Report

Current Biology

Positive genetic covariance between male sexual ornamentation and fertilizing capacity

Highlights

- Genetic covariance among traits is a requirement for indirect selection to operate
- In *Drosophila bipectinata*, ejaculate traits coevolve with a secondary sexual trait
- Sexual trait size is positively genetically correlated with fertilizing ability
- Postcopulatory sexual selection may magnify net selection on sexual trait size

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In Brief

Using artificial selection, transcriptomic profiling, and laser phenotypic engineering, Polak et al. document a positive genetic correlation between a Darwinian precopulatory "ornament," the male sex comb in *Drosophila bipectinata*, and competitive fertilizing ability. Two models potentially explain how such genetic covariance may evolve.

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Positive genetic covariance between male sexual ornamentation and fertilizing capacity

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SUMMARY

Postcopulatory sexual selection results from variation in competitive fertilization success among males and comprises powerful evolutionary forces that operate after the onset of mating.[1,2] Theoretical advances in the field of sexual selection addressing the buildup and coevolutionary consequences of genetic coupling[3–5] motivate the hypothesis that indirect postcopulatory sexual selection may promote evolution of male secondary sexual traits—those traits traditionally ascribed to mate choice and male fighting.[6,7] A crucial prediction of this hypothesis is genetic covariance between trait expression and competitive fertilization success, which has been predicted to arise, for example, when traits subject to pre- and postcopulatory sexual selection are under positive correlational selection.[8] We imposed bidirectional artificial selection on male ornament (sex comb) size in *Drosophila bipectinata* and demonstrated increased competitive fertilization success as a correlated evolutionary response to increasing ornament size. Transcriptional analyses revealed that levels of specific seminal fluid proteins repeatedly shifted in response to this selection, suggesting that properties of the ejaculate, rather than the enlarged sex comb itself, contributed fertilizing capacity. We used ultraprecise laser surgery to reduce ornament size of high-line males and found that their fertilizing superiority persisted despite the size reduction, reinforcing the transcriptional results. The data support the existence of positive genetic covariance between a male secondary sexual trait and competitive fertilization success, and suggest the possibility that indirect postcopulatory sexual selection may, under certain conditions, magnify net selection on ornamental trait expression.

RESULTS AND DISCUSSION

Darwin[7] proposed the theory of sexual selection to account for the evolution of male weaponry and extravagant ornamental displays seen throughout the animal kingdom. He defined sexual selection as "the advantage which certain individuals have over other individuals … in exclusive relation to reproduction" and believed it to arise wholly from differences in male fighting and mate attraction; that is, from *pre*copulatory sexual competition. Although enormously successful in explaining major trends in animal evolution,[6,9,10] Darwin's theory nevertheless was incomplete because it failed to recognize that sexual selection also operates during and/or after insemination.[11] This omission perhaps is unsurprising, because in Darwin's day it was generally assumed that female promiscuity—the acceptance by a female of more than one sexual partner during a reproductive cycle—was rare among animals, and hence unimportant in evolution.[12]

Female promiscuity, however, is taxonomically widespread and far more prevalent than previously believed.[12,13] Importantly, when females mate with multiple males, and when this behavior results in the overlap of ejaculates from different males that vie for fertilizations, powerful selective forces may be triggered within the female reproductive tract in the form of sperm competition and "cryptic" female choice.[8] These and related processes are evolutionary drivers of a variety of reproductive traits closely tied to insemination and fertilization; viz., aspects of genital morphology,[14] chastity enforcement mechanisms such as mating plugs and the guarding of the female by the male,[15] sperm form and related female anatomies,[16,17] and seminal plasma composition.[18,19] Despite intense research in the field,[8,20] it remains an open question whether this selection can also propel the evolution of male secondary sexual traits—traits whose functions are ascribed exclusively to the precopulatory arena.

The mechanism that we expect could actuate such evolution is indirect selection, a ubiquitous form of selection that operates when alleles for a given trait evolve owing to their coupling with other alleles that are the direct targets of selection.[21,22] Indirect selection is a foundation stone of popular coevolutionary models of sexual selection, the Fisherian and "good genes" processes,[6] which require strong genetic covariation between the male ornamental trait and female preference, or between male trait and viability, respectively.[3,23] In the present context, for indirect postcopulatory sexual selection to promote secondary sexual trait

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Figure 1. Interspecific variation in sex comb size and geometry

Sex combs in a sample of four *Drosophila* species within the *melanogaster* species group of the subgenus *Sophophora*, illustrating interspecific diversification in the size and shape of this sexual ornament, typical of secondary sexual traits in other animal groups.[6] *D. bipectinata* and *D. malerkotliana* are closely related taxa belonging to the *bipectinata* complex.[28,29] All flies from Thailand, Khao Sok region, Phanom District. Determinations and images (with a Leica M205 stereomicroscope, Leica Microsystems, Buffalo Grove, IL) by M. Polak, University of Cincinnati.

evolution, positive genetic covariation between trait expression and male competitive fertilization success is required. Whereas positive *phenotypic* correlations between ornament expression and relevant ejaculate characteristics have been predicted,[24] and observed in some species[25] such as guppies, *Poecilia reticulata*,[26] convincing demonstrations of the crucial prediction of genetic covariance are lacking.[27]

Here, we present evidence for positive genetic covariation between a secondary sexual trait and competitive fertilizing capacity, focusing on the male sex comb in *Drosophila bipectinata*. Within the genus *Drosophila*, the sex comb is comprised of strong, heavily melanized bristles or "teeth" on the front tarsal segments of males (it is absent in females) and exhibits striking and rapid evolutionary diversification among species (Figure 1),[30] a signature feature of secondary sexual traits in other animal groups.[6] Sex comb size (as tooth number) in *D. bipectinata* is undergoing incipient biogeographic differentiation throughout the species' range and among closely related taxa.[31,32] It is known to be the target of precopulatory sexual selection in some field populations[31] where males with larger combs independently of body size enjoy a mating advantage over their smaller-combed counterparts, an effect unlikely to be solely a function of differential grasping ability.[33] Males grasp females with their sex combs and press them against either side of the female's abdomen before the onset of copulation, at which stage the combs in *D. bipectinata* may deliver the sensory (tactile) signals that influence the female mating response in favor of a larger comb. Whereas comb size as tooth number has been linked to mating success in the wild, other comb attributes may play a role as well. For example, in *D. melanogaster*, mutations in the *yellow* gene reduce mating success likely as a result of structural deficits in the sex comb.[34] In addition to being the target of precopulatory sexual selection, sex comb size in *D. bipectinata* has previously been shown to exhibit family-level covariation with competitive fertilization success.[35] Sex comb size is condition dependent[36,37] and significantly heritable within natural populations.[31,38] Thus, the *D. bipectinata* sex comb (Figures 2A and 2B) shares key features with ornamental traits of animals in general[6,39,40] and is a suitable model for the evolutionary analysis of such traits.

The present work is predicated upon the study of correlated responses to artificial selection; measurement of correlated responses to selection is an established quantitative genetics tool for uncovering the existence of genetic correlations among traits.[3,22,41] We exerted bidirectional artificial (truncation) selection for comb size independently of body size in replicate lines of *D. bipectinata* derived from a common, field-fresh base population from Taiwan. Comb size responded to selection strongly and consistently across all three replicates in both the "up" and "down" directions over 11 consecutive generations of selection (Figure 2C). At the terminus of selection, divergence in comb size was highly significant ($F_{2,6.392}$ = 144.418; $p < 0.0001$; Table S2A), showing 58.5% divergence in tooth number between low ($\bar{x}$ ± SE; 10.589 ± 0.258 teeth) and high (16.781 ± 0.258 teeth) lines. Control, unselected lines (n = 3) exhibited intermediate mean comb size (13.424 ± 0.273 teeth). Realized heritability estimates (±SE; Table S2B) averaged across the high and low lines were 0.451 ± 0.0395 and 0.434 ± 0.0371, respectively, and comparable to an independent estimate from a New Caledonian population.[38] Male body size did not differ between high and low treatments post selection ($F_{2,6.164}$ = 1.024; $p = 0.413$), indicating that it did not exhibit a correlated evolutionary response. This outcome was expected as selection pressure was expressly applied on the ornament independently of body size to decouple these traits during the course of selection. As condition (an index of nutritional history) can influence ejaculate traits,[42] and since body size reflects condition in holometabolous insects,[43] we deemed it appropriate to control for body size during the course of

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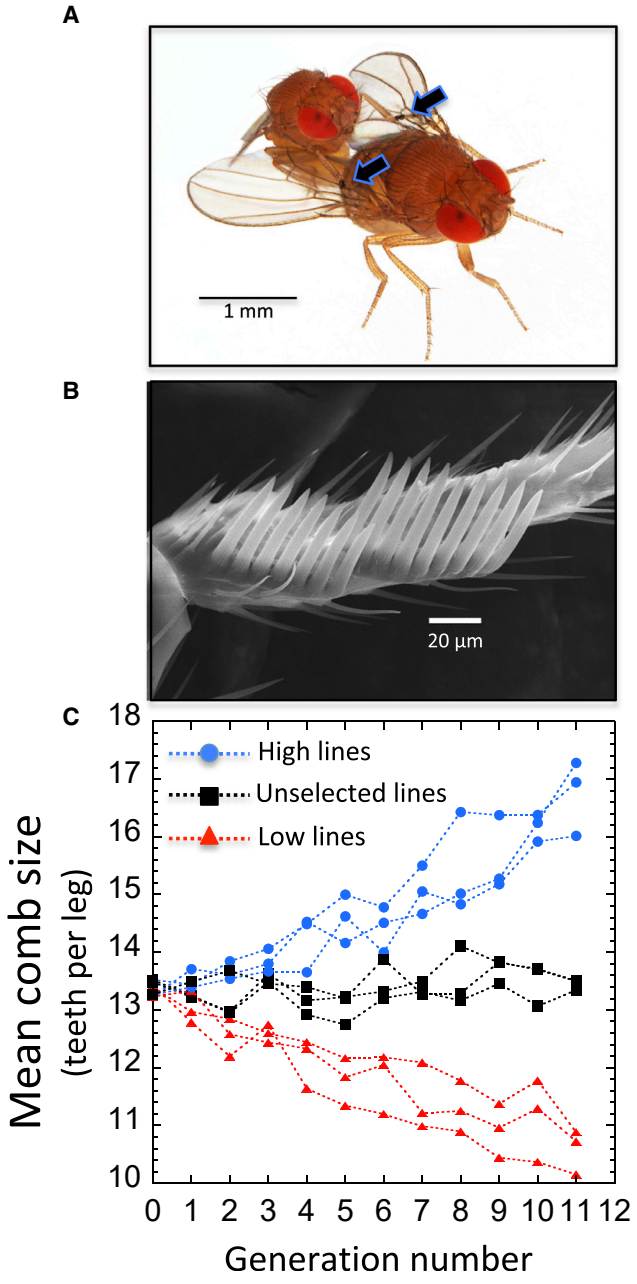

Figure 2. The sex combs in *D. bipectinata* and results of bidirectional artificial selection on sex comb size

(A) Mating pair with male sex combs indicated by arrows.

(B) Environmental scanning electron micrograph (650×) of the sex comb, consisting of two rows of teeth on the metatarsus (16 total teeth in this specimen).

(C) Steady and progressive divergence in sex comb size resulting from 11 consecutive generations of bidirectional artificial selection for body-size-specific comb size (as teeth per leg) (see Tables S1 and S2A). Trait values for unselected lines remained virtually unchanged, confirming minimal genetic drift effects on the trait over the course of selection. Heritability estimates are provided in Table S2B.

selection so that any correlated change in fertilizing capacity could more confidently be attributed to the genetic evolution of the sex trait per se. We note that this procedure, however, would not control for correlated shifts in a narrower set of potential condition factors not captured by body size variation.[39]

Competitive fertilizing success of all lines (n = 9) was measured post selection. Base population females were first each mated to a base population "competitor" male that had been previously irradiated using a ^{60}Co source to allow offspring paternity to be assigned. Females were then mated a second time to either a high, low, or control male, and the proportion of progeny sired by the second male (P_2) calculated for each mating. In 5% of cases (n = 8 out of 148), second matings yielded zero fertilizations by the second male (where $P_2 = 0$), likely as a result of "dry" copulations, that is, failures of the second male to transfer any ejaculate. The frequency of zero P_2 values was slightly but significantly ($p < 0.05$) overrepresented among low lines (see STAR methods), suggesting that selection for smaller comb size resulted in impaired ability to transfer sperm. Among the second matings that yielded fertilizations, high-line males had significantly greater P_2 than low-line males (Figure 3A). There was a significant positive effect of male body size (measured as thorax length) on P_2 (Figure S2; Table S3), but no statistical interaction between selection treatment (i.e., high, low, and control categories) and male body size ($F_{2,106.8} = 0.914$; $p = 0.404$). By using females and males sourced from different populations in this experiment, sperm competition outcomes could not be the result of coevolved genetic associations between the sexes.[16] We also tested whether high-line females coevolved higher remating rates; if this were the case, and even though line females were not used in the P_2 determination assay, a coevolutionary increase in female remating rate in high lines could have intensified post-copulatory sexual selection in these lines, potentially driving the shift in fertilization capacity of high-line males we detected. This also does not appear to be the case, however, as propensity to remate did not differ between high- and low-line females (Figure S3). Collectively, these results provide evidence for a positive genetic correlation between a male secondary sexual trait and competitive fertilization success.

We conducted a transcriptional comparison of high and low lines for insight into the putative cause of the enhanced fertilizing capacity. The adult males used in this investigation were 4 days old, similar in age to those used to propagate each new generation of the artificial selection experiment and for P_2 determination. Among 45 differentially regulated genes, three that encode male seminal fluid proteins (SFPs) were consistently upregulated in high lines (Figure 3B; Data S1A). Two of these SFPs are serine proteases previously linked to male fertility.[44,45] Another encodes sex peptide, a seminal protein known to bind to sperm; when released, sex peptide binds targets in the female reproductive tract and nervous system.[46,47] qPCR was used to validate the expression of SFPs, showing that all had increased expression in the high lines compared to the low lines (Data S1B); there was no evidence for increased SFPs in the low lines. A further comparison of these lines[48] found that the viability of sperm, a trait that may be modulated by SFPs,[49] showed enhancement in the high lines. Differences among lines in sperm viability were evident for sperm sampled from the female reproductive tract (the ventral receptacle), but not from the male seminal vesicles (where sperm have yet to be combined with accessory gland products), consistent with the finding that SFPs were

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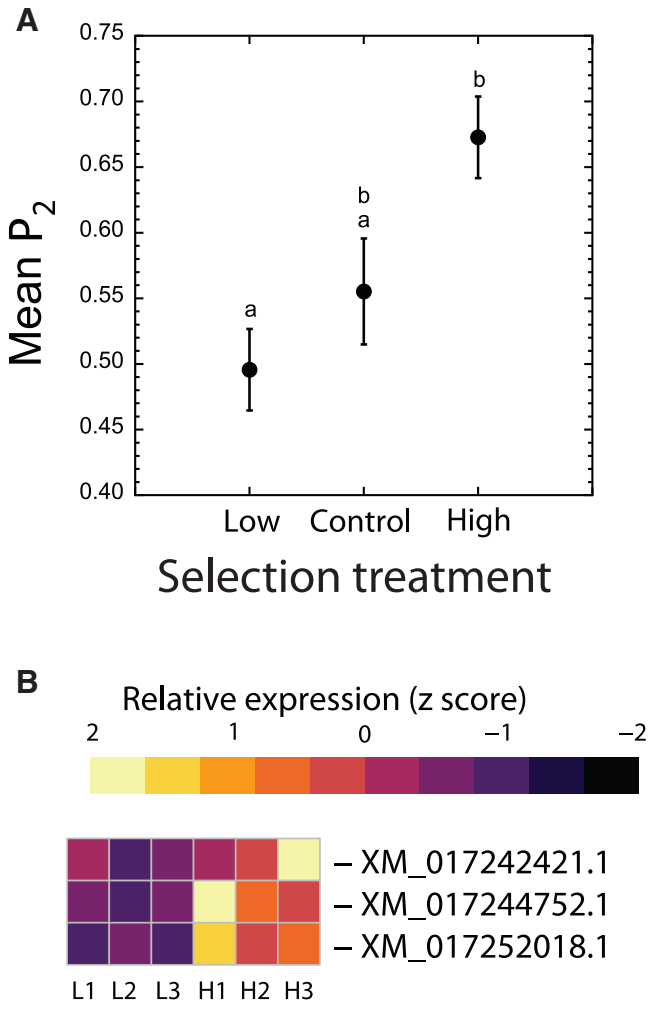

Figure 3. Fertilizing advantage of high-line male *D. bipectinata*

(A) Mean (±1 SE) competitive fertilization success (P_2) across selection treatments. Means not sharing a letter are significantly different ($\alpha < 0.05$). P_2 (proportion) data were analyzed using a restricted maximum likelihood mixed model with replicate line (treated as a random effect) nested within selection treatment (Table S3) and appropriate covariates (Figure S2). Results were confirmed using a generalized linear mixed model with a binomial data structure.

(B) Of 45 genes with significant differential expression between high (H1–H3) and low (L1–L3) lines (Data S1A), increased transcript levels for predicted seminal fluid proteins (SFPs) identified with NCBI (GenBank) gene identification codes are shown (see Data S1B).

increased in high lines. No statistical differences between high and low lines in other ejaculate investment traits, including testis size and sperm number, were found.[48]

At least two mechanisms could explain the correlated shift in fertilizing superiority of high-line males. The sperm competition hypothesis proposes that high-line males coevolved superior competitive fertilization success, resulting from a positive genetic correlation between sexual trait size and specific aspects of the ejaculate. Alternatively, the direct stimulation hypothesis posits that females selectively use sperm of high-line males in response to enhanced sensory inputs received from a relatively large sex comb. Whereas the transcriptional results favor the sperm competition hypothesis, to discriminate these competing hypotheses experimentally, we performed a manipulative test with an ultraprecise surgical laser,[50] where we phenotypically altered high-line males to resemble low-line males in comb size (Figures 4A–4D; Tables S4A and S4B). The sperm competition hypothesis predicts that the fertilizing advantage of surgically treated high-line males would persist, whereas the direct stimulation hypothesis predicts that the fertilizing advantage would be reduced. The relative fertilizing superiority of high line persisted post surgery (Figure 4E; Table S4C), aligning with the transcriptional results in favor of the sperm competition hypothesis. It remains a possibility that a correlated response to selection on comb size involved other changes, such as to aspects of male genital morphology,[51] copulatory courtship behavior,[52] female preference,[53] or ecological ("magic") traits,[54] that conceivably could have influenced fertilization. However, what we know about the function of semen components[44] suggests that the enhanced fertilization success came about through shifts in ejaculate quality, as pointed to by the transcriptional results.

How might positive genetic covariance between ornament and fertilizing capacity become established in natural populations? We first recognize that genetic covariance between traits may result from epistasis, linkage, or pleiotropy (Bell, p. 167[55]), and from this starting point suggest two scenarios. The first is based on the theoretical notion of a relationship between fitness-related traits and the underlying physiological state or condition of an organism.[56,57] Condition dependence of secondary sexual traits is well established in many systems,[6] and there is growing evidence for condition dependent expression of postcopulatory traits as well.[42] Since high genetic variance for condition is expected,[56] positive genetic covariance between the ornament and competitive fertilization success could thus arise when sets of variable genes for condition exert pleiotropic effects on the two traits.[3]

According to a second model, the development of genetic covariance is promoted when the most highly adorned (sexually attractive or coercive) males in the population on average experience greater levels of sperm competition, which would favor alleles conferring higher "fertilizing power"[58] in males with the most sexually successful phenotypes. In *Drosophila* and other species where females store sperm, the most attractive males' ejaculates may encounter an intensified sperm-competitive environment when such males induce previously inseminated females to mate sooner in their sperm use cycle than less attractive males; or in other words, are more efficient at overcoming nonvirgin female resistance to mate. The ejaculates of the most successful males in this way could, on average, encounter a greater density of non-self sperm in storage, favoring ejaculatory traits conferring superior "offensive" competitive capability. In *Drosophila*, when a male mates with a previously inseminated female, release of previous sperm is initiated even before the sperm of the second male begins to enter storage, suggesting that the release may be triggered by SFPs of the second male, although copulatory courtship may also play a role in inducing the release of stored sperm.[52] Theoretical models indicate that increased intensity of sperm competition may promote the evolution of increased ejaculate expenditure,[59] and evidence from a

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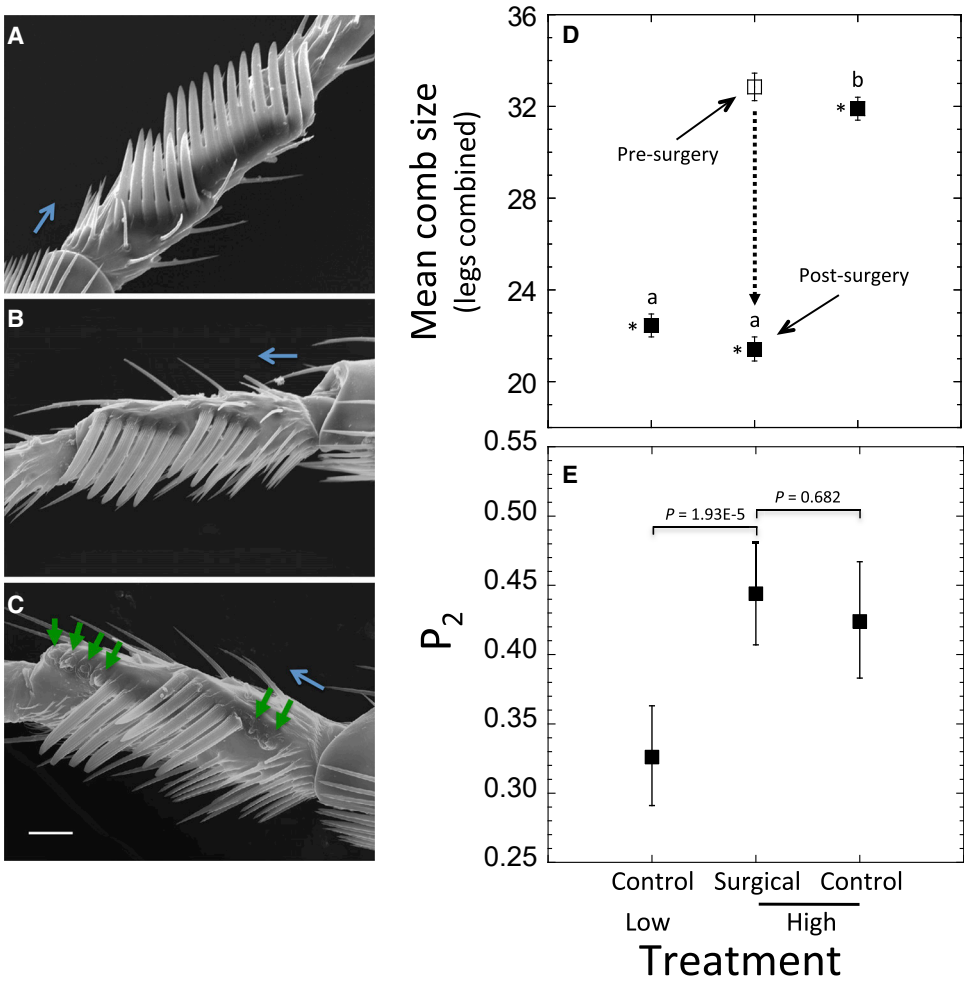

Figure 4. Results of the phenotype engineering experiment

Environmental scanning electron micrographs (650×) of male sex combs in *D. bipectinata*, showing an exemplar of (A) an intact high-line sex comb (6 teeth in C1, 11 in C2, 17 total), (B) an intact low-line sex comb (4 teeth in C1, 6 in C2, 10 total), and (C) a high-line sex comb reduced in tooth number using laser surgery. Green arrows indicate insertion points of six previous teeth. Blue arrows point distally. Scale bar, 20 μm.

(D) Mean comb size (as total tooth number per male) (±1 SE) of the three experimental groups in the laser phenotypic engineering experiment. Sex comb sizes before and after surgery of a group of high-line males are shown (see Tables S4A and S4B). Asterisks indicate the groups subjected to P_2 determination.

(E) Mean (±1 SE) P_2 of the three experimental groups demonstrating that high-line surgical males maintained their relative fertilizing superiority over low-line males despite sharply reduced ornament size via laser surgery (Table S4C). p values are from specific linear contrasts (low versus surgical high, $\chi^2 = 18.253$, d.f. = 1, p = 1.934×10^{-5}; surgical high versus control high, $\chi^2 = 0.167$, d.f. = 1, p = 0.682).

variety of species confirms that males are indeed capable of remarkably fine-scale adjustments in ejaculate characteristics (in both sperm traits and chemical composition of the seminal fluid) in response to differing levels of risk and intensity of sperm competition.[18,42,59] In fowl *Gallus gallus*, dominant and subordinate males face different levels of sperm competition and tailor their ejaculates accordingly.[60] In neriid flies *Telostylinus angusticollis,* high-condition males elevate rate of ejaculate transfer only when perceiving risk of sperm competition, that is, when the male is second in the mating sequence.[61]

According to this second model, which we are inclined to favor as the basis for the genetic correlation reported herein, co-occurrence of alleles for sexual trait attractiveness and superior ejaculate potency in males confers disproportionately high fitness, and positive epistasis[62] results in the buildup of linkage disequilibrium between them in the population.[63] The resultant genetic covariation could, ceteris paribus, engender further evolutionary change in the "original Darwinian" precopulatory trait under conditions of a shifting postcopulatory fitness landscape, so long as the genetic coupling between segregating factors for trait and fertilizing power is sustained. We note that the evolutionary mechanisms through which any positive genetic correlation becomes established in a particular species will surely vary, and in the present case remain unknown. Moreover, the above scenarios for how this covariance could arise, though conceptually distinct, are not mutually exclusive. Here, we have used a set of integrated tests leveraging leading-edge experimental techniques to establish the existence of positive genetic covariance between secondary sexual trait size and fertilizing capacity in a well-characterized insect model.

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STAR★METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

M.P. designed the research, contributed to conducting the research, analyzed the data, and wrote the manuscript. J.L.H.-G. contributed to the design of the research and conducted experiments. K.J.H. contributed to data collection, artificial selection, and line maintenance. J.B. conducted RNA-seq, bioinformatics analyses, and data interpretation. F.T. conducted female remating experiments and data analysis.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
TRIzol reagent	ThermoFisher	15596026
Ambion DNase	ThermoFisher	AM2222
DyNAmo cDNA Synthesis Kit	ThermoFisher	F470L
KiCqStart SYBR Green qPCR ReadyMix	SigmaAldrich	KCQS00
Deposited data		
Raw fertilization success data	This study	https://doi.org/10.5061/dryad.37pvmcvjd
Experimental models: organisms/strains		
Drosophila bipectinata Duda	Wild population, Taiwan	N/A
Oligonucleotides		
Tubulin-F 5′ TCGTAACTTGGACATTGAGC 3′	This study	N/A
Tubulin-R 5′ GGAATTCAGTCAGATCCACG 3′	This study	N/A
XM_017244752.1-F 5′ TTCAATGGTGGCATCTCAAG 3′	This study	N/A
XM_017244752.1-R 5′ TAGATTTAGTCGGCACCACT 3′	This study	N/A
XM_017252018.1-F 5′ ATTGCTCTCTCCATATCCGG 3′	This study	N/A
XM_017252018.1-R 5′ AAGCCGTTGAAGTGACATTT 3′	This study	N/A
XM_017242421.1-F 5′ CCATTTGTGCAGAGGAGTTT 3′	This study	N/A
XM_017242421.1-R 5′ GATCCATTGCAGCCATTGTA 3′	This study	N/A
Software and algorithms		
CLC Genomics Workbench	QIAGEN	https://digitalinsights.qiagen.com/
FastQC	69	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
Trimmomatic	70	http://www.usadellab.org/cms/?page=trimmomatic
Flybase	Flybase	https://flybase.org/
OrthoDB10	OrthoDB	https://www.orthodb.org/
R 3.6.1	R Core Team	https://www.r-project.org/
coxme	R package	https://cran.r-project.org/web/packages/coxme/index.html
glmm	R package	https://cran.r-project.org/web/packages/glmm/index.html
JMP® Pro Version 14.0.0	SAS	https://www.jmp.com/en_us/software/predictive-analytics-software.html
Drosophila bipectinata genome	NCBI	https://www.ncbi.nlm.nih.gov/genome/?term=txid42026[orgn]
pheatmap	R package	https://cran.r-project.org/web/packages/pheatmap
Other		
Leica M205 Stereomicroscope	Leica	https://www.leica-microsystems.com/
Agilant Bioanalyzer 2100	Agilant	https://www.agilent.com
Illumina Eco quantitative PCR system	Illumina	https://www.illumina.com/documents/documentation/user_guide/Eco_System_User_Guide_15017157_F.pdf
Vector 532-1000-20 Q-switched laser	Coherent	https://www.coherent.com/
IX71 inverted light microscope	Olympus	https://www.olympusamerica.com/

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RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Michal Polak (polakm@ucmail.uc.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The data pertaining to the main conclusions of the study are available at Dryad: https://doi.org/10.5061/dryad.37pvmcvjd. Other datasets are available from the lead author upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

A large outbred base population of *Drosophila bipectinata* Duda (Diptera: Drosophilidae) was established in the laboratory with 300 field-caught females and an approximately equal number of males captured from the surface of fruit substrates in Taiwan (25°2′30.24″ N, 121°36′39.37″ E).

Prior to commencing artificial selection, the base population was mass bred in the laboratory for 4 generations in an environmental chamber under controlled light and temperature conditions (12 h light (24°C):12 h dark (22°C)). We consider this number of generations sufficient to "wash out" field-environmental/maternal influences on phenotype, with negligible consequences for genetic parameters.[64] Flies were cultured in 10 half-pint glass culture bottles, containing standard cornmeal-agar medium, and seeded each new generation with 30 adult females and 30 males. Maintaining sexual selection in the population was desired to limit loss of linkage disequilibrium and genetic correlations.[3] Flies were allowed to reproduce in culture bottles for 36-48 h, after which time adults were removed. Ample pupations sites were provided.

METHOD DETAILS

Bidirectional artificial selection

Artificial selection was exerted on body-size specific comb size for 11 consecutive generations in three replicate "high" lines (increasing comb size) and three replicate "low" lines (decreasing comb size) simultaneously. Three control, unselected, lines were maintained in parallel to the selection lines throughout the experiment. Thus, the selection program generated 9 distinct genetic lines originating from a common base population recently derived from the wild. For each selected line, 105 males were individually anesthetized one at a time with CO_2, and under an Olympus SZX12 stereomicroscope the number of teeth in both comb segments (C1 and C2) on both legs were counted, and thorax length measured with an ocular micrometer. We statistically related comb size data to thorax length using general linear models (linear models were always appropriate), and the GLM residuals were extracted and sorted. Selection for increasing comb size was applied by choosing the 30 males with the largest residual comb size in each line, and for decreasing comb size by choosing the 30 males with the smallest residual comb size. Critically, therefore, the protocol selected on sex comb size independently of condition,[56] as body size in insects strongly reflects nutritional history.[39,43] The 30 selected males in each line were paired with 30 randomly chosen virgin females from within their respective lines, and cultured in bottles containing standard cornmeal-agar food. Females were allowed to lay eggs for 36-48 h in a first culture bottle, and then transferred to a fresh bottle for an additional 36 h of reproduction. This procedure maintained consistent and moderate larval densities across bottles and lines. Ample pupation sites were provided.

Heritability of sex comb size

Response to bidirectional selection was tracked in all lines (Table S1), and realized heritability (h^2) of sex comb size were determined for each line as twice the regression slope relating cumulative response on selection differential.[22,65] Mean realized heritability was calculated separately for high and low lines. The standard error of each estimate was taken as the empirical standard error, estimated directly from the variance of the replicate estimates.[66] Three control lines were propagated each generation with exactly the same numbers of flies as selected lines. To estimate the effects of drift over the course of selection on the focal trait,[67] we tracked comb size in control lines by measuring comb size and thorax length in a random sample of 30 males from each of the three control lines each and every generation of the selection regimen.

Competitive fertilization success

We assayed the competitive fertilization success of males in the 9 lines (three high, three low, three control lines) using a standard sterile male technique[15,68] optimized for *D. bipectinata*.[35] Two blocks of this experiment were performed immediately after the terminus of selection, with all 9 lines assayed simultaneously in each block. Time blocks were conducted at generations 12 and 13. Lines were reared in multiple replicate culture bottles under density-controlled conditions described above.

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In each block, 5-day old virgin females sourced from the base-population were each first mated to a 3-day old base-population virgin male that had been irradiated at 24 h of age with a 150 Gy sublethal dose of gamma radiation from a ^{60}Co source at the University of Cincinnati. These first males, referred to as irradiated (IR) males, were donors of "defensive sperm," which are able to fertilize eggs, but the zygote dies and fails to hatch into a larva as a result of lethal mutations. Previous work with *D. bipectinata*[35] has demonstrated that this dose is ideal to achieve essentially complete sterility of the males. To achieve matings with IR males, we followed the procedure in.[35] Briefly, virgin females were individually introduced to food vials on the evening of their 4th day of age, and upon turning on the lights the next morning, randomly selected IR males were individually loaded into vials. Vials were continually scanned in sequential order. Copulation duration was determined as the time from the onset of mating to when the pair disengaged.

Females that had mated to IR males were individually housed in oviposition vials (containing an agar-grape juice substrate), and all females were transferred to fresh oviposition vials every 2 d. All eggs deposited by females were counted, and referred to as "pre-P_2 eggs." On day 5 after mating with the IR males (females were allowed 5 d to lay eggs), females were individually mated to 4 d old test males sourced from the different lines. In each block an approximately equal number of test males were taken from 4 culture bottles of each selection line. In block 1, there were 20 test males per selection line, for a total of 120 test males (6 lines x 20 males). From each control (i.e., unselected) line in block 1, 2-3 males were chosen from each of two bottles of each line. In block 2, 3-4 test males were randomly sourced from each of 4 bottles of each selection line, for a total of 90 selection-line males (6 lines x 15 males). For each control line, 3-4 males were sourced from each of 3 bottles.

All doubly-mated females were transferred to a fresh vial containing oviposition substrate and allowed to lay eggs for 24 h, after which time all eggs were counted. The proportion of eggs that hatched into larvae (P_2) was attributed to the second male to mate.[15] The frequency distribution of P_2 values is provided in Figure S1. Copulation duration, sex comb size (as tooth number) of IR and test males, and thorax length (mm) (as an estimate of body size) of all males and females were measured. For females that mated twice, mean copulation durations (s.e.) for first and second matings were 10.61 (0.22) min (n = 146), and 9.32 (0.20) min (n = 144), respectively.

Female remating

Virgin females were sourced from high and low lines and housed at a density of 10 flies in vials containing standard cornmeal medium and active yeast until their first mating. Virgin males were sourced from the base population and likewise housed in groups of 10 flies per vial with standard food (without yeast), then separated into individual food vials the evening prior to mating trials. All females were 5 d old at their first mating. All males were 6 d old when first exposed to females. The experiment was conducted across two successive blocks, both starting with 150 mating pairs, with high and low line females equally represented.

Females were added to the males' vials and allowed 3 h to mate. All males that successfully mated were preserved in ethanol for later characterization. Females were housed in vials containing an oviposition medium, and transferred every 2 d onto fresh food until their second mating. All eggs laid during this period were counted.

Females were exposed to their second males after 2 d. Due to their reluctance to re-mate, three attempts were made to mate the females, using the same males at each attempt. These were spaced at two-day intervals, and so the interval between the first and second matings ranged from 2 to 6 d. The time that each of the females was exposed to a male during these successive attempts was recorded. Latency to re-mate was calculated as the time (in minutes) a female was exposed to a male before onset of copulation, summed across successive attempts. Males and females were preserved in ethanol for later sex comb size (males) and thorax length determination.

Transcriptional comparisons

Males were reared under density-controlled conditions as above, and aged in standard cornmeal food vials for 3 d. On their third day, total RNA was extracted with TRIzol reagent (Life Technologies) and RNA quality was examined with an Agilant Bioanalyzer 2100. DNA was removed through DNase treatment (Turbo DNA-free, Ambion) according to manufacturer's protocols and quality of the remaining RNA was assessed with an Agilant Bioanalyzer 2100. cDNA libraries are prepared with a Illumina TruSeq cDNA synthesis kit. Each library was barcoded to distinguish each library within a single lane and poly-A purified to increase the proportion of mRNA. Sequencing was performed at Cincinnati Children's Hospital Medical Center (CCHMC) Genetic Variation and Gene Discovery Core. Illumina sequencing machines at this facility can generate at least 180-200 million reads per lane. Six barcoded samples per lane, yielding ~25-35 million reads per sample. Read files have been deposited to the NCBI SRA archive under the Bioproject PRJNA607084.

Data quality of the RNA-seq sets was assessed using FastQC.[69] Ambiguous or low quality reads were trimmed or removed through the use of Trimmomatic[70] or CLC Genomics (CLC Bio). The predicted CDS sequences for *D. bipectinata* (version 2.0, GCF_000236285.1) were acquired from the *Drosophila* modENcode project and NCBI.[70] RNA-seq reads were mapped to the predicted genes through the use of CLC Genomics with with 80% coverage and two nucleotide mismatches for each read mapped. Differentially expressed genes were determined using the RNA-seq package of CLC Genomics with 40% of each read matching the gene at the level of 90% with no more than two mismatches. Significance was noted with an EDGE test followed by a false detection rate of 0.05 based on comparisons among all genes.[71] These analyses indicated that 45 genes were differentially expressed between high and low lines. Functional annotation of the genes was accomplished using tblastx (E-value cut-off of 1e-3) to a previously annotated to *D. melanogaster* gene sets from FlyBase.[72,73] Orthology analysis was accomplished through the use of OrthoDB10.[74]

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Specifically, we compared our set to male associated genes identified in *D. melanogaster* based on previous proteomic and transcriptomic studies that examined expression in males and male reproductive organs.[75–77] On the basis of these criteria, three genes associated with male reproduction have differential expression between the selected and control lines (Data S1A). Most of the differentially expressed genes are spread across multiple genomics scaffolds and the orthologs in *D. melanogaster* are spread across different chromosomes and arms.

Quantitative PCR of male-associated genes

To validate genes of interest, we used quantitative PCR to measure the expression of the three genes associated with male fertility. Methods were based on our previously developed methods.[78] RNA was extracted as described previously for independent biological replicates from those used in the RNA-seq analyses. DyNAmo cDNA Synthesis Kit (Thermo Scientific) was used to generate complementary DNA (cDNA). Each reaction used 300 ng RNA, 50 ng oligo (dT) primers, reaction buffer containing dNTPs and 5 mmol•l-1 MgCl2, and M-MuLV RNase H^+ reverse transcriptase. KiCqStart SYBR Green qPCR ReadyMix (Sigma Aldrich) along with 300 nmol l-1 forward and reverse primers, cDNA diluted 1:20, and nuclease-free water were used for all reactions. Primers were designed using Primer3.

qPCR reactions were conducted using an Illumina Eco quantitative PCR system. Three biological replicates were examined for each line. Expression levels were normalized to tubulin using the DDCq method. Fold change was compared between control and selected lines (Data S1B), which verified that the three SFPs consistently express increased transcript levels in the high line males compared to their low line counterparts. The expression of tubulin was consistent between the RNA-seq samples (less than 5% difference among samples), indicating that this is a quality housekeeping gene for our qPCR. The expression changes based on RNA-seq analysis of the three SFPs were compared to those based on qPCR with the use of the Pearson correlation coefficient (Data S1B).

Laser phenotypic engineering

The laser surgical protocol is described in detail elsewhere.[50] Briefly, males were collected as virgins, and at 24 h of age, anesthetized under a light, humidified stream of CO2 in an acrylic (plexiglass) chamber with a thin glass bottom. The male was positioned ventral side down in the chamber, so the sex combs were visible from below and accessible to the laser light. The chamber was mounted on a Prior (Rockland, MA, USA) H117 motorized stage fitted to an Olympus (Center Valley, PA, USA) IX71 inverted light microscope. Individual pulses of laser light (λ = 532 nm) from a Vector 532-1000-20 Q-switched laser (Coherent, Santa Clara, CA, USA) focused through an Olympus UPlanApo 20x objective were used to ablate individual sex comb teeth one at a time.

We generated three treatment groups, for which P_2 values were determined as described above. One group (“Surgical High”) consisted of high-line males whose sex combs were phenotypically engineered with the laser to approximately match comb size of low line males. Surgery was performed by ablating teeth from both C1 (first comb segment) and C2 (second comb segment). Teeth were ablated one at a time by directing a single laser shot to the base of each tooth. For C1, teeth were removed in the distal direction along each front tarsus, while C2 teeth were removed in the proximal direction. Table S4A provides details on the magnitude of the comb size reduction in this group, in terms of the number of teeth removed from each comb segment per male. The two groups whose sex combs were *not* altered consisted of both high line surgical control males (“Control High”) and low line surgical control males (“Control Low”). Males in these groups were handled in a similar manner to the combs-altered group, and had an approximately equal number on large non-sex comb bristles on the foretarsi of the males removed with laser shots. Table S4B provides average comb size and n’s of the three experimental groups whose competitive fertilizing ability was assayed. The P_2 assay was conducted as previously described except that 4 days elapsed between a female’s first and second matings. In the assay, the initial number of treatment males paired with non-virgin females previously mated to IR males was 95. Of these, 75 females mated with treatment males. One female of these 75 subjects failed to lay any eggs after her second mating and was discarded.

QUANTIFICATION AND STATISTICAL ANALYSIS

Response to artificial selection

To test the effectiveness of the artificial selection program on sex comb size divergence, we analyzed comb size data with a REML (restricted maximum likelihood) mixed model using JMP® Pro[79] at the terminus of the 11 generations of selection. Selection treatment (high versus low) and replicate line (nested within treatment and treated as a random effect) were entered as explanatory factors, and thorax length, the measure of body size,[80] as covariate (Table S2A). Effect of selection treatment on male thorax length was evaluated with a REML mixed model with selection treatment and replicate line treated as above.

Competitive fertilization success

Variation in competitive fertilization success of the second (test) male was modeled with two approaches, using REML mixed models and generalized linear mixed models (glmm’s). REML models were conducted in JMP® Pro[79] and glmm’s in R[81] implemented with the ‘lme4’ package.[82] Prior to these analyses, we first eliminated cases (n = 8) for which $P_2 = 0$, as zero values may often be the result of failure to transfer ejaculate by the second male and therefore not reflect sperm precedence as an outcome on inter-ejaculate dynamics.[83,84] The distribution of zero values was: 6 cases in low lines, 1 case in high lines, and 1 case in control lines, representing a marginally significant overrepresentation of zero values in low lines ($\chi^2 = 6.23$, df = 2, $0.025 < p < 0.05$). In our first analysis, we constructed a REML mixed model on fertilization success expressed as the proportion (P_2), calculated as the number of hatched eggs

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deposited by a given doubly mated female divided by the total number of eggs laid. P_2 values were not arcsine-square root transformed, as the distribution of untransformed values (Figure S1) provided an adequate fit to the normal (Shapiro-Wilk W = 0.979, p = 0.032) and marginally better than for transformed values (Shapiro-Wilk W = 0.96, p = 0.0016). The REML model contained the following terms: time block (1 and 2, fixed effect), selection treatment (high, low and control, fixed effect), replicate line (1, 2 and 3, treated as a random effect and nested within selection treatment), and the following mean-centered[85] covariates: pre-P_2 eggs (the total number of eggs laid between the female's first and second mating), sex comb size of the IR male, thorax length of the IR male, thorax length of the test male (male 2), copulation durations with the IR and test males. Block ($F_{1,118.6}$ = 0.0214, p = 0.884) and the block-by-treatment interaction ($F_{2,118.6}$ = 1.915, p = 0.152) did not explain a significant portion of paternity share variation, so they were removed from the model and not considered further. Also examined was the selection treatment-by-thorax length of the test male interaction, which was not significant ($F_{2,106.8}$ = 0.914, p = 0.404), and excluded. Finally we note that the number of eggs laid by the doubly mated females did not predict P_2 values ($F_{1,120.39}$ = 0.0007, p = 0.9785). Plotted residuals (e_i's) against predicted values ($\hat{y}$'s) formed a roughly horizontal band around the zero line and revealed no outliers; residuals were normally distributed (Shapiro-Wilk W = 0.99, p = 0.56).

In a second approach, we modeled variation in paternity using a generalized linear mixed model with a binomial error structure and logit link function, where the response variable was the number of eggs that hatched for each female and the total number of unhatched eggs deposited the binomial denominator. This analysis has the advantage of accounting for variation in sample size associated with individual paternity share (P_2) values. Results were qualitatively identical to the REML model, identifying the same explanatory terms with significant effects, including the effect of Selection treatment ($\chi 2$ = 46.14, df = 2, p < 0.0001), of primary interest here. As above, inspection of the plot of residuals against predicted values showed no outliers, and resisuals were normally distributed (Shapiro-Wilk W = 0.99, p = 0.53). Given the similar model outcomes, we report only the results of the REML model (Table S3).

Female remating

All analyses of female remating data were conducted in R.[81] A female's propensity to re-mate was assessed using a Cox proportional hazards survival analysis (*coxph:* R 'survival' library).[86] A mixed effects model (*coxme*: R 'coxme' library),[87] which included the nested structure of the experimental design (replicate selection line nested within selection treatment) as well as experimental block as random effects did not provide a better fit compared to one excluding this structure. Therefore we continued without the addition of random effects, using *coxph.* Latency to re-mate was entered as the response. Females that did not re-mate after the three attempts were excluded from the analysis. Female treatment ('high' or 'low), female thorax size, and number of eggs laid within two days of first mating were entered as explanatory variables, as well as all two- and three-way interactions. Male thorax lengths and male comb sizes were also entered as explanatory variables. All terms were tested for conformity to proportional hazards assumptions. Significance of terms was determined through likelihood ratio tests. All terms were non-significant (p > 0.1). The proportion females yet to remate over time is plotted in Figure S3.

Laser phenotypic engineering

We first tested for an overall effect of treatment on competitive fertilization success using a generalized linear model with a binomial error structure and logit link function, where the number of fertilized eggs laid by each female after her second mating was the response and the total number of eggs laid the binomial denominator. The factor in the final model was treatment (laser treated high-line males, control high-line males, and control low line males), and the following mean-centered covariates: pre-P_2 eggs, thorax length of the IR male, thorax length of the treatment male (i.e., a given female's second male), and copulation durations of IR and treatment males (Table S4C). This experiment was designed to evaluate whether the lines expressing genetically enlarged combs but whose combs were surgically reduced in size would maintain their fertilizing superiority over low line males. Therefore, our post hoc procedure consisted of 2 focal contrasts which were sufficient to evaluate the prediction: One contrasted fertilization success of the surgically altered high line males versus control of high line males, and the second contrasted surgically altered high line males versus control low line males. Statistical models were run in JMP®.[79]

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Supplemental Information

Positive genetic covariance between male sexual ornamentation and fertilizing capacity

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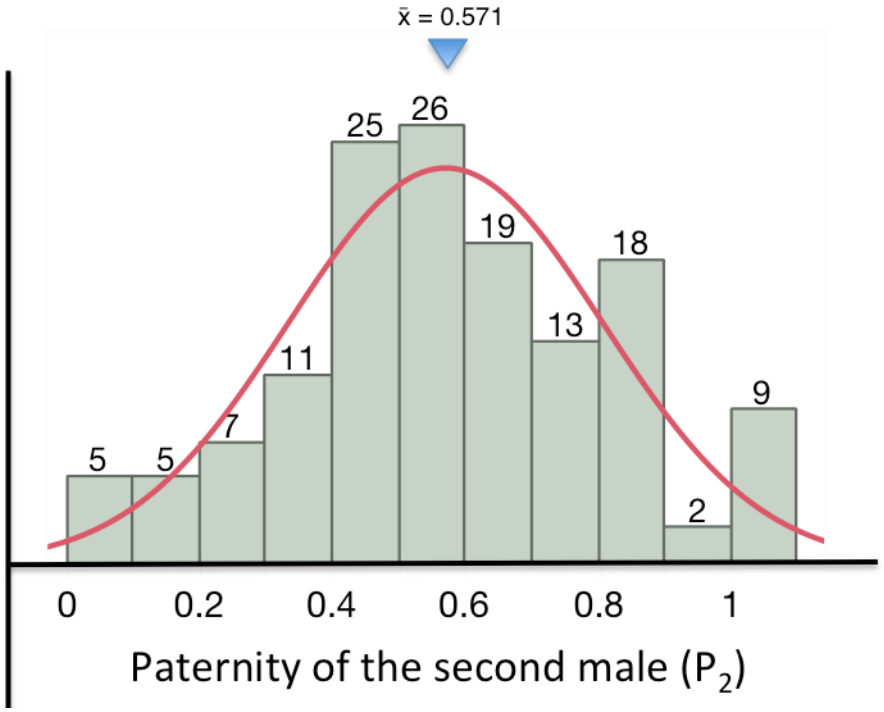

Figure S1. Frequency distribution of observed paternity share (P_2) values, related to STAR Methods.

Data pertain to the competitive fertilization success experiment testing for differences among Selection treatment categories post-selection. Red line is the expected Normal distribution for the data. The observed data fit reasonably well to the Normal (Shapiro-Wilk W=0.979, *P*=0.032).

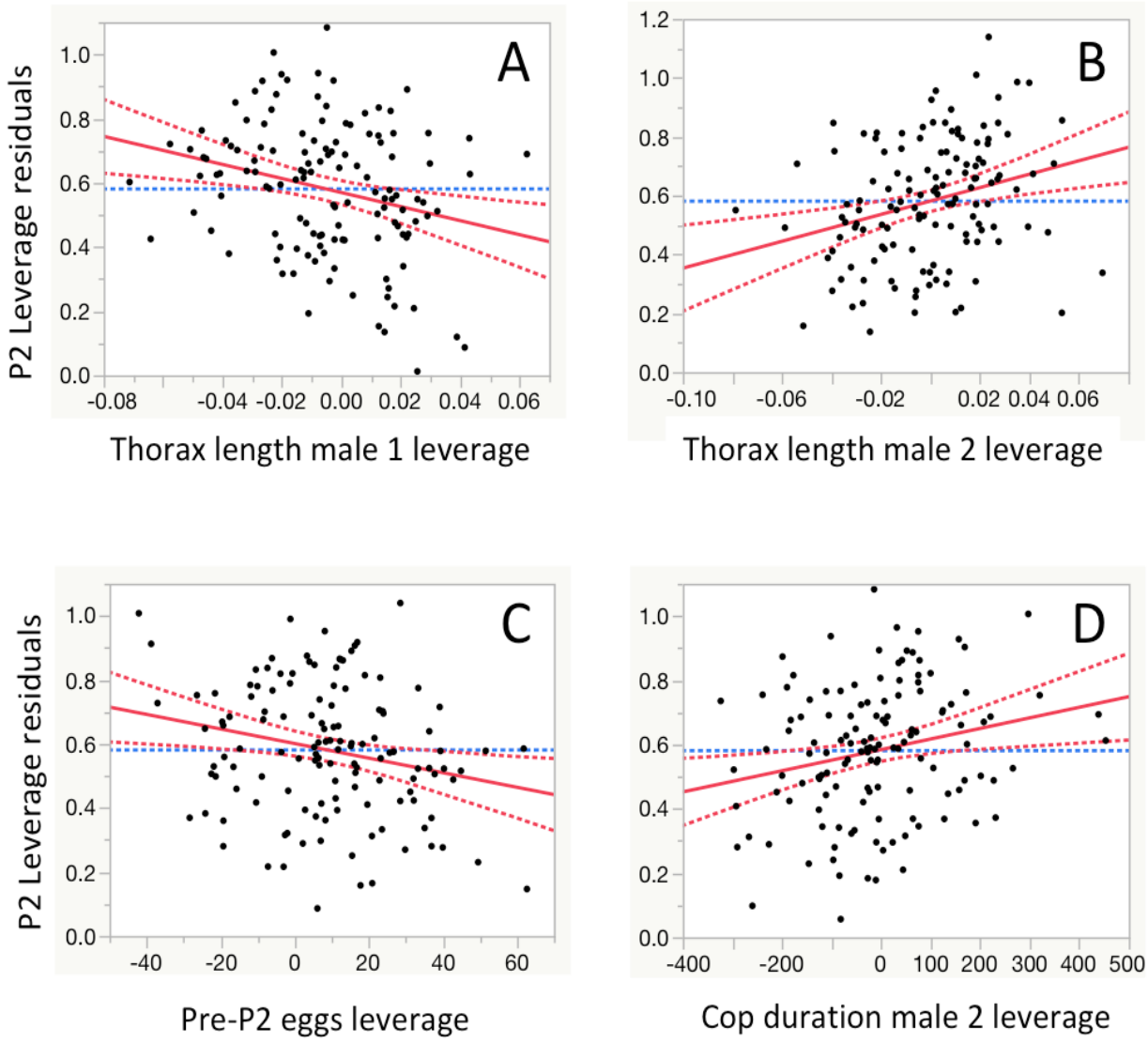

Figure S2. Leverage plots for (mean-centered) covariates that reached statistical significance ($\alpha < 0.05$) in the REML mixed model on P_2, related to Figure 3.
Each plot shows the influence of adding the covariate to the model, given the other effects are already included. Covariate in **A,** thorax length of the 1st (irradiated, IR) male; **B**, thorax length of the 2nd (test) male; **C**, eggs laid by females between their first and second matings; **D**, mating duration of the 2nd male. Dotted lines represent 95% confidence curves.

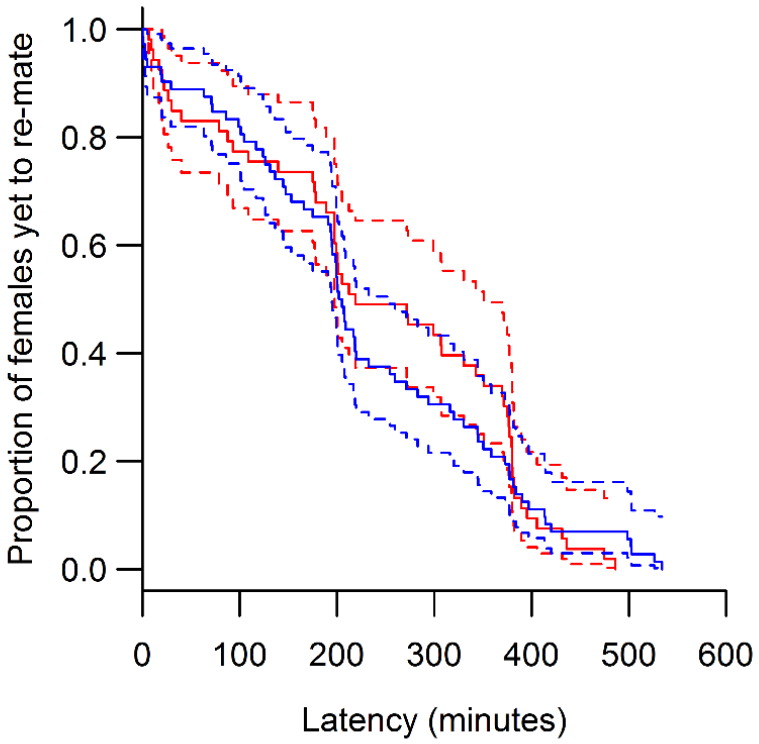

Figure S3. **Proportion females yet to remate over time, related to STAR Methods**.
The effect of selection treatment on the propensity of females to remate was not significant ($\chi^2 = 0.007$, d.f. = 1, $P = 0.935$); high line females are shown in red, and low line females are shown in blue. Dashed lines are 95% confidence curves.

Line	Generation	Rep	Mean	s.e.	Rep	Mean	s.e.	Rep	Mean	s.e.
High	0	1	13.262	0.1142	2	13.239	0.1262	3	13.522	0.1101
	1	1	13.390	0.1153	2	13.707	0.1264	3	13.441	0.1097
	2	1	13.541	0.1145	2	13.615	0.1244	3	13.843	0.1108
	3	1	13.667	0.1187	2	13.804	0.1239	3	14.059	0.1090
	4	1	13.654	0.1143	2	14.526	0.1253	3	14.489	0.1093
	5	1	14.623	0.1147	2	14.161	0.1235	3	15.000	0.1089
	6	1	14.000	0.1143	2	14.512	0.1263	3	14.780	0.1098
	7	1	15.055	0.1143	2	14.666	0.1239	3	15.503	0.1097
	8	1	14.833	0.1156	2	15.017	0.1236	3	16.429	0.1108
	9	1	15.178	0.1142	2	15.276	0.1242	3	16.376	0.1102
	10	1	15.918	0.1162	2	16.243	0.1244	3	16.381	0.1091
	11	1	16.017	0.1153	2	17.282	0.1240	3	16.944	0.1093
Unselected	0	1	13.269	0.2097	2	13.479	0.2131	3	13.512	0.1954
	1	1	13.487	0.1842	2	13.224	0.1971	3	13.245	0.1926
	2	1	13.682	0.1932	2	12.955	0.1956	3	12.969	0.1908
	3	1	13.453	0.1956	2	13.481	0.1990	3	13.626	0.1829
	4	1	13.399	0.1935	2	12.917	0.1958	3	13.162	0.1911
	5	1	13.203	0.1976	2	12.746	0.1988	3	13.224	0.1907
	6	1	13.881	0.1934	2	13.206	0.1973	3	13.325	0.1908
	7	1	13.268	0.1934	2	13.316	0.1962	3	13.493	0.1922
	8	1	13.288	0.1934	2	13.158	0.1958	3	14.107	0.1921
	9	1	13.832	0.1946	2	13.458	0.2012	3	13.818	0.1908
	10	1	13.693	0.1933	2	13.070	0.2075	3	13.714	0.1910
	11	1	13.500	0.1938	2	13.343	0.1956	3	13.504	0.1912
Low	0	1	13.232	0.0892	2	13.425	0.0992	3	13.378	0.1011
	1	1	13.334	0.0887	2	12.784	0.1088	3	12.969	0.0964
	2	1	12.578	0.0879	2	12.197	0.0991	3	12.847	0.0955
	3	1	12.435	0.0880	2	12.747	0.0992	3	12.596	0.0958
	4	1	12.331	0.0880	2	11.642	0.1016	3	12.441	0.0952
	5	1	11.835	0.0879	2	11.343	0.0997	3	12.164	0.0951
	6	1	12.048	0.0880	2	11.196	0.0991	3	12.181	0.0952
	7	1	11.218	0.0881	2	10.985	0.0990	3	12.090	0.0961
	8	1	11.250	0.0881	2	10.894	0.0991	3	11.775	0.0949
	9	1	10.958	0.0896	2	10.441	0.0998	3	11.371	0.0968
	10	1	11.294	0.0879	2	10.367	0.0990	3	11.776	0.0951
	11	1	10.720	0.0884	2	10.150	0.0990	3	10.882	0.0949

Table S1. Mean sex comb sizes (as tooth number per leg) and standard errors (s.e.), related to Figure 2 and STAR Methods.

Means are for each line across 11 consecutive generations of artificial selection in *Drosophila bipectinata* for replicate high, unselected, and low selection lines.

A: REML mixed model results

Source	Numerator d.f.*	Denominator d.f.	*F*	*P*
Thorax length	**1**	**729**	**8.047**	**0.0047**
Selection treatment	**2**	**6.392**	**144.418**	**<0.0001**
Interaction	2	728.6	0.7478	0.474

*degrees of freedom

B: Heritability (h^2) estimates

Replicate High Line	h^2 estimate	s.e.	Replicate Low Line	h^2 estimate	s.e.
1	0.3778	0.0358	1	0.4618	0.0372
2	0.4082	0.0440	2	0.4796	0.0432
3	0.5664	0.0386	3	0.3612	0.0308
Mean	0.4508	0.0395	Mean	0.4342	0.0371

Table S2. **Divergence in sex comb size and realized heritability estimates, related to Figure 2.**

A) Results of a REML mixed model following 11 generations of bidirectional artificial selection on sex comb size in *Drosophila bipectinata*. Significant effects highlighted in bold. The cumulative effect of selection on the size of the secondary sexual trait is reflected in the Selection treatment term. Replicate line, modeled as a random effect nested within selection treatment, was not significant (variance component = 0.1866, s.e. = 0.1159, 95% CI: -0.04059, 0.4138).

B) Realized heritability estimates (h^2) and standard errors for body-size specific sex comb size in *Drosophila bipectinata*. Replicate estimates from high and low selection lines are provided. Artificial (truncation) selection was applied for 11 consecutive generations. All lines are derived from a common, field-fresh population from Taiwan.

Source	Num d.f.*	Den d.f.**	*F*	*P*	*Estimate*	*s.e*
Selection treatment	**2**	**6.40**	**7.846**	**0.019**	.	.
Pre-P_2 eggs	**1**	**121.5**	**6.487**	**0.012**	**-0.00227**	**0.00089**
Sex comb male 1	1	121.2	0.6461	0.423	0.00601	0.00747
Thorax length male 1	**1**	**121.6**	**8.709**	**0.0038**	**-2.188**	**0.741**
Thorax length male 2	**1**	**104.8**	**9.438**	**0.0027**	**2.288**	**0.745**
Cop duration male 1	1	121.9	0.445	0.506	-7.414e-5	1.11e-4
Cop duration male 2	**1**	**115.9**	**6.262**	**0.0137**	**3.281e-4**	**1.310e-4**

*Numerator degrees of freedom, **Denominator degrees of freedom

Table S3. Results of a REML mixed model on P_2 (proportion eggs fertilized by a female's second mate), related to Figure 3 and STAR Methods.

Significant effects are highlighted in bold. Replicate line, treated as a random effect nested within Selection treatment, is not significant (var (s.e.) = 0.0001053 (0.00174); 95% C.I., -0.0033, 0.0035). Parameter estimates for the covariates are provided.

A: Teeth ablated from each comb segment

C1, Left	C1, Right	C2, Left	C2, Right	Total teeth ablated per male	Mean % total teeth ablated per male
2 (1-3)	2 (1-5)	3 (2-5)	3 (2-4)	11 (7-13)	33.54% (23.3 -38.7%)

B: Resultant comb sizes

High line pre-surgery males (n=30)	*High line post-surgery males (n=30)	*High line surgical controls (n=18)	*Low line surgical controls (n=26)
31.533 (0.486)	20.967 (0.391)	32.833 (0.809)	22.462 (0.373)

*Groups whose fertilization success was measured

C: Generalized linear model

Source	d.f.**	χ^2	*P*	Estimate	s.e.
Surgical treatment	**1**	**21.370**	**<1.0e-4**	.	.
Pre-P_2 eggs	**1**	**44.339**	**<1.0e-4**	**-0.00974**	**0.00150**
Thorax length male 1	**1**	**4.668**	**0.031**	**-0.0401**	**0.0186**
Thorax length male 2	**1**	**9.006**	**0.0027**	**0.0724**	**0.0242**
Cop duration male 1	1	0.597	0.440	-0.0184	0.0239
Cop duration male 2	**1**	**17.950**	**<1.0e-4**	**0.0730**	**0.0174**

**Degrees of freedom

Table S4. Laser ablation of teeth, resultant comb sizes of experimental groups, and analysis, related to Figure 4 and STAR Methods.

A) Median number of teeth (range in parentheses) ablated from the sex comb of high line males in the laser phenotypic engineering experiment. Surgical ablation of teeth was performed one tooth at a time from comb segments, C1 and C2, on both the left and right foretarsus of males. Median total teeth removed per male is also provided. The extent of tooth number reduction is also expressed as a mean percentage of the total pre-surgical number of teeth (range in parentheses); n = 30 males. Pre- and post-surgery mean tooth numbers for these males are provided in B.

B) Comb size (s.e.), as mean total number of teeth, in high and low line males used in the laser phenotypic engineering experiment. Pre- and post-surgery means for the high line surgical treatment group are provided. Asterisks indicate the groups whose competitive fertilization success was measured and analyzed.

C) Results of a generalized linear model on P_2 in the experiment where sex comb size of high line males was surgically reduced in size. Significant effects are highlighted in bold. Parameter estimates for covariates are provided.