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Evolution of *Drosophila* resistance against different pathogens and infection routes entails no detectable maintenance costs

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Pathogens exert a strong selective pressure on hosts, entailing host adaptation to infection. This adaptation often affects negatively other fitness-related traits. Such trade-offs may underlie the maintenance of genetic diversity for pathogen resistance. Trade-offs can be tested with experimental evolution of host populations adapting to parasites, using two approaches: (1) measuring changes in immunocompetence in relaxed-selection lines and (2) comparing life-history traits of evolved and control lines in pathogen-free environments. Here, we used both approaches to examine trade-offs in *Drosophila melanogaster* populations evolving for over 30 generations under infection with *Drosophila* C Virus or the bacterium *Pseudomonas entomophila*, the latter through different routes. We find that resistance is maintained after up to 30 generations of relaxed selection. Moreover, no differences in several classical life-history traits between control and evolved populations were found in pathogen-free environments, even under stresses such as desiccation, nutrient limitation, and high densities. Hence, we did not detect any maintenance costs associated with resistance to pathogens. We hypothesize that extremely high selection pressures commonly used lead to the disproportionate expression of costs relative to their actual occurrence in natural systems. Still, the maintenance of genetic variation for pathogen resistance calls for an explanation.

KEY WORDS: Costs, *Drosophila*, experimental evolution, host–parasite, immunity, trade-offs.

Several studies have shown that resistance to pathogens evolves rapidly in host populations (Boots and Begon 1993; Kraaijeveld and Godfray 1997; Lohse et al. 2006; Zbinden et al. 2008; Martins et al. 2013). This indicates that standing genetic variation (SGV) for host resistance to parasites is maintained in most systems. However, parasites are ubiquitous and they pose a strong fitness cost upon hosts. Hence, high resistance should be fixed in host populations. In other words, the seemingly paradoxical occurrence of SGV for traits involved in fighting pathogenic infections calls for an explanation. Such maintenance is often attributed to the occurrence of a trade-off between resistance to pathogens and other fitness-related traits (for a review, see McKean and Lazzaro 2011).

Experimental evolution allows for robust tests of the occurrence of evolutionary-relevant genetic trade-offs. Indeed, with this methodology, the ancestral state is known, hence comparisons between control and evolved lines allow identifying traits

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modified by a specific selection pressure as well as correlated responses to selection. Moreover, the method avoids spurious correlations due to individuals (or their parents) having been in different conditions, or subject to different recent evolutionary histories (Kawecki et al. 2012; Magalhães and Matos 2012).

Trade-offs between immunity and fitness-related traits in experimentally evolving lines are tested using two main approaches. The first consists in creating lines of relaxed selection (Lenski 1988; Ye et al. 2009; Meyer et al. 2010; Duncan et al. 2011). These lines derive from populations evolving in the presence of the pathogen and are then placed for several generations in pathogen-free conditions. The occurrence of a trade-off is inferred if individuals from these lines show a lower performance when exposed to pathogens, as compared to the pathogen-resistant ancestral population they were derived from. In short, a costly defense is expected to be rapidly lost in the absence of the pathogen it targets. This logic is appealing but may not be universal. Indeed, reverting to the ancestral state may be prevented by the loss of genetic variation allowing for such a reversion, although this possibility is seldom tested (but see Teotónio and Rose 2000). Alternatively, resistance may be costly but evolution in a pathogen-free environment selects for mutations that compensate such cost. This is widely shown in antibiotic-resistant bacteria (reviewed in MacLean et al. 2010) but has never been tested in multicellular sexual species, possibly because it relies upon the appearance of novel mutations, which require large populations and a high number of generations.

Another possible approach to test such costs is by measuring the performance of individuals from lines selected for pathogen resistance when placed in a pathogen-free environment (Boots and Begon 1993; Kraaijeveld and Godfray 1997; Lohse et al. 2006; Schwarzenbach and Ward 2006; Luong and Polak 2007; Cotter et al. 2008; Zbinden et al. 2008; Vijendravarma et al. 2009; Koskella et al. 2012; cf. review in Duncan et al. 2011). Under such an approach, several life-history traits, thought to correlate with fitness, can be measured. Moreover, these tests can be done in several environments.

Irrespective of the method used, all studies addressing the consequences of the evolution of pathogen resistance have found a cost for this trait, with two exceptions. First, using both methods described above, adaptation of the cabbage looper to a virus was found to be free of cost (Milks et al. 2002). Second, Meyer et al. (2010) found no cost in *Escherichia coli* resistance to phage T6 (but a cost in resistance to other phages). Therefore, such costs seem to be the rule, with few exceptions. This ubiquity of costs to immunity lends support to the hypothesis that such costs underlie the maintenance of SGV for host resistance (Antonovics and Thrall 1994).

Experimental evolution using *Drosophila* as a model host has repeatedly shown that the evolution of resistance to pathogens is costly (Kraaijeveld and Godfray 1997; Fellowes et al. 1998; Luong and Polak 2007; Vijendravarma et al. 2009; Ye et al. 2009). In our previous work, we have performed experimental evolution of an outbred population of *Drosophila melanogaster* adapting to infection with different pathogens, Drosophila C Virus (DCV) or the gram-negative bacterium *Pseudomonas entomophila*, the latter being administrated via either an oral or a systemic route (Martins et al. 2013, 2014).

We found that these populations increased resistance against these challenges within few generations, thereby demonstrating the presence of ample SGV for this trait. Here, we took advantage of this resource to test whether *Drosophila* resistance to such immune challenges entailed a cost. We did this using the two approaches mentioned above: (1) we created relaxed-selection lines, that is, lines in which selection for pathogen resistance was relaxed, and tested for its maintenance over several generations; and (2) we compared the values of several life-history traits in control and evolved lines in several pathogen-free environments, including the ancestral environment.

Materials and methods

PATHOGEN STOCKS AND CULTURES

Pseudomonas entomophila (a generous gift of B. Lemaitre) was grown in LB inoculated with a single bacterial colony, taken from glycerol stocks kept at −80°C and streaked in fresh Petri dishes. Bacteria were prepared from an overnight culture grown at 30°C, centrifuged, and adjusted to the desired optical density (OD) using fresh Luria Broth medium (LB). Virus aliquots were grown and titrated as described elsewhere (Teixeira et al. 2008), kept at −80°C and thawed prior to infection.

EXPERIMENTAL EVOLUTION LINES

From a highly outbred population of *D. melanogaster* (Martins et al. 2013), we derived 20 lines corresponding to three distinct immune challenges and two matched controls with four replicate lines each: (1) oral infection with *P. entomophila* (BactOral), (2) systemic infection by pricking flies with *P. entomophila* (BactSys), (3) systemic infection by pricking flies with DCV (VirSys), (4) controls under standard conditions (Control), and (5) blank injected controls (ControlSys). At each generation, 600 flies were exposed to each challenge, and the survivors used to form the next generation. We selected an initial concentration of pathogens that killed approximately 66% of the fly population. At each generation, survival to infection was monitored by following the survival of 100–120 adults challenged with the same pathogen they were exposed to during selection every day until at least the 10th day postinfection. Flies were maintained under constant temperature (25°C), humidity (60–70%) and light-darkness cycle (12:12),

and fed with standard cornmeal-agar medium. Detailed protocols for the selection experiment can be found in our previously published work (Martins et al. 2013, 2014). We hereafter refer to lines continuously exposed to the parasites as "Selection lines", to distinguish them from "Relaxed-Selection lines," see below.

RELAXED-SELECTION LINES (AND TESTS TO THEIR IMMUNOCOMPETENCE)

We first established that a plateau of resistance was reached in each selection regime. This was estimated to occur whenever no difference in the response to pathogen infection was found in five consecutive generations, which took place at different periods for each selection regime. BactOral reached this plateau from generation 9 onwards, VirsSys from generation 21 onwards, and BactSys from generation 25 onwards (Martins et al. 2013, 2014). We then derived Relaxed-Selection lines, one per each Selection line (i.e., four per selection regime, cf. Fig. 1A). To do this, 600 individuals of each population of a given selection regime were placed in new population cages. Reproduction took place at the same days as the matching Selection lines, and in the subsequent generations, the Relaxed-Selection population sizes (600 individuals) mirrored those of the Control lines. Survival of Relaxed-Selection following exposure to the parasites/route of infection matching to the corresponding Selection lines was monitored daily until at least the 10th day postinfection at each generation, in parallel with the Selection and Control lines.

FITNESS COSTS IN PARASITE-FREE ENVIRONMENTS

Fitness-related traits in parasite-free environments were compared between individuals from Selection and Control lines. To avoid possible artifacts due to maternal effects, flies used in these tests were the progeny of flies that spent at least one generation in a common environment without pathogens, that is, in the standard environment of the base population. These assays were performed at generation 23 or 24 for reproductive output, development time, and resistance to desiccation and starvation. Nutritional restriction and competition assays were done more than 30 generations after the end of the selection experiment (between generations 64 and 75 for all lines), hence evolved lines had been under a Relaxed-Selection regime for 30 generations. Therefore, a test for the maintenance of immunocompetence was performed on those lines at that moment, to ensure that differences between control and evolved lines were still present. This test was done as described in the last section.

Reproductive output

Reproductive output assays were designed to mimic the procedure followed during experimental evolution. Fifteen male–female pairs from each Selection and Control lines were transferred to fresh food vials eight to 10 days posteclosion and let to lay eggs for 48 h. Reproductive output was assayed as the number of adults emerging from pupae 12 days after oviposition.

Development time

To determine the mean fly development time, 10 replicate groups of five uninfected females (10–11 days old) were let to lay eggs for 1 h in standard food vials. Egg never exceeded 52 per vial (mean density 17). The assay conditions mimic the experimental evolution procedure. The number of emerging adults was counted every 3 h after the ninth day postoviposition.

Resistance to starvation and desiccation

For the desiccation assay, 100 individuals (males and females) from each population were placed in groups of 10 in empty vials, and mortality was scored every 3 h. For the starvation assay, 100 individuals (males and females) from each population were placed in groups of 10 in empty vials, with water supplied ad libitum by moisturizing the vial plugs.

Nutritional restriction

For each assay, 200 eggs from each population were placed in 10 groups of 20 eggs, both in standard food vials and nutritionally restricted food (standard food diluted 1:8 with water maintaining the agar concentration). Viability in both conditions was estimated as the number of adults emerging from pupae. To determine the mean fly development time, the number of emerging adults was counted every 12 h after the ninth and 14th day postoviposition for standard and restricted food, respectively.

Larval competitive ability

Finally, we tested whether populations that had evolved increased immunocompetence against each pathogen had lower larval competitive ability compared to control lines. To this aim, we competed first instar larvae of the evolved populations (and their controls) against the same outbred control population carrying an introgressed white mutation. Pharates were weighted and classified as males or females, red eyes or white eyes.

STATISTICAL ANALYSES

Relaxed selection

To compare survival across generations in the different Selection and Relaxed-Selection lines, the proportion of individuals surviving at day 10 after infection in each vial was first estimated using the Kaplan–Meier method. Subsequently, a generalized linear mixed model (GLMM) was fitted to the data, assuming a binomial distribution and an underlying logit link function. The proportion of survivors, weighted by the number of individuals in each vial as dependent variable was fitted in a model with sex, generation, and regime (Control, Selection, or Relaxed-Selection)

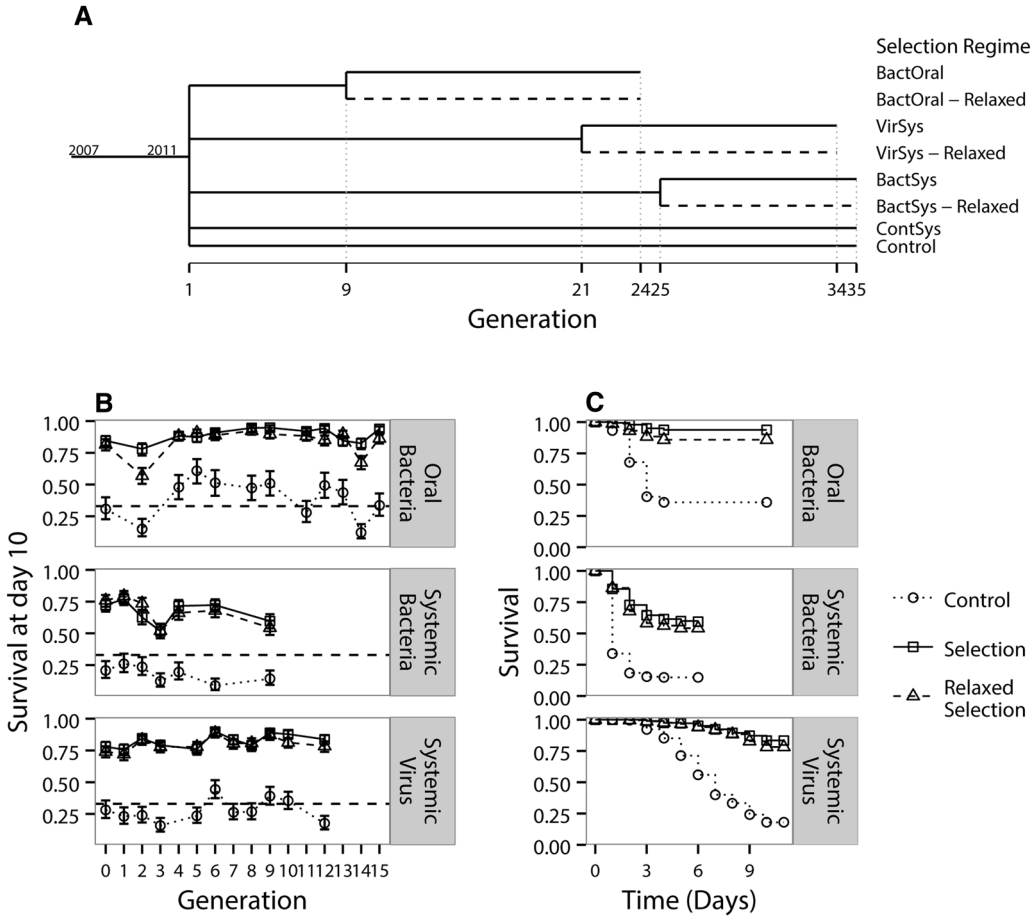

Figure 1. **Increased immunocompetence is maintained in relaxed-selection populations. (A) Diagram representing the different selection regimes used in this study. Lines were challenged with a pathogen at every generation (Selection) or kept unchallenged (Control). From each Selection line, a line was derived and maintained in the ancestral environment (dashed lines, Relaxed-Selection). (B) Mean survival (±95% CI) 10 days postinfection of individuals from Control (circles), Selection (squares), and Relaxed-Selection (triangles) lines, across 10–15, starting at the moment at which relaxed selection lines were initiated generations (see Materials and Methods). (C) Dynamics of survival after infection at the last generation of relaxed selection.**

as fixed factors. Line nested within selection regime and sex at each generation was considered a random factor.

Subsequently, we tested for differences in survival between lines, both overall and across generations. When differences in survival between Selected and Relaxed-selection lines were found, we then tested for changes in the mean difference between Control and Selection or Relaxed-Selection lines, between the first and subsequent generations after the derivation of the Relaxed-Selection lines. In addition, we also tested if there was a linear trend for change (increase or decrease) across generations in the mean survival of the different lines, by considering generation an ordered factor.

Moreover, we tested for differences in the slope of the mean survival across generations, by fitting a logistic regression mixed model with generation as a continuous variable, assuming a binomial distribution and an underlying logit link function. The proportion of survivors, weighted by the number of individuals in each vial as dependent variable was fitted to a model with sex and regime (Control, Selection, or Relaxed-Selection) as fixed factors and generation of relaxed selection as a continuous covariate.

To compare survival among Control, Selection, and Relaxed-Selection lines in the last generation of selection, we used a Cox's proportional hazards mixed-effect model for each treatment, with survival time of individual flies as the dependent variable, selection regime and sex as fixed factors and replicate vial nested within line as a random factor.

In the tests for maintenance of immunocompetence, done at generations 60–75, we used a GLMM identical to that used for the relaxed-selection analysis, comparing survival after infection between Control and Relaxed-Selection lines.

Life-history traits in parasite-free environments

To compare reproductive output in the Control and Selection lines in the absence of infection, we used a linear mixed model (LMM),

with the number of hatching eggs within 48 h by a single female as dependent variable, selection regime and generation as fixed factors, and replicate vial nested within line and generation as a random factor.

To compare development time among lines, we fitted an LMM with days to eclosion of individual flies as dependent variable, selection regime as fixed factor, and replicate vial nested within line as a random factor.

To compare survival under starvation and desiccation conditions, we used a Cox's proportional hazards mixed-effect model for each treatment (starvation or desiccation), with survival time of individual flies as the dependent variable, selection regime and sex as fixed factors, and replicate vial nested within line as a random variable. We also compared differences in the mean time to death (TTD) between selection regimes. For this, TTD was calculated for each vial, using the Kaplan–Meier method, and was fitted as a dependent variable in a GLMM with sex and selection regime as fixed factors and line nested within each selection regime and sex as random factor.

To compare viability in nutrient-limiting conditions, we used a GLMM with the number of eclosing versus noneclosing individuals as a binomial variable, selection regime and food type (Regular vs. Nutrient limited) and their interaction as fixed factors, and test vials nested into line as random factors, with an underlying logit link function. Development time was compared as above, including food type as an additional fixed factor and removing egg density as covariate. Least-square estimates of viability and development time were then compared between selection regimes, independently for each food type.

To test for differences in larval competitive ability, the variable weight was log-transformed to comply with normality. To confirm that a higher density implied a cost in larval weight, we compared the weight in each density using a generalized mixed model with competition level (either 15 or 30 flies from each line), selection regime and sex, and their interactions, as fixed factors and replicate as random factor. Following a significant effect of the density (cf. results), we then performed the analysis at the highest density, to address potential costs in flies derived from the selection lines. To this aim, we compared the weight of individuals from each selection regime to that of tester individuals from the same assay using a general linear model (GLM) with selection regime (either BactSys, BactOral; ContSys, VyrSys; or Tester populations), sex and their interaction as factors.

All statistical analyses were done in R (version 3.1.2). LMMs were fitted using the *lmer* function and GLMMs with the *glmer* function, both in the "lme4" package in R. The effects of the fixed factors and of the hierarchical interaction terms were compared using Type II Wald χ^2 tests (*Anova* function in the "car" package). Contrasts of least-square means estimates and of regression coefficients were done on the most parsimonious model, that is, in models including only significant ($P < 0.05$) factors and interactions, using the *lsmeans* and *lstrends* function in the "lsmeans" package. Survival data were compared using the *coxme* function in "coxme" package. Hierarchically nested models were compared using likelihood ratio tests. The sex-averaged hazard ratios were then compared, using the *glht* function in the "multcomp" package in R. The reported *P*-values for tests involving multiple comparisons were adjusted using a sequential Bonferroni correction.

Results

MAINTENANCE OF RESISTANCE UNDER RELAXED SELECTION

For all pathogen challenges, significant differences in survival were found among Control, Selection, and Relaxed-Selection lines (Fig. 1B and Table S1). This effect was mainly caused by the difference between Control and either Selection or Relaxed-Selection lines (Fig. 1B). To get a more detailed description of mortality dynamics upon infection of the different selection lines, we also measured survival over 10 days after infection in flies from the last generation of selection (Fig. 1C and Table S5).

Differences between both Selection and Relaxed-Selection lines to Controls were always significant in the BactSys, BactOral, and VirSys lines (Fig. 1B and C), either globally ($|z|>$ 23.5, $P < 0.001$, $|z| > 29.3$, $P < 0.001$ and $|z| > 37.2$, $P < 0.001$, respectively), at each generation ($|z| > 7.31$, $P < 0.001$, $|z| > 5.7$, $P < 0.001$ and $|z| > 9.46$, $P < 0.001$, respectively, for all comparisons), or when comparing mortality dynamics in the last generation of selection ($|z| > 5.58$, $P < 0.001$, $|z| > 10.06$, $P < 0.001$ and $|z| > 6.30$, $P < 0.001$, respectively, for all comparisons). Excluding in the third generation of relaxed selection, where the Relaxed-Selection lines showed significantly lower mortality than the Selection lines ($|z| = -2.87$, $P = 0.029$), we did not observe significant differences between these lines at different generations ($|z| <$ 1.38, $P > 0.999$, for all comparisons), nor in the mortality dynamics in the last generation of selection ($|z| = 0.83$, $P = 0.405$). In the VirSys versus VirSys-Relaxed comparisons, no differences were found when comparing survival at each generation ($|z| < 2.49$, $P > 0.4$, for all comparisons), nor when comparing the mortality dynamics in the last generation ($|z| = 0.38$, $P = 0.704$; Tables S2 and S6). We also did not find a significant difference in the linear slope of survival across generations between the different selection regimes (GLMM, Generation × Selection regime effect, $\chi^2_2 < 3.79$, $P > 0.150$), despite a significant generation effect (Generation effect, $\chi^2_1 > 18.67$, $P < 0.001$), indicating no differences between the regimes in the overall trend in survival across generations (Tables S3 and S4).

In contrast, there was a significant difference, between the BactOral lines and their matched Relaxed-Selection lines ($|z|$ = 5.8, $P < 0.001$), in four generations across the experiment,

including in the last generation of selection ($|z| = 3.63$, $P < 0.001$; Tables S2 and S4). This difference cannot be attributed to either an increased relative mortality in the Relaxed-Selection lines (comparison between Control and Relaxed-Selection lines remained constant across generations, $|z| < 1.74$, $P > 0.9$) or a decrease relative mortality in the Selection lines (comparison between Control and Selection lines remained constant, $|z| < 2.76$, $P > 0.53$).

To explore the reason for this difference, we tested changes in absolute survival across generations, separately for the Selection, Control, and Relaxed-Selection Lines. In this analysis, whereas in the Selection lines survival increased significantly ($|z| = 3.74$, $P < 0.001$), this trait did not change significantly in Relaxed-Selection and Control lines over 11 generations ($|z| = 1.44$, $P = 0.450$ and $|z| = 1.29$, $P = 0.595$, respectively). In agreement with this finding, we also did not find a significant difference in the linear slope of survival across generations among selection regimes (GLMM, Generation × Selection regime effect, $\chi^2_2 = 2.91$, $P = 0.233$), again indicating no differences among regimes in changes in survival across generations (Tables S3 and S4). Therefore, we attribute the small but significant differences between Selection and Relaxed-Selection lines (less than 7% in the last generation of selection) to a marginal increase in survival in the former (approximately 9%), where selection was continued, whereas there was no increase (or decrease) in mortality in the latter.

At generations 60 and 70, at the moment we tested for larval competitive ability, relaxed-selection lines were still significantly more immunocompetent than control lines (lme, BactOral vs. Control: $z = 3.04$, $P = 0.0002$; BactSys vs. ContSys: $z = 8.28$, $P < 0.0001$; VirSys vs. ContSys: $z = 9.48$, $P < 0.0001$).

COSTS OF RESISTANCE IN PARASITE-FREE ENVIRONMENTS

We also tested for the occurrence of trade-offs by comparing several life-history traits between Selection and Control lines. We started by measuring the reproductive output (Fig. 2A) and developmental time at generations 23 and 24 (Fig. 2B) in these lines in the absence of infection. We found no effect of Selection Regime in the reproductive output ($\chi^2_4 = 0.640$, $P > 0.959$).

For developmental time (Fig. 2B), and despite a statistically significant selection regime by egg density interaction ($\chi^2_4 = 12.20$, $P = 0.016$, Table S7), no difference between any Selection line and their matched Controls was detected ($|t_{22}| < 2.21$, $P > 0.114$, Table S8).

Next, we measured desiccation resistance and starvation resistance in Control versus Selection lines. These stressors that have putative ecological importance for *Drosophila* (David et al. 1983). For both traits, we failed to detect statistically significant differences between selection regimes (Table S9, Selection regime effect, $\chi^2_4 < 5.21$, $P > 0.266$; $\chi^2_4 < 9.3$, $P > 0.053$ for both starvation and desiccation assays, considering either the mean TTD or the full mortality dynamics, respectively). This indicates an absence of a correlated response between adaptation to infection and both stress-related traits (Fig. 3).

Moreover, because it has been often argued that costs are more easily revealed in nutrient-limited environments (McKean et al. 2008), we measured egg-to-adult viability and developmental time under these conditions (Fig. 4A, B). Since these tests were done in lines that derived from the Selection lines in the end of the selection experiment, but maintained in control conditions (without selection) for >30 generations, these lines represent a second set of Relaxed-Selection lines.

Although we detected increased mortality and developmental time in individuals raised on nutritionally limited food relative to those raised on standard food (Food-type effect, $\chi^2_2 > 141.3$, $P < 0.001$, for both traits), no differences were detected in either viability or development time among selection regimes (Selection regime effect, $\chi^2_4 < 7.4$, $P > 0.11$, in both traits; Table S10). Since we observed a significant Regime by Food interaction in the viability assay ($\chi^2_4 < 12.99$, $P < 0.05$, Table S10), we tested for differences between Selection and their matched Control lines independently in the different food types. The absence of differences in viability among selection regimes was confirmed in both food types ($|z| < 1.94$. and $|z| < 2.14$ for comparisons in standard and nutritionally limited food, respectively, $P > 0.194$, Table S11).

Concerning differences in weight following larval development at high or low densities, the final model retained sex, density, selection regime, the interaction between sex and each of the other factors, and the triple interaction. Overall, adults were smaller at the highest density relative to the lowest, indicating an effect of competition on this trait (GLM, effect of density: $F_{1,165} = 74.99$, $P < 0.0001$, Fig. 4C). We then compared the weight of flies from each selection regime to that of tester flies from the same assay, at the highest density. No differences were found between tester flies and flies from ContSys, BactOral, or VirSys regimes ($F_{1,24} = 1.996$, $P = 0.158$; $F_{1,52} = 0.938$ $P = 0.333$; $F_{1,38} = 2.311$ $P = 0.128$, for ContSys, BactOral, and Virsys, respectively, Fig. 4C). In contrast, flies from the BactSys selection regime were on average bigger than tester flies ($F_{1,41} = 5.916$, $P = 0.015$, Fig. 4C). Although the interaction between sex and selection regime was never significant ($F > 1.562$, $P > 0.211$), the factor sex was always significant ($F < 8.22$, $P < 0.004$), as males were on average lighter than females.

Discussion

In this study, we used a large-scale experimental evolution study addressing host adaptation to pathogen infection to test

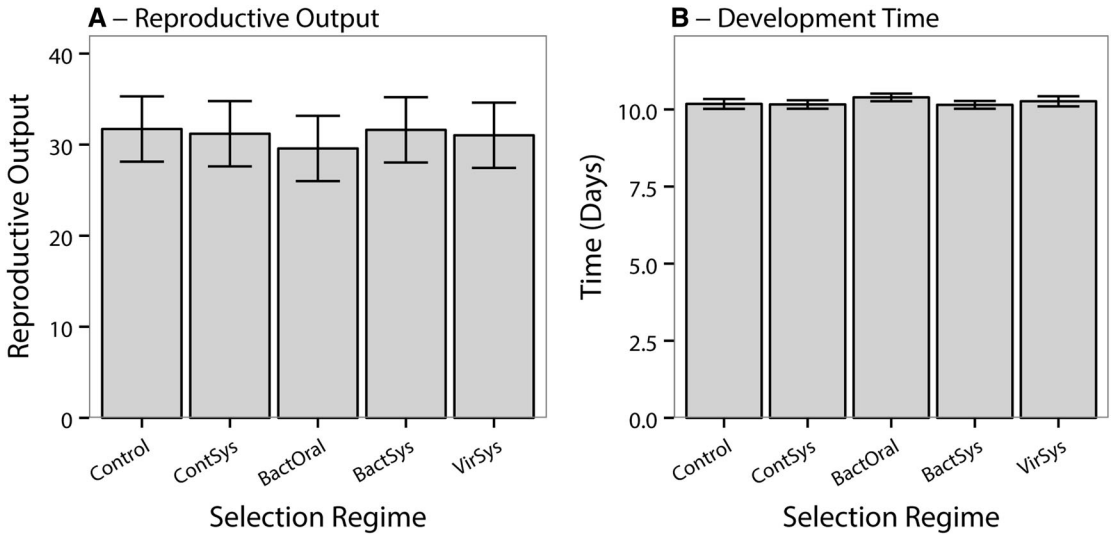

Figure 2. **Reproductive output and developmental time of individuals from Control and Selection lines in the absence of pathogens. (A) Mean (±95% CI) reproductive output eight to 10 days after females reached adulthood. (B) Mean egg-to-adults developmental time from egg to adult.**

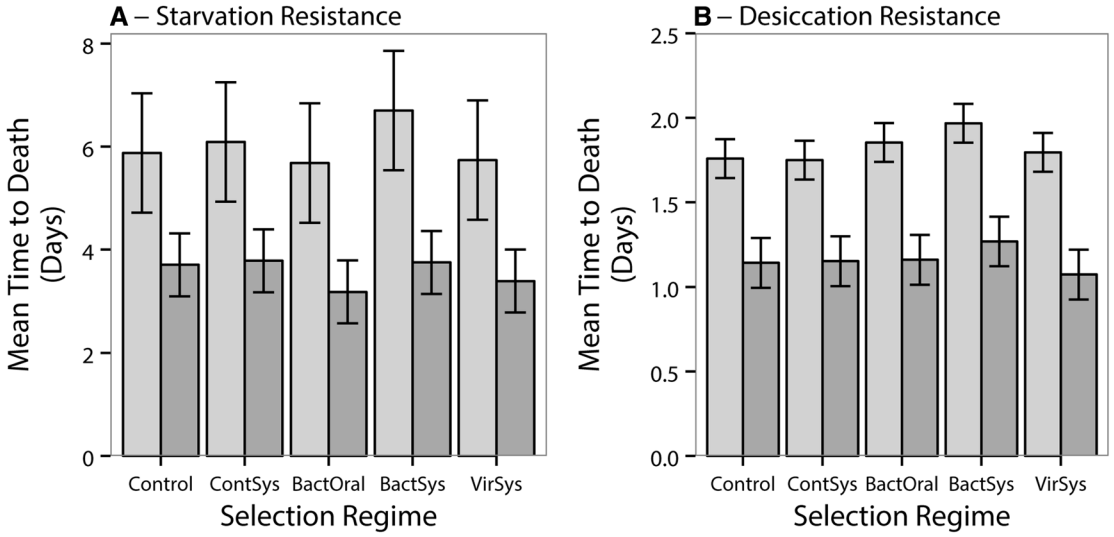

Figure 3. **Starvation and desiccation resistance of individuals from Control and Selection lines. Mean time to death (±95% CI) after (A) starvation or (B) desiccation of males (dark gray bars) and females (light gray bars).**

for the occurrence of trade-offs between immunity and other traits. We used two complementary methodologies (relaxation of selection and direct measurements of costs in selected lines), and tested 12 Selection lines, distributed over three different selection regimes, encompassing two distinct parasites (viruses and bacteria) and two infection routes (oral or systemic). Taken together, our observations support the absence of maintenance costs in *Drosophila* populations evolved for higher immunocompetence against pathogens.

Using lines subject to relaxed selection allows testing the response as a whole. That is, had we observed a decrease in immunocompetence in individuals stemming from those lines, we would have concluded that a trade-off with some fitness-related trait existed. Nonetheless, we would not attribute this trade-off to a particular trait. The fact that none of the lines in this study has lost its immunocompetence suggests that these trade-offs with fitness traits are absent in ancestral environment conditions. Still, this pattern could have also been explained by a loss of genetic variation in the selection lines, such that relaxed-selection lines would be stuck in a maladaptive peak (Teotónio and Rose 2000). However, two lines of evidence suggest that this is not the case: first, whole genome sequencing revealed that genetic variation in a subset of these lines was the same in Control and Selection lines, and that even loci under selection did not reach fixation (Martins

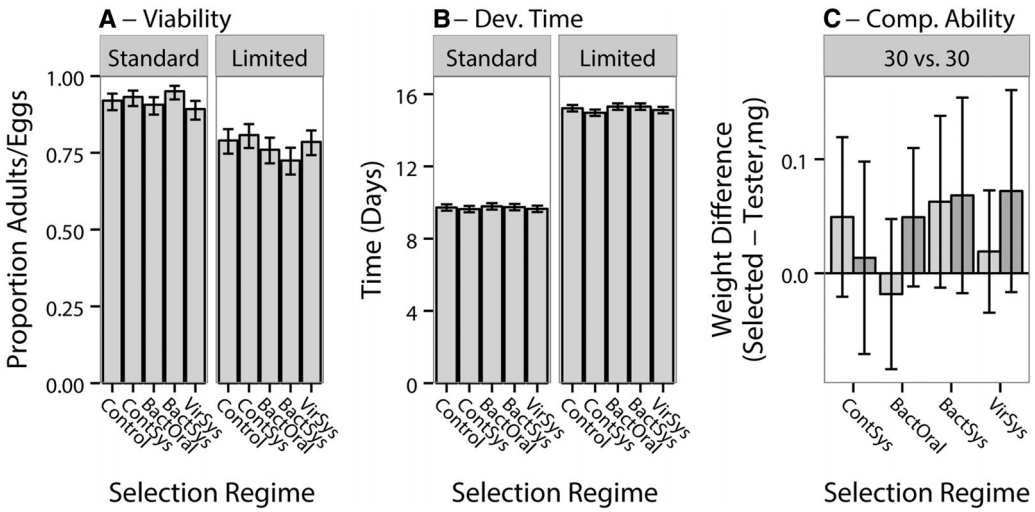

Figure 4. Survival, developmental time and competitive ability of individuals from Control and Selection lines in nutrient-limiting conditions. Mean (±95% CI) (A) egg-to-adult viability and (B) development time of individuals developing in standard (left subpanel) and nutrient-limited (right subpanel) medium. (C) Mean (±95% CI) weight difference between individuals from the experimental lines and Tester mutants (outbred [w1118]), at high larval competition conditions (30:30 larvae in 0.5 mL of food); light gray bars: females; dark gray bars: males.

et al. 2014). Second, the performance of relaxed-selection lines in the ancestral, pathogen-free environment, showed no difference to Control for the fitness traits measured. Together, these results indicate that adaptation of our populations to pathogen infection entails no maintenance costs in conditions pertaining to the ancestral environment.

To further understand how our evolved populations respond in different pathogen-free environments, we performed direct tests for the occurrence of trade-offs between immunity and several life-history traits. The problem with this approach is that we may miss the trait in which the cost is expressed. However, we tested a comprehensive set of classical life-history traits, namely reproductive output, developmental time, starvation resistance, desiccation resistance, and larval competitive ability, to maximize the possibility of detecting trade-offs. Moreover, we measured these traits in both males and females, thereby discarding the possibility of sexual antagonism for such costs (Vincent and Sharp 2014). This further reinforces the notion that, in the pathogen-free environment, evolution for increased survival upon infection by *P. entomophila* or DCV has no observable costs.

Given that the large majority of studies using experimental evolution detected trade-offs between immunity and life-history traits (reviewed in Duncan et al. 2011), the absence of such a trade-off calls for an explanation. First, although we can state that maintenance costs were not present and that we did not find trade-offs related to the tested traits, some costs in other traits or environments might exist. Indeed, we did find a (relatively minor) cost of BactSys lines in presence of viruses: they performed worse than control lines (Martins et al. 2013). The reverse, however, was not found: no costs were detected of VirSys lines in presence of other pathogens when testing the performance of these lines in presence of other pathogens (Martins et al. 2014). Moreover, apart from survival (Martins et al. 2013, 2014) and reproduction after infection (Fig. S1), we did not test for the occurrence of deployment costs, or of costs in many other environments. Second, a cost may have occurred at a transient state then be compensated for during evolution. Although we know much about compensatory evolution in bacteria, we know little about its occurrence and dynamics in sexual organisms, with some remarkable exceptions in extensively studied systems (e.g., Labbé et al. 2007). However, compensatory evolution is not likely in the system used here because the performance of relaxed-selection lines does not decrease and recovers across generations: it is always similar to that of evolved lines. This suggests that no transient cost was compensated for.

We hypothesize that the probability of finding a cost hinges on the selection pressure posed on the populations: a high selection pressure may sweep away most of the genetic variation that would allow for adaptation to the challenge posed, leaving only the most effective but most costly alleles. Indeed, the selection protocol we used was such that 33% of the population survived in the first generations (this percentage then increased due to adaptation). In the other studies of adaptation to pathogens, the selection pressure, when reported, was much higher, ranging from 90 to 95% mortality (Kraaijeveld and Godfray 1997; Fellowes et al. 1998; Ye et al. 2009). In contrast, in the single study that has also reported no cost in multicellular organisms, the selection procedure was such that 20–30% of the hosts

(a cabbage looper) survived (Milks and Myers 2000). This reasoning may also explain why some studies failed to find a trade-off with immunity when selecting for other life-history traits (Sanders et al. 2005; Kolss et al. 2006; Hangartner et al. 2013). In particular, the results reported in Sanders et al. (2005) are surprising, as the relaxed-selection process (i.e., selection for immunity and measuring consequences in life-history traits) did reveal a trade-off. The traits selected in these experiments (larval competitive ability, learning, and reproductive investment, respectively) have a looser link to survival than resistance to pathogens. Hence, it may well be that the selection pressure that populations were exposed to in these studies was lower than that of studies selecting for increased immunocompetence, and this may account for the absence of a trade-off. Clearly, this hypothesis calls for a direct test. For example, one could set up selection lines evolving in presence of the same parasite but at different doses, and test whether trade-offs appeared in the treatments with higher selection pressures only. In any case, the lack of symmetry in the trade-off between immunity and other life-history traits suggests that the trade-off is not a universal genetic characteristic of the organisms under study, but a conditional property, which may hinge upon the selection pressure posed.

Unfortunately, it is not possible to validate this hypothesis with studies that have used other approaches to test the occurrence of a cost of immunity. A cost was found in circa 50% of such studies (reviewed in Labbé et al. 2010). However, either the evolutionary trajectories leading to host resistance are unknown or resistant clones have been generated via artificial selection, which may lead to spurious correlations among traits (Rose 1984). Hence, these data cannot be used to test whether the strength of selection underlies the probability of finding a cost (see also the discussion in Labbé et al. 2010 for other potential confounding factors in that dataset).

Our hypothesis, however, is congruent with data concerning pesticide resistance. Indeed, in one of the best-documented examples of allele replacement in the wild, Labbé et al. (2009) have shown that pesticide resistance in the mosquito *Culex pipiens* in Southern France first evolved via a highly resistant but highly costly allele. When mosquito populations were established in the treated area (hence selection for increased pesticide resistance was weaker), this allele was replaced by one conferring a lower cost. Similarly, Lopes et al. (2008) found no cost for resistance to levamisole in experimentally evolving *C. elegans* lines in which a dose killing initially 25% of individuals was used. This contrasts with most studies of natural populations, in which a cost for pesticide resistance was found (Coustau et al. 2000).

Given the low prevalence of costs in this system, the question remains: what maintains genetic diversity for resistance to pathogens in our system? One possibility is that alleles conferring resistance have a large effect, such that susceptibilities differ widely in the population. This has been shown to allow for the maintenance of polymorphisms for resistance even when the cost is negligible (Antonovics and Thrall 1994). In line with this, we have found that the majority of the selection response for increased resistance to DCV could be attributed to alleles of three genes in our populations, all of which with a considerable effect upon host survival (Martins et al. 2014). Moreover, we have shown that adaptation to all immune challenges occurred via resistance, rather than tolerance. Models predict that the maintenance of genetic variation for resistance is more likely than for tolerance mechanisms, although a cost is still necessary (Roy and Kirchner 2000). Another possibility is that the maintenance of genetic diversity in host populations in the field is due to coevolutionary dynamics. In that case, diversity for pathogen resistance may be maintained for a wider range of parameters than contemplated in models that consider host evolution alone (Sasaki 2000; Best et al. 2010). Coevolution in natural populations of *Drosophila* could have maintained the SGV present in our populations at the onset of experimental evolution.

Overall, this study suggests that the occurrence of maintenance costs for immunity traits is not a universal feature of organisms, raising questions as to (1) under which conditions such costs evolve and (2) what maintains genetic diversity for costless immunity traits.

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DATA ARCHIVING

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Supporting Material

Table S1. Results of generalized linear mixed models testing differences in survival of individuals of Control, Selection, and Relaxed-Selection lines (i.e., the factor Regime), 10 days after infection with the pathogen treatment used in the Selection lines.

Table S2. Pairwise estimates of the differences in survival of individuals of Selected and Relaxed-Selection lines, for each generation after the beginning of the relaxation of selection, 10 days after infection with the pathogen treatment used in the Selection lines.

Table S3. Results of the logistic regression mixed models for differences in the slope across generations of survival of individuals of Control, Selection, and Relaxed-Selection lines (i.e., the factor Regime), 10 days after infection with the pathogen treatment used in the Selection lines.

Table S4. Estimated coefficients for the generation effect in the logistic regression mixed models in Table S3.

Table S5. ANOVA tables for the mixed-effects Cox models on the survival dynamics of individuals of Control, Selected, and Relaxed-Selection lines in the last generation of selection, after infection with the pathogen treatment used in the Selection lines.

Table S6. Pairwise comparisons between survival of individuals of Selection, Relaxed-Selection, and matched Control lines in the last generation of selection, after infection with the pathogen treatment used in the Selection lines.

Table S7. ANCOVA table for the linear mixed model for differences in mean developmental time between individuals from Selection and Control lines.

Table S8. Pairwise estimates of differences between mean developmental time of individuals of Selection and their matched Control lines.

Table S9. ANOVA table for the mixed-effects Cox-model (LMM) on the survival dynamics of individuals from Selection and Control lines, after desiccation and starvation stress.

Table S10. ANOVA table for differences in mean developmental time and egg-to-adult viability of individuals of Selection and Control lines, in standard and nutrient restricted conditions.

Table S11. Pairwise estimates of differences between egg-to-adult viability of individuals of Selection and their matched Control lines, in standard and nutrient restricted conditions.

Figure S1. Mean (±95% CI) reproductive output of females from the Selected (gray) and respective Control (white) populations, 5-7 days post-infection with the pathogen treatment used in the Selection lines.