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Sexual selection reveals a cost of pathogen resistance undetected in life-history assays

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Mechanisms of resistance to pathogens and parasites are thought to be costly and thus to lead to evolutionary trade-offs between resistance and life-history traits expressed in the absence of the infective agents. On the other hand, sexually selected traits are often proposed to indicate "good genes" for resistance, which implies a positive genetic correlation between resistance and success in sexual selection. Here I show that experimental evolution of improved resistance to the intestinal pathogen *Pseudomonas entomophila* in *Drosophila melanogaster* was associated with a reduction in male sexual success. Males from four resistant populations achieved lower paternity than males from four susceptible control populations in competition with males from a competitor strain, indicating an evolutionary cost of resistance in terms of mating success and/or sperm competition. In contrast, no costs were found in larval viability, larval competitive ability and population productivity assayed under nutritional limitation; together with earlier studies this suggests that the costs of *P. entomophila* resistance for nonsexual fitness components are negligible. Thus, rather than indicating heritable pathogen resistance, sexually selected traits expressed in the absence of pathogens may be sensitive to costs of resistance, even if no such costs are detected in other fitness traits.

KEY WORDS: **Costs of resistance, experimental evolution, good genes, Hamilton–Zuk, immunocompetence, trade-offs.**

Resistance to parasites and pathogens is thought to be costly—defensive mechanisms (including behavioral avoidance, barriers to infection, immune defense, and physiological tolerance of damage) may be energetically costly, inflict collateral damage, and/or interfere with resource acquisition and other functions of the organism (Sheldon and Verhulst 1996; Lazzaro and Little 2009; Schulenburg et al. 2009). Such costs are expected to result in evolutionary trade-offs, where evolution of higher resistance is associated with reduced fitness in the absence of the pathogen. This notion underlies the theory of optimal immune defense (e.g., Viney et al. 2005; Donnelly et al. 2017), as well as some of the theories linking heritable pathogen resistance with sexually selected traits (Folstad and Karter 1992; Westneat and Birkhead 1998; Adamo and Spiteri 2005). It is supported by extensive evidence from genetic analyses and selection experiments that found negative genetic correlations between pathogen resistance and survival, growth, developmental rate, fecundity or longevity, often amplified under nutritional or other stress (Kraaijeveld and Godfray 2008; Vorburger et al. 2008; Modak et al. 2009; Ye et al. 2009; Hall et al. 2010; Boots 2011; Duncan et al. 2011; Auld et al. 2013; Vijendravarma et al. 2015; McGonigle et al. 2017; McNamara and Simmons 2017; Bartlett et al. 2018; older studies reviewed in Lazzaro and Little 2009).

Against this background of apparently ubiquitous trade-offs of pathogen resistance, two recent studies that found no such trade-offs in *Drosophila melanogaster* stand out. Faria et al. (2015) studied replicated populations experimentally evolved for 30 generations under three different pathogen regimes: intestinal infection with *Pseudomonas entomophila*, systemic infection with *P. entomophila*, and systemic infection with the C-virus. Although all populations evolved high resistance to their specific pathogenic regimes, no correlated responses were detected in larval viability or developmental time of either sex (on either standard or nutrient-poor diet), larval competitive ability, adult reproductive output, or starvation or desiccation resistance. In an independent evolution experiment starting from a different base population, populations selected for resistance to systemic infection by *P. entomophila* showed no detectable changes in developmental time, body size,

fecundity, life span, egg viability or starvation resistance (Gupta et al. 2016). Furthermore, in both experiments, the evolved resistance persisted for 30 (Faria et al. 2015) and 15 (Gupta et al. 2016) generations after exposure to the pathogen had ceased. Interpretation of these results is subject to epistemological caveats: it is based on nonrejection of null hypothesis and it remains possible that a trade-off would be revealed under some other environmental conditions. Nonetheless, these studies provide as strong a support for the absence of significant life-history costs of pathogen resistance as one could reasonably hope for. (For another example of no apparent costs of resistance, in *Caenorhabditis elegans*, see Penley et al. 2018.)

The present study used the *D. melanogaster–P. entomophila* system to test if a cost of evolved pathogen resistance is revealed by sexual selection even if no trade-offs with life-history or stress resistance traits are detected. Male sexually selected traits have been predicted to be particularly sensitive to physiological condition (Rowe and Houle 1996; Getty 2002). Thus, if enhanced pathogen resistance has subtle negative effects on condition in the absence of pathogens, they might make more difference to male success in sexual competition than to life-history traits. On the other hand, some theoretical arguments for the evolution of sexual display traits posit that the genetic correlation between those traits and pathogen resistance is positive even in the absence of pathogens (Westneat and Birkhead 1998). If so, evolution of higher pathogen resistance should rather lead to enhanced sexual traits, making it less likely for costs of resistance to manifest in sexual success.

Two previous studies in *D. melanogaster* allow a direct comparison of correlated responses of life-history traits and male mating success to selection for resistance. The success of males from populations selected for resistance to *Pseudomonas aeruginosa* was indistinguishable from that of unselected controls (Ye et al. 2009), whereas populations selected for parasitoid resistance even evolved a higher competitive mating success (Rolff and Kraaijeveld 2003). Both studies found clear trade-offs of resistance in life-history traits (Kraaijeveld and Godfray 1997; Ye et al. 2009), consistent with the notion that traits under sexual selection may be protected from costs of pathogen or parasite resistance. In contrast, Joye and Kawecki (2019) found that *Drosophila* males carrying alleles that improved resistance to intestinal infection with *P. entomophila* were less likely to win mating contests in the absence of the pathogen (although they were more likely to win contests that took place after pathogen exposure). This suggests a cost of heritable *P. entomophila* resistance that reduces male sexual competitiveness and/or attractiveness. However, this result is based on variation within a population with no history of *P. entomophila* exposure; it also likely represents a net effect of resistance alleles at multiple loci that might vary in their side effects. It is conceivable that the response to selection for *P. entomophila* resistance would be based on those alleles that do not impose costs on mating success, or that such costs would be ameliorated by compensatory changes. Examples of such breaking of genetic correlations are known from laboratory selection experiments (Phelan et al. 2003; Fischer et al. 2007).

Therefore, to test whether evolution of improved *P. entomophila* resistance is costly in terms of male sexual success even in the apparent absence of trade-off with life-history traits, I used the resistant populations of Martins et al. (2013), in which Faria et al. (2015) detected no trade-offs. Because *P. entomophila* is a natural intestinal pathogen of *Drosophila* (Vodovar et al. 2005), and because intestinal infection was applied in the study that found the within-population trade-off mentioned above (Joye and Kawecki 2019), I focused on the four populations of Faria et al. (2015) that had evolved resistance to intestinal infection and the corresponding four Control populations. I compared their sexual success by quantifying paternity when competing with a standard competitor strain over a period of 4 days corresponding to the time of reproduction under their evolutionary regime. To verify that Faria et al.'s (2015) finding of no life-history trade-offs holds under the conditions of my laboratory, I compared larval survival and competitive ability, as well as a measure of productivity that combines larval survival, fecundity, and fertility. I also verified that the focal populations actually had remained resistant despite selection having been relaxed for multiple generations.

Methods

FLY MAINTENANCE

The origin of the flies and the selection regimes are described in detail in Martins et al. (2013). I used their four populations of *D. melanogaster* subjected to selection for 24 generations for resistance to intestinal infection (i.e., by feeding) with the Gram-negative bacterium *P. entomophila* (BactOral populations, further referred to as BO1–BO4) and the four Control populations maintained with no exposure to pathogen under otherwise identical conditions (Control populations, further referred to as C1–C4). Under the selection regime the populations were maintained on a 3-week cycle; the eggs for the next generation were collected over the last 2 days (i.e., days 20–21 counted from egg laying). The selection and the experiments described in Martins et al. (2013) and Faria et al. (2015) were carried out at the Gulbenkian Institute for Science, Portugal. Selection for pathogen resistance was discontinued in July 2012 and the populations have subsequently been maintained on a 3-week cycle. In September 2015, populations derived from them were established at the University of Lausanne, Switzerland, and maintained on a 3-week generation cycle at the density of approximately 250 eggs per 30 mL of a cornmeal–sugar–yeast medium (20 g of yeast per liter), at 25°C and 12:12 L:D cycle. These conditions correspond to Martin et al.'s (2013)

Control regime, but the food recipe and detailed handling were somewhat different. Flies used in the assays described below were raised under the same conditions unless specified otherwise.

RESISTANCE TO *P. ENTOMOPHILA*

To verify that the BactOral populations retained their superior resistance to *P. entomophila* in spite of relaxed selection I assayed their mortality upon oral infection with this pathogen. Adult flies reared as described above were collected 14 days after egg laying and transferred in single-sex groups of 10 individuals to food vials with standard food (10 replicate vials per population and sex). Six days later, these fly groups were transferred to infection vials. The infection vials contained agarose covered by filter paper soaked with 0.1 mL of bacterial suspension. The suspension was a 50:50 mix of 5% sucrose solution and *P. entomophila* pellet obtained from a 48 h liquid culture by centrifugation and resuspended to $OD_{600\ nm} = 200$ in 0.9% NaCl solution. After 30 h in the infection vials, the flies were transferred to new vials with standard food. The number of dead and alive flies in each vial was scored 42 h later (72 h after the onset of *P. entomophila* exposure). In some vials the total of dead + alive flies was less than 10 because a few flies died before the transfer to the infection vials and some were lost during the transfer. This assay was performed in December 2016, when the populations have been maintained without selection for over 4 years.

COMPETITION FOR PATERNITY

The aim of this assay was to test whether evolutionary trade-offs of pathogen resistance affect male competitive paternity success. In the absence of markers that would allow one to distinguish offspring sired by BactOral and Control males from each other, paternity success was measured in competition with males from a "standard competitor" strain carrying a recessive genetic marker (*ebony*). The males competed for females form the same *ebony* strain, allowing one to distinguish offspring sired by the BactOral or Control males (which would be wild-type) from those sired by the *ebony* competitors (which would show the *ebony* phenotype). Studies relying on such "standard competition" approach to assess male sexual competitiveness usually use a marker strain backcrossed to the ancestral population (e.g., Pischedda and Chippindale 2006; Bretman et al. 2009; Hollis et al. 2019). However, here the ancestral gene pool might have been more similar to the Control populations (which continued to evolve under the same conditions) than to the BactOral (which were subject to a strong novel selection). Therefore I used an *ebony* strain backcrossed to an unrelated outbred genetic background (the "IV" population), which is likely to be similarly distant from both Control and BactOral populations.

Males of each BactOral and Control population were raised under standard conditions (three bottles per population) and collected within 24 h of emergence 11 days after egg laying and subsequently maintained in male-only groups. In parallel, I collected freshly emerged males and virgin females from multiple bottles of the *ebony* competitor strain. Five days later the competition vials were established, whereby four males from a given BO or C line were put together with eight *ebony* males and seven *ebony* females in a vial with 12 mL of food (12 replicates per line). A male-biased sex ratio was used to increase the strength of sexual selection, the numerical superiority of the *ebony* males compensated for their lower competitive strength. The flies were allowed to mate and oviposit in those vials for 4 days, that is, on days 18–21 counted from eggs collection. The adults were then discarded and the vials were incubated for 14 days to allow the offspring to develop. Wild-type and *ebony* offspring (the former fathered by the BactOral or Control males, the latter by the competitors) were subsequently counted in each vial. This experiment was carried out in June 2017.

Because the offspring phenotype was only assessed at the adult stage, the paternity estimates could potentially be confounded by differences in competitive survival of larvae sired by BactOral versus Control males. Although the number of eggs laid per vial could not be controlled, *ebony* females have a rather low fecundity and the number of offspring per vial was moderate (see Results), unlikely to induce substantial density-dependent mortality. Nonetheless, the statistical analysis tested for this possibility (see below). Furthermore, one of the experiments described in the next subsection directly tested for differences in larval competitive ability under a more severe food limitation.

VIABILITY, PRODUCTIVITY, AND LARVAL COMPETITIVE ABILITY

Previous thorough experiments by Faria et al. (2015) found no evidence for an inferiority of the BactOral populations relative to the Controls in any nonsexual life-history or stress resistance traits (see Introduction). However, detection of differences in fitness components may be sensitive to specific laboratory conditions and procedures (Ackermann et al. 2001). Thus, it was in principle possible that life-history trade-offs undetected by Faria et al. (2015) would be expressed under the conditions of the laboratory at which the present study has been conducted. If so, a difference in competitive paternity between the BactOral and Control populations could still be part of a more general difference in performance rather than being specific to sexual selection. I addressed this alternative explanation with three experiments, carried out between December 2015 and June 2016.

First, I quantified egg-to-adult developmental time and dry body weight of females and males from the BactOral and Control populations. The flies were raised on standard diet at the density of approximately 200 eggs per bottle (four replicates per population). Because of a technical issue (thermostat drift), this experiment

was carried out at about 23–24°C rather than the 25°C used in other assays. Emergence of adults was scored daily to obtain the mean time from egg to adult in each replicate and sex. Ten haphazardly picked flies of each sex emerged on the peak day of emergence were dried for 24 h at 60°C and weighed as a group to the nearest 0.01 mg on a precision balance to provide a single weight estimate for each replicate and sex.

Second, I performed a noncompetitive assay of egg-to-adult survival and the capacity of the population to grow. Reasoning that trade-offs between immunity and reproduction may be more strongly expressed under nutrient limitation, I quantified these traits on a diet that contained half of yeast compared to our standard diet (i.e., 10 g rather than 20 g/L). Flies of each BactOral and Control population were allowed to oviposit on an orange juice and agar medium and 60 eggs were transferred to a bottle with 30 mL of food (four replicates per population). Sixteen days later, the adults that developed from these eggs were counted to estimate egg-to-adult survival. Over the three following days, these surviving adults were allowed to oviposit in three consecutive bottles with fresh food, being transferred from one bottle to the next at 24 h intervals, before finally being discarded. Their offspring were allowed to develop and those that survived to adulthood were counted 21 days after the eggs were laid. The number of surviving offspring, pooled over the three consecutive bottles and divided by the number of first-generation adults that produced them was used as a measure of per-generation productivity (productivity per adult). I also analyzed the product of first-generation egg-to-adult survival and productivity per adult. This "productivity per egg" quantifies population growth from egg in generation 1 to adult in generation 2, and thus should be more sensitive to small cumulative differences.

Third, in a competition experiment BactOral and Control larvae were pitted against numerically prevailing *ebony* competitors. Approximately 300 eggs of *ebony* flies were transferred to each of 32 bottles with 30 mL of food (with 10 g of yeast per liter); the bottles were assigned randomly to the BactOral and Control populations. A day later, 60 eggs of a given BactOral or Control population were added to the bottles. Thus, the *ebony* larvae were not only initially fivefold more numerous, but also had a day of head start; this was done to compensate in part for the somewhat slower growth and development of the *ebony* larvae. Twenty-one days later the wild-type (i.e., BactOral or Control) and *ebony* adults were counted. Following the standard approach in such competition experiments (e.g., Kraaijeveld and Godfray 1997; Vijendravarma et al. 2008; Tobler et al. 2015), I used the proportion of wild-type adults among all adults that completed development as a measure of larval competitive ability of the respective BactOral or Control populations. This measure is analogous to that used in the competitive paternity assay above; it combines the ability of the focal populations to withstand competition and their ability to suppress the competitor, both of which contribute to their relative fitness. However, I also analyzed the two components of competitive ability separately: egg-to-adult survival of the BactOral versus Control populations, and the egg-to-adult survival of the *ebony* strain when competing with BactOral versus Control larvae.

STATISTICAL ANALYSIS

The analysis was carried out with SAS version 9.4 statistical software. The analysis reflects the two levels of replication in the experiment, the four populations per selection regime and the replicates (vials) within each population. Binomial response variables (the number of dead versus alive or wild-type vs. *ebony* flies) were analyzed with a generalized mixed model (procedure Glimmix) with logit link and binomial error distribution. Developmental time, weight, and productivity were analyzed with a general mixed model (procedure Mixed; normality of residuals was confirmed with a Q–Q plot). Denominator degrees of freedom for the *F*-tests were calculated using the Satterthwaite approximation. Selection regime (BactOral vs. Control) was the key fixed factor; population was a random factor nested in selection regime. For developmental time and dry weight, the model also included sex. The model for binomial variables also included replicate (nested in population and regime) as a random factor.

To address the potential confounding effect of larval competition on the outcome of the competitive paternity assay I also fitted additional models including the total number of adult offspring as a covariate, with and without heterogeneity of slopes between the selection regimes. At low to moderately high densities, the number of surviving adults increases with initial larval density (Sang 1949; Clark and Feldman 1981); thus, a greater number of surviving adults is predictive of a greater initial number of eggs and thus stronger larval competition. If the competitive paternity estimates were confounded by differences in density-dependent survival of larvae sired by BactOral versus Control males, the relationship between their competitive paternity and the total number of surviving offspring should have different slopes.

Results

RESISTANCE TO *P. ENTOMOPHILA*

Males from the BactOral populations were highly resistant to intestinal infection by *P. entomophila*, with only about 5% on average dying within 72 h of infection, whereas the Control males suffered between 40% and 75% mortality, depending on the population ($F_{1,8.1} = 54.5$, $P < 0.0001$; Fig. 1A). For females the difference was less obvious; in particular, several replicate vials of population BO4 suffered 30–70% mortality. A join analysis of both sexes confirms that the effects of selection was less pronounced in females (sex × regime interaction $F_{1,13.6} = 12.8$,

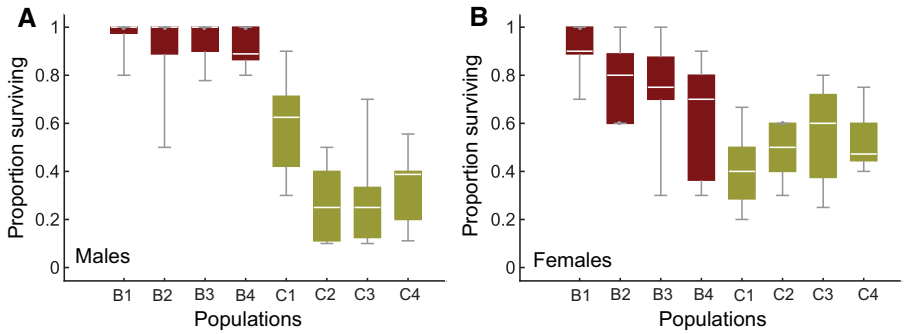

Figure 1. Resistance to intestinal infection with *P. entomophila*. The proportion of male (A) and female (B) flies from the resistant BactOral populations (B1–B4) and the Control populations (C1–C4) that remained alive 72 h from the onset of exposure to the pathogen ($N = 10$ replicates of approximately 10 flies per population and sex).

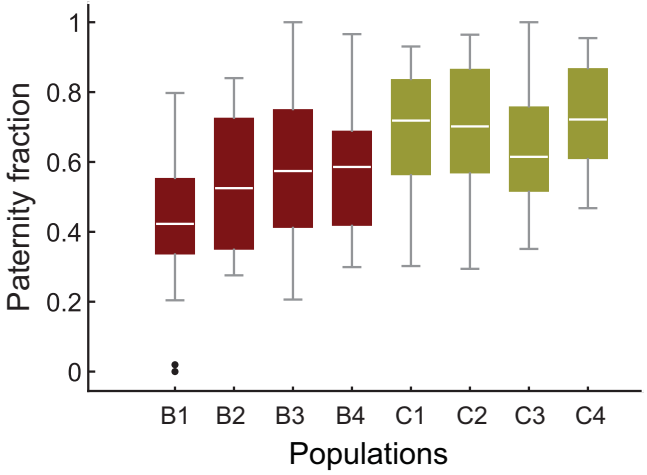

Figure 2. Competitive paternity success (proportion of offspring fathered) of males from the BactOral and Control populations; $N = 12$ replicates per population. Dots indicate extreme outliers in population B1.

$P = 0.0032$). However, the difference in female survival between the selection regimes was still detectable ($F_{1,5.4} = 10.3$, $P = 0.021$; Fig. 1B).

COMPETITION FOR PATERNITY

Males from the BactOral populations fathered a substantially smaller fraction of the offspring than those from the Control populations (53.4 ± 5.3% vs. 72.9 ± 4.2%, least-square means ± SE; $F_{1,5.9} = 8.2$, $P = 0.029$; Fig. 2). Two replicates of the BO1 population had extremely low paternity scores (dot symbols in Fig. 2). When these outliers were removed, the signal of an inferior paternity success of the BactOral relative to Control populations became stronger (main effect of selection regime: $F_{1,6} = 10.7$, $P = 0.017$).

The total number of emerged adult offspring ranged between 39 and 132 (mean = 77.8, SD = 18); the mean was virtually identical for the two regimes (77.9 vs. 77.8). When the total number of offspring per vial was included as a covariate in the analysis of the proportion of wild-type offspring, there was no heterogeneity of slopes between the regimes ($F_{1,92} = 0.1$, $P = 0.81$), and the common slope was not detectably different from zero ($b = -0.0017$ on logit scale, $F_{1,93} = 1.3$, $P = 0.26$; Supporting Information Fig. S1). Thus, the proportion of offspring sired by BactOral or Control males was independent of the total number of offspring per vial.

VIABILITY, PRODUCTIVITY, AND LARVAL COMPETITIVE ABILITY

I found no differences between BactOral and Control populations for either developmental time ($F_{1,6} = 0.1$, $P = 0.79$) or dry body weight ($F_{1,6} = 0.2$, $P = 0.67$), with 95% confidence intervals for the difference between the regimes [−0.40, 0.50] days and [−0.018, 0.026] mg, respectively (Supporting Information Fig. S1). This held for both sexes (sex × regime interaction $P > 0.6$ for both traits).

I also detected no differences between the BactOral and Control populations in egg-to-adult survival and productivity (survival: $F_{1,5.5} = 0.6$, $P = 0.45$; productivity per adult: $F_{1,6} = 0.12$, $P = 0.74$). Although the BactOral populations tended to have a slightly lower egg-to-adult survival and a slightly higher productivity (Fig 3A,B), the least-square means of the two selection regimes were very close to each other (survival: 86.4 ± 1.6% vs. 88.1 ± 1.5%; productivity 8.3 ± 0.6 vs. 8.0 ± 0.6). The same conclusion was reached for "productivity per egg," which combines the effects of egg-to-adult survival in the first-generation and the productivity of the surviving adults (7.1 ± 0.5 vs. 7.0 ± 0.5; $F_{1,6} = 0.02$, $P = 0.89$; Supporting Information Fig. S2A). The 95% confidence intervals for the difference between the regimes (survival: [–7.4, 3.8%], productivity per adult: [–1.7, 2.2], productivity per egg: [–1.8, 2.0]) leave some scope for undetected differences. However, combined with the absence of regime effects in the numerous noncompetitive assays reported by Faria

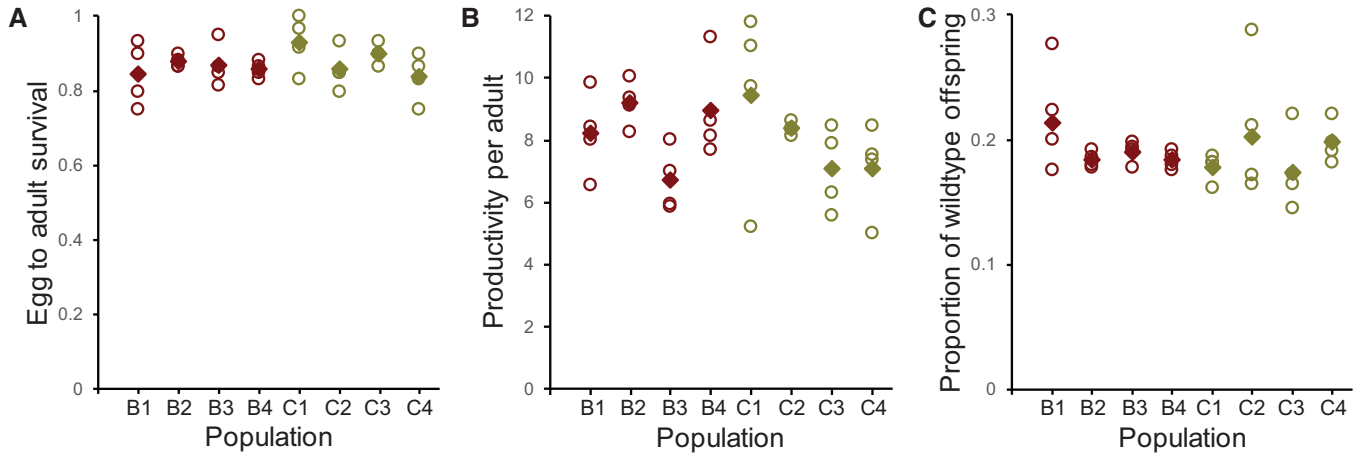

Figure 3. **Performance of the BactOral (B) and Control (C) populations in assays not involving sexual selection. (A) The probability of surviving from egg to adult. (B) Productivity, defined as the number of surviving offspring at generation 2 per adult at generation 1. (C) The outcome of larval competition with ebony standard competitor, quantified as the proportion of adults of a given BactOral or Control population (i.e., wild-type phenotypes) among all individuals surviving to adulthood. Open circles indicate replicate values and diamonds indicate population means.**

et al. (2015), the existence of substantial undetected differences is unlikely.

Similarly, the selection regime did not detectably affect larval competitive ability against numerically superior *ebony* larvae: the proportion of wild types among flies that completed development was similar between BactOral and Control populations (19.3 ± 0.7% vs. 18.8 ± 0.7%, $F_{1,5.8} = 0.2$, $P = 0.67$; confidence intervals for the difference [–2.0%, 2.8%]; Fig. 3C). I also detected no difference in the two components of competitive ability when analyzed separately: the egg-to-adult survival of BactOral versus Control populations in competition with *ebony* larvae (72.1 ± 2.2% vs. 75.8 ± 2.0%, $F_{1,5.4} = 1.6$, $P = 0.26$; Supporting Information Fig. S2B), and the egg-to-adult survival of the *ebony* strain when competing against BactOral versus Control larvae (60.6 ± 2.1% vs. 65.7 ± 2.0%, $F_{1,5.4} = 3.05$, $P = 0.09$; Supporting Information Fig. S2C). The total number of surviving offspring (out of the initial 360 eggs per bottle) ranged from 155 to 272 (mean 233, SD 25).

Discussion

This study has shown that the evolution of enhanced resistance to intestinal infections with *P. entomophila* in *D. melanogaster* was associated with a reduction of male competitive paternity success in the absence of the pathogen. Although the competitive paternity was assessed based on the phenotype of surviving offspring, its outcome is unlikely to be confounded by differences in larval survival between offspring of the BactOral and Control males. First, I found no difference in egg-to-adult survival of BactOral and Control larvae under low-density conditions, implying no density-independent difference in larval viability. Second, in the competitive paternity assay itself there was no relationship between the proportion of larvae sired by the focal males and the number of emerging adults, nor any indication that the slope of this relationship might differ between the BactOral and Control populations. Such a difference would be expected if the two sets of populations differed in density-dependent larval mortality. Therefore, the lower proportion of offspring sired by BactOral males most likely indicates their lower sexual competitiveness compared to Control males, mediated by lower success in acquiring matings, preventing females form remating with other males and/or sperm competition.

I found no evidence of trade-offs in nonsexual traits such as development time, adult weight, larval viability, competitive ability, and population reproductive rate. Although these negative results alone could only provide weak support for absence of nonsexual trade-offs of *P. entomophila* resistance, they are consistent with previous extensive assays of these populations (Faria et al. 2015) and the independent experimental evolution study by Gupta et al. (2016), which likewise found no such evidence. This suggests that, at least in some cases, sexually selected traits are more sensitive to subtle physiological trade-offs than life-history traits, as has been proposed on theoretical grounds (Rowe and Houle 1996; Getty 2002).

During their experimental evolution, the BactOral populations were bred from survivors of 620 adults exposed to infection, with mortality in the first-generation reaching 77% (Martins et al. 2013). This raises the possibility that these populations suffered from inbreeding, which could explain their lower sexual competitiveness. However, even in this first generation, the number of surviving adults was about 140, which is not a severe bottleneck. Furthermore, the response to selection was swift, with mortality

below 50% after one generation, less than 20% after 4 generations and less than 10% after 15 generations (Fig. 1A in Martins et al. 2013). Using demographic data from Martins et al. (2013) and assuming an effective population size of half of the number of breeding adults (Hardy et al. 2018), I estimated that after the 24 generations of selection the BactOral populations should have retained about 95% of their initial heterozygosity. This is only slightly less that the corresponding estimate of 96% for the Control populations (see Supporting Information Appendix). Genomic data that could be used to support these rough estimates are not published. However, the estimates are consistent with estimates based on genomic data from analogous populations selected in the same laboratory for resistance to a virus, starting from the same initial gene pool and following the same protocol (Martins et al. 2014). Those virus-resistant (VirSys) populations retained 93% of their initial heterozygosity despite being under selection for 10 more generations than the BactOral populations, and despite taking longer to evolve reduced mortality. The corresponding sham Control populations retained 95% of the initial heterozygosity (Supporting Information Fig. S2B in Martins et al. 2014). Naturally, both BactOral and Control populations would continue to lose genetic diversity to drift after the selection has been relaxed, but once under the same demographic regime the ratio of their heterozygosities would remain unchanged (Crow and Kimura 1970). It is hard to conceive of a genetic architecture under which such a small difference in heterozygosity ($<2\%$) would cause a 20% difference in competitive paternity reported above. Thus, greater inbreeding (i.e., genome-wide loss of heterozygosity) is unlikely to be a major contributor to the sexual inferiority of the BactOral populations.

The lower number (and thus population density) of breeding adults in the first few generations of experimental selection might also have to some degree reduced the intensity of sexual selection in the BactOral populations. However, even with the minimum number of 140 adults the density would have been high enough to ensure multiple intra- and intersexual interactions, as well as ample opportunity for sperm competition. Once the infection-induced mortality decreased, the adult density has been similar in BactOral and Control populations, both during the last 15 generations of experimental selection, and during the subsequent >4 years (>70 generations) without a selection treatment. It seems unlikely that a few generations at a lower adult density could on their own have led to a substantial reduction in male sexual success that would not be reversed by subsequent >70 generations at the high density.

It is also in principle possible that the differences in male sexual success evolved after the selection for pathogen resistance was discontinued, in particular following the transfer of the populations between laboratories, which likely triggered some adaptation to the new laboratory conditions. However, both BactOral and Control populations have been exposed to the same conditions, and preliminary analysis of their whole genome sequence data (from samples taken before the transfer) indicates that their gene pools were very similar (E. Sucena and N. Martins, pers. comm.). They would thus be expected to adapt to the new conditions similarly. Furthermore, I found no difference in their life-history traits under the new conditions that would suggest different degree of laboratory adaptation.

It is thus more parsimonious to interpret the lower sexual competitiveness of the BactOral populations as a correlated response to selection, reflecting an additive genetic correlation between sexual competitiveness and resistance to *P. entomophila* (Falconer and Mackay 1996). It could be mediated by pleiotropic effects of alleles improving resistance to *P. entomophila* or by hitchhiking of linked deleterious alleles; both would result in a microevolutionary trade-off constraining short-term evolution (Lande 1982; Stearns 1989). This result is consistent with the finding that, within an unrelated population, *Drosophila* males that sire more *P. entomophila* resistant sons are less successful in competition for mates in the absence of the pathogen (Joye and Kawecki 2019).

Although the physiological and molecular mechanisms of pathogen virulence and host defense in the *D. melanogaster–P. entomophila* system are relatively well known (Lemaitre and Hoffmann 2007; Buchon et al. 2009; Lemaitre and Girardin 2013), the mechanisms of the improved resistance of the BactOral populations have not been addressed. Two other studies associated natural genetic variation in *P. entomophila* resistance with differences in the production of reactive oxygen species (ROS), which are candidates for a mechanism mediating costs of resistance due to oxidative damage. However, in both studies resistant genotypes showed lower ROS levels, making it an unlikely candidate to explain costs of resistance (Sleiman et al. 2015; Vijendravarma et al. 2015). Neither study detected any differences in the expression of antimicrobial peptides. There is also no evidence of resistant genotypes ingesting less bacteria (Sleiman et al. 2015; Vijendravarma et al. 2015), despite the fact that flies can learn to avoid food contaminated with *P. entomophila* (Babin et al. 2014). Resistant genotypes are better able to maintain gut wall integrity (Vijendravarma et al. 2015), which depends on the activity of intestinal stem cells and the ability to resist pathogen-induced inhibition of protein synthesis (Sleiman et al. 2015). Maintenance of this ability might reduce the efficiency of digestion and nutrient absorption, a hypothesis supported by experimental evolution on nutrient-poor diet having led to enhanced protein digestion and lower resistance to *P. entomophila* as a correlated response (Vijendravarma et al. 2015; Erkosar et al. 2017). None of these hypothetical mechanisms would explain why costs of resistance should be expressed in traits affecting male success and not in life-history traits. However, the fact that the effect of selection

on resistance (in terms of mortality difference between BactOral and Control flies) was greater in males than in females might be an explanation as to why the costs were detected in a male trait, but not in female traits, or population traits largely determined by female life history (like productivity).

It is also conceivable that BactOral males were selected to reduce their investment in courtship and mating because these activities reduce resistance. Rather than poorer male success being a side effect of immune defense mechanisms, in this scenario susceptibility to pathogens would be a cost of elevated sexual activity (Fedorka et al. 2004). Thus, in the presence of pathogens a reduced rate of sexual activity might facilitate a higher lifetime reproductive success via a longer reproductive life span. However, the evidence for the effects of sexual activity on resistance in *Drosophila* males is mixed, with one study reporting a negative effect (McKean and Nunney 2001) and another a positive effect (Gupta et al. 2013).

A trade-off with male sexual success would be expected to select against resistance alleles in the absence of the pathogen. Yet, the BactOral populations maintained their resistance for over 70 generations after exposure to *P. entomophila* had ceased. Several potential explanations can be proposed. Possibly, the strong selection had led to fixation of alleles for resistance in the BactOral populations and thus prevented reversal after selection was relaxed. It is also possible that the consequences of the trade-off for male fitness under the actual culture conditions were small and only became magnified under the conditions of the experiment. The experiment necessitated use of virgin flies, which then mated and oviposited over 4 days under a male-biased sex ratio. By comparison, under the relaxed selection regime flies were maintained from emergence in large groups with natural (i.e., approximately 50:50) sex ratio, and eggs for the next generation were collected on day 21. The experiment also involved competition with a standard competitor strain carrying a genetic marker to enable scoring of paternity rather than a direct competition between BactOral and Control males. The outcome of sexual competition and mate choice may be affected by specific genotypes of males and females involved, and thus the results might not be representative of sexual selection within populations (Chapman 2018). Finally, having evolved under pathogen pressure, females from the BactOral populations could have been selected for preference to mate with and use the sperm of genetically resistant males (Hamilton and Zuk 1982; Beltran-Bech and Richard 2014). One might thus speculate that BactOral females have evolved a preference for BactOral males, which could compensate for inferiority of the latter in general sexual competitiveness. If so, the trade-off would be of little consequence for fitness under the conditions under which these populations evolved. Nonetheless, it is interesting that it affects male sexual success rather than other aspects of performance that were not under direct selection, such as starvation and desiccation resistance or ability to develop on nutrient-poor diet (Faria et al. 2015).

Apparently very few previous studies quantified correlated responses of male mating success to selection on pathogen resistance or immune response. Bank vole males selected for high humoral immune response had lower mating success (Mills et al. 2010), in qualitative agreement with the present study. However, in *Drosophila*, Ye et al. (2009) found no correlated response of male mating success in *Drosophila* selected for resistance to systemic infection by *P. aeruginosa*, whereas Rolff and Kraaijeveld (2003) found that selection for parasitoid resistance increased male competitive mating success. Of experimental evolution studies that manipulated the strength of sexual selection, some found that evolution of greater male sexual competitiveness was associated with reduced immunity (McKean and Nunney 2008; van Lieshout et al. 2014) while others found no effect (Hangartner et al. 2013; McNamara et al. 2013; Hangartner et al. 2015). Estimates of within-population genetic correlations between male success or attractiveness in the absence of pathogens and pathogen resistance or immunity traits obtained from breeding designs are likewise variable. Some are positive (Birkhead et al. 2006; Svensson et al. 2009), some negative (Simmons and Roberts 2005; Simmons et al. 2010), others found no correlation or correlations that were inconsistent across different immune or sexual traits (Kurtz and Sauer 1999; Kurtz 2007; Bonato et al. 2013; Guncay et al. 2017). (Barber et al. 2001 found a positive genetic correlation between a sexual ornament and resistance to a parasite, but in this case the males had most likely been exposed to the parasite while developing the ornament; Barber and Scharsack 2010.) The interpretation of these inconsistent results is further hampered by most of them being based on quantification of immune effectors or pathogen load as a measure of resistance. Such proxies may not be good predictors of resistance defined as reduction of fitness costs of pathogen exposure, which is what matters from the viewpoint of the host (Getty 2002; Adamo 2004; Viney et al. 2005; Raveh et al. 2014). Those that did measure resistance in terms of host survival (Rolff and Kraaijeveld 2003; Ye et al. 2009; Guncay et al. 2017) found no trade-off with male sexual success. Thus, the present study and Joye and Kawecki (2019) may be the first to document a genetically based cost to male sexual success of being able to survive pathogen exposure.

Several of the studies cited above were motivated by the "general immocompetence" theory linking sexually selected traits with pathogen resistance (Fig. 2B of Westneat and Birkhead 1998). It proposes that resistance to diverse pathogens and parasites is determined by a general immunocompetence that is linked to sexually selected traits by shared dependence on the physiological condition of the organism. Although there is an allocation trade-off between them, genetic variation corresponding to this trade-off is assumed to be small relative to genetic variation in condition,

which has synergistic effects on both types of traits. This leads to the prediction that the genetic correlation between immunity and sexually selected traits should be positive irrespective whether the individuals have been exposed to pathogens or parasites (Westneat and Birkhead 1998; Roberts et al. 2004; Tomkins et al. 2004; Birkhead et al. 2006; Hill 2011). The present results contradict this prediction. One potential explanation is that the genetic variance that affected allocation to sexual traits versus immunity was substantial and selection for *P. entomophila* resistance mainly acted on this antagonistic variance rather than on loci affecting both traits synergistically via condition. Alternatively, resistance to *P. entomophila* might largely be condition-independent but rather affect aspects of condition relevant to male sexual success through immunopathology or other pleiotropic effects ("resistance and immunopathology" model, Fig. 2C of Westneat and Birkhead 1998). In support of the latter hypothesis, flies raised on an extremely poor diet have similar resistance as flies raised on standard diet despite being half the normal size, and the difference between resistant and susceptible populations is not affected by diet (Vijendravarma et al. 2015).

Irrespective of the underlying mechanisms, the present results, combined with the literature reviewed above, indicate that the genetic and evolutionary relationships between pathogen resistance and major nonsexual and sexual fitness components may be highly variable, even within the same host species. These relationships may depend in idiosyncratic ways on the specific pathogen or parasite and the environmental context, or on the initial gene pool. Thus, rather than being universal indicators of pathogen resistance, sexually selected traits may indicate heritable susceptibility because resistant males suffer physiological costs in the absence of pathogens.

AUTHOR CONTRIBUTIONS

TJK designed the study, performed the experiments (with the help of persons mentioned in Acknowledgments), analyzed the results, and wrote the article.

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DATA ARCHIVING

Data are available on Dryad (https://doi.org/10.5061/dryad.9p8cz8wbv).

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary Figure S1. Egg-to-adult developmental time and dry body weight of females and males from the BactOral and Control populations (the symbols correspond to replicate means).

Supplementary Figure S2. The relationship between the proportion of offspring sired by males from the BactOral and Control populations in the competitive paternity assay and the total number of offspring emerging from a given vial.

Supplementary Figure S3. Alternative measures of success of BactOral and Control populations in the productivity and larval competition assays.

Appendix. Estimation of the loss of heterozygosity during experimental evolution.