EVOLUTIONARY BIOLOGY

Evolutionary divergence in competitive mating success through female mating bias for good genes

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Despite heritable variation for univariate sexually selected traits, recent analyses exploring multivariate traits find evidence consistent with the lek paradox in showing no genetic variation available to choosy females, and therefore no genetic benefits of choice. We used the preferences of *Drosophila melanogaster* females to exert bidirectional selection on competitive male mating success to test for the presence and nature of genetic variation underlying this multivariate trait. Male mating success diverged between selection regimens, and flies from success-selected lines had a smaller burden of deleterious, recessive mutations that affect egg-to-adult viability, were better sperm competitors (sperm offence), and did not demonstrate reduced desiccation resistance or components of female fitness (traits thought to trade off with attractiveness) relative to flies from failure-selected populations. Mating success remained subject to inbreeding depression in success-selected lines, suggesting that variation in mating success remains, thanks to numerous genes of small effect. Together, our results provide unique evidence for the evolutionary divergence in male mating success, demonstrating that genetic variation is not exhausted along the axis of precopulatory sexual selection and that female mating biases align with the avoidance of bad genes.

INTRODUCTION

Persistent female mate choice for male sexual traits that confer genetic benefits should erode genetic variation among males and preclude choice from providing genetic benefits [the lek paradox; (*1*)]. The lek paradox is a pertinent example of the long-standing evolutionary question of what maintains genetic variation in fitness and fitness-related traits (*2*). Therefore, abundant theoretical and empirical attention has gone toward explaining the persistence of female mate choice in light of the lek paradox. From this, it is clear that (i) ample heritable genetic variation is maintained in male sexually selected traits (*3*), (ii) genetic variation could be maintained by many mechanisms [for example, negative frequency-dependent selection or mutation; reviewed by Radwan (*4*)], and (iii) variation in male traits can correlate with offspring fitness, particularly in the form of Fisherian benefits [for example, attractive sons; (*5*)]. The conclusion from this body of research is that choosy females can receive genetic benefits. However, if male attractiveness is a multivariate trait, then genetic correlations among traits will influence the amount of genetic variation, as well as any genetic benefits, available to choosy females (*6*), thereby challenging these conclusions. If correlations are negative, then the genetic variation available to females is reduced and any genetic benefits based on univariate traits may be overestimated (*7*). Only two experiments (*8*, *9*) have directly selected on male mating success or male attractiveness (which incorporates all components of precopulatory sexual selection) and neither identified a response to selection. For example, in a laboratory system, genetic variation in nine cuticular hydrocarbons under strong female choice in *Drosophila bunnanda* was almost completely oriented away from the direction of female choice (*10*), and neither the attractive combination of hydrocarbons nor mating success itself evolved under bidirectional selection (*9*). Similarly, evidence from long-term field studies indicates that traits under strong sexual selection might not evolve despite maintaining ample heritable variation (*11*). Focusing on a single trait can produce heavily biased estimates of overall genetic benefits (*7*). Empiricists must provide evidence for heritable genetic benefits of female choice, and the mechanisms maintaining these benefits, with regard to multivariate male attractiveness or mating success to explain the selective pressures acting on female choice (*7*).

Here, we use the mating biases of female *Drosophila melanogaster* in binomial mate choice trials to identify whether there is genetic variation available in male mating success and, if there is, identify the nature of this genetic variation. Although there is some evidence for the evolution of mating success through artificial selection for components of attractiveness (*12*), we use the outcome of mating trials to evolve populations, thereby incorporating all traits important to female mating decisions [as with the studies of Hall *et al.* (*8*) and McGuigan *et al.* (*9*)]. We artificially selected on male mating success directly by allowing nonvirgin females to "choose" between two competing males and used males successful at mounting females to generate four replicate success-selected lines ($n = 25$ males and 25 virgin females) and males that failed to mount females to generate four replicate failure-selected lines ($n = 25$ males and 25 virgin females). This selection protocol allows active female mate choice but does not exclude male-male competition. Both processes could lead to evolutionary divergence in competitive mating success; however, if females mate with competitively superior males to secure genetic benefits in the form of competitively superior sons or offspring with good genes, then the evolutionary problem of the maintenance of genetic variation remains. In the context of this study, where we address the question of whether there is genetic variation in a multivariate trait, it is more important to incorporate all components of precopulatory sexual selection than to identify the underlying mechanisms of any female mating bias [note that, here, "bias" does not equate to active mate choice, but the propensity of females to mate more readily with males of certain phenotypes; (*13*)]. We applied 14 rounds of selection across 17 generations, generating lines with ancestries of success or failure in mate acquisition trials. Four control lines were established and maintained with males not exposed to mate choice trials. We then compared key components of fitness after at least two generations of relaxed selection to identify what maintains variation in male mating success.

On the one hand, competitive mating success may be dependent on the underlying condition of males, where condition itself is dependent on any trait that affects resource acquisition and utilization (*14*, *15*). Condition provides a large target for mutations, and hence, genetic variation is maintained in mutation-selection balance, where choosy

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females benefit by producing offspring with increased fitness (*16*–*20*). On the other hand, competitive mating success may be subject to trade-offs with life history (*21*, *22*), sexually selected (*23*), or female (*24*, *25*) traits, thus maintaining genetic variation through balancing selection [reviewed by Radwan (*4*)]. To assess mutation-selection balance and balancing selection, we quantified a suite of important life history traits (egg-to-adult viability, fecundity, desiccation resistance, sperm competitiveness, and male harm) to test for evolutionary trade-offs and assessed differences in the load of deleterious recessive mutations between selection regimens (through inbreeding) in two traits (male mating success and egg-to-adult viability) that show substantial inbreeding load in *D. melanogaster* (*26*, *27*). Mutations affecting both traits are, on average, partially recessive (*26*), making inbreeding a pertinent assay for quantifying load. Negative genetic correlations between mating success and other fitness-related traits might indicate a constraint on genetic benefits to choosy females. Conversely, if mutations supply variance in mating success, then mutational load should evolve and we need not expect evolutionary trade-offs; females will benefit by producing offspring with good genes.

RESULTS

Evolutionary response

To identify whether there had been an evolutionary response to selection, we assayed the male mating success of 100 outbred males from each selection line against standard competitor males from the base population. We also measured the mating success of 100 inbred males from each line in the same way to quantify the load of deleterious recessive mutations affecting mating success. Test females were nonvirgins that rejected both males in 57.6% of mating trials, demonstrating that they were capable of choosing whether to bear the costs of remating. Males from success-selected lines obtained significantly more matings than males from failure-selected lines (21.1 ± 7.1% mean difference across the 4 days; see Materials and Methods, Fig. 1A, and Table 1) and control lines (13.1 ± 5.0%; fig. S1A and table S1; see fig. S2 for the mating success of all 12 lines), but failure-selected and control lines were not significantly different (fig. S1A and table S1). Inbred males secured fewer matings than outbred males (Fig. 1A and Table 1), demonstrating the genotypic condition dependence of mating success. However, failure-selected males did not have heightened inbreeding depression for male mating success (that is, there was no regimen-by-cross interaction; Fig. 1A) that would have suggested that mutations affecting mating success had differentially accumulated in these lines.

Good genes

Traditional "good genes" benefits invoke viability or mutational load benefits of sexual selection. Therefore, we assayed the egg-to-adult viability of inbred and outbred flies from each line. We identified a significant regimen-by-cross interaction for egg-to-adult viability, where flies from failure-selected lines suffered inbreeding depression and flies from success-selected lines did not (Fig. 1B and Table 1; see fig. S1B for controls). Furthermore, mating success and inbreeding depression (for viability) were significantly correlated across the 12 selection lines ($\rho = 0.72$, $P = 0.011$; fig. S3), demonstrating that mating success correlates with the load of deleterious recessive alleles that directly affect viability.

Trade-offs

We then tested for trade-offs with four key fitness-related traits that the literature suggests might trade off with attractiveness. First, we assayed desiccation resistance because desiccation stress affects fitness in natural populations, and cuticular hydrocarbons influence both male attractiveness and desiccation resistance. Recent evidence suggests that high-signaling males are more vulnerable to desiccation, indicating that there may be a trade-off (*28*, *29*), and sexual dimorphism in desiccation resistance was evident in our stock population (fig. S1C and table S2). However, this sexual dimorphism did not evolve, and there was no significant difference between selection regimens for desiccation resistance (Fig. 1C and Table 1). Second, we quantified female fecundity and productivity because both intralocus (*30*) and interlocus (*24*) sexual conflict are well documented for this species, particularly in laboratory populations, and this conflict could maintain genetic variation in mating success. However, female fecundity was not significantly different between selection regimens (Table 1), and when experimental males were mated with standard females, productivity was not lower for females mated with males from success-selected lines (Table 1), suggesting that there was no evidence for intra- or interlocus sexual conflict. Finally, we assayed one component of sperm competitive ability because female fruitflies are polyandrous, and pre- and postcopulatory traits have been known to trade off (*23*). Our measure of sperm competitiveness was P2, the proportion of offspring sired by experimental males mated second (*31*) and in competition with males from a brown-eyed recessive, isogenic strain. P2 was significantly higher for success-selected lines (Fig. 1D and Table 1). To ensure that this result was not simply due to differences in larval competitive ability (that is, that larvae from success-selected lines are competitively superior), we reared 50 experimental larvae and 50 brown-eyed larvae in each of five replicate vials per line. We found no significant difference between success- and failure-selected lines (generalized linear mixed model; $\chi^2 = 0.98$, df = 1, $P = 0.321$), indicating that greater viability of success-selected flied did not explain the difference in the proportion of adults in the P2 experiment.

Body size

Body size is an important predictor of mating success in *D. melanogaster* (*32*) and might explain any response to mating success selection. Success-selected flies were larger than failure-selected flies (wing area of 0.91 ± 0.02 mm^2 and 0.87 ± 0.01 mm^2, respectively; Table 1). Therefore, we might expect that body size is a simple proxy for attractiveness that is inversely proportional to mutational load. To test this hypothesis, we performed a separate artificial selection experiment on body size with flies derived from the same stock population. Wing size diverged significantly (0.90 ± 0.01 and 0.78 ± 0.02, respectively; table S3), and large flies were more viable than small flies (table S3). However, both selection regimens (large- and small-selected flies) suffered inbreeding depression for viability (that is, no significant interaction; table S3); only success-selected flies were purged of inbreeding depression.

DISCUSSION

We provide the first empirical evidence of the evolutionary response of male mating success, demonstrating that genetic variation along the axis of precopulatory sexual selection was present in the base population or that variation rapidly accumulated through new mutations or the recruitment of new loci (*19*, *33*). Our supporting experiments show that success-selected populations have purged their load of recessive mutations that affect viability, with no negative consequences for the other life history traits that are likely candidates for a trade-off with mating success.

Our results contrast with the only two previous laboratory studies that have directly selected on male mating success (*8*, *9*). Although Hall *et al.*

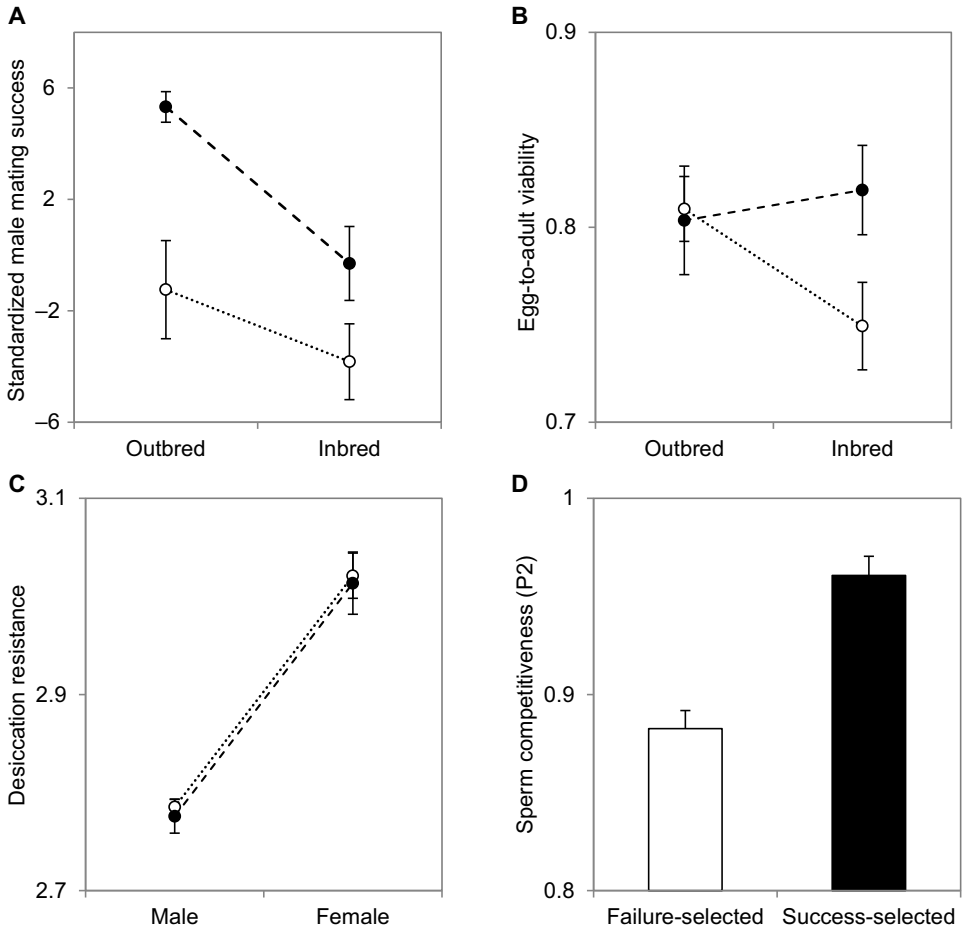

Fig. 1. Effect of selection history (success-selected, solid; failure-selected, open), cross (outbred and inbred), and sex on fitness-related traits. (**A**) Standardized male mating success. Ten inbred and 10 outbred males from each of 10 replicate families per line were assayed (n = 678 matings total from 1600 mating trials). We conducted mating trials on four consecutive days, with one line from each treatment performed on each day. The day on which we conducted the mating trials significantly influenced the proportion of experimental males that acquired a mate, and therefore, we standardized mating success to have a mean of zero and SD of one for each day. (**B**) Egg-to-adult viability. We assayed the egg-to-adult viability of offspring from up to 25 brother-sister and 25 unrelated pairs across five families for each line (n = 362 crosses total). (**C**) Desiccation resistance. For each line, approximately 100 males and 100 females were desiccated until death in 10 replicate vials (n = 1546 total). Units are log minutes to death. (**D**) Sperm competitiveness (P2). Sperm competitiveness was quantified against a brown-eyed mutant fly strain as the proportion of offspring sired by the experimental male (n = 28 success-selected and 34 failure-selected males). Means and SEs are of four replicate selection lines for each selection regimen. Joining lines are for illustrative purposes only.

(*8*) only selected for three generations, which may contribute to the lack of a response to selection, McGuigan *et al.* (*9*) applied an approach similar to ours for 10 generations. One potential explanation for the contrast is that we established our experimental populations from a newly formed stock population compared to the long-standing stock of *D. bunnanda* used by McGuigan *et al.* (*9*). "Off-peak" populations—those not yet near their adaptive peak—are expected to harbor more additive genetic variation with good genes potential as they rapidly respond to their novel environment compared to "peak" populations—those near their adaptive peak—where such benefits have been exhausted over time (*34*). Hence, starting our experiment with a newly formed stock population may have contributed to the observed evolutionary response to selection.

We show that success selection on mating success purged recessive mutations that affect viability, indicating that female preferences avoid "bad" genes and that genetic variation in mating success is supplied, in part, by mutations that affect viability. However, inbreeding depression in success-selected lines was completely purged (Fig. 1B), suggesting that viability benefits may be rapidly exhausted under elevated sexual selection. In contrast, male mating success remained subject to inbreeding depression, suggesting that the load of recessive mutations affecting mating success (and therefore condition) is too large and individual effects are too small to purged, in line with the genic capture hypothesis (*19*, *33*). We provide a nuanced view of the genetic architecture; evidently, success in mate acquisition is due not only to recessive viability genes (that is, good genes) but also to additive gene action affecting mating success genes (Fisherian-type). Our results also support recent studies in other taxa that show that sexual selection can purge deleterious mutations (*35*–*38*).

We found no evidence for trade-offs between mating success and four important fitness traits of *Drosophila*. Sexual conflict is well documented in *D. melanogaster* (*24*) and is particularly related to body size (*39*), so it is perhaps surprising that we found no evidence for sexual conflict in this study. The lack of observed sexual conflict may reflect reduced sexual conflict over attractiveness compared to postcopulatory

Table 1. Effect of selection regimen, inbreeding (cross), and sex on fitness-related traits. Model 1 is a generalized linear mixed model, whereas model 2 is a linear mixed model. Each model was also analyzed with a randomization test (1000 permutations). Bold font indicates significance at $\alpha = 0.05$.

Trait	Source	df	χ^2	*P*		
				Model 1	Model 2	Randomization
Male mating success	Regimen	1	10.46	**0.001**		**0.002**
	Cross	1	4.75	**0.029**		**0.029**
	Regimen × Cross	1	1.40	0.237		0.129
Egg-to-adult viability	Regimen	1	1.89	0.169		0.174
	Cross	1	3.12	0.078		0.081
	Regimen × Cross	1	4.63	**0.031**		**0.035**
Wing size (squared)	Regimen	1	3.99		**0.046**	**0.034**
Sperm competitiveness	Regimen	1	5.91	**0.015**		**0.021**
Desiccation resistance (log-transformed)	Regimen	1	0.10		0.758	0.755
	Sex	1	1379.09		**<0.001**	**<0.001**
	Regimen × Sex	1	0.06		0.805	0.791
Female fecundity	Regimen	1	0.51		0.476	0.426
	Cross	1	0.56		0.455	0.430
	Regimen × Cross	1	0.07		0.793	0.793
Female productivity	Regimen	1	0.05		0.816	0.811

fitness-related traits and body size or may be that we have not adequately measured sexual conflict. For desiccation resistance, although there is some evidence to suggest that mating success and resistance might trade off (*28*, *29*), this relationship is not well understood for *D. melanogaster*. Finally, we found that male mating success and sperm offence appeared to be positively correlated, with males from success-selected lines having higher P2. Although this may reflect a general good genes benefit, where mutations negatively affecting condition-dependent mating success also reduce sperm competitive ability, there are a number of alternative explanations for this pattern. For example, a similar pattern could emerge if females biased paternity toward attractive males or away from unattractive males (*40*) or if the larger, success-selected males transferred a higher volume of sperm or seminal fluids. In addition, we only measured P2 (sperm offence) and therefore cannot conclude that success-selected males are better sperm competitors overall. It is unlikely that the observed difference between selection regimens is a consequence of more intense sperm competition in success-selected lines, given that females used to establish each generation were virgins that were paired with males for only 24 hours, and female remating within this time is rare (*41*). Regardless of the mechanism, our results show that male mating success and P2 are unlikely to be strongly negatively correlated.

One explanation for the lack of observed trade-offs is that genetic correlations among traits vary depending on environmental conditions (*42*), and novel environmental conditions can cause an alignment between natural and sexual selection when it is otherwise antagonistic in well-adapted populations (*34*). Most evidence for sexual conflict in *D. melanogaster* comes from long-standing laboratory populations (*24*, *25*), where synergistic variation is largely eroded (because selection can act efficiently on synergistic variation in a stable environment) and only antagonistic variation persists. We have assessed the traits that are most likely to be involved in a trade-off with male attractiveness and to have profound fitness consequences. Nevertheless, there are limitations to a trait-based approach to quantifying trade-offs—namely, that the key trait has been missed. Although it seems unlikely that "hidden" trade-offs would counter the substantial positive effects on viability and competitive mating success, only an assay of total fitness would provide definitive support for a complete rejection of the trade-off hypothesis. The positive effects that we document agree with Houle's (*43*) finding of substantial positive genetic correlations between life history traits, as well as with the genic capture hypothesis in general (*18*, *19*), where trade-offs are not necessary for maintaining variation in condition-dependent, fitness-related traits.

Rather than being limited by trade-offs, we suggest that the presence of genetic variation in male mating success is related to the ecological opportunity for sexual selection. In the context of female choice, standing genetic variation might reflect a balance between the ecological opportunity for females to make reliable mate choice decisions (inclusive of the direct costs of choice) and the indirect fitness benefits of being choosy versus the direct benefit of being less choosy and its associated indirect fitness costs. In success-selected populations in this study, we enhanced the opportunity for females to make a reliable choice by providing them with two competing males in a confined space. This resulted in an evolutionary increase in male attractiveness and elevated the indirect fitness benefits to females.

Finally, we showed that artificial selection on both male mating success and body size increased the size of male flies. However, only sexual selection was associated with the purging of recessive mutations that affect viability, with large-selected flies still suffering inbreeding depression.

This suggests that multivariate selection on mating success is different from artificial selection on body size, although the changes in body size are similar. This also shows that body size is not a simple proxy for mutational load, demonstrating that precopulatory sexual selection is crucial to purging deleterious recessive alleles affecting viability.

CONCLUSIONS

Our results provide novel evidence for the evolutionary divergence in competitive mating success, which has important implications for understanding the selective pressures acting on female mate choice. Moreover, the experiments show how increasing the strength of sexual selection on competitive mating success removes deleterious recessive mutations that affect viability. This is a result that has important implications for conservation because it reveals the positive effects that sexual selection can have (purging inbreeding depression) under conditions of inbreeding that are commonly found in small, captive populations (*44*, *45*). Consistent with genic capture models (*18*, *19*, *43*), we found no evidence that genetic variation in attractiveness is maintained by trade-offs with other important life history traits. Together, these results reveal the indirect Fisherian and good genes benefits of female mating bias.

MATERIALS AND METHODS

Experimental design

A stock population of *D. melanogaster* was established in December 2012 from wild-caught flies collected en masse from Innisfail, Queensland, Australia and maintained in a 9-liter (270 cm × 180 cm × 180 cm) population cage with overlapping generations. Twelve selection lines were generated from the stock approximately 10 generations later. We expect that this stock was experiencing some adaptation to the laboratory environment at the start of our experiment (*46*). However, we chose to establish our populations after 10 generations to balance adaptation with the accumulation of deleterious mutations in the benign environment (*45*, *47*). All flies were maintained on standard agar-maize-yeast medium at 25°C with a 12:12 light-dark cycle. Experimental flies were maintained in 50-ml vials with 10-ml standard food media and kept at no more than 50 individuals per vial and equal sex ratio when in a mixed-sex milieu.

Artificial selection on male mating success

To initiate the experiment, flies were collected across 2 days of emergence and allowed to mate freely for this time plus an additional 24 hours, ensuring that all individuals had ample time to mate at least once. Flies were then separated by sex and held in vials of no more than 50 individuals, with female food supplemented with live yeast. Binomial mate choice trials were carried out 5 days later, where, for each trial, two males were placed in a 50-ml vial with 10-ml standard food for 20 min before being presented with a nonvirgin female of the same age. The use of nonvirgin females followed Rundle *et al.* (*48*) and was due to virgins potentially being less discriminatory. Vials were then scanned for up to 90 min between 0800 and 1400 hours. Males that succeeded in mounting females (that is, copulated) were separated from the female under cold anesthesia and allocated to one of four success-selected lines, whereas the remaining males were similarly anesthetized and allocated to one of four failure-selected lines. If neither male mated within 90 min, then all flies from that vial were discarded. Four control lines were established, with unselected males not exposed to the binomial mating trials. All males were given a 3-day respite before being used to initiate the next generation. The census population size of each selection/control line was 25 males and 25 virgin females, which were left to lay for 24 hours in five replicate vials, each with five males and five females, before being discarded. For each subsequent round of selection, screen (that is, nonvirgins used in mate choice trials) and virgin females came from the line rather than the base population, and males from success-selected lines that failed to mate and males from failure-selected lines that acquired a mate were discarded. Selection continued for 14 rounds across 17 generations (we applied 7 generations of selection, followed by 3 generations of relaxed selection, followed by 7 generations of selection), generating populations with ancestries of success or failure in mate acquisition. Flies used in all subsequent assays were reared at standard density (50 per vial), were collected as first-instar larvae, and had not been passed through the selection protocol.

Male mating success assay (generation 18)

To assay male mating success, 10 single-pair crosses were established per line (10 families). For each family, emerging adults were collected, with one female paired with a brother (generating inbred offspring) and another paired with an unrelated male from a different family (generating outbred offspring). Pairs were allowed to mate and lay for 24 hours before being discarded. Emerging adults from these crosses were then collected across 48 hours of emergence and held for a further 24 hours, as with the selection protocol. Males were then collected under cold anesthesia and held in vials for 5 days. Females were discarded. Standard females and standard competitor males used in the mating trial assay were collected as first-instar larvae from the base population and raised at standard density. As with the experimental flies, standard males and females were collected across 48 hours and allowed to mate freely for a further 24 hours before being separated by sex and held for 5 days. Three hours before the mating trials were conducted, standard males were placed into small population cages (125 ml; ~200 individuals per cage) and supplied with live yeast impregnated with blue food dye that, upon consumption, is visible as a blue marking in the abdomen. Ten males from each family were then exposed to mating trials, which were similar to the selection protocol, although here they were competed against standard blue males and presented to standard females. Vials were then scanned visually by two observers. Copulating pairs were removed by aspiration and transferred to new vials until the conclusion of the mating trial period. Family integrity of the males was maintained by transferring copulating pairs from each family to separate vials. If neither male had mated after 90 min, then both individuals were discarded. All remaining males were checked for the blue marking on the abdomen, and thus, the number of experimental males that succeeded or failed in mate acquisition was obtained for each family. Mating trials were conducted across four consecutive days, with one line from each treatment performed on each day.

Egg-to-adult viability and fecundity assays (generation 18)

We quantified the egg-to-adult viability of inbred and outbred flies from each of five families per line. For each family, virgin flies were collected and separated by sex for 9 days. Crosses were then established by pairing females (sisters) with either a brother (five replicates) or an unrelated male from the same selection line (five replicates; producing a total of 50 crosses per line), with a comparison used to estimate segregating mutational load. Protocol followed Robinson *et al.* (*27*), where pairs were held together for 48 hours in vials with a standardized scoop of live yeast before being transferred to new vials for egg laying. Pairs were then held together for exactly 24 hours on standard food before

being discarded. The number of eggs within each vial was counted within 4 hours of the adults being removed, and this value was scored as female fecundity. After 14 days, vials were inverted and frozen at −20°C. Adult flies were then counted, and the number of eggs laid and the number of adults that emerged for each vial was used in analyses. Vials in which fewer than 10 adults had emerged were excluded from analyses.

Direct male harm assay (generation 20)

We quantified the female productivity of standard females from the stock population exposed to success- and failure-selected males from each experimental line to assess male harm. If attractive males are more harmful to females, then female productivity should be lower when mated to males from success-selected lines. To measure this, flies were reared at standard density, and five virgin males from each of six replicate vials per line were collected (30 males per line). Standard, virgin females were collected from the base population, reared at standard density, and held in vials of no more than 20. Flies were aged to 11 to 12 days before line males were paired with standard females in individual vials and scanned for copulations. At the end of copulation, females were transferred to a fresh vial with live yeast for 24 hours (males were discarded) and transferred to another yeasted vial for a further 48 hours before being discarded. All vials were frozen after 14 days, and the number of adults that had emerged was counted.

Sperm competitive ability assay (generation 22)

To quantify sperm competitive ability, we followed a similar protocol to Travers *et al.* (*49*), with minor technical differences. Briefly, we allowed competitor strain males and females to mate freely in vials (10 males and 10 females) for 2 hours before isolating 550 females into individual vials for 48 hours. Five males from each of 10 families per line were then paired with females. After a further 24 hours, females were transferred to a fresh vial with live yeast and held for 72 hours to lay before being discarded. Twelve days later, vials were inverted and frozen and the numbers of wild-type and brown-eyed offspring that emerged were counted. Only females that produced wild-type and brown-eyed offspring were included in analyses.

Our estimate of sperm competitive ability was simply the proportion of wild-type adults produced and therefore included sperm competition between wild-type and brown-eyed flies as well as any larval competition. If, for example, larvae from success-selected lines were more competitive than larvae from failure-selected lines, then the resultant proportion of wild-type adults would reflect higher sperm competitiveness in success-selected lines. To ensure that the effect was due to sperm competition and not competitive larval viability, we reared 50 experimental larvae and 50 brown-eyed larvae in each of five replicate vials per line. The number of experimental flies that emerged (or not) was counted and scored as larval competitive ability.

Desiccation resistance assay (generation 21)

To assess desiccation resistance, we followed a protocol similar to Kennington *et al.* (*50*). Flies from each experimental population were held in a controlled temperature room at 25°C and ambient humidity in the absence of water, and the time taken to death was the measure of resistance. To measure desiccation resistance, standard density–reared flies were collected as they emerged over 48 hours and allowed to mate freely for a further 24 hours. Flies were then separated by sex and held in vials of 10 individuals for 10 replicate vials per sex per line (that is, 100 males and 100 females per line). Flies were held on standard food for 5 days before being transferred, at random, to empty 50-ml vials with a mesh lid secured with an elastic band. Vials were held under constant light and checked for dead flies 7 hours later and at half hourly intervals thereafter until all flies had died. The time to death of each fly was calculated.

We similarly measured the desiccation resistance and dry weight of flies (100 males and 100 females) from the stock population to see if there was a general size effect on desiccation resistance and identify whether there was sexual dimorphism in the base population. Dry weight was obtained by placing flies into an oven at 60°C for 48 hours and then under ambient conditions for 24 hours before weighing them with a *Sartorius* SE3 micro-balance.

Wing size of success/failure-selected lines (generation 18)

To measure wing size, we reared flies at standard density in five replicate vials for each line. From each vial, we measured five males' wings (25 males per line) by removing them with forceps and mounting them on slides with Histo-Clear (National Diagnostics Inc.) and Aqua-Mount (Thermo Fisher Scientific Inc). Photos were taken and analyzed using Object-Image (*51*), and the wing area was computed using landmark analysis as described by Gilchrist and Partridge (*52*).

Artificial selection on body size

In addition to simply measuring the wing sizes of flies from our experimental populations, we performed a separate artificial selection experiment on body size with flies derived from the same stock population. If mating success is simply due to size divergence, then this becomes an important adjunct for assessing the correlated responses—do correlated responses differ between size-selection and success/failure-selection lines? To assess this, nonvirgin adults were collected 4 to 5 days after the start of eclosion and separated into single-sex vials for 4 days. Selection involved aligning 100 anesthetized males under a dissecting microscope and selecting, by eye, the 25 largest and 25 smallest individuals. This was repeated for females, and then both steps were repeated for replicate lines. Three large-selected lines were generated by combining five large females with five large males in five replicate vials ($n = 50$) for each line and allowing flies to mate for 24 hours before being transferred to fresh laying vials for a further 24 hours to produce the next generation. Four small-selected lines were similarly established. Emerging adults from these crosses were used for the next generation of selection, where only the 25 largest (for large-selected lines) or the 25 smallest (for small-selected lines) flies were used to initiate further generations. Selection continued for 11 generations.

We then measured the wing size and egg-to-adult viability of size-selected flies for comparison with our success/failure-selected flies. The viability assay protocol was the same as with selection for success/failure, with five inbred/outbred pairs assayed from each of five families per line. Wing size was measured (as above) for five males from each of four standard density picks.

Statistical analyses

All statistical tests were performed using the R statistical platform (http://cran.r-project.org/). Generalized/linear mixed models (G/LMMs) were performed using the lme4 package (*53*).

The effects of regimen, cross, and their interaction on male mating success were analyzed using a generalized linear mixed effects model (GLMM) on the number of wins and losses for each family. The model included regimen, cross, and regimen-by-cross interaction as fixed effects, and line (nested within regimen) and day (nested within line) as random effects. Day was included as a random effect because the

proportion of experimental males that mated varied significantly across the four days of mating trials (0.49, 0.59, 0.70, and 0.69) and this could have been explained by, for example, variation in the amount of blue dye consumed by competitor males between days. An observation-level random effect was added because data were overdispersed (residual deviance of 202.5 on 143 degrees of freedom).

The effects of regimen, cross, and their interaction on egg-to-adult viability were analyzed using a GLMM for the number of adults and dead flies (dead = eggs − adults). Because there were replicate vials within a family, family was added as a random effect in the model (nested within line). Data were overdispersed (residual deviance of 2081.1 on 356 degrees of freedom), and an observation-level random effect was added to the model.

The effects of selection regimen, cross, and their interaction on fecundity were analyzed using a linear mixed effects model (LMM) on the number of eggs. As with viability, line (nested within regimen) and family (nested within line) were included as random effects in the model.

The effect of selection regimen on productivity was analyzed using an LMM on the number of adults, with line (nested within regimen) and vial (nested within line) included as random effects.

The effect of regimen on sperm competitive ability (number of emerging wild-type versus brown-eyed flies) was analyzed using a GLMM, with regimen (fixed), line (random; nested within regimen), and family (random; nested within line) all included in the model. An observation-level random effect was added to correct for overdispersion (residual deviance of 210.9 on 58 degrees of freedom).

Competitive larval ability (number alive versus number dead) was analyzed using a GLMM, with regimen (fixed), line (random; nested within regimen), and vial (random; nested within line) included in the model. An observation-level random effect was added to correct for overdispersion (residual deviance of 46.4 on 37 degrees of freedom).

The effects of regimen, sex, and their interaction on desiccation resistance were analyzed with a linear model on the log-transformed survival times (minutes to death) of each individual fly. Because there were up to 10 flies in a vial, the model included regimen, sex, and their interaction as fixed effects, and line (nested within regimen) and vial (nested within line) as random effects. For the base population, we analyzed the effect of sex, weight, and the interaction of the two on desiccation resistance using a linear model.

Wing size was analyzed using an LMM, with the effects of selection regimen (fixed), line (random; nested within selection regimen), and vial (random; nested within line) included in the model. Data were squared before analysis.

Analyses of wing size and egg-to-adult viability for size-selected lines followed that of success/failure-selected lines. An observation-level random effect was added to correct for overdispersion when analyzing egg-to-adult viability (residual deviance of 3252.0 on 288 degrees of freedom).

For each of the analyses described above, we then performed randomization tests by randomly assigning trait values across the explanatory variables and recalculating the χ^2 value. The observed χ^2 value was then compared to the distribution generated from 1000 randomizations, and a *P* value was obtained as the proportion of times that $\chi^2_O > \chi^2_P$ (where χ^2_O is the observed χ^2 value, and χ^2_P is the permuted χ^2 value).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/4/5/eaaq0369/DC1

fig. S1. Effect of selection regimen, inbreeding, and sex on fitness-related traits from experimental, control, and stock populations.
fig. S2. Standardized male mating success of outbred (black) and inbred (white) flies from the 12 selection/control lines.
fig. S3. Relationship between standardized male mating success inbreeding load.
fig. S4. Male mating success of outbred and inbred flies from success-selected (black circles) and failure-selected (white circles) populations.
table S1. Effect of selection regimen, inbreeding (cross), and their interaction on male mating success of all lines, success-selected *v* control lines, and failure-selected *v* control lines.
table S2. Effect of sex, weight, and their interaction on the desiccation resistance of males and females from the stock population.
table S3. Effect of selection regimen, inbreeding (cross), and their interaction on wing size and egg-to-adult viability of size-selected lines.
table S4. Sample sizes for all measured traits.

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