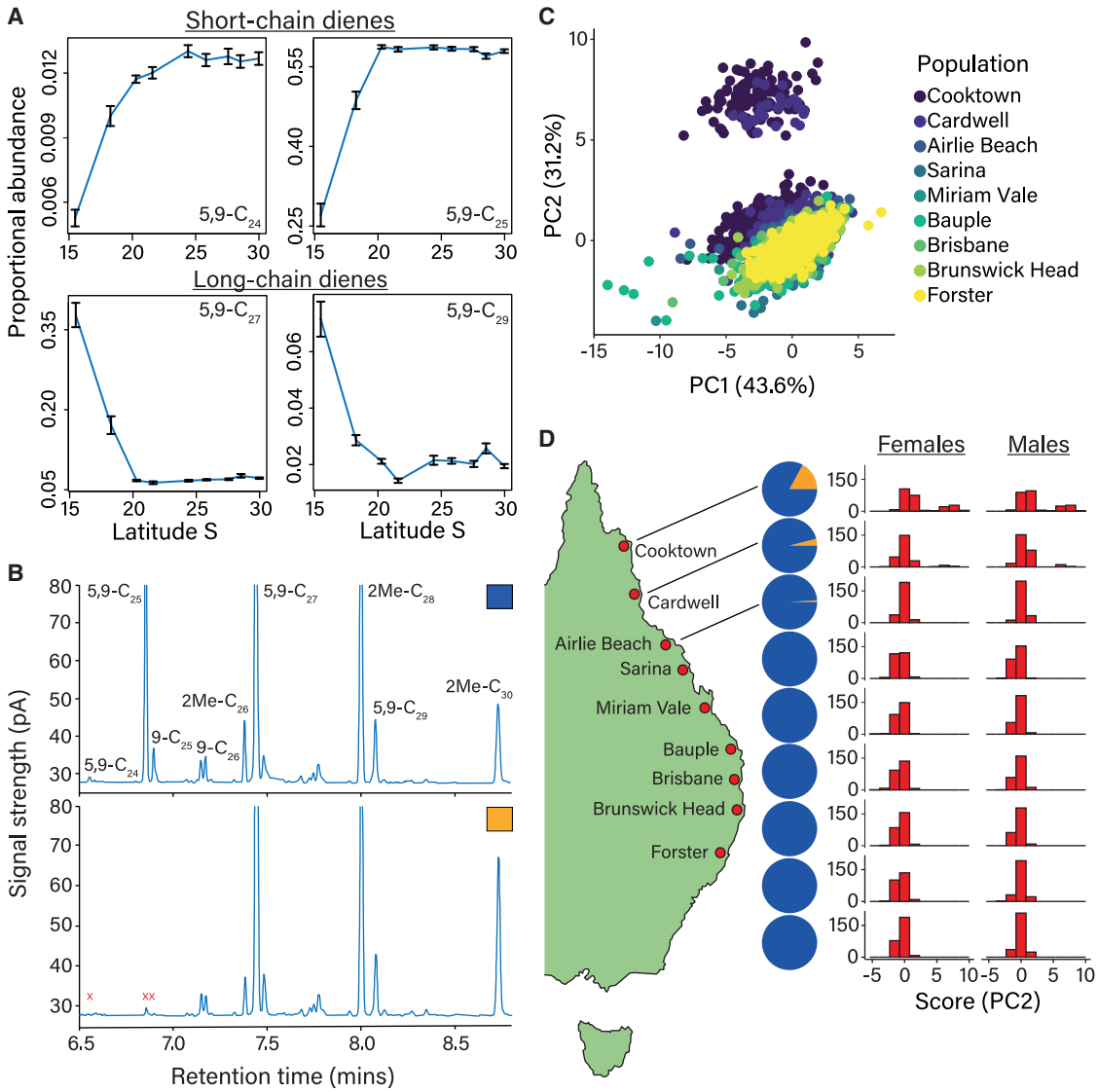

Figure 1. Clinal and polymorphic cuticular hydrocarbons

(A) Latitudinal clines in the *D. serrata* short-chain (<26 carbon) and long-chain (>25 carbon) dienes along the eastern Australian coastline show a reciprocal pattern. Shown are population means (+/− 1 s.e.) (after Chenoweth and Blows[26]).

(B) Two CHC phenotypes occur in far northern populations of flies: one where the short-chain CHCs 5,9-C24, 5,9-C25, and 9-C25 are expressed only in trace amounts (*northern* phenotype) and another, more and widespread, phenotype where these compounds are expressed at normal levels (*common* phenotype).

(C) Principal components analysis of the clinal CHC data displays two distinct phenotypes that are divided by the second principal component (PC2), one that is exclusive to the northern population of Cooktown and Cardwell and another that is found in all populations.

(D) The geographic distribution of *common* (blue) and *northern* (orange) phenotype flies along the eastern Australian coastline. The proportion of *northern* phenotype flies increases towards the equator.

significantly lower amounts of short-chain CHCs (25 or fewer carbon atoms), while long-chain CHCs, such as (*Z*,*Z*)-5,9 heptacosadiene (5,9-C_{27}), are expressed in higher amounts (Figure 1A). Examination of individual variation within populations revealed that some flies from northern populations express only trace amounts of all three shortest-chain CHCs: (*Z*,*Z*)-5,9-tetracosadiene (5,9-C_{24}), (*Z*,*Z*)-5,9-pentacosadiene (5,9-C_{25}), and (*Z*)-9-pentacosene (9-C_{25}) (Figure 1B). This can be considered an extreme phenotype because 5,9-C_{25}, which is virtually nonexistent in some *northern* flies, is usually the most abundant CHC in *common* phenotype individuals, comprising over 55% of the total CHC blend. Like the CHC cline itself, the frequency of these *northern* phenotype flies is also clinal, increasing in populations near the equator (Figure 1C). Furthermore, a principal components analysis clearly displays two distinct groups that are driven by a distinct phenotype in northern populations (Figures 1C and 1D). These observations point to a major segregating factor with phenotypic effects closely aligned with the latitudinal cline that contrasts short- with long-chain CHC expression.

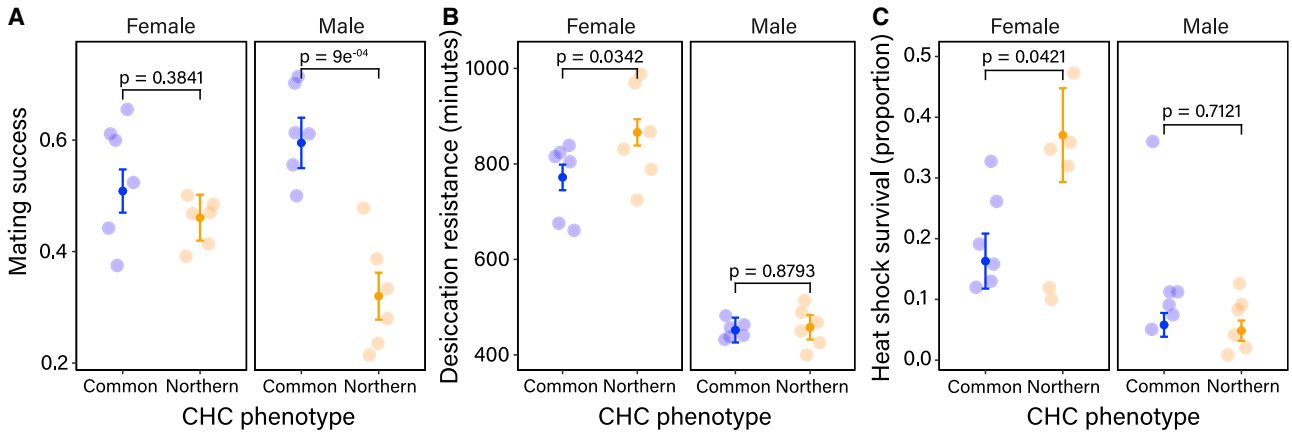

Figure 2. Sexually antagonsic fitness effects of CHC phenotypes

(A–C) Male and female mating success (A), desiccation resistance (B), and heat shock resistance (C). Points represent genotypic means, and bars are standard errors. Shown are overall CHC phenotype mean trait values +/− 1 s.e. Colored dots show individual F1 genotype mean trait values.

To investigate potential fitness effects of this CHC variation, we tested for differences in mating success and abiotic stress resistance between *northern* and *common* phenotype flies from Cooktown, Australia, where the *northern* phenotype occurs with highest frequency. Because the *northern* phenotype appears to be recessive and we needed to assay fitness in an otherwise outbred random genetic background, we created multiple F1 crosses between wild-derived inbred lines that were fixed for each phenotype. While CHC phenotype did not affect female mating success (generalized linear mixed model [GLMM]: $F_{1,10}$ = 0.83, p = 0.3841, n = 377), there was a significant effect of CHC phenotype on male mating success (GLMM: $F_{1,10}$ = 21.89, p = 0.0009, n = 308), regardless of the female choosers' CHC phenotype (GLMM: $F_{1,10}$ = 3.12, p = 0.1078, n = 308), suggesting that female *D. serrata* prefer males with the *common* CHC blend (Figure 2A; Table S1). *Common* CHC males thus have a significant mating advantage (~50%) when competing against *northern* CHC males. In contrast, the *northern* CHC phenotype was beneficial to female abiotic stress resistance. Desiccation resistance of *northern* females was significantly greater than that of *common* females (Table S1; sex × genotype: $F_{1,10}$ = 5.29, p = 0.0442, n = 157; within female effect: t_{10} = 2.45, p = 0.0342, n = 70; Figure 2B), but there was no detectable effect on males (t_{10} = 0.16, p = 0.8793, n = 87). There were also sex-dependent effects on heat shock resistance (Table S1; sex × genotype: $F_{1,10}$ = 5.64, p = 0.0389, n = 2,683; Figure 2C). Heat shock resistance of *northern* females was significantly greater than that of *common* females (within female effect: t_{10} = 2.33, p = 0.0421 n = 1,374; Figure 2B), but there was no detectable effect on males (t_{10} = −0.38, p = 0.7121, n = 1,309). The combined results across these three traits indicate a relatively complex form of sexually antagonistic selection on the CHC polymorphism that plays out across the fitness components of natural and sexual selection (Figures 2A–2C).

Discovery of a sexually antagonistic phenotypic polymorphism underlying clinal variation in CHCs provided a rare opportunity to dissect its genetic basis and to examine molecular population genetic processes at the underlying loci. We began by generating an F2 intercross between an inbred line from Cooktown (CTN42) at the northern end of the cline and an inbred line from Forster (FORS4) at the southern end, with each line fixed for the two alternate CHC phenotypes (Figure S1). Quantitative trait locus (QTL) mapping revealed a major recessive QTL on the right arm of the third chromosome, which is homologous to *D. melanogaster* 3R (Figures 3A and 3B).[30] To improve mapping resolution, we created a highly advanced 60-generation mass bred population founded from the same two inbred lines (Figure S1). We took a bulked segregant approach to fine mapping, applying Illumina whole-genome resequencing to DNA pools of flies displaying either the *common* or *northern* CHC profiles. The fine mapping revealed a single narrow QTL peak, again on 3R (Figure 3C; Table S2).

Because our initial mapping cross was between quite distant geographical populations, results might have been complicated by population structure. To circumvent this complication and replicate the association on a within-population scale, we sequenced the genomes of 10 wild-derived inbred lines collected from Cooktown, where the *northern* phenotype is most common. Four lines were fixed for the *common* phenotype while six were fixed for the *northern* phenotype. Window-based summation of the fixed SNPs between the two CHC phenotypes across the genome revealed a peak of maximal differentiation at the same location on 3R identified by the bulked segregant analysis (Figure 3D).

The highest 30 kb interval of the QTL peak identified via bulked segregant and the genomic window of maximal differentiation in the ten Cooktown genomes contained five clustered genes with predicted fatty acyl-CoA reductase (FAR) activity (Figure 4A). FARs play a role in CHC synthesis by converting long-chain fatty acyl-CoA to long-chain fatty alcohols before converting them to hydrocarbons via a P450 decarbonylase.[31] FAR genes have been shown to be important for pheromone biosynthesis in other insects,[32] for example moths, where changes in a single gene can produce very different pheromone blends.[33] Moreover, a recent genome-wide association study (GWAS) implicated FAR genes as part of the genetic basis of CHC variation in *D. melanogaster*.[34]

Genomic clustering of FAR genes is common in *Drosophila*, and they are thought to evolve via duplication and subsequent gene loss.[35] In the homologous genome region underlying the QTL peak, orthologs of the *DsFAR* genes in *D. melanogaster* are tightly

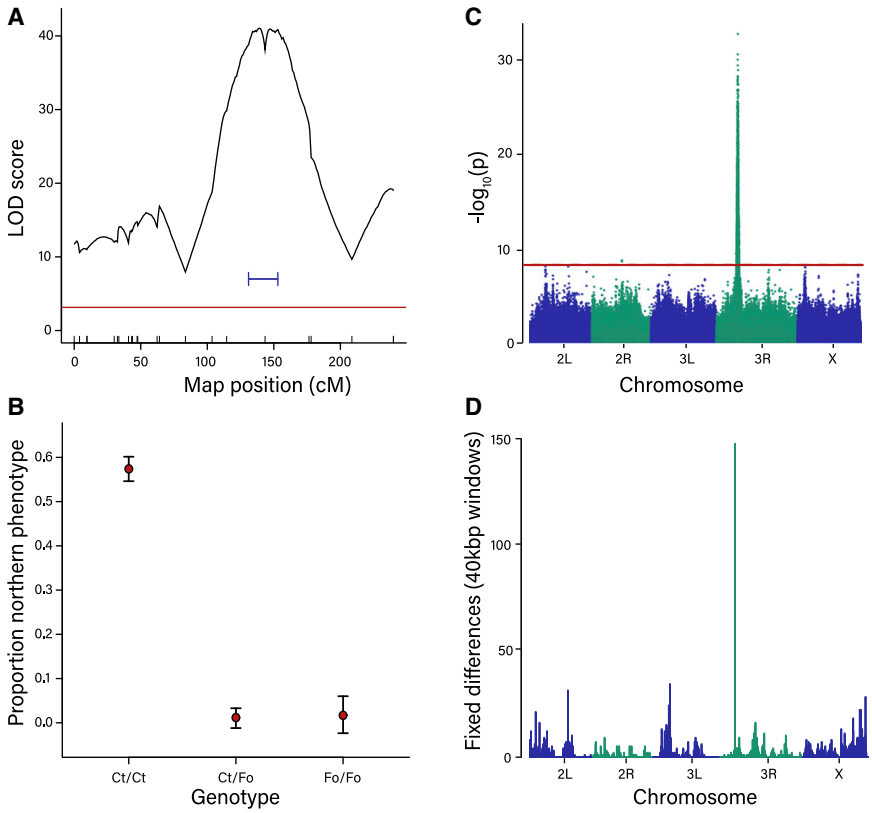

Figure 3. CHC polymorphism maps to a single major locus

(A) Binary trait QTL analysis revealed a major effect QTL on chromosome 3R in an F2 mapping cross between inbred *northern* (CTN42, genotype Ct/Ct) and *common* (FORS4, genotype Fo/Fo) phenotype lines. The location of the QTL confidence interval is shown in blue, and the 95% permutation-based genome-wide threshold is highlighted in red.

(B) A cross between these same two inbred lines indicated that the nearest marker genotype effects for the major CHC QTL on chromosome 3R had a recessive mode of action. Shown are genotype means +/− 1 s.e.

(C) Illumina-sequenced bulk segregant analysis (BSA) of an F60 intercross between CTN42 and FORS4 lines. Each blue dot represents the −log10 p value for a Fisher's exact test, and the red line represents a 5% false discovery rate threshold. See also Figure S1.

(D) Replication of the association between FAR loci implicated in the BSA using whole-genome sequences of ten inbred lines from Cooktown in far northern Queensland. Shown are the number of fixed differences between four *common* and six *northern* lines within 40 kbp non-overlapping windows.

clustered in a similar manner. However, *D. melanogaster* only harbors three consecutive FAR genes in this region (*CG17562*, *CG17560*, and *CG14893*). In *D. serrata*, two of these FAR genes appear as duplicates in the reference genome (Figure S2),[36] which was generated by long-read sequencing of an inbred line displaying the *common* CHC phenotype. Interestingly, while all *common* genomes from Cooktown contained at least one copy of the duplication and therefore all five *DsFAR* genes, all *northern* genomes from Cooktown lacked the duplication and possessed only *DsFAR1*, *DsFAR2-B*, and *DsFAR3-B* (Figure 4A). However, this association between the duplication and CHC phenotype was not present in ten inbred lines from a different population; several lines from the *D. serrata* genomic reference panel (DsGRP)[37] that display the *common* CHC phenotype also lack the duplication and only possess *DsFAR1*, *DsFAR2-B*, and *DsFAR3-B*. This observation excluded *DsFAR2-A* and *DsFAR3-A* as candidate genes.

To further narrow our search, we turned to *in situ* hybridization of the DsFAR candidates. CHC synthesis occurs in specialized cells called oenocytes. *In situ* hybridization of *DsFAR1*, *DsFAR2-A/B*, and *DsFAR3-A/B* in *D. serrata* showed that only *DsFAR2-A/B* is expressed in oenocytes of both *common* and *northern* adults (Figure 4B), narrowing the search to a single candidate gene. To verify the function of *DsFAR2* in CHC synthesis, we silenced expression of *DsFAR2*'s ortholog (*CG17560*) in *D. melanogaster* oenocytes by RNA interference. Silencing *CG17560* in *D. melanogaster* produced a strikingly similar phenotypic effect to that seen in *D. serrata.* Production of short-chain CHCs was dramatically reduced, while many long-chain compounds significantly increased in production (Figures 4C, S3, and S4).

Both the *northern* and *common* versions of *DsFAR2-B* appeared to be functional. The distinction between the *northern* and *common* versions of *DsFAR2-B* was a large number of non-synonymous substitutions. Of the 33 fixed differences between the *northern* and *common* alleles, 27 (~82%) were non-synonymous. Furthermore, the majority of the non-synonymous fixed differences (24 of 27, ~89%) were concentrated in the second half of the coding sequence, especially exon 4. This suggests that, as seen for pheromones in moths,[33] evolutionary changes within a single FAR gene are a simple mechanism capable of generating diverse CHC blends in flies that may, in turn, have varied fitness effects through mate choice and stress resistance. Taken together, our results strongly implicate *DsFAR2-B* in generating a sexually antagonistic CHC polymorphism in *D. serrata* and provide much needed knowledge about how sexually antagonistic selection manifests at the genome level.

Sexually antagonistic selection can sometimes lead to a form of balancing selection that may be detectable using population genetic analyses.[15] We estimated Tajima's D for all five *DsFAR2-B* exons in two natural populations: Cooktown, where the polymorphism is most frequent and balancing selection could be occurring, and a population approximately 2000 km further south in Brisbane, which displays only the *common* CHC phenotype,[37] where balancing selection is less likely. Tajima's D was a significantly positive genome-wide outlier for exons 2 ($D = 1.59$, $p = 0.03$), 4 ($D = 2$, $p = 0.007$), and 5 ($D = 1.6$, $p = 0.03$) of the *DsFAR2-B* locus in the Cooktown population, a pattern consistent with balancing selection. In contrast, Tajima's D was not consistent with balancing selection at any *DsFAR2-B* exon in the Brisbane population (all $p > 0.769$), with

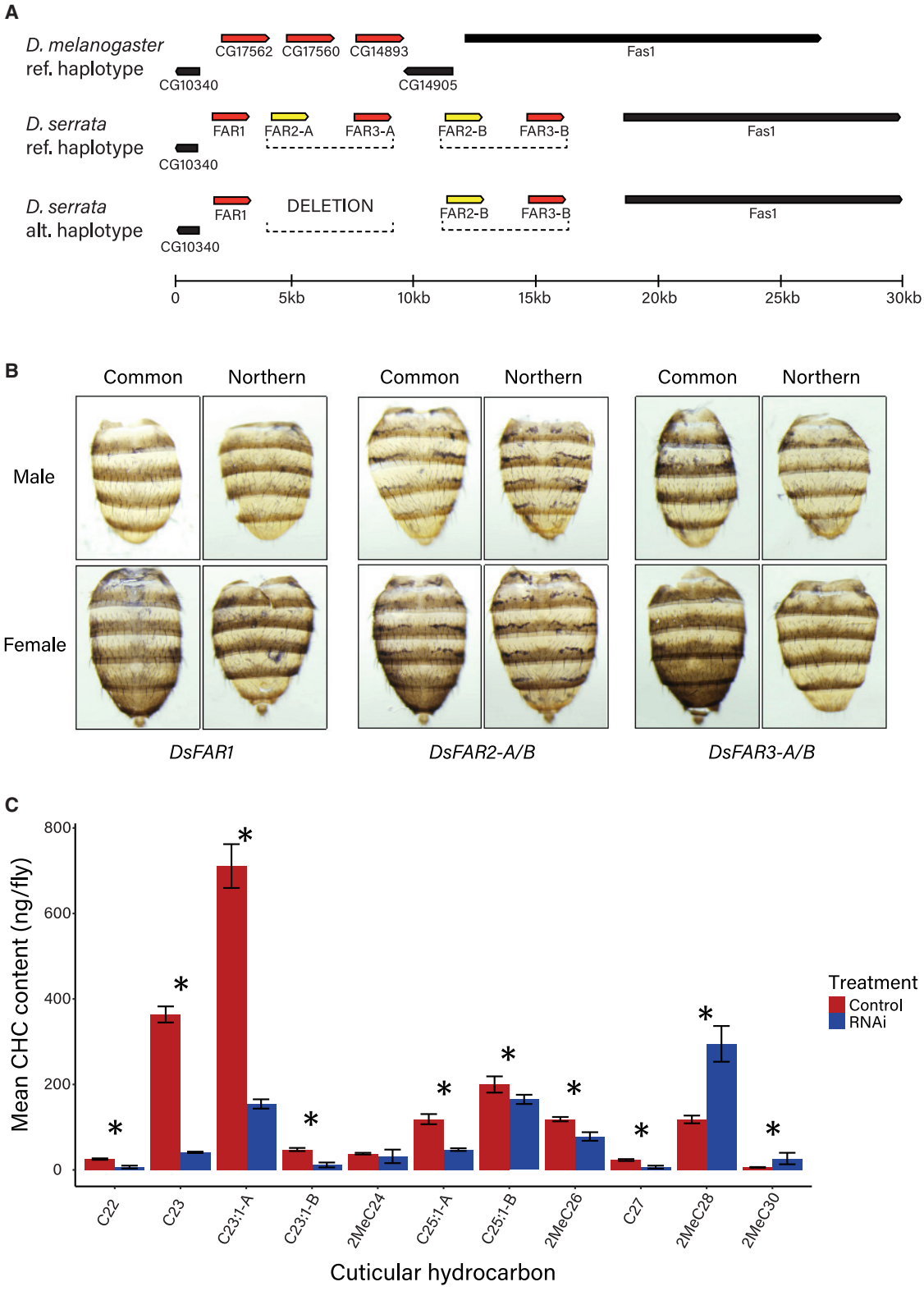

Figure 4. A fatty acyl-CoA reductase affects CHC phenotype

(A) Comparison of the orthologous region of *D. melanogaster* where the major QTL was mapped in *D. serrata*. The *D. melanogaster* reference genome contains three fatty acyl-coA reductase genes (*CG17562*, *CG17560*, and *CG14893*), which are orthologous to *D. serrata DsFAR1*, *DsFAR2*, and *DsFAR3*, respectively. The

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all D values negative and ranging from −0.59 to −1.16. Positive Tajima's D for *DsFAR2-B* in Cooktown strongly suggests balancing selection at this locus but only in populations where the CHC polymorphism is present.

For phenotypic traits with different fitness optima in males and females, sexual antagonism arises when mutations affect traits in similar ways in the two sexes.[1,2] An interesting question is how the polymorphism, which affects the CHCs of both sexes, generates the sex-specific fitness effects observed. In terms of mating success, there is evidence for both divergent fitness optima and sex differences in the strength of sexual selection on CHCs in *D. serrata*. While CHCs are phenotypically correlated with male and female mating success in *D. serrata,* the associations are always far stronger in males than in females.[38–41] Recent experiments suggest phenotypic CHC-mating success associations may be causal only in males.[42] Together, these results may help to explain why the *DsFAR2-B* polymorphism affected male, but not female mating success. With respect to abiotic stress, the increase in long-chain CHCs that accompanies the short-chain CHC reduction in the *northern* phenotype may account for increased desiccation resistance,[43] but it does not account for the effect being restricted to females. Interestingly, responses of males and females to desiccation and thermal stress are often very different in *Drosophila* flies. For example, short-term exposure of *D. melanogaster* to low relative humidity conditions leads to a plastic response that increases female, but not male, desiccation resistance.[44] Moreover, sex-dependent responses of CHCs have been detected during experimental adaptation to low relative humidity environments in *D. melanogaster* [27] and to higher temperatures in *D. simulans*.[28] These results point to sexually dimorphic physiology in *D. serrata* and suggest that CHCs may differ in their importance for abiotic stress resistance in males and females. Future physiological studies will be required to understand how such sex differences arise.

The long-chain versus short-chain phenotypic effect of the *DsFAR2-B* polymorphism closely aligns with the *D. serrata* latitudinal cline, which suggests that expression of low amounts of short-chain but high amounts of long-chain CHCs is indeed beneficial in warmer tropical populations. In the far north, where temperatures are higher, this benefit to female stress resistance may outweigh the cost to decreased male sexual attractiveness, setting up a scenario of sexual antagonism. In contrast, the cost-benefit relationship likely changes moving south to cooler populations, where *northern* phenotype individuals have not been observed. It therefore appears that climatic variation is key to determining whether sexual antagonism may or may not arise in a given population. Another potentially influential factor affecting maintenance of the polymorphism is ongoing migration. It is possible that recurrent gene flow of common alleles into Cooktown also helps to prevent fixation of the northern allele. Developing a better understanding the relative contributions of sexual antagonism and migration will require further investigation but will be important to understand how this polymorphism has been maintained.

There is considerable interest in understanding how sexually antagonistic selection manifests at the genome level.[6,15] However, to date, very few investigations have provided gene-level resolution of experimentally confirmed sexually antagonistic loci.[16] Our detailed analysis, which spans from phenotypic variation in the wild to identifying the underlying genetic mechanism, confirms the existence of large-effect sexually antagonistic loci in nature. Further, it highlights how a variant with similar phenotypic effects on shared traits can generate a sexual antagonism that plays out across multiple components of fitness, involving both natural and sexual selection. The interplay between clinal variation and the presence of the polymorphism suggests that population differences in sex-specific natural and sexual selection may be important determinants of the sexually antagonistic loci segregating in any given population. The present study provides us with a platform upon which to reconstruct the evolutionary history of a sexually antagonistic locus and investigate how these sex-specific fitness effects arise.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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D. serrata reference genome contains a duplication of *DsFAR2* and *DsFAR3* that is not present in some sequenced *D. serrata* genomes, which contain an alternate haplotype in this region. This duplication is not associated with the CHC polymorphism (see results and discussion) (see also Figure S2).
(B) *In situ* hybridization indicating expression of *DsFAR2-A/B* in *D. serrata* oenocytes in *common* and *northern* males and females (indicated by the ribbon-like bands) and lack of expression for *DsFAR1* and *DsFAR3-A/B*.
(C) RNAi knockdown of the *DsFAR2* ortholog (*CG17560*) in *D. melanogaster* oenocytes indicates a major effect on CHCs. Shown are mean +/− 95% confidence interval for male CHCs expressed at a minimum of 20 ng/fly. Asterisks indicate significant differences in mean CHC expression ($p < 0.05$) between the control and RNAi treatment within each reciprocal cross (t test).
See Figures S3 and S4 for all CHCs in both sexes.

- Bulked segregant analysis
- Genome resequencing
- Genotyping the DsFAR2 duplication using short-read data
- Population genetic analysis
- In situ hybridisation to adult D. serrata oenocytes
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- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Desiccation resistance
  - Heat shock stress survival
  - Competitive mating success
  - QTL mapping
  - Bulked segregant analysis
  - Genome resequencing
  - Population genetic analysis

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2022.05.038.

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AUTHOR CONTRIBUTIONS

B.B.R. and S.F.C. designed the study. B.B.R. and F.D.F. performed the QTL analysis. H.C. performed the RNAi experiments, the *in situ* hybridizations, and phylogenetic analysis of the DsFAR genomic region. S.L.A. and B.B.R. performed the fitness assays and BSA study. S.L.A. analyzed the bulk segregant and ten genome association analyses, annotated the DsFAR2 locus, and conducted molecular population genetic analyses. B.B.R. and S.F.C. drafted the manuscript, which was reviewed and edited by all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
D. serrata reference genome (NCBI)	[36]	GCA_002093755.1
D. serrata Hi-C scaffolded genome	This paper	https://doi.org/10.5061/dryad.0p2ngf245
D. serrata Annotation Release 100 (NCBI)	N/A	GCF_002093755.1
Bulked segregant sequence data (NCBI)	This paper	PRJNA603628
Cooktown inbred lines sequence data (NCBI)	This paper	PRJNA604036
Brisbane inbred lines sequence data (NCBI)	[37]	PRJNA419238
Clinal CHC data	[26]	https://doi.org/10.5061/dryad.0p2ngf245
Mating success	This paper	https://doi.org/10.5061/dryad.0p2ngf245
Heat shock	This paper	https://doi.org/10.5061/dryad.0p2ngf245
Desiccation	This paper	https://doi.org/10.5061/dryad.0p2ngf245
QTL data	This paper	https://doi.org/10.5061/dryad.0p2ngf245
Aligned DsFAR2B orthologs	This paper	https://doi.org/10.5061/dryad.0p2ngf245
RNAi CHC data	This paper	https://doi.org/10.5061/dryad.0p2ngf245
Experimental models: Organisms/strains		
D. serrata common Inbred line from Forster (FORS4)	This paper	FORS4
D. serrata common Inbred line from Cooktown (CTN44)	This paper	CTN44
D. serrata common Inbred line from Cooktown (CTN21)	This paper	CTN21
D. serrata common Inbred line from Cooktown (134)	This paper	134
D. serrata common Inbred line from Cooktown (145)	This paper	145
D. serrata northern Inbred line from Cooktown (CTN42)	This paper	CTN42
D. serrata northern Inbred line from Cooktown (CTN10)	This paper	CTN10
D. serrata northern Inbred line from Cooktown (CTN18)	This paper	CTN18
D. serrata northern Inbred line from Cooktown (CTN32)	This paper	CTN32
D. serrata northern Inbred line from Cooktown (CTN34)	This paper	CTN34
D. serrata northern Inbred line from Cooktown (180)	This paper	180
D. serrata common Inbred line from Brisbane (12)	[37]	DsGRP12
D. serrata common Inbred line from Brisbane (28)	[37]	DsGRP28
D. serrata common Inbred line from Brisbane (49)	[37]	DsGRP49
D. serrata common Inbred line from Brisbane (63)	[37]	DsGRP63
D. serrata common Inbred line from Brisbane (133)	[37]	DsGRP133
D. serrata common Inbred line from Brisbane (165)	[37]	DsGRP165
D. serrata common Inbred line from Brisbane (171)	[37]	DsGRP171
D. serrata common Inbred line from Brisbane (193)	[37]	DsGRP193
D. serrata common Inbred line from Brisbane (195)	[37]	DsGRP195
D. serrata common Inbred line from Brisbane (228)	[37]	DsGRP228
D. melanogaster oenocyte GAL4 driver line (oenoGAL4)	[45]	oenoGAL4
D. melanogaster RNAi line (UAS-CG17560RNAi-KK)	VDRC Stock Center	104756
D. melanogaster y,w[1118] with attP landing site	Bloomington Drosophila Stock Center	
D. serrata used for *in situ* hybridisation	Drosophila Species Stock Center	14028-0681.05
Oligonucleotides		
DsFAR2-A/B (CG17560) Forward in-situ hybridization probe SerCG17560-probeF (5′-TGGCCTGTGCCTGGCACACGGG-3′)	This paper	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
DsFAR2-A/B (CG17560) Reverse in-situ hybridization probe SerCG17560-probeR (5′-GGGATGGTGTGGAAAATTAAGGC-3′)	This paper	N/A
DsFAR1 (CG17562) Forward in-situ hybridization probe SerCG17562-probeF (5′-ATGGATACTACCCATATTCAAAAG-3′)	This paper	N/A
DsFAR1 (CG17562) Reverse in-situ hybridization probe SerCG17562-probeR (5′-ATGGGAGAAACTCGCTGAAGTGCC-3′)	This paper	N/A
DsFAR3-A/B (CG14893) Forward in-situ hybridization probe SerCG14893-probeF (5′-TGGCCAGCATCTGGAAAACGGC-3′)	This paper	N/A
DsFAR3-A/B (CG14893) Reverse in-situ hybridization probe SerCG14893-probeR (5′-GGTATTGTGTGATAAAAGAAGGC-′)	This paper	N/A
Software and algorithms		
Agilent ChemStation Software	Agilent	https://www.agilent.com/en/product/software-informatics/analytical-software-suite/chromatography-data-systems
fastQC	[46]	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
Burrows Wheeler Alignment (BWA)	[47]	https://github.com/lh3/bwa
Samtools	[48]	https://sourceforge.net/projects/samtools/files/samtools/
PoPoolation2	[49]	https://sourceforge.net/p/popoolation2/wiki/Main/
Genome Analysis Tool Kit	[50]	https://github.com/broadinstitute/gatk/releases
Integrative Genomics Viewer (IGV)	[51]	https://software.broadinstitute.org/software/igv/download
Molecular Evolutionary Genetic Analysis (MEGA X)	[52]	https://www.megasoftware.net/download_form
Statistical Analysis Software (SAS)	SAS Institute Inc.	https://www.sas.com/en_us/contact/form/register.html
The R Project for Statistical Computing (R)	R Core Team, 2013	https://www.r-project.org/
R/qtl	[53]	https://rqtl.org/
PopGenome	[54]	https://cran.r-project.org/web/packages/PopGenome/index.html

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Stephen F. Chenoweth (s.chenoweth@uq.edu.au).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Raw gas chromatography traces of CHCs used for the clinal analysis of phenotypes have been deposited at Dryad and are publicly available as of the date of publication. DOIs are listed in the key resources table. Mating and abiotic stress trait data used to assay fitness have been deposited at Dryad and are publicly available as of the date of publication. DOIs are listed in the key resources table. *D. serrata* reference genome: Genome (Dser1.0) has been deposited at GenBank and are publicly

available as of the date of publication. Accession numbers are listed in the key resources table. Hi-C based scaffolds have been deposited at Dryad and are publicly available as of the date of publication. DOIs are listed in the key resources table. Genotypic and phenotypic data used for the F2 binary QTL analysis have been deposited at Dryad and are publicly available as of the date of publication. DOIs are listed in the key resources table. Bulked segregant sequence data have been deposited at SRA and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. Cooktown population sequence data have been deposited at SRA and are publicly available as of the date of publication. Accession numbers are listed in the key resources tableBrisbane population sequence data have been deposited at SRA and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. Aligned FAR gene sequences used for phylogenetics have been deposited at Dryad and are publicly available as of the date of publication. DOIs are listed in the key resources table. RNAi CHC data have been deposited at Dryad and are publicly available as of the date of publication. DOIs are listed in the key resources table.

- This paper does not report original code.
- Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Fitness assays

We examined the fitness effects of the CHC polymorphism by assaying mating success and two stress traits; desiccation resistance and heat shock resistance on male and female flies. To create flies that displayed either the *northern* or *common* phenotype (the *northern* form is recessive), but at the same time avoid the effects of inbreeding, we created a series of F1 genotypes formed from crosses within two panels of wild-derived inbred lines from Cooktown (15°28'11.62 "S, 145°14'55.27 "E) that were fixed for the different CHC phenotypes. This approach allowed us to generate a large number of flies for a set of 12 *northern* CHC expressing genotypes and a set of 12 *common* CHC expressing genotypes.

Parental flies were sexed as virgins under light CO_2 anaesthesia and then held individually in vials until they were 4 days old. Mating vials were established by pairing 3 males and 3 females from different lines in vials containing 10mL of food media sprinkled with live yeast. Flies were allowed to mate for 4 days after which the vials were cleared. The F1 offspring flies were sexed as virgins and held at a density of five flies per vial before the mating, desiccation and heat tolerance trials commenced. All flies were reared at a constant temperature of 25°C on a 12h:12h light:dark cycle. Flies were maintained on the same standard fly diet comprising 36g agar, 108g raw sugar and 74g yeast, cooked in 2L of water combined with 24mL propionic acid and 12mL nipagin.

QTL mapping

To create a mapping population, an F2 intercross was made between two highly inbred lines (20 generations of full-sib mating) of *D. serrata* that were derived from two natural populations at opposing ends of the species' eastern Australian distribution (Cooktown: CTN42, Forster: FORS4). CTN42 expresses the *northern* CHC phenotype where the production 5,9-C_{24}, 5,9-C_{25} and 9-C_{25} is massively reduced relative to the FORS4 line which expresses the *common* CHC phenotype. A number of chromosomal inversions have been documented in *D. serrata* and so to minimise their potential effects in the QTL mapping, the founder lines were first confirmed to be homosequential using established protocols for polytene chromosome squashes.[55] Newly emerged virgin F2 flies were sexed (180 males, 203 females) and held singly in fresh vials for five days before being assayed for their CHC profiles.

Bulked segregant analysis

The same parental lines used to establish the F2 intercross (FORS4 *common* phenotype, CTN42 *northern* phenotype) were used to establish an advanced mass-bred population. We again performed reciprocal crosses to generate a very large population. The population was maintained in 32 glass bottles (500mL) for 60 non-overlapping generations resulting in a census size of approximately 6500 flies. We favoured the "large outbred" approach over randomly crossing pairs of flies every generation because a larger population size could be maintained with this setup, as it does not require sexing of flies at each generation. Flies were maintained on standard laboratory yeast medium, at 25°C with a 12h:12h light:dark photoperiod. We sampled flies for CHC phenotyping and subsequent bulked segregant analysis at generation 60. Newly emerged virgin flies were sexed (n = 600 female flies) under light CO_2 anaesthesia and held singly in food vials for five days before being assayed for their CHC profile through gas chromatography as described above. All of the flies phenotyped were individually preserved in 95% ethanol and stored at -80°C for subsequent DNA extraction.

Genome resequencing

As our mapping crosses were geographically broad, we attempted to replicate the association at *DsFAR2* by sequencing genomes from a sample of ten wild-derived inbred lines (17 generations of full-sib mating) from a single population in Cooktown where the *northern* phenotype has been found previously with highest frequency. CHC phenotyping after inbreeding confirmed that the *northern* phenotype also occurred at high frequency in this sample. Six lines (CTN10, CTN18, CTN32, CTN34, and 180) expressed the *northern* phenotype whereas the *common* phenotype occurred in four lines (CTN44, CTN21, 134, 145).

Genotyping the DsFAR2 duplication using short-read data

The inbred lines used for genome resequencing detailed above were used to genotype the DsFAR2 locus.

Population genetic analysis

Population genetic analyses for the Cooktown population where the *northern* phenotype occurs at highest frequency were performed using the genome resequencing data from 10 wild-derived inbred lines detailed above. We also performed the same analyses in a random sample of ten lines sampled from the *D. serrata* Genomic Reference Panel (DsGRP)[37] (lines 12, 28, 49, 63, 133, 165, 171, 193, 195, and 228), which is a sample from a single endemic population in Brisbane where the *northern* phenotype has not been observed.

In situ hybridisation to adult D. serrata oenocytes

In situ hybridisation were performed on both FORS4 and CTN42 males and females that are detailed above in the QTL mapping section.

D. melanogaster RNAi and CHC analyses

Oenocyte specific RNAi of *CG17560* (the DsFAR2 ortholog, see Figure S2) in *D. melanogaster* was performed at 25°C by crossing an oenocyte GAL4 driver line, oenoGAL4 (PromE(800) line 2M)[45] and a RNAi line, UAS-CG17560RNAi-KK (obtained from the Vienna Drosophila RNAi center). Controls were performed by crossing oenoGAL4 to *y,w[1118]* with attP landing site VIE-260B (line which the RNAi line was generated from). Reciprocal crosses were performed for each condition and flies are collected and separated by sex within eight hours of eclosion.

METHOD DETAILS

Clinal patterns

Figures 1A, 1C, and 1D were drawn after reanalysis of raw gas chromatography traces from flies analysed in the multi-population dataset in Chenoweth and Blows.[26] The principal component analysis of log contrast CHC expression was performed via the *prcomp* function in R with scaling to unit variance set to TRUE.[56,57]

Desiccation resistance

A modified version of published procedures[58,59,] was followed in this experiment. Desiccation resistance was measured by enclosing 20 flies of each sex from each F1 genotype in a standard 30ml *Drosophila* vial without any medium. Approximately 3g of fresh Drierite desiccant was suspended on the top end of the vial on cotton gauze. The vial was then sealed off with parafilm to sustain low humidity. Trial runs indicated that this set up reduced humidity in the vial to 10% in 30 minutes. Flies were observed at hourly intervals for death, as indicated by failure to right themselves or to move their legs when their vials were tapped or inverted. Because temperature parameters alone can account for up to 97% of variability in desiccation tolerance,[60] all tests were carried out at 25°C.

Heat shock stress survival

Heat tolerance was measured as survival after a high (potentially lethal) temperature stress[61] at 4-6 days post-eclosion. Twelve replicates of twenty flies for each sex per line cross were enclosed in an empty stoppered vial and placed in a constant temperature cabinet set at 38°C for 30 minutes. Flies were then transferred to fresh media vials and left to recover at 25∘C for 24 hours before being scored for survival.

Competitive mating success

Disparities in male and female mating success between phenotypes were assessed by determining whether a focal *northern* or *common* fly preferred to mate with a *northern* or *common* suitor when potential mates of both types were availed to them. Briefly, individuals were collected as virgins soon after eclosion and held separately by sex in yeasted food vials for four days. On the fourth day, a focal fly (*northern*/*common*) was presented with two flies of the opposite sex, one from its own phenotype (not from the same F1 genotype as the focal fly) and another from a contrasting phenotype. Once copulation commenced, the unsuccessful fly was taken out of the vial and identified. To facilitate identification of the two potential suitors, one of either fly phenotype was slightly wing-clipped. The number of wing-clipped competing flies (left-wing clip and right-wing clip) was kept even between the two phenotypes to balance out any effects of wing clipping on courtship success.

QTL mapping

CHCs were extracted from individual flies by washing each fly in 100mL of hexane in a microvial insert for three minutes and then vortexing for one minute.[62] The CHC samples were run on an Agilent Technologies 6890N gas Chromatograph (Wilmington, Delaware, United States). Individual fly CHC profiles were derived by integrating the area under the following nine peaks using the Agilent ChemStation Software (version Rev B.04.02), in the order of their retention times: 5,9-C_{24}, 5,9-C_{25}, 9-C_{24}, 9-C_{26}, 2-MeC_{26}, 5,9-C_{27} heptacosadiene, 2-MeC_{28}, 5,9-C_{29} and 2-MeC_{30}.[63] All the flies phenotyped were then individually preserved in 95% ethanol and kept in a -80°C freezer pending DNA extraction.

SNP discovery and genotyping for the F2 cross has been previously described.[30] Briefly, SNP genotyping of individual flies on 61 SNPs was carried out on a SEQUENOM© MASS ARRAY platform at the Australian Genome Research Facility (AGRF) using two multiplexes of 30 and 31 SNPs, with approximately 10ng of genomic DNA for each multiplex assay. Out of the 61 markers, three were discarded (s10, s36, and s37) because no difference in genotype calls was detected between the parental lines.

Bulked segregant analysis

A total of 85 *northern* phenotype flies and 85 *common* flies were selected for DNA extraction. Two DNA bulks were made, one containing DNA from *northern* flies and the other DNA from *common* flies, effectively resulting in two types of pooled DNA sample that were expected to differ genetically at the underlying QTL, but were theoretically undifferentiated for all other regions.[64] The sequencing of these DNA pools was technically replicated using two independent sequencing libraries. Phenol-chloroform extraction was used to obtain fly genomic DNA.

Sequencing of the DNA bulks was performed using an Illumina HiSeq 2000 sequencing machine which produced 90 base pair paired-end reads with a median insert size of ~ 490 base pairs. Quality control of the DNA sequence data was performed using fastQC,[46] and no problems were detected. The *D. serrata* reference genome is based on one of the parental lines used in this experiment, FORS4 Allen et al. 2017,[36] so we did we not need to re-sequence this line. We sequenced the CTN42 line so that the parentage of different SNPs could be determined. The parental CTN42 line was sequenced at the Australian Genome Research Facility (AGRF), whereas the F60 samples were sequenced at The Beijing Genomics Institute (Hong Kong, China). Table S1 provides details of the sequencing libraries, numbers of reads and raw coverage for each pool.

Common and *northern* pool sequence reads from this experiment were aligned to a version of the *D. serrata* reference available on NCBI (BioProject: PRJNA355616) that we have scaffolded using Dovetail™ Hi-C technology (Dovetail genomics). The scaffolding greatly increased the contiguity of the assembly from an N50 of just under 1 Mbp[36] to an N50 of 30.3 Mbp. To place the scaffolds onto chromosomes, we used 78 physical and linkage markers for *D. serrata* that have known chromosomal locations.[30] Mapping was performed using the bwa-mem algorithm of the Burrows Wheeler Alignment (BWA) software (version 0.7.12).[47] Samtools[48] was used to convert the reference genome-mapped reads of the DNA sequence pools (generated as output of BWA) to mpileup format for subsequent SNP calling. We then used the PoPoolation2 pipeline[49] to call SNPs and generate final output files with read counts for each variable site in the northern and common pools. To determine the genes residing within the bulked segregant analysis defined QTL interval, we used the *Drosophila serrata* Annotation Release 100 provided by the NCBI Eukaryotic Genome Annotation Pipeline (GCF_002093755.1), which built upon the previously published and annotated genome.[36]

Genome resequencing

Paired-end Illumina HiSeq DNA sequencing was done for each of the DNA samples from the 10 lines at BGI-Hong Kong Co. Limited (China); subsequent analysis of the re-sequenced genomes was conducted as follows. Quality control of the reads was performed using FastQC,[46] no problems were detected. Reads for each line were initially aligned to the *D. serrata* reference genome[36] using the bwa-mem algorithm of the Burrows Wheeler Alignment (BWA) software (version 0.7.12).[47] Local realignment was then performed around indels using the Genome Analysis Tool Kit (GATK, version 2.5-2-gf57256b)[50] following best practices.[65,66] Average depth of coverage across the 10 genomes was 22x. SNPs relative to the reference genome were called for each of the 10 inbred lines using samtools and bcftools.[48,67]

Genotyping the DsFAR2 duplication using short-read data

We were able to determine whether a line contained the *DsFAR* duplication using only short-read data through visual inspection of reads that were mapped to the *D. serrata* reference genome for genome resequencing, which has both copies of the gene using IGV.[51] It was apparent that some lines were missing the duplication containing DsFAR2-A and DsFAR3-A. For these lines, mapping of the region was dominated by reads with an ~8 Kbp insert length.

Population genetic analysis

We estimated Tajima's D[68] to gain insights into the population genetic processes shaping variation around the *DsFAR2-B* locus. The six inbred lines with the northern phenotype were remapped to a version of the *D. serrata* genome that had the ~8 kbp duplicated region masked to improve mapping quality. Mapping details for all other lines are as in the genome resequencing section above. We also performed the same analyses in a random sample of ten lines sampled from the *D. serrata* Genomic Reference Panel (DsGRP)[37] which is a sample from a single endemic population in Brisbane where the *northern* phenotype has not been observed.

In situ hybridisation to adult D. serrata oenocytes

In situ hybridisation to four- to five-day old adult oenocytes were performed with RNA probes as described previously.[69] Probes for *in situ* hybridisation were synthesised from *D. serrata* (UCSD Stock no. 14028-0681.05) cDNA using primers SerCG17560-probeF (5′-TGGCCTGTGCCTGGCACACGGG-3′) and SerCG17560-probeR (5′-GGGATGGTGTGGAAAATTAAGGC-3′) for DsFAR2-A/B (*CG17560*), SerCG17562-probeF (5′-ATGGATACTACCCATATTCAAAAG-3′) and SerCG17562-probeR (5′-ATGGGAGAAACTCGCT GAAGTGCC-3′) for DsFAR1 (*CG17562*), and SerCG14893-probeF (5′-TGGCCAGCATCTGGAAAACGGC-3′) and SerCG14893-probeR (5′-GGTATTGTGTGATAAAAGAAGGC-3′) for DsFAR3-A/B (*CG14893*).

D. melanogaster RNAi and CHC analyses

For quantitation of CHCs, five 5-day old flies were placed in 100μL of hexane spiked with 50μg/mL of C26 as an internal standard and analysed using a Hewlett-Packard (now Agilent; Santa Clara CA) 6890 gas chromatograph (GC) interfaced to an H-P 5973 mass selective detector. The GC was fitted with a DB-17 30m × 0.25 mm i.d. capillary column (J&W Scientific, Folsom CA) with splitless injections and a temperature program of initial temperature 100°C/0 min, 20°C per min to 160°C/0 min, 4°C per min to 280°C, hold 25 min. Detailed methods were described previously.[70] Each individual CHC peak was quantified using peak area.

Phylogenetic relationships of reductases at the DsFAR locus

Amino acid sequences were downloaded from Flybase (www.flybase.com), based on reported homologues from Finet et al.[35] and aligned using MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/). Evolutionary history of these reductases was inferred by using the Maximum Likelihood method and performing bootstrapping and obtain 1000 bootstrapped trees. All analyses were conducted in MEGA X.[52]

QUANTIFICATION AND STATISTICAL ANALYSIS

Desiccation resistance

The time elapsed until 10 flies were dead (completely immobile) was recorded and defined as LT_{50}. As the number of dead flies neared 10, vials were checked for death every 20 min. In total 78 trials were conducted for the *northern* phenotype (females: n = 33, males: n = 45) and 79 trials for the *common* phenotype (females: n = 37, males: n = 42). Desiccation resistance was analysed using mixed effects linear models fitted using the MIXED (Gaussian response variables) procedures of SAS (version 9.3, SAS Inst. Cary, NC). Desiccation resistance was modelled as:

$$Y = \mu + S + C + [S \times C] + [S \times G_C] + \varepsilon \quad \text{(Equation 1)}$$

where, Y was the time taken for 50% of the test flies to die, μ is the intercept, S is the sex of the flies, C is the CHC phenotype of the fly, $G_{(C)}$ is the random effect of genotype nested within CHC phenotype and ε is the random error. When the sexes are analysed together, the experiment is effectively a split-plot design and so line nested within CHC phenotype and an interaction between sex and line within CHC phenotype were also fitted. The error term for the CHC polymorphism effect is ε in this linear model. The sex × genotype interaction was tested via an F-test and within-sex effects via t-tests. Statistical significance was defined as a p-value < 0.05.

Heat shock stress survival

The number of survivors per replicate vial was scored. In total 118 trials were conducted for the *northern* phenotype (females: n = 71, males: n = 47) and 130 trials for the *common* phenotype (females: n = 69, males: n = 61). Heat shock stress survival was analysed using mixed effects linear models fitted using the GLIMMIX (binary responses) procedures of SAS (version 9.3, SAS Inst. Cary, NC). Heat shock stress survival was modelled as:

$$Y = \mu + S + C + [S \times C] + G_C + [S \times G_C] + \varepsilon \quad \text{(Equation 2)}$$

where Y was survival (dead or alive) after 24 hours post-exposure to heat shock, μ is the intercept, S is the sex of the flies, C is the 'northern' status of the flies, G_C is the genotype for the 'northern' status of the flies and ε is the random error. The sex × genotype interaction was tested via an F-test and within-sex effects via t-tests. Statistical significance was defined as a p-value < 0.05.

Competitive mating success

A total of 785 mating trials were conducted; 308 female choice trials and 377 male choice trials. Mating success was analysed using mixed effects linear models fitted using the GLIMMIX (binary responses) procedures of SAS (version 9.3, SAS Inst. Cary, NC). The mating assay data was analysed in males and females separately. Mating success was assessed using a binary mixed model through Proc GLIMMIX as:

$$Y = \mu + SC + FC + [SC \times FC] + G_{(SC)} + G_{FC} + \varepsilon \quad \text{(Equation 3)}$$

where Y was mating success of the focal fly (accepted or rejected), μ is the intercept, SC is the CHC phenotype of the suitor flies, FC is the CHC phenotype of the focal fly, $G_{(SC)}$ is the random effect of genotype nested within the suitor CHC phenotype, $G_{(FC)}$ is the random effect of genotype nested within focal fly CHC phenotype and ε is random error. The interaction was tested via an F-test and within-sex effects via t-tests. Statistical significance was defined as a p-value < 0.05.

QTL mapping

QTL mapping was performed by analysing the CHC polymorphism as a binary trait. The trait 5,9-C_{25} was used to categorise flies as having either the *northern* or *common* phenotype; it is the largest CHC peak in *D. serrata* and is the easiest to score. For QTL analysis, we recoded the data, classifying flies as either *northern* (1) or *common* phenotype (0) based on their 5,9-C_{25} phenotype. 5,9-C_{25} had a clear bimodal distribution in the F2 cross with two distinct phenotypes. A 3:1 segregation ratio could not be rejected using Chi-Square analysis (χ^2 = 2.181, p = 0.14), which is consistent with a single recessive segregating factor. Although the F2 progeny came from a

reciprocal cross that included both male and female offspring, we found no effect of either factor (cross or sex) on variability in the binary phenotype score. Thus, they were not fitted in subsequent QTL analyses. Standard interval mapping in R/qtl[53] was then performed using the *scanone()* function and the model="binary" option.[71] Genome-wide significance thresholds (95%) for the detected QTLs were determined through permutation (n=1000) tests.[72]

Bulked segregant analysis

Read counts for each variable site in the northern and common pools were analysed in R where we assessed genetic differentiation between the pools for each SNP using Fisher's exact tests and a false discovery rate threshold of 5%.[73]

Genome resequencing

As the northern allele is recessive to the common type and we were working with inbred lines, we developed a diagnostic criterion for whether a SNP could be the causal variant. First, SNPs had to be fixed and identical across all *northern* lines. Second, the homozygous genotype of all *northern* lines must differ from any genotype of the *common* lines. Although in the cases of a tri-allelic SNP, it was possible for multiple *common* lines to possess different bases at a potentially causal variant as long as they differed from the *northern* lines, we applied the conservative test of homozygous fixed differences only. This rule was applied and we summed the number of candidate SNPs within 40kbp windows along the genome to look for an overrepresentation of positive hits using R.[57]

Population genetic analysis

Because Tajima's D is affected by both selection and demography, we estimated Tajima's D at all polymorphic exons within the genome using the PopGenome package in R[54] and used this empirical distribution as a point of comparison. The expectation was that a locus under balancing selection would be in the 95th percentile of the empirical distribution, that is, 95% or more of the genome-wide Tajima's D values would be less than the value for the locus. One-tailed significance (p-values) of Tajima's D being a genome-wide outlier was therefore assessed by calculating that proportion of all exons that were greater than the value of interest.