Sexually antagonistic co-evolution at the molecular level predicts phenotypic outcomes of mating interactions.

Alma Thorson[1], Rhonda R. Snook[1,+], R. Axel W. Wiberg[1,*,+]

1. Department of Zoology, Stockholm University, Svante Arrhenius väg 18B, Stockholm, 106 91, Sweden.

*** Correspondence:** raw.wiberg@protonmail.com

+ RAWW and RRS should be considered shared senior authors.

Keywords: population genomics, sexual selection, sexual conflict, co-evolution

Summary

Sexually antagonistic co-evolution (SAC) is a potent driver of phenotypic change, with males and females engaged in cycles of co-evolution in which sex-specific trait expression enhances their own fitness at the expense of the other[1,2]. The cyclical process results in transient mismatches between male and female traits wherin the relative exaggeration of sex-specific phenotypes predicts which sex has the upper-hand during mating interactions[3,4]. Parallel genetic signatures of exaggeration of SAC traits should be observed, but due to a poor knowledge of genes underlying SAC traits this remains untested, impeding understanding of how SAC shapes genetic variation and the phenotypic consequences[5,6]. Here we show the first evidence that SAC theory readily extends to the molecular level and classic patterns of phenotypic SAC are recapitulated. We exploit the iconic sex-peptide (SP) network in *Drosophila melanogaster*, which comprises male seminal fluid proteins and female reproductive tract proteins that interact to influence sexually antagonistic female post-mating behaviours[7,8]. Using genomic data from ~150 populations[9,10], we show that genetic differentiation at loci of the male- and female- components of the SP-network loci co-vary as expected under SAC. We use relative levels of genetic differentiation between male- and female- components of the SP-network to infer which sex has the advantage in mating interactions. Mating and survival assays using populations established from wild-derived isofemale lines[11] show populations with inferred male-advantage have reduced female remating and earlier peaks of egg production compared to populations with inferred female-advantage. Thus, relative levels of genetic differentiation in the classic SP-network with known SAC phenotypes accurately predicts outcomes of mating interactions within populations. Our findings shed light how SAC operates at the molecular level, and sets a standard for future investigations.

Main text

Sexual conflict arises when the fitness optima of the sexes are not aligned[1,2,12,13]. interlocus sexual conflict occurs when different loci in males and females influence the phenotypic value of a trait that depends on contributions from both partners. A classic trait influenced by interlocus sexual conflict is the female remating rate[1,14]. Males benefit when they can prevent future inseminations by rival males, for example by reducing female receptivity to further matings. In contrast, females may gain fitness, either *via* direct or indirect benefits, from controlling when, how often, and with whom to remate[15–19]. This does not preclude strong selection on males to court females aggressively, if the opportunity exists for them to accrue benefits from such courting, and thereby increase the overall female mating rate (e.g. [3,4]; see below). Such conflicts can result in sexually antagonistic co-evolution (SAC), an escalating arms race between the sexes leading to novel and increasingly exaggerated persistence and resistance traits in males and females[2,3,13,20]. On average, males and females should be evenly matched in this arms race, making conflicts difficult to detect[3]. However, a central prediction of SAC theory is that due to the cyclical nature of SAC, transient mismatches can occasionally result wherein for some populations (or species) either males or females have the upper-hand. Which sex has the advantage can be detected from patterns of relative phenotypic exaggeration of male and female traits[2,3].

The most well-documented example of SAC influencing phenotypic traits is among *Gerris* water striders[3,21]. Intense mating struggles between the sexes generates strong selection for male persistence and female resistance morphologies and behaviours[3,21]. Across both species and populations, the relative level of exaggeration between male and female morphologies predicts the outcome of mating interactions, reflecting which sex has an advantage, in agreement with SAC theory[3,4]. Relatively more exaggerated male morphologies result in, for example, shorter pre-mating struggles, and higher male mating success[3,4]. Patterns of co-variation in antagonistic male and female traits in other taxa also likely reflect SAC[13,22–25], making this a compelling hypothesis explaining phenotypic diversity across several systems. As genetic evolution underlies phenotypic

evolution, the logic of SAC should also extend to the genetic level at loci underlying male and female conflict traits. That is, these loci (e.g. [26], and reviewed in [5,27]) should show patterns of co-varying "molecular exaggeration" between male- and female- beneficial loci across populations and species. More critically, the relative degree of "molecular exaggeration" of genes under interlocus sexual conflict should predict which sex has the upper-hand and therefore fitness-related outcomes of mating interactions, just as for phenotypic traits.

However, to-date no studies have tested these predictions. This is because the loci underlying most conflict traits remain unknown (but see [28–30]). Meanwhile, classes of loci proposed to be associated with sexual conflicts and SAC, such as genes encoding male seminal fluid proteins (SFPs), show rapid evolutionary change across species[26,31]. These SFPs, transferred to females during mating, mediate female reproductive physiology, manipulating female traits such as receptivity to further mating attempts, egg production, and survival[27]. While SFPs are relatively well-described genetically with many having known phenotypic functions, their co-evolution with loci underlying female resistance traits is unexplored and how genetic (co-)variation at these loci impacts fitness outcomes of male-female interactions is unknown. Thus, unlike the progress of the "genomics revolution" in understanding the genetics of ecological adaptations over the past quarter century, studies of the genomics of sexually selected and sexual conflict traits have lagged behind[5,6,32]. Empirical tests of SAC theory require integrating genetic (co-)variation at known SAC loci and the consequence of the co-variation for outcomes of mating interactions across multiple populations and species. Such tests are critical for understanding how many loci, and what proportion of those underlying a given trait, are involved in this ubiquitous process in sexually reproducing taxa. And also how sex-specific selection and co-evolution between males and females shapes patterns of genetic variation across populations and species, and what the fitness consequences of this genetic (co-)variation are[5,6,32].

We capitalize on the iconic "sex peptide" (SP) network of *Drosophila melanogaster* to explicitly test SAC theory in a molecular framework for the first time. The SP-network includes a

male component, the SP protein and a suite of other SFPs, that interact with a female component, "sex-peptide receptor" (SPR) and other proteins expressed in the female reproductive tract (FRT), to influence a wide range of female post-mating behaviours[7,8,33,34]. SP has been shown to depress the female remating rate, induce a higher ovulation rate, shorten female lifespan, and reduce reproductive success (reviewed in [7,8]). As such, these traits are likely under sexual conflict and therefore also potentially subject to SAC in *D. melanogaster*[1,14–17,35]. However, whether the evolution of *SP* and *SPR* is driven by SAC is controversial[8,36]. Controversy arises because studies are mixed in whether SP receipt consistently results in reductions in lifespan or lifetime reproductive success for females[8]. Additionally, across species, molecular evolution of *SP* and *SPR* seems uncorrelated[33,36] contrary to SAC theory[1,2]. However, other traits influenced by SP, such as female remating rate and oviposition patterns, may be equally important sources of sexual conflict in natural populations (e.g. [37]). These traits are frequently ignored in assessment of the role of sexual conflict and SAC in SP and SPR co-evolution, as are the other loci of the male-and female-components of the SP-network.

Leveraging the known loci of the male- and female- components of the SP-network, we use genomic data across ~150 natural populations of *D. melanogaster*[9,10] to test co-evolutionary patterns within and between these components. Our analysis determines the relative molecular exaggeration of the male- and female- components in natural populations, which we then use to infer the relative advantage of the two sexes in each population. If SAC is a driver of molecular evolution at these loci, then the relative molecular advantage of each sex should predictably influence female post-mating behaviour. We then test this critical prediction using lab populations established from a resource of isofemale lines[11] established from natural populations that vary continuously in the degree of predicted advantage to one or the other sex.

To assess molecular evidence for SAC in the male- and female- components of the SP-network across *D. melanogaster* populations, we used population genomic data generated by the DEST consortium, which comprises a total of 530 pool-seq samples of ~50 flies from >170

populations world-wide sampled across years[9,10]. We first used the full, recently published DEST v2.0 dataset[10] to establish whether a single pool-seq sample per population could be used for further analysis. To this end, we performed hierarchical Fst analysis in poolfstat[38,39]. Samples from the same population showed overall low within-population Fst (Fsg = 0.01, [0.0096, 0.0104]), compared to between-population Fst (Fgt = 0.04 [0.038, 0.045]), in agreement with previous results[10]. Thus, we chose a single pool-seq sample per population for further analysis, favouring samples with highest coverage, to ensure the highest quality data and estimates. Populations from the ancestral range in Africa were excluded due to very few samples, different sequencing strategies, and a large impact on overall diversity and differentiation so that the final set of samples represented 149 populations (Extended Data Table 1). We next established a data-set of male- and female- components of the SP-network, by partitioning the union of known loci of the SP-network[33,34], on the basis of sex-specific gene expression patterns (Extended Data Table 2 and Extended Data Table 3). We also established a set of 100 randomly selected loci from the rest of the genome as genomic background comparison.

We first evaluated the overall population genetic signature of selection at the loci of the SP-network. Comparative work has found rapid evolution of *Drosophila* SFPs generally, and co-variation in the evolutionary rates across species among the loci of the SP-network in particular[31,33,36]. However, *SP* and *SPR* seem to have uncoupled evolutionary rates across species[33,36]. These patterns are consistent with either relaxed- or positive- selection, and have not been evaluated across populations. Selection (whether positive, in favour of new beneficial alleles, or purifying, against new deleterious mutations) should leave a characteristic signal of reduced non-synonymous diversity (πN) relative to synonymous diversity (πS), comparable to other protein-coding genes subject to selection. We find that nucleotide diversity (π) for SP-network loci is no different from the set of randomly selection loci (figure 1A) and $\pi N/\pi S$ values are well below 1 (figure 1A), patterns of strong evidence of selection. Combined with the observed rapid evolution across species at SP and other SFPs[31,33,36], our results support a process of recurrent selective sweeps. Such a

dynamic genomic signature is expected for SAC traits, in which alleles beneficial to one sex rapidly spread, but which are then countered by new alleles beneficial to the other sex.

We next found strong evidence for co-variation in population genetic differentiation between the male- and female- components of the SP-network from analysis of population-specific Fst estimates of each locus (Phylogenetically corrected 2-Block Partial Least Squares (2B-PLS) analysis[40,41]; r-PLS = 0.44, Z = 4.17, p = 0.001; figure 1B). The male axis of this co-variation loads most heavily for SP network genes, *SP* and *Sems*, while the female axis loads most heavily for *Esp* and *hdly* (Extended Data Table 4). There is relatively low loading of *SPR* on the female axis, which is consistent with the weak patterns of covariation between *SP* and *SPR* observed across species[33,36] (Extended Data Table 4). In summary, across 149 natural *D. melanogaster* populations, our results suggest ongoing selection at SP-network loci, and natural genetic co-evolution between male- and female- components of the network consistent with SAC. However, the pattern of co-variation is weak between the most directly interacting *SP* and *SPR*, and strongest between *SP*, *Sems* and other indirectly interacting loci of the female component of the SP-network (*Esp* and *hdly*). These also show the highest correlations in Fst in simple pairwise tests (Extended Data Fig. 1). Our results highlight that the most obvious candidate loci underlying conflict traits may not always be the strongest drivers of SAC, and that a only a subset of the loci underlying a suite of sexually antagonistic traits may be contributing to SAC. By including multiple interacting loci of SP-network, rather than focusing on the one from each sex, we are able to detect molecular signatures of co-evolution that may otherwise be elusive.

The second main prediction of SAC is that relatively more exaggeration in male- compared to female- traits infer a fitness advantage in sexual conflicts to males and *vice versa*[2,3]. We extend this prediction to the molecular level, defining the relative degree of “molecular exaggeration” as the difference in genetic differentiation between male- and female- components of the SP-network, expressed as a continuous measure using the second principle component (PC2) of the male- and female- axes of co-variation derived from the 2B-PLS[3,4,21] (figure 1B). Higher scores indicate more

exaggeration, or greater genetic differentiation in allele frequencies from the overall average, in the male- compared to the female- component, and thus a more derived state, and an inferred advantage to males. If these patterns of genetic co-variation between components of the SP-network, with previously described phenotypic effects ([7] but see [8]) reflect a process of SAC, then populations with an inferred male-advantage should have: lower female remating rates and an earlier peak in offspring production (faster initial rate of offspring production) than populations with an inferred female-advantage. If SP harms females by reducing lifespan, then we also expect shorter total lifespan and lower survival in populations with inferred male advantage.

We tested these predictions using six populations along the axis of relative genetic differentiation (PC2; figure 1B), representing a continuum from strong inferred male-advantage to strong inferred female-advantage, for which isofemale line resources are available; these represent a small proportion of the original 149 populations[11] (figure 1B, Extended Data Table 5). As our aim was to investigate population-level phenotypic effects, we reconstructed each of the six populations by combining individuals from all isofemale lines for each population in population cages, with three replicate cages per population. Flies within each population cage mated freely (allowing recombination) for 4-8 generations. We then assayed female remating rates in each population by first randomly pairing virgin males and females from the same population in a vial for two hours. Vials in which mating occurred had males removed and, 48 hours later, these mated females were given an opportunity to remate with a new virgin male from the same population for three hours, and we recorded whether females remated. All mated females were transferred individually into new vials 5 times over the next 30 days for a total of 7 vials. Across this time, we measured survival and offspring production/vial and total offspring number.

Remating rates varied across populations, from 14% (Uman, Ukraine) to 40% (Munich, Germany) (figure 2A; Extended Data Table 6; total N = 765, n= 113-140 females per population). Critically, the variation in remating rates confirmed the predictions from SAC theory: populations with relatively more genetic differentiation in the male component of the SP-network, i.e. greater

inferred male-advantage, had lower female remating rates (figure 2A and 2B). Over 94% of the posterior distribution of the relationship between relative genetic differentiation and female remating rates was < 0, indicating a clearly negative relationship, as predicted by SAC theory (figure 2B). The model in which relative genetic differentiation (PC2) as the explanatory variable was represented by the degree of male- and female- advantage was preferred over a model that did not include this explanatory variable ($ELPD_{w/\ term}$ = -399.3 ±14.7, $ELPD_{w/o\ term}$ = -399.4 ±14.6, ELPD difference = -0.1 ±0.7). These effects are even stronger when contrasting female remating rates from populations with the greatest inferred male- and female- advantage, with 99% of the posterior distribution supporting a lower remating rate for populations with an inferred male advantage (Extended Data Fig. 2).

Molecular exaggeration of SP-network loci was also associated with patterns of oviposition as predicted by SAC theory. Male-advantaged populations had peak oviposition earlier than female-advantaged populations (figure 3; Extended Data Fig. 3). This resulted in females from populations with inferred female advantage producing 68% of their total offspring within the first 7 days after mating, on average, compared to 71% for females from populations with inferred male advantage. Male induction of faster egg-laying rates and earlier peaks of offspring production bias paternity to the most recently mated male and may be counter to female interests insofar as they override female-beneficial determination of the timing of reproductive investment[2,42–44].

Contrary to SAC predictions, we found no relationship between molecular exaggeration at SP network loci and total number of offspring (Extended Data Fig. 4). Similarly, while there is strong evidence that survival differs between populations (Cox regression survival analysis; X^2 = 27.03, d.f. = 5, $p < 0.001$; Extended Data Fig. 5A), there was no evidence that this varied based on which sex was advantaged (HR = 1.23, Z-ratio = 1.04, p = 0.3). Moreover, there was no evidence that either female remating status (X^2 = 0.36, d.f. = 1, p = 0.55) or the interaction between remating status and population (X^2 = 4.66, d.f. = 5, p = 0.46) influenced survival. Survival results were

similar in a second experiment tracking survival over ~80 days, while also allowing for continuous mating in groups of 10 males and 10 females (Extended Data Fig. 5B).

By using patterns of natural genetic (co-)variation of loci of multiple male- and female- SP network components across multiple populations, we explicitly test and support the role of SAC. We show that both the female remating rate and egg production timing are predictable based on relative molecular exaggeration of SP-network genes. Our results are in complete agreement with predictions from SAC theory for these traits and extend SAC theory to the molecular level for the first time. Although Interest in *Drosophila* SFPs as mediators of sexual conflict arose because of their apparent toxic effects to females, observed as reduced lifetime reproductive success and survival[7,8], the role of sexual conflict in the (co-)evolution of SP and SPR has been questioned. This is due to inconsistent observations for the expected reductions in female longevity or lifetime reproductive success, leading to speculation that these costs may be condition or environment dependent, or that these traits are not sexually antagonistic at all[8]. Fewer studies have focussed on female remating rates and the timing of offspring production, despite these traits likely also being under sexual conflict and highly relevant in nature[2,18,44]. However, we found no relationship between this exaggeration on total offspring production or lifespan, suggesting that these traits, in fact, are not driven by SAC at these loci.

Our study also addresses critical shortcomings in previous work and illuminates seemingly contradictory results. SAC theory predicts that sex-specific effects will be likely be highly dependent on genotype-by-genotype interactions[37,45] so mixed results across studies using either different populations or inter-population crosses in different ways[37,46] is unsurprising. Our study, by taking an explicitly comparative approach and using within-population crosses, avoids this unpredictability, and instead focusses on patterns of co-evolution between males and females. Additionally, our study also highlights the perils of a specific focus on only genetic variation in, and the direct interactions of, *SP* and *SPR;* the SP-network consists of several other male and interacting female proteins that may play a more important role in the co-evolution between males and females.

We show that effects of molecular exaggeration at SPR is relatively small and that, instead, other loci of the female component of the SP-network is driving female resistance evolution. Not accounting for the effects of variation at these loci will miss much of the relevant biology.

In sum, we extend SAC to the molecular level for the first time. We predict and test outcomes of mating interactions by documenting patterns of natural genetic co-evolution and molecular exaggeration at interacting loci of the classic *Drosophila* SP-network. Our results show that relatively few interacting loci can drive SAC, and predict sexually antagonistic phenotypes, which, coupled with strong selection, may explain the rapid pace of this evolutionary process, at least for some traits and genes. This also offers encouragement to other studies of sexually antagonistic complex behavioural and morphological traits in that, while good knowledge of the relevant interacting loci is needed, not all loci underlying these traits need be accounted for. Our approach offers a standard that can be extended to ongoing work identifying loci underlying conflict traits in other systems to yield important insights into how SAC can shape patterns of genetic variation (e.g. [5,28–30]).

References

1. Parker, G. A. Sperm competition and its evolutionary consequences in the insects. *Biol. Rev.* **45**, 525–567 (1970).
2. Arnqvist, G. & Rowe, L. *Sexual Conflict*. (Princeton University Press, Princeton, N.J., 2005).
3. Arnqvist, G. & Rowe, L. Antagonistic coevolution between the sexes in a group of insects. *Nature* **415**, 787–789 (2002).
4. Perry, J. C. & Rowe, L. Sexual conflict and antagonistic coevolution across water strider populations. *Evolution* **66**, 544–557 (2012).
5. Rowe, L., Chenoweth, S. F. & Agrawal, A. F. The Genomics of Sexual Conflict. *Am. Nat.* **192**, 274–286 (2018).
6. Wilkinson, G. S. *et al.* The locus of sexual selection: moving sexual selection studies into the post-genomics era. *J. Evol. Biol.* **28**, 739–755 (2015).
7. Kubli, E. & Bopp, D. Sexual Behavior: How Sex Peptide Flips the Postmating Switch of Female Flies. *Curr. Biol.* **22**, R520–R522 (2012).
8. Hopkins, B. R. & Perry, J. C. The evolution of sex peptide: sexual conflict, cooperation, and coevolution. *Biol. Rev.* **97**, 1426–1448 (2022).
9. Kapun, M. *et al.* Drosophila Evolution over Space and Time (DEST): A New Population Genomics Resource. **38**, 5782–5805 (2021).
10. Nunez, J. C. B. *et al.* Footprints of worldwide adaptation in structured populations of *D. melanogaster* through the expanded DEST 2.0 genomic resource. *Mol. Biol. Evol.* **42**, msaf132 (2025).
11. Durmaz Mitchell, E. *et al.* Continent-wide differentiation of fitness traits and patterns of climate adaptation among European populations of *Drosophila melanogaster. Evol. Lett.* **9**, 473–490 (2025).
12. Parker, G. A. Sexual selection and sexual conflict. in *Sexual Selection and Reproductive Competition in Insects* 123–166 (Academic Press, New York, 1979). doi:10.1016/B978-0-12-108750-0.50010-0.
13. Perry, J. C. & Rowe, L. The evolution of sexually antagonistic phenotypes. *Cold Spring Harb. Perspect. Biol.* **7**, a017558 (2015).

14. Parker, G. A. Conceptual developments in sperm competition: a very brief synopsis. *Philos. Trans. R. Soc. B Biol. Sci.* **375**, 20200061 (2020).

15. Rundle, H. D., Ödeen, A. & Mooers, A. Ø. An experimental test for indirect benefits in Drosophila melanogaster. *BMC Evol. Biol.* **7**, 36 (2007).

16. Priest, N. K., Galloway, L. F. & Roach, D. A. Mating Frequency and Inclusive Fitness in *Drosophila melanogaster. Am. Nat.* **171**, 10–21 (2008).

17. Priest, N. K., Roach, D. A. & Galloway, L. F. Cross-generational fitness benefits of mating and male seminal fluid. *Biol. Lett.* **4**, 6–8 (2008).

18. Arnqvist, G. & Nilsson, T. The evolution of polyandry: multiple mating and female fitness in insects. *Anim. Behav.* **60**, 145–164 (2000).

19. Fromonteil, S., Marie-Orleach, L., Winkler, L. & Janicke, T. Sexual selection in females and the evolution of polyandry. *PLOS Biol.* **21**, e3001916 (2023).

20. Holland, B. & Rice, W. R. Perspective: Chase-Away Sexual Selection: Antagonistic Seduction Versus Resistance. *Evolution* **52**, 1–7 (1998).

21. Arnqvist, G. & Rowe, L. Correlated evolution of male and female morphologies in water striders. *Evolution* **56**, 936–947 (2002).

22. Tatarnic, N. J. & Cassis, G. Sexual coevolution in the traumatically inseminating plant bug genus *Coridromius*. *J. Evol. Biol.* **23**, 1321–1326 (2010).

23. Rönn, J., Katvala, M. & Arnqvist, G. Coevolution between harmful male genitalia and female resistance in seed beetles. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 10921–10925 (2007).

24. Bergsten, J., Töyrä, A. & Nilsson, A. N. Intraspecific variation and intersexual correlation in secondary sexual characters of three diving beetles (Coleoptera: Dytiscidae). *Biol. J. Linn. Soc.* **73**, 221–232 (2001).

25. Bergsten, J. & Miller, K. B. Phylogeny of diving beetles reveals a coevolutionary arms race between the sexes. *PLoS ONE* **2**, e522 (2007).

26. Swanson, W. J. & Vacquier, V. D. The rapid evolution of reproductive proteins. *Nat. Rev. Genet.* **3**, 137–144 (2002).

27. Sirot, L. K., Wong, A., Chapman, T. & Wolfner, M. F. Sexual Conflict and Seminal Fluid Proteins: A Dynamic Landscape of Sexual Interactions. *Cold Spring Harb. Perspect. Biol.* **7**, a017533 (2015).

28. Khila, A., Abouheif, E. & Rowe, L. Evolution of a Novel Appendage Ground Plan in Water Striders Is Driven by Changes in the Hox Gene Ultrabithorax. *PLoS Genet.* **5**, e1000583 (2009).

29. Crumière, A. J. J. & Khila, A. Hox genes mediate the escalation of sexually antagonistic traits in water striders. *Biol. Lett.* **15**, 20180720 (2019).

30. Pruvôt, C. *et al.* A shared developmental genetic basis for sexually antagonistic male and female adaptations in the toothed water strider. *Evol. Lett.* **9**, 13–23 (2025).

31. Haerty, W. *et al.* Evolution in the Fast Lane: Rapidly Evolving Sex-Related Genes in Drosophila. *Genetics* **177**, 1321–1335 (2007).

32. Kasimatis, K. R., Nelson, T. C. & Phillips, P. C. Genomic Signatures of Sexual Conflict. *J. Hered.* **108**, 780–790 (2017).

33. Findlay, G. D. *et al.* Evolutionary Rate Covariation Identifies New Members of a Protein Network Required for Drosophila melanogaster Female Post-Mating Responses. *PLoS Genet.* **10**, e1004108 (2014).

34. Singh, A. *et al.* Long-term interaction between Drosophila sperm and sex peptide is mediated by other seminal proteins that bind only transiently to sperm. *Insect Biochem. Mol. Biol.* **102**, 43–51 (2018).

35. Smith, D. T., Clarke, N. V. E., Boone, J. M., Fricke, C. & Chapman, T. Sexual conflict over remating interval is modulated by the *sex peptide* pathway. *Proc. R. Soc. B Biol. Sci.* **284**, 20162394 (2017).

36. Hopkins, B. R. *et al.* Decoupled evolution of the *Sex Peptide* gene family and *Sex Peptide Receptor* in Drosophilidae. *Proc. Natl. Acad. Sci.* **121**, e2312380120 (2024).

37. Wensing, K. U. & Fricke, C. Divergence in sex peptide-mediated female post-mating responses in *Drosophila melanogaster. Proc. R. Soc. B Biol. Sci.* **285**, 20181563 (2018).

38. Hivert, V., Leblois, R., Petit, E. J., Gautier, M. & Vitalis, R. Measuring Genetic Differentiation from Pool-seq Data. *Genetics* **210**, 315–330 (2018).

39. Gautier, M., Coronado-Zamora, M. & Vitalis, R. Estimating hierarchical *F* –statistics from Pool–Seq data. Preprint at https://doi.org/10.1101/2024.11.22.624688 (2024).

40. Rohlf, F. J. & Corti, M. Use of Two-Block Partial Least-Squares to Study Covariation in Shape. *Syst. Biol.* **49**, 740–753 (2000).

41. Adams, D. C. & Felice, R. N. Assessing Trait Covariation and Morphological Integration on Phylogenies Using Evolutionary Covariance Matrices. *PLoS ONE* **9**, e94335 (2014).

42. Crudgington, H. S., Beckerman, A. P., Brüstle, L., Green, K. & Snook, R. R. Experimental Removal and Elevation of Sexual Selection: Does Sexual Selection Generate Manipulative Males and Resistant Females? *Am. Nat.* **165**, S72–S87 (2005).

43. Edward, D. A., Fricke, C. & Chapman, T. Adaptations to sexual selection and sexual conflict: insights from experimental evolution and artificial selection. *Philos. Trans. R. Soc. B Biol. Sci.* **365**, 2541–2548 (2010).

44. Eberhard, W. G. *Female Control: Sexual Selection by Cryptic Female Choice*. (Princeton University Press, 1996).

45. Rowe, L., Cameron, E. & Day, T. Detecting sexually antagonistic coevolution with population crosses. *Proc. R. Soc. Lond. B Biol. Sci.* **270**, 2009–2016 (2003).

46. Smith, D. T., Hosken, D. J., Ffrench-Constant, R. H. & Wedell, N. Variation in sex peptide expression in *D. melanogaster. Genet. Res.* **91**, 237–242 (2009).

Figures

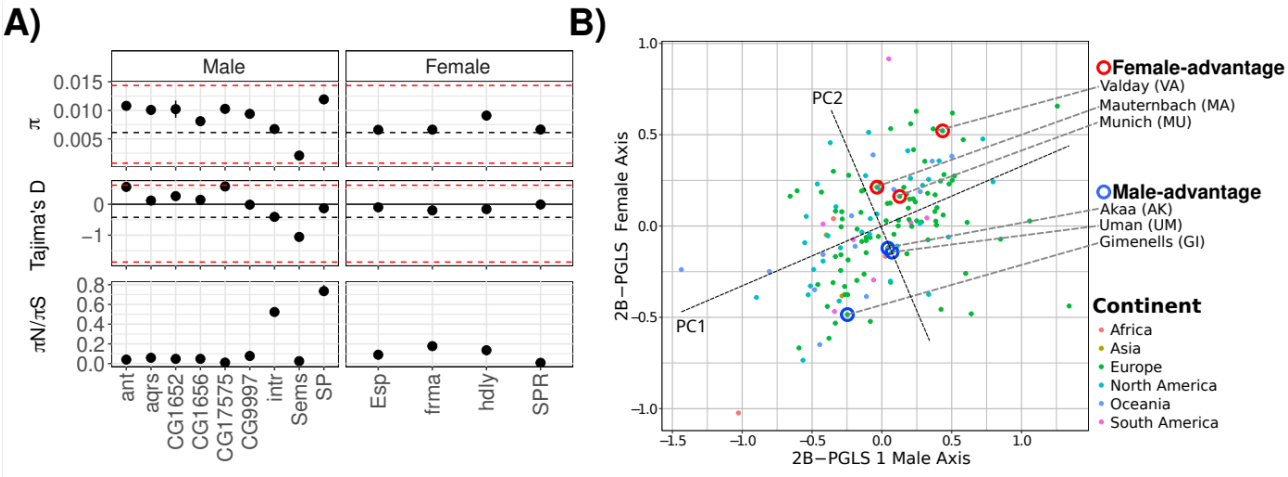

Figure 1. A). Estimates of π (top), Tajima's D (middle), and πN/πS (bottom) for loci in the male- and female-components of the SP-network. Red dashed lines show the maximum and minimum values obtained for a set of 100 randomly sampled genes from the rest of the genome. **B)** Male and female axes of co-variation in Fst at SP-network loci. Points are coloured by their continent of origin. Populations chosen for behavioural experiments, and the sex with inferred advantage are highlighted with coloured circles. The dashed lines labelled "PC1" and "PC2" illustrate the first two principle components from a PCA of the male and female axes of co-variation.

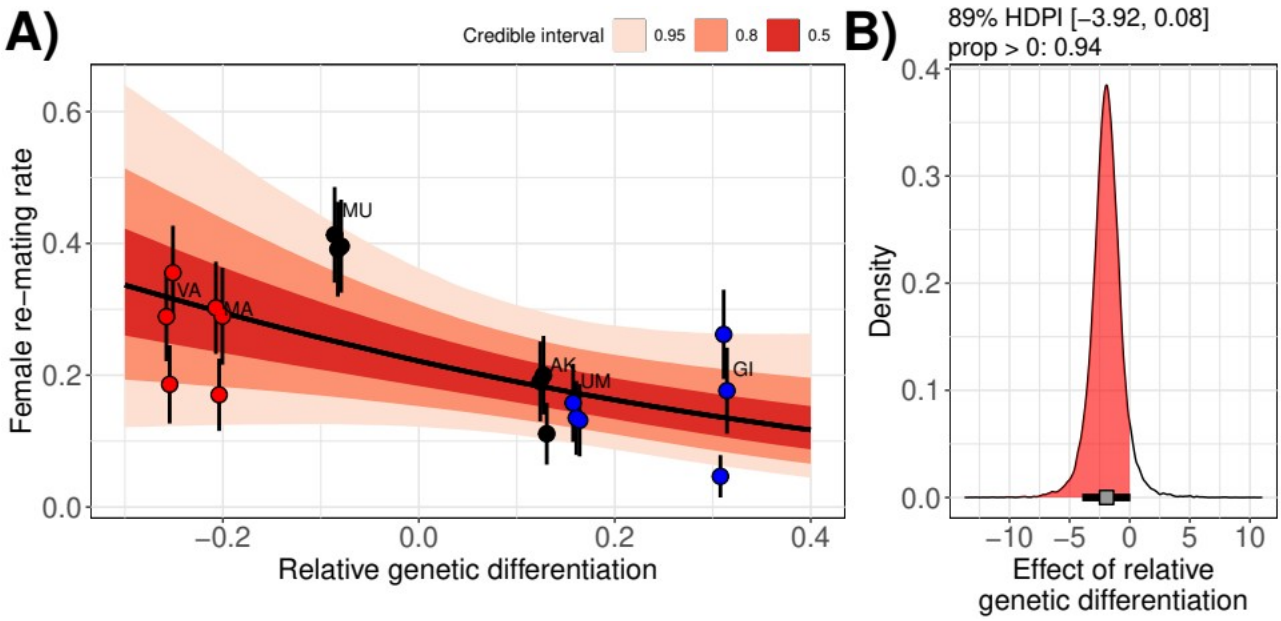

Figure 2. A) Female re-mating rates as a function of a continuous measure of relative genetic differentiation at female- and male- components of the SP-network. Negative values indicate relatively more genetic differentiation in the female- *versus* the male-component. Each individual point is a replicate population cage, points are coloured by which sex is inferred to have an advantage in sexual conflicts (red – female-advantage, blue – male advantage, black - intermediate). The slope and error ribbons represent the posterior mean and credible intervals for the slope. **B)** Posterior distribution of the slope estimate. The area shaded in red indicates the proportion of the posterior distribution with slope estimates < 0, that supports a negative relationship between relative genetic differentiation and the female re-mating rate, the grey box and bar indicate the posterior mean and 89% HDPI respectively.

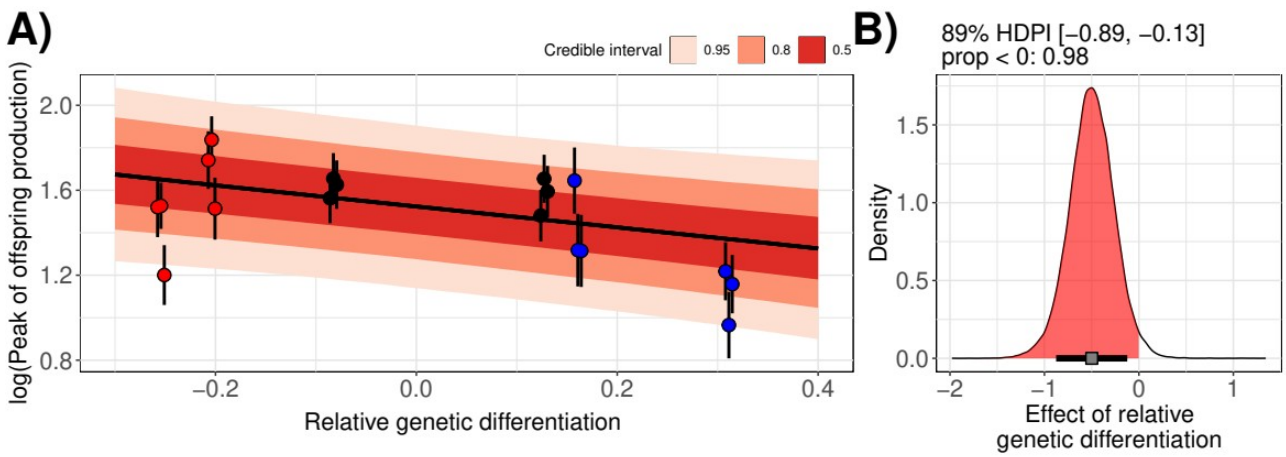

Figure 3. A) The log(number of days) until the peak of offspring production as a function of a continuous measure of relative genetic differentiation at female- and male- components of the SP-network. Negative values indicate relatively more genetic differentiation in the female- *versus* the male-component. Each individual point is a replicate population cage, points are coloured by which sex is inferred to have an advantage in sexual conflicts (red – female-advantage, blue – male advantage, black - intermediate). The slope and error ribbons represent the posterior mean and credible intervals for the slope. **B)** Posterior distribution of the slope estimate. The area shaded in red indicates the proportion of the posterior distribution with slope estimates < 0, that supports a negative relationship between relative genetic differentiation and the peak of offspring production, the grey box and bar indicate the posterior mean and 89% HDPI respectively.

Methods

Genetic data

Population genomic data were obtained from the publicly available DEST dataset (https://dest.bio/)[9,10]. We ascertained that individual samples from each population can serve as representatives on the basis of hierarchical Fst analysis of a neutral set of SNPs with poolfstat[38,39] (see the Supplementary Methods).

A set of interacting loci that make up the SP-network have been described from several studies. Here we use the union of loci described in Findlay et al.[33] and Singh et al.[34], because these represent the current most complete understanding of the system (see also[47]). Several loci are poorly characterised in this context, but all have been shown to be necessary for the observation of SP-network mediated post-mating behaviours (such as the reduced female receptivity to re-mating, oviposition rates), as well as longevity.

These loci can be divided into “male”- and “female”-components of the SP-network based on sex-specific reproductive tissue expression (Extended Data Tables 2 and 3) although this does not necessarily preclude expression of those loci in the other sex. For example, SPR is expressed only in the female reproductive tract (FRT) but is expressed in the nervous system of both sexes (Extended Data Table 3). We consider two different definitions of the SP-network loci, depending on the analysis. Where only coding sites are analysed (πN/πS), we limit ourselves to the coding sequence (CDS) of each gene. In other analyses (Fst, π, and Tajima’s D) we include introns and sites 2,000 bp up-/down-stream of the locus. This is to strike a balance between a focus on the loci of interest while ensuring enough data for accurate population genetic estimates and inclusion of potential regulatory elements. To obtain genomic background estimates as a comparison, we also sampled 100 randomly selected loci from the *D. melanogaster* annotation.

To investigate patterns of genetic diversity and evaluate the role of selection at SP-network loci, population genetic summary statistics were computed (π, Tajima’s D, and πN/πS), with npstat (v. 1.0)[48] and SNPGenie (v. 2019.10.31)[49]. For πN/πS, we summed the mean number of pairwise

(non-)synonymous differences and the number of (non-)synonymous sites, across all populations for each gene. We then computed overall πN/πS for each locus using these sums. To derive confidence intervals we bootstrap sampled populations with replacement and re-computed πN/πS 100 times. We also computed population-specific Fst values (see Supplementary Methods) for each locus in the SP-network with poolfstat (v. 2.1.2)[8].

Analysis of molecular co-evolution

Population-specific Fst estimates (see the Supplementary Methods) were analysed to characterise patterns of co-variation in Fst between male- and female- components of the SP-network. We use 2-Block Partial Least Squares (2B-PLS) to identify male and female axes that describe the largest amount of co-variance in Fst between male- and female- components of the SP-network. 2B-PLS is well suited for analyses of co-evolution across species or populations[40] and has been employed to study complex multivariate morphologies in the context of SAC (e.g.[3,4,21]). 2B-PLS produces two main reduced axes that describe co-variation between the two blocks, as well as individual loading scores for the components of those blocks on the axes. A permutation test of rows within the two blocks tests whether the degree of co-variation is greater than expected by chance. This permutation test conserves relationships between the variables (in this case the loci of the SP-network) within blocks, but should break any signal of co-variation across blocks.

Populations are not independent but genetically related to varying degrees. Such relatedness structure is expected to produce correlations between loci due purely to demographic processes, and should therefore be accounted for in analyses[50–53]. 2B-PLS also integrates phylogenetic or relatedness information using a phylogenetic tree[54]. We first computed a relatedness matrix for all populations using BayPass (v. 2.4)[55] (see the Supplementary Methods) and then converted the summarised omega matrix of BayPass to a dendrogram using the neighbour-joining method in the "ape" R package (v. 5.8)[56]. Phylogenetically corrected 2B-PLS was used to test for and describe the main axes of co-variation between male and female components of the SP-network. 2B-PLS

analysis was performed with the "geomorph" R package (v. 4.0.8)[57,58]. Because the sign of the strongest loadings on both axes was negative, we switched the sign of all values on both axes for ease of interpretation and plotting, such that higher values on the 2B-PLS axes indicated higher differentiation at the loci with strongest loadings on these axes. The main axes of co-variation from the 2B-PLS analysis can be further processed into two principle components by PCA, where the first PC describes the absolute degree of genetic differentiation across both male- and female-components of the SP-network. Meanwhile, the second PC axis describes the *relative* degree of genetic differentiation and therefore provides a continuous measure of the degree of mismatch between males and females[3,21].

Behavioural experiments

We set up stock population cages using isofemale line resources available through the DrosEU consortium[11] (see Supplementary Materials). Briefly, we created three replicate population cages using isofemale lines from each of six natural populations (18 cages in total). For each population, we took 15 males and 15 females of each isofemale line, and split them across the three replicate cages. Populations were allowed to mate freely and recombine for 4-9 generations prior to the experiment. Experimental flies were obtained from each population cage using egg laying plates consisting of a molasses, agar, and water mix (in 1L dH_20: 16.7g agar, 159.7g molasses) set in a small Petri dish lid (4 cm in diameter) and topped with a small amount of yeast paste (in 10mL dH_20: 3g dried yeast) placed in each cage overnight. 100 eggs from each cage were collected and distributed across 5 vials (20 eggs / vial) containing standard fly medium (see Supplementary Materials). These were left to develop for 9-11 days in an incubator (Panasonic MIR-154-PE, Panasonic Healthcare Co. Ltd., 25º C, 12h:12h L:D). On the days of eclosion, virgin flies were collected from each vial throughout the day, ensuring collected flies were no older than 2 hours post-eclosion and thus virgin. Flies were kept in single-sex groups of up to 10 flies in vials containing standard fly medium for 3 days prior to mating and re-mating assays. Collections of eggs

and virgins for mating and re-mating assays were staggered in order to obtain, as far as possible, males of controlled ages. All males were 2-5 days old at the time of mating and re-mating assays.

On the day of the mating assay, a female and male from the same population were loaded into individual vials. We recorded the time at which pairs entered the vial, and both the start and end time of copulation. The assay was run for 2 hours from the time of the last pair entering the vial. Flies that did not mate in that time were discarded (~3-12% of all flies across populations). There was no relationship between the percentage of “failed” pairings and the relative degree of genetic differentiation (Pearson’s correlation: cor = -0.17, t = -0.34, d.f. 4, p = 0.75). Flies that were observed to mate later than 2 hours after pair formation were censored and discarded. Mated males were removed from their vial and females were left to lay eggs for 2 days. A female re-mating assay was performed the second day after the mating assay in which each female was transferred to a new vial and paired with a new male from the same population of approximately equal age to the initial male to isolate the effect of re-mating. We again recorded the time at which pairs entered the vial, and, if re-mated, copulation start and end times. To account for female re-mating refraction, we ran the assay for 3 hours from the time of the last pair entering the vial, pairs that were observed to mate later than 3 hours after pair formation were censored and discarded.

We collected data on two fitness related traits for each female – mortality and egg production. Mortality was assessed for each female up to 30 days after the first mating day. All vials were checked every day (except weekends) and the date of death recorded for each female. Females that died due to handling error or escaped were censored as were all females that survived past the 30-day cut-off. Throughout the experiment, females were provided with fresh vials of fly medium on days 2, 4, 7, 11, 17, and 24 after the mating assay, producing 7 vials of offspring in total. Total reproductive success was scored by counting offspring from each vial after 14 days, to allow ample time for the majority of offspring to hatch, pupate, and eclose as adults. Total reproductive success, was measured as either the total offpsring in vials 2 and 3, or the total offspring across all 7 vials. This was done to keep a maximum amount of data, as some females died during the experiment and

did not have an opportunity to lay eggs in later vials. We also measured timing of peak offspring production computing the time, in days, until the vial with the maximum number of offspring. The full experiment was conducted over five batches to obtain suitable sample sizes and batch was included as a random factor in analyses.

In a second mortality experiment, experimental flies were collected from each cage using egg laying plates as above. Upon collection of emerged adults, virgin flies were distributed across vials in groups of 10 females and 10 males per vial. Vials were then kept in a controlled temperature room (25º C, 12h:12h L:D) for the duration of the experiment. Survival of females was checked every day, except weekends, until less than 10% of flies from each cage were remaining.

See the Supplementary Materials for the details of all statistical analyses.

Data and code availability

Data that support the findings of this study have been deposited in Zenodo with the accession code:10.5281/zenodo.17520634

References

47. McGeary, M. K. & Findlay, G. D. Molecular evolution of the sex peptide network in *Drosophila*. *J. Evol. Biol.* **33**, 629–641 (2020).
48. Ferretti, L., Ramos-Onsins, S. E. & Pérez-Enciso, M. Population genomics from pool sequencing. *Mol. Ecol.* **22**, 5561–5576 (2013).
49. Nelson, C. W., Moncla, L. H. & Hughes, A. L. SNPGenie: estimating evolutionary parameters to detect natural selection using pooled next-generation sequencing data. *Bioinformatics* **31**, 3709–3711 (2015).
50. Stone, G. N., Nee, S. & Felsenstein, J. Controlling for non-independence in comparative analysis of patterns across populations within species. *Philos. Trans. R. Soc. B Biol. Sci.* **366**, 1410–1424 (2011).

51. Castillo, D. M. Factors contributing to the accumulation of reproductive isolation: A mixed model approach. *Ecol. Evol.* **7**, 5808–5820 (2017).

52. Chelini, M. C., Brock, K., Yeager, J. & Edwards, D. L. Environmental drivers of sexual dimorphism in a lizard with alternative mating strategies. *J. Evol. Biol.* **34**, 1241–1255 (2021).

53. Raas, M. W. D. & Dutheil, J. Y. The rate of adaptive molecular evolution in wild and domesticated *Saccharomyces cerevisiae* populations. *Mol. Ecol.* **33**, e16980 (2024).

54. Adams, D. C. & Felice, R. N. Assessing Trait Covariation and Morphological Integration on Phylogenies Using Evolutionary Covariance Matrices. *PLoS ONE* **9**, e94335 (2014).

55. Gautier, M. *et al.* Estimation of population allele frequencies from next-generation sequencing data: pool-versus individual-based genotyping. *Mol. Ecol.* **22**, 3766–3779 (2013).

56. Paradis, E., Claude, J. & Strimmer, K. APE: Analyses of Phylogenetics and Evolution in R language. *Bioinformatics* **20**, 289–290 (2004).

57. Baken, E. K., Collyer, M. L., Kaliontzopoulou, A. & Adams, D. C. geomorph v4.0 and gmShiny: Enhanced analytics and a new graphical interface for a comprehensive morphometric experience. *Methods Ecol. Evol.* **12**, 2355–2363 (2021).

58. Adams, D., Collyer, M., Kaliontzopoulou, A. & Baken, E. Geomorph: Software for geometric morphometric analyses.

Acknowledgements

We are grateful to the DrosEU and DrosRTEC consortia for their heroic efforts of sampling, sequencing, and establishing of isofemale line resources of *Drosophila melanogaster*, and also for helpful feedback. In particular we are grateful to Thomas Flatt, Pau Carazo, Mathieu Gautier for advice in the planning of experiments; to Élio Sucena for maintaining and shipping us isofemale lines. We are also grateful to Nadeem Naber, Caja Samsen, and Matilde Stella for help in the lab during experiments and to Luc Bussiere and Mike Ritchie for comments that improved the manuscript. The computations and data handling was enabled by resources provided by the National Academic Infrastructure for Supercomputing in Sweden (NAISS), partially funded by the Swedish Research Council through grant agreement no. 2022-06725. This work was funded by a Starting Grant from the Swedish Research Council (Vetenskapsrådet) [grant no. 2022-03701], and a European Commission Marie Sklodowska-Curie Actions Independent Postdoctoral Fellowship [grant no. 101061664] to RAWW, as well as a Project Grant from the Swedish Research Council (Vetenskapsrådet) to RRS [2018-04598].

Author contributions

R.A.W.W. contributed with the conception of the study, funding acquisition, data collection and analysis, and led the writing of the manuscript. A.T. contributed with experimental design, collection and analysis of data, R.R.S. contributed with the conception of the study and analysis, funding acquisition, and writing of the manuscript.

Competing interest declaration

The authors declare no competing interests.

Additional information

The online version of this article contains supplementary material. Correspondence and requests for materials should be addressed to R. Axel W. Wiberg.