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COSTS OF RESISTANCE IN THE *DROSOPHILA–MACROCHELES* SYSTEM: A NEGATIVE GENETIC CORRELATION BETWEEN ECTOPARASITE RESISTANCE AND REPRODUCTION

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Genetic variation for parasite resistance occurs in most host populations. Costs of resistance, manifested as reduced fitness of resistant genotypes in the absence of parasitism, can be an important factor contributing to the maintenance of this variation. One powerful tool for detecting costs of resistance is the study of correlated responses to artificial selection. Provided that experimental lines are recently derived from large outbreeding populations, and that inbreeding is minimized during the experiment, correlated responses to selection are expected to be strong indicators of pleiotropy. We artificially selected for elevated behavioral resistance against an ectoparasitic mite (*Macrocheles subbadius*) in replicate populations of the fly *Drosophila nigrospiracula*. Resistance was modeled as a threshold trait, and the realized heritability of resistance was estimated to be 12.3% (1.4% SE) across three replicate lines recently derived from nature. We contrasted the longevity and fecundity of resistant and control (unselected) flies under a variable thermal environment. We report that reduced fecundity is a correlated response to artificial selection for increased resistance, and that the strength of this effect increases from 25° to 29°C. In contrast, longevity differences were not detected between resistant and control lines at either temperature. These findings are robust as they were confirmed with an independent set of experimental lines. Thus, our results identify a negative genetic correlation between ectoparasite resistance and an important life-history trait. That a correlated response was only detected for fecundity, and not longevity, suggests that the genetic correlation is attributable to pleiotropic effects with narrower effects than reallocation of a general resource pool within the organism, although other interpretations are discussed. Combined with fluctuating parasite-mediated selection and temperature, the presence of this trade-off may contribute to the maintenance of genetic variation for resistance in natural populations.

KEY WORDS: ***Drosophila,*** **ectoparasitism, fecundity costs, genetic variation, host behavior, *Macrocheles* mites, negative genetic correlation, trade-offs, parasite resistance.**

Parasites may impose significant fitness costs on their hosts and, therefore, can represent potent agents of selection in natural populations (Ewald 1980, 1995; Price 1980; Godfray 1994). Although strong parasite-mediated directional selection is expected to fix genes conferring resistance, and thereby reduce standing genetic variation (Fisher 1930; Falconer and Mackay 1996), the presence of heritable genetic variation for resistance is common in field populations (Wakelin 1978; Parker 1991; Henter and Via 1995; Kraaijeveld et al. 1998; Brown 2003). One important theory for the maintenance of this variation involves costs of resistance (Henter

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and Via 1995; Ebert and Hamilton 1996; Woolhouse and Webster 2000; Rigby et al. 2002). If resistance imposes pleiotropic costs on the host such that resistant genotypes suffer decrements in fitness traits such as growth rate, fecundity, or survival in the absence of parasites, then resistance may be constrained from evolving to ever-higher values, and equilibrate at intermediate levels (Anderson and May 1983; Simms and Rausher 1987; Simms 1992; Mitchell-Olds and Bradley 1996; Gemmill and Read 1998). Indeed, several models of natural selection confirm that a stable polymorphism can arise in a population wherein resistant genotypes pay a fitness toll, relative to susceptible forms, in the absence of parasitism (Gillespie 1975; Anderson and May 1982; Kemper 1982; Antonovics and Thrall 1994; Sasaki and Godfray 1999).

Studies of *Escherichia coli* and their T4 phage parasites provide some of the strongest evidence that resistance mutations pleiotropically impose fitness costs, in this case, by compromising nutrient assimilation (Lenski and Levin 1985; Bouma and Lenski 1988). But much of the empirical evidence for costs of resistance comes from studies of plants (Bergelson and Purrington 1996; Brown 2003). These studies represent an extensive and wide-ranging body of literature, providing data on resource-based and genetic trade-offs between resistance against herbicides, pathogens and insect herbivores, and plant fitness traits such as growth rate, dry weight, and seed set (Simms 1992; Mitchell-Olds and Bradley 1996; Hare et al. 2003; Tian et al. 2003). Many studies, however, have failed to detect costs of resistance, an inconsistency that has fueled debate about the prevalence of costs, and of the generality of the pleiotropy hypothesis for resistance polymorphisms in natural populations (Parker 1991; Simms 1992; Antonovics and Thrall 1994; Bergelson and Purrington 1996; Rigby et al. 2002).

One popular prediction emerging from this discussion is that costs should be more prevalent under stressful conditions, but which has also received mixed empirical support (Bergelson and Purrington 1996; Sandland and Minchella 2003). In general, fitness cost–benefit ratios of resistance can vary under some forms of stress (Service and Rose 1985; Bowers and White 2002), including abiotic factors such as desiccation and resource limilation (Moret and Schmid-Hempel 2000; Hoang 2001), or biotic factors such as competition and predation (Kraaijeveld and Godfray 1997; Rigby and Jokela 2000). In studies of animal hosts, costs and their environmental sensitivity have been documented in the form of trade-offs involving the body's immunological defense mechanisms (Boots and Begon 1993; Yan et al. 1997; Fellowes et al. 1998; Hurd et al. 2005; Simmons and Roberts 2005; and see reviews in Lochmiller and Deerenberg 2000; Rigby et al. 2002).

Here we focus on the naturally occurring *Drosophila* host–*Macrocheles* mite association (Polak 1996) in which the interaction is mediated by behavioral forms of defense (Polak 2003). Once infested, flies suffer reductions in female longevity and fecundity, and body condition (Polak 1996; 1998). Moreover, Polak and Markow (1995) have demonstrated that ectoparasitism can drive host sexual selection in the wild.

The host in this system is therefore selected to avoid mites; pronounced, dose-dependent fitness consequences accrue from ectoparasitism. Yet heritable genetic variation for resistance has been documented in natural populations; Polak (2003) demonstrated significant responses to artificial selection for increased behavioral resistance in replicate lines recently derived from the field. This selection was applied on preattachment defensive behaviors, as in the present study. Here we test for fitness costs of resistance by measuring correlated responses to selection for ectoparasite resistance in two major life-history traits of the host; correlated responses to selection reflect genetic correlations, and thus the likely possibility of genetically based trade-offs among traits (Stearns 1992; Mitchell-Olds and Bradley 1996; Sgrò and Hoffmann 2004; Fuller et al. 2005). Genetic costs associated with behavioral defenses against ectoparasites are so far unknown.

We tested for costs of resistance reflected in adult fecundity and longevity. Longevity was measured in both males and females, and in the presence and absence of reproduction. We also evaluated the possibility that costs of resistance may be context dependent; these fitness-related traits were assayed at 25° and 29°C, with the latter value being demonstrably stressful to flies. Temperature is an especially relevant ecological stress factor in this system, as desert flies experience dramatic daily and seasonal fluctuations in their thermal environment (Gibbs et al. 2003).

Methods

STUDY SYSTEM

In the Sonoran desert, the facultative ectoparasite *Macrocheles subbadius* Berlese (Acari: Macrochelidae) colonizes and reproduces in necrotic cacti in which *Drosophila nigrospiracula* Patterson and Wheeler (Diptera: Drosophilidae) also breeds. Mites disperse by attaching to fly abdomens whereby they penetrate fly cuticle and consume host hemolymph (Polak 1996). Intensity and prevalence of parasitism reach high levels in nature, and are variable in both space and time (Polak and Markow 1995). Infested females suffer greater mortality, experience delays in the onset of oviposition, lay fewer eggs, produce fewer progeny over their lives, and produce progeny with significantly greater developmental abnormalities. The extent of the host pathology depends on the number of mites per host and on the duration of infestation (Polak 1996, 1998).

BASE POPULATIONS AND MITE CULTURES

All laboratory populations were established using flies collected in the field at necrotic saguaro cacti (*Carnegiea gigantea*). Flies returned to the laboratory were cleared of any mites they carried,

and mass cultured under laboratory light and temperature conditions (12 h light:12 h dark, 26°C). The flies from which mites were removed were combined with unparasitized hosts to found base populations. The food medium is described elsewhere (Polak 1996).

We conducted two studies testing for costs of resistance, each based on a set of lines independently derived from nature. For the first study, the two host selection lines were each derived from a separate base population (Table 1). These populations were initiated from cacti in close proximity to each other east of Phoenix, Arizona (collection localities are given in table 1 of Polak 2003), and so represent samples of one panmictic field population (and see Sluss 1975; Johnston and Heed 1976). For the second study, flies were collected at two different cacti approximately 115 km apart (Freeman Road, 38 km S of Florence, Pinal County, AZ [32° 50′ N, 111° 9′ W]; Salome Creek Road, 60 km N of Globe, Gila County, AZ [33° 40′ N; 111° 2′ W]), and in the same general vicinity as those for the first study. From each cactus, > 250 females and an approximately equal number of males were collected. Flies were cultured in the laboratory at standard conditions for two generations and combined in equal proportions into one base population. This pooled population was mass cultured for an additional two generations prior to commencing artificial selection for increased resistance.

Colonies of mites were initiated at the time base populations of flies were collected. Mites were removed from the bodies of the flies and cultured (Polak 1996). All mites were discarded following use in artificial selection to avoid reciprocal selection on the mite stock.

ARTIFICIAL SELECTION FOR BEHAVIORAL RESISTANCE

The artificial selection protocol described in Polak (2003) was used to generate resistance lines of flies. Briefly, 250 male flies of a given line were exposed each generation to mites in four experimental infestation chambers (see fig. 1 in Polak 2003). Fly–mite interactions occur in a space within the chambers that mimic the pockets inside necrotic cactus tissue where interactions between these organisms occur naturally. Within this space, mites approach flies from all sides, so that flies are not selected to avoid any particular surface within chambers, or to exhibit geotactic behavior that could incidentally protect them from mites.

Following about 48 h of exposure, flies were removed from the chambers, and those that carried neither mites nor scars were used as sires to seed each subsequent generation of the selected line. In this way, selection was applied on premite attachment defensive behaviors only. A control line was maintained in parallel to each selected line throughout the experiment; any one pair of lines was derived from the same base population. Each control line was propagated with exactly the same number of males and females as its selected line, thus minimizing inbreeding differences between them.

In the first study, an equal number of females as males seeded each new generation. Lines 1 and 2 were backcrossed to their respective base populations after 25 and 16 generations of selection, respectively. Lines were then cultured for two generations without selection, and reselected for resistance for eight generations. The minimum number of flies ever used to seed a next generation was 53 and 49, for lines 1 and 2, respectively. Tests for costs of resistance (see below) for this study were conducted after these eight generations of reselection.

In the second study, exactly 75 females from within each line were combined with the selected males each generation. In neither study were females exposed to mites. Table 1 provides the number of generations of selection used to generate the resistant lines, as well as the mean number of males selected each generation. The minimum number of flies ever used to seed a next generation was 100, 100, and 105, for lines 1, 2, and 3, respectively.

In both studies, lines were mass cultured (without selection) for at least one generation before commencing the fitness assays. Each line was cultured in four bottles, each seeded with exactly 25 flies of each sex. Moreover, in study 1 in which tests for fitness

Table 1. Number generations of artificial selection for increased resistance prior to commencing tests for costs of resistance and mean number selected males used to seed subsequent generations, by experiment and selection line.

Study	Selection line	Date of field collection	Location of field collection[1]	Generations of selection	Mean # males out of 250 (SD)
1	1	August 1995	Peralta Rd	25	36.4 (13.7)
1	2	March 1999	Peralta Rd	16	35.3 (14.7)
2	1	April 2003	Freeman Rd,[2] Salome Creek Rd	12	61.75 (16.7)
2	2	April 2003	Freeman Rd, Salome Creek Rd	12	45.5 (12.2)
2	3	April 2003	Freeman Rd, Salome Creek Rd	12	54.4 (10.8)

[1] Latitude and longitude provided in text.

[2] Populations from these two sites were combined into one base population prior to initiating the three selection lines.

differences consisted of three blocks, lines were similarly cultured for one to two generations between blocks in the absence of selection. Base populations in both studies were cultured as above but in eight bottles over the course of the selection experiment for later incorporation into the cost assays (see below).

RESPONSE TO SELECTION AND REALIZED HERITABILITY

Resistance assays tracked response to selection in each resistant line; the full protocol is provided in Polak (2003). Briefly, assays consisted of aspirating flies from the selected line and its paired control into a common infestation chamber. Multiple chambers per line were used, and the sexes were assayed separately. Groups of resistant and control flies within any chamber were equal in size and ranged from 10 to 70 flies. Flies were distinguished by treatment (i.e., selected vs. control) with a small clip to the tip of a wing. Which wing received the clip was alternated between treatment categories across assay chambers. Wing clips were applied 24 h prior to commencing the resistance assay. Following exposure, living flies were recovered from chambers, checked for the presence of mites and scars under a stereomicroscope, and identified by their wing clip. The death of any flies was attributed to parasitism; negligible mortality occurs in chambers without mites (Polak 2003).

Prevalence for a given group within a chamber was calculated as the proportion of flies that became parasitized, divided by the number of flies exposed. Resistance was modeled as a discontinuous or threshold trait, with an expected underlying continuous variable called the liability, influenced by both genetic and environmental factors (Falconer and Mackay 1996). It was assumed that a single threshold separates resistant and susceptible forms (Polak 2003). Thus, mean prevalence across chambers of a given assay was transformed to "mean liability" for each treatment category (Falconer and Mackay 1996, p. 301), and the difference in mean liability (in standard deviation units) between categories was taken as the strength of divergence due to selection.

The two selected lines in the first study were assayed for their degree of resistance 12 and five times, respectively, prior to the backcross. The realized heritability of resistance based on these assay data was estimated previously from the slope of the regression of divergence on generation number (Polak 2003). However, for the three lines in the second study, resistance assays were conducted after the ninth and 12th (final) generations of selection only. Thus, for these lines the slope of the function relating divergence to generation number was calculated as the ratio of net divergence to total number of generations up to the ninth and final generations (Hill 1971), and averaged so that there was one value per selected line. The grand mean across the three replicate lines was calculated, and the standard error was the empirical standard error of the response, estimated from the variance of the replicate estimates (Falconer and Mackay 1996; Hill 1971). Realized heritability of ectoparasite resistance was calculated as twice the average value of the slope (Falconer and Mackay 1996).

The results of resistance assays following the final generation of selection were analyzed in detail and presented graphically to illustrate the degree of resistance divergence between selected and control lines at the time fitness traits were measured. In the first study, the fitness assay was repeated across three time blocks so that three resistance assays were deemed necessary, one at the onset of each time block to verify that the degree of divergence remained stable across the entire experiment. Logistic regression was performed in which the categorical response variable was whether or not a fly became infested (carried a mite(s) or scar(s). Predictor variables were block (1–3), line (1, 2), selection treatment (selected, control), gender, and infestation chamber (there were seven to 13 chambers per line). The interaction between time block and selection treatment was specifically examined to evaluate the stability of resistance divergence. In an alternate analysis, analysis of variance (ANOVA) analyzed the effect of the above predictor variables on prevalence data, but which were first arcsine-transformed to meet assumptions of parametric analysis; residuals were normally distributed and error variances similar across experimental lines. Chamber was nested within line and modeled as a random factor. In the second study, four replicate infestation chambers (two per sex) were used to assess divergence in resistance for each of the three replicate selection lines. Logistic regression and ANOVA analyzed the data in this second study, as above.

COST ASSAYS

Female fecundity and longevity

This section describes protocol for measuring female fecundity and longevity in studies 1 and 2. Resistant and control (unselected) flies were simultaneously measured in the absence of parasitism. Adult females were harvested no more than 24 h postemergence and allowed to mature for four to five days (study 1) or five to seven days (study 2). Twenty females per treatment by line combination, along with 30 females from the base population, were assayed per temperature. Each adult female was then placed with two virgin males (from a separate, standard outbred stock) into an oviposition vial with banana–agar media (0.67% agar, 4.3% unsulphured molasses, 1.2% live Brewer's yeast, and 4.6% banana). Expired males were replaced continuously throughout the experiment with fresh individuals; all males were replaced every two weeks so that each female had ad libitum access to sperm.

Experimental vials were randomly assigned to one of two temperature treatments and placed at different, random locations within incubators to avoid systematic environmental effects. The low and high temperature regimes were 23°C night: 25°C day and 27°C night: 29°C day, respectively. Flies were checked daily at the

same time for mortality and vials containing eggs were vacated; eggs were counted under a stereomicroscope. All flies were transferred to fresh vials every three days to minimize bacterial growth on the food substrate. Thorax length was measured to estimate body size.

Longevity in the absence of reproduction

In study 1, longevity without reproduction was measured in both sexes. However, in study 2, longevity without reproduction was measured in males only. Table 2 summarizes the condition under which longevity was measured in the two studies. Male and female flies were collected and allowed to mature in same sex vials as described above. Each fly was subsequently transferred to an individual agar vial and maintained alone until time of death. For each of the three blocks of the first study, 10 flies per selection treatment × temperature combination were tested per line. In the second study, 20 males per selection treatment × temperature combination were tested per line; there was a single time block in this study.

ANALYSES OF LIFE-HISTORY DATA

Mixed-model analysis of covariance (ANCOVA), with longevity as a covariable, tested for effects of the selection treatment on lifetime fecundity. Longevity effects were similarly analyzed except fecundity was the covariable. Analyses of covariance were used because lifetime fecundity and longevity were strongly positively correlated in both studies (study 1: slope = 9.40, SE = 0.80, $F_{1,236} = 204.9$, $P < 0.0001$; study 2: slope = 7.18, SE = 1.01, $F_{1,285} = 27.7$, $P < 0.0001$). Selection treatment, temperature, and block were fixed factors, whereas replicate line (e.g., 1, 2, and 3 in study 1) was modeled as random. Both the dependent variable and covariable were square-root transformed to satisfy the ANCOVA assumptions, including normality and homogeneity of variances and slopes across treatments. Figures present back-transformed means, and standard errors were computed on untransformed data. Body size was generally not related to fecundity or longevity in either sex, so it was excluded as a covariable.

The base populations (one per study), which were maintained in mass culture throughout the selection experiments, were assayed along with resistant and control categories, allowing us to contrast the performance of each base population to its respective controls. In study 1, the base population was not assayed in the first block, so when effecting tests involving the base, a separate ANCOVA, identical in structure to that described in the preceding paragraph, analyzed data for blocks 2 and 3 only. Dunnett's test was used to test for differences between the base and each control line when the selection treatment was found to have significant effects.

We also examined differences between selected and control flies in net fecundity (average daily egg production at age x, weighted by the probability of surviving to that age) at high temperature (this is because significant fecundity costs were only detected at 29°C, see Results). Net fecundity was calculated using females pooled across lines of a given selection treatment and study; pooling was justified as the effect of the selection treatment on fecundity was homogeneous across lines in each study (see Results). Net fecundity was plotted against age to visualize the *pattern* of the difference between selection treatments.

Where longevity was assessed in the absence of mating (see Table 2), the data were first square-root transformed, and analyzed using ANOVA (selection and temperature were fixed factors; line was a random factor). However, male longevity data in study 2 were not normally distributed (Shapiro–Wilk normality test), despite efforts to transform them. So we used nonparametric statistical analyses, including the Spearman's rank correlation to test for an association between longevity and body size, which was not significant, as above. Kruskal–Wallis and Wilcoxon–Mann–Whitney tests were used to test for treatment effects.

Survivorship analyses were conducted with either the Kaplan–Meier or Cox's proportional hazard regression method. The Cox's proportional hazard model estimates the relative risk of death per unit time while controlling for a time-dependent covariable, such as cumulative fecundity in this study. The Cox model is a powerful tool for analyzing survivorship data because it accounts for covariables that change over time, or for covariables whose effects changes over time. In addition to generating chi-square and P-values, the analysis also provides a hazard ratio, which estimates the relative risk of death at time t for a unit increase in the covariable. Here the hazard ratio is a measure of the relative risk of mortality for resistant over control treatment groups; a value > 1 would indicate a higher probability of mortality in the resistant group. All statistical analyses were performed using SAS (SAS Institute 2002), except that Kaplan–Meier analyses were run in JMP (SAS Institute 2002).

Table 2. The different conditions under which longevity was measured in studies 1 and 2.

Condition/sex	Study 1	Study 2.
With reproduction		
Females	yes	yes
Without reproduction		
Females	yes	no
Males	yes	yes

Results

RESPONSE TO SELECTION, STABILITY OF RESISTANCE AND HERITABILITY

Flies selected for increased resistance were significantly less susceptible to ectoparasitism than controls. In the first study, logistic

regression revealed a significantly reduced probability of parasitism in the selected categories compared to their paired controls (estimate: −0.93, 0.22 SE, $\chi^2 = 18.8$, $P < 0.0001$). The interaction between selection treatment and time block was nonsignificant ($\chi^2 = 0.95$, $P = 0.62$), indicating that divergence was stable across the three time blocks of study 1. Interactions between selection treatment and either sex ($\chi^2 = 1.81$, $P = 0.18$) or line ($\chi^2 = 3.0$, $P = 0.083$) were likewise nonsignificant.

ANOVA confirmed this strong difference in the rate of parasitism ($F_{1,86} = 61.24$, $P < 0.0001$): selected lines 1 and 2 were 37% and 44% more resistant than their paired controls (Fig. 1). All interactions were not significant, including the block × selection treatment effect, which confirmed the stability of the divergence.

In the second study, assays performed after generations 9 and 12 of selection likewise revealed positive divergence (control minus resistant) in prevalence (Fig. 2; here we chose to report the *difference* in prevalence as the metric of divergence to simplify the presentation of the data). Logistic regression analyses following the 12th generation of selection (i.e., when cost assays were conducted) revealed a significant reduced probability of parasitism among the selected flies (estimate: −0.55, 0.10 SE, $\chi^2 = 32.2$, $P < 0.0001$). The interactions between selection treatment and sex ($\chi^2 = 1.03$, $P = 0.31$) or line ($\chi^2 = 0.02$, $P = 0.99$) were nonsignificant. ANOVA showed that prevalence of parasitism (pooled across lines) was significantly lower (by 26%) for resistant compared to control flies ($F_{1,26} = 48.4$, $P < 0.0001$). All interactions were nonsignificant. Thus, selected flies in both studies were significantly and consistently more resistant than controls throughout all cost work.

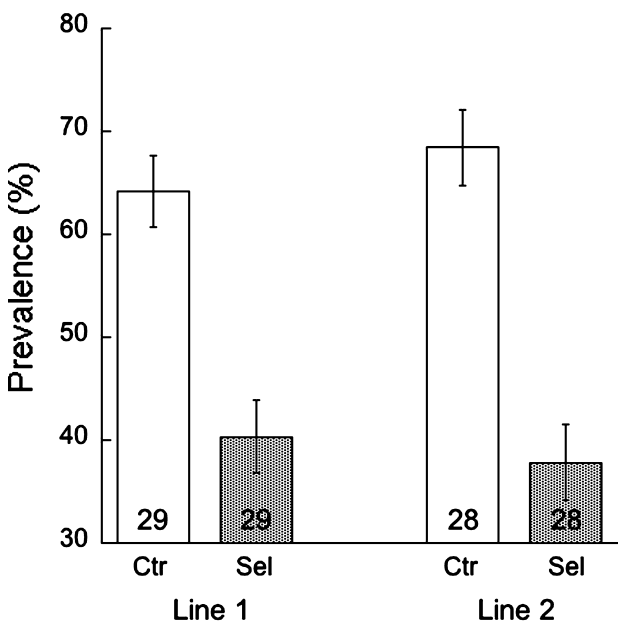

Figure 1. Mean prevalence (% individuals infected by mites) in selected and control categories of the first study at the onset of tests for costs of resistance. Error bars represent ± SE, and numerals within the bars of the figure represent sample sizes (numbers of assay chambers).

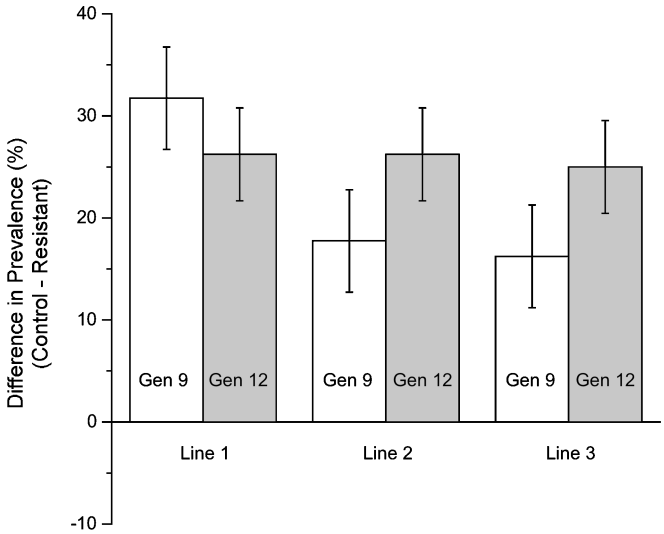

Figure 2. Outcome of artificial selection for increased behavioral resistance across replicate lines in study 2. Resistance assays were conducted after nine ($n = 4$ infestation chambers per line) and 12 ($n = 4$ chambers per line) generations of selection. Bars represent *differences* in mean prevalence of infestation between control (unselected) and selected categories (± SE); thus, values above the zero line indicate a higher prevalence in controls.

The mean realized heritability of ectoparasite resistance calculated across the three replicates of the second study was 12.3% (1.4% SE, $t = 8.72$, df = 2, $P < 0.01$), which is similar to a previously reported parameter estimate of 15.2% (1.4% SE) based on an average across four selection lines (Polak 2003).

COSTS OF RESISTANCE

Study 1: Fecundity

ANCOVA revealed significant effects of the selection treatment on fecundity (Table 3): in the absence of parasites, resistant females deposited fewer total eggs than control females (Fig 3A). The magnitude of this reduction was repeatable across the three time blocks; the percent decrease in fecundity was 31.3%, 26.5%, and 36.5% for blocks 1, 2, and 3, respectively. Temperature effects were also significant (Table 3), such that more eggs were deposited at high than at low temperature (Fig. 3A).

At low temperature resistant flies deposited on average 22.7% fewer eggs than control females, but this difference was not significant (Tukey–Kramer post hoc test, $P = 0.46$). The difference at high temperature, however, was significant. Here, resistant flies experienced a 35.4% reduction in fecundity (Tukey–Kramer, $P = 0.02$). Fecundity costs therefore appear to be somewhat magnified at the higher temperature, although we note that the selection

Table 3. Results of ANCOVA on female fecundity and longevity, study 1. All interaction terms are nonsignificant; only interactions of specific interest are shown.

Trait	Source	df	MS	*F*	*P*
Fecundity	block	2	337.6	8.46	0.0003
	line	1	3.7	0.09	0.76
	treatment[1]	1	397.0	9.94	0.002
	temperature	1	359.1	8.99	0.003
	temp×trtmt	1	46.2	1.16	0.28
	line×trtmt	1	62.8	1.57	0.21
	Error	229	39.9		
Longevity	block	2	0.25	0.25	0.78
	line	1	0.20	0.20	0.66
	treatment[1]	1	1.45	1.45	0.23
	temperature	1	57.5	57.5	<0.0001
	temp×trtmt	1	0.65	0.65	0.42
	line×trtmt	1	0.31	0.31	0.58
	Error	189	1.00		

[1] Selection treatments: resistant and control.

treatment × temperature interaction was not significant (Table 3). Figure 3B. shows that net fecundity was consistently lower among resistant flies compared to control flies throughout much of the flies' lifetimes.

Lifetime fecundity for each of the two controls did not differ from the base population (Dunnett's post hoc tests, α level = 0.05), as expected in the absence of inbreeding effects, and of genetic drift in the control lines.

Study 1: Female longevity (with reproduction)

ANCOVA on longevity revealed nonsignificant effects of selection treatment (Table 3), and post hoc tests confirmed that this effect was nonsignificant at either temperature (Tukey–Kramer test, $P = 0.99$ and $P = 0.52$, respectively). The analysis did, however, detect a significant effect of temperature on female longevity; at low temperature flies lived on average 1.4 times longer (12.0 days) than at high temperature. The selection × temperature and the selection × line interactions were not significant.

Cox's proportional hazards analysis, with cumulative fecundity as a time-dependent covariable, revealed no significant difference in the mortality risk between selection treatment groups at either the low ($\chi^2 = 0.87$, df = 1, $P = 0.35$, hazard ratio = 0.834) or high ($\chi^2 = 1.79$, df = 1, $P = 0.18$, hazard ratio = 0.775) temperatures. Hence, these analyses indicate a lack of a survivorship cost of resistance.

Study 1: Longevity in the absence of mating (both sexes)

ANOVA on female longevity revealed no effect of either selection treatment ($F_{1,230} = 2.05$, $P = 0.15$) or line ($F_{1,230} = 1.53$,

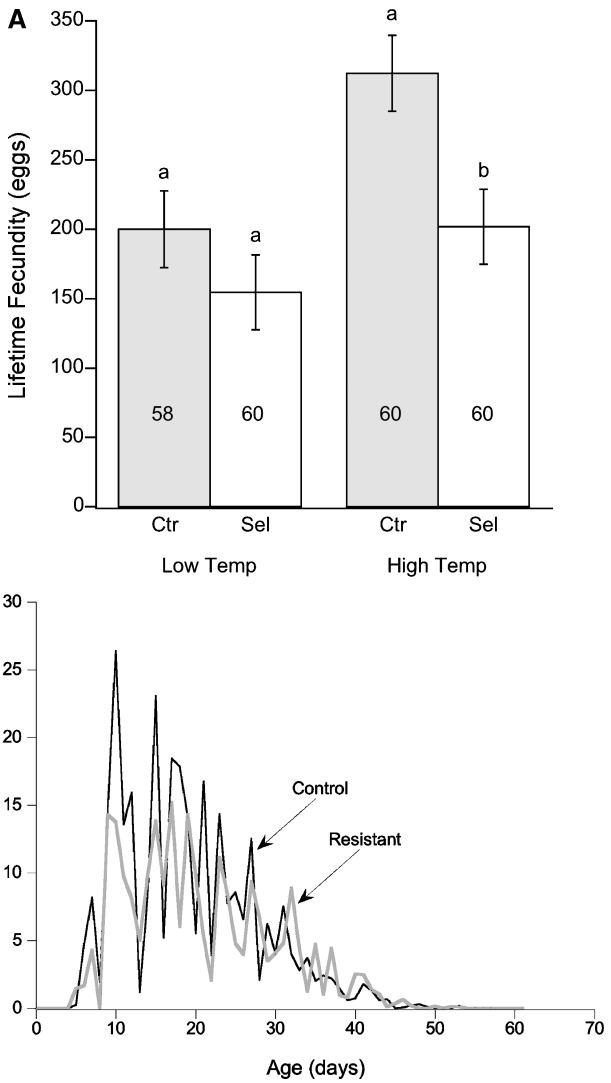

Figure 3. Fecundity results from study 1. (A) Least-squares mean lifetime fecundity for females at the two experimental temperatures. For each temperature treatment, bars sharing the same letter are not significantly different from each other (Tukey–Kramer test). Numerals inside bars represent total number of flies assayed. (B) Net female fecundity with age for resistant and control categories at high temperature.

$P = 0.22$). Temperature did again have a significant effect, such that female flies lived 1.8 times (33.2 days) longer at 25°C than at 29°C ($F_{1,230} = 96.9$, $P < 0.0001$); the temperature × treatment interaction was nonsignificant ($F_{1,230} = 0.29$, $P = 0.59$). The Kaplan–Meier analysis reinforced this result: no significant difference in survivorship was detected between the control and resistant categories at either of the low ($\chi^2 = 0.18$, df = 1, $P = 0.68$) or high ($\chi^2 = 3.17$, df = 1, $P = 0.08$) temperatures.

For male longevity, in turn, neither selection treatment ($F_{1,227} = 0.82$, $P = 0.37$) nor line ($F_{1,227} = 1.84$, $P = 0.18$) effects were significant. However, males lived 2.5 times (61.1 days) longer at low temperature than high temperature ($F_{1,227} = 210.4$,

$P < 0.0001$); the temperature × selection treatment interaction was not significant ($F_{1,227} = 0.28$, $P = 0.60$). Survivorship for control and resistant categories was not significantly different at the low (Kaplan–Meier $\chi^2 = 3.18$, df = 1, $P = 0.07$) and high ($\chi^2 = 3.12$, df = 1, $P = 0.08$) temperatures. Given these near-significant results, we report the relevant untransformed means (SE, *n*). At low temperature, resistant and control males lived 111.5 days (3.89, 58) and 104.1 days (3.83, 60), respectively; whereas at high temperature, these values were 45.6 days (3.89, 58) and 43.6 days (3.89, 58), respectively.

Study 2: Fecundity

ANCOVA again revealed significant effects of the selection treatment on fecundity (Table 4) such that the average lifetime fecundity for resistant flies was lower compared to controls (Fig. 4A). At low temperature, resistant flies produced approximately 41.5% fewer eggs than controls, and this difference was not significant (Tukey–Kramer, $P = 0.29$). At high temperature, there was a 50.6% reduction in fecundity among resistant lines, which was significant (Tukey–Kramer, $P < 0.0001$). Thus, the reduction in fecundity among resistant lines is magnified at the high temperature (Fig. 4A), as in study 1. Net fecundity was consistently lower among resistant flies compared to control flies across the life span of flies (Fig 4B).

There were also significant effects of temperature and line on fecundity, but not of the treatment × line and treatment × temperature interactions (Table 4). Females produced approximately twice as many eggs in their lifetimes at the high temperature than at the low temperature (Fig. 4A). Post hoc analysis showed that for each of the three control lines, mean fecundity did not differ significantly from the base population (Dunnett's tests, $\alpha = 0.05$).

Table 4. Results of ANCOVA on female fecundity and longevity, study 2. All interaction terms are nonsignificant; only interactions of specific interest are listed.

Trait	Source	df	MS	*F*	*P*
Fecundity	line	2	217.6	5.04	0.007
	treatment[1]	2	926.4	21.45	<0.0001
	temperature	1	3730.5	86.38	<0.0001
	trtmt×temp	2	93.0	2.15	0.12
	line×trtmt	4	60.3	1.40	0.24
	Error	274	43.2		
Longevity	line	2	5.80	4.81	0.009
	treatment[1]	2	6.34	5.26	0.006
	temperature	1	66.4	55.1	<0.0001
	trtmt×temp	2	0.47	0.39	0.68
	line×trtmt	4	1.12	0.93	0.45
	Error	274	1.20		

[1] Selection treatments: resistant, control, and base.

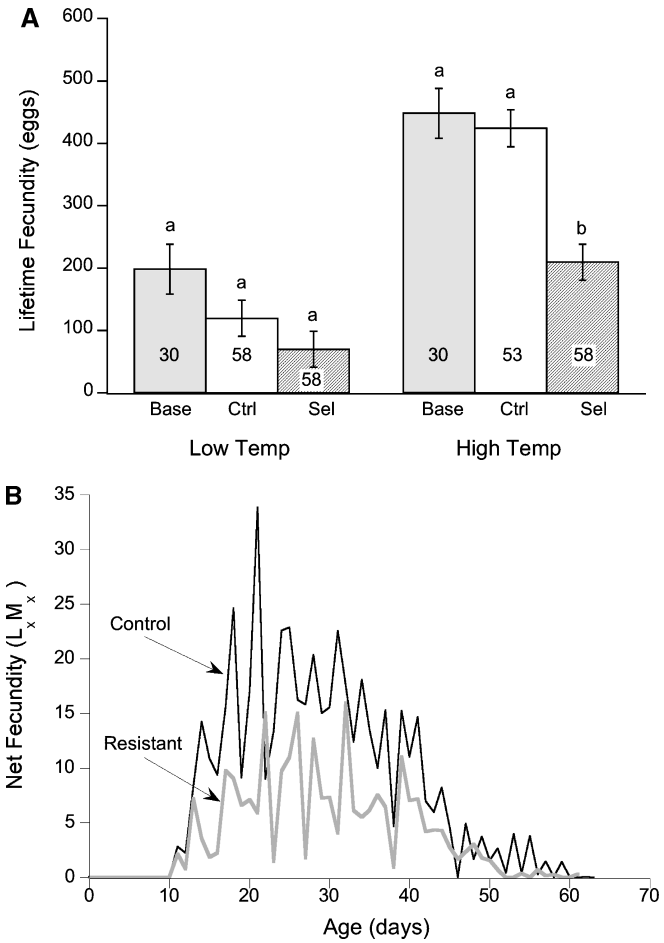

Figure 4. Fecundity results from study 2. (A) Least-squares mean lifetime fecundity at two temperature treatments. Within each treatment, bars sharing the same letter are not significantly different from each other (Tukey–Kramer test). Numerals inside bars represent number of flies assayed. (B) Net female fecundity with age, contrasting resistant and control lines at high temperature.

Study 2: Female longevity (with reproduction)

ANCOVA using fecundity as a covariable revealed a significant effect of selection treatment on female longevity (Table 4). This effect is attributable to the difference between the base and resistant flies (Tukey–Kramer, $P = 0.005$). But there was no significant difference between the control and resistant categories (Tukey–Kramer, $P = 0.12$) or the base and control categories (Tukey–Kramer, $P = 0.25$). We found a significant effect of temperature, such that females at low temperature lived 1.4 times longer (13.1 days) than at high temperature. Interactions were not significant.

Results of the Cox's proportional hazards analysis, using fecundity as a time-dependent covariable, showed that selection treatment did not have an effect on survival at either the low ($\chi^2 = 2.16$, df = 1, $P = 0.14$, hazard ratio = 1.34) or high ($\chi^2 = 0.19$, df = 1, $P = 0.66$, hazard ratio = 1.09) temperatures.

Study 2: Longevity in the absence of mating (males only)

Male flies lived approximately 1.4 times longer at low (median = 92.5, range = 111 days, $n = 144$) than at high (median = 68.0, range = 163 days, $n = 146$) temperature (Wilcoxon–Mann–Whitney test, $Z = -6.94$, $P < 0.0001$). At the low temperature, mean longevity in days did not differ significantly between the base (median = 80.0, range = 142, $n = 30$), control (median = 109.5, range = 158, $n = 60$) and resistant (median = 81.5, range = 163, $n = 56$) lines (Kruskal–Wallis test, $\chi^2 = 4.91$, $P = 0.09$). The mean longevity for base (median = 56.0, range 93, $n = 28$), control (median = 70.0, range = 111, $n = 57$), and resistant (median = 69.0, range = 100, $n = 59$) males were also comparable at the high temperature (Kruskal–Wallis test, $\chi^2 = 5.61$, $P = 0.061$). The Kaplan–Meier analysis showed a lack of survivorship differences between control and resistant males at the low ($\chi^2 = 2.53$, df = 1, $P = 0.11$) and high ($\chi^2 = 2.44$, df = 1, $P = 0.12$) temperatures.

Discussion

Artificial selection applied to host populations sampled from nature resulted in significant and repeatable increases in ectoparasite resistance. By modeling resistance as a threshold trait, we estimated the realized heritability for resistance in the second study to be 0.12, consistent with the value of 0.15 (reported in Polak 2003) from the first set of lines. Collectively, these parameter estimates encompass seven selection lines derived from field samples collected over eight years, and demonstrate the existence of significant additive genetic variation for ectoparasite resistance.

Resistant genotypes suffered reductions in fecundity compared to unselected control lines in the absence of parasitism. As illustrated by plots of net fecundity with age, fecundity differences were manifested across most of the life span of females. Thus, because flies undoubtedly live much shorter lives in the field than the laboratory, this pattern of expression of the fecundity cost suggests that it may indeed be of evolutionary significance in natural populations. One alternative, for example, is that if this fitness cost was manifested only late in life, its contribution to the maintenance of genetic polymorphism would be diminished because individuals in old age classes presumably are rare or absent in the field.

An important feature of our work that distinguishes it from previous studies (Kraaijeveld and Godfray 1997; Yan et al. 1997; Fellowes et al. 1998; Webster and Woolhouse 1999) is that the targeted host traits were behavioral in nature (Polak 2003). By using flies that successfully evaded mite attachment (determined by the absence of mites and feeding scars), selection was applied to traits that minimized host contact with, and colonization by, mites. Flies actively avoid contact with mites by engaging in sudden reflex movements and bursts of flight, and by rapidly redirecting their path of locomotion. Once a mite has made contact and seized hold of a leg, the host can still dislodge the mite by vigorous grooming and tarsal flicking. Indeed, when host behavioral defenses were experimentally attenuated, resistance divergence disappeared (Polak 2003). Thus, the traits we targeted represent "front line" forms of defense (Rigby et al. 2002), in contrast to the internal cellular encapsulation mechanisms of *D. melanogaster* against parasitoids that entail complex physiological and biochemical events within the insect's haemocoel (Fellowes et al. 1998). Collectively these fly studies indicate that different forms of defense (i.e., internal vs. external) can be associated with evolutionary significant costs in natural systems, and that these costs can be context dependent and involve damage to very different fitness-related traits. Future work should examine whether the fecundity costs we detected translate to differences in number of adult offspring (a more precise measure of fitness) between relatively resistant and susceptible genotypes.

The fecundity cost detected in our study is unlikely the result of greater inbreeding depression suffered by resistant lines. Each selected line had a paired control line maintained in parallel throughout the experiment. These paired lines were seeded each generation with an equal number of individuals of similar age, thus minimizing the possibility of differential inbreeding between them. Additionally, the consistent *lack* of a difference in reproductive output between the base and control lines further suggests that inbreeding depression was not a significant factor in our experimental system, and moreover, that fecundity did not evolve in the unselected flies during the course of the experiment. In other words, comparisons involving the base, which is not common practice in such work (e.g., Boots and Begon 1993; Kraaijeveld and Godfray 1997; Fellowes et al. 1998; Webster and Woolhouse 1999; Lohse et al. 2006), verify that divergence between the resistant and control lines is a result of a relative decline in fecundity among the selected lines and not of an unexpected rise in fitness of the control lines (Harshman and Hoffmann 2000), which could occur by genetic drift.

The negative genetic correlation we uncovered may be attributed to either pleiotropy or linkage disequilibrium (Crow and Kimura 1970; Parker 1991). Linkage can result in observable costs if selecting for resistance-conferring genes results in the "hitchhiking" of deleterious mutation(s) at linked loci. Alternatively, costs due to genetic pleiotropy arise when the same gene(s) influencing resistance disrupts other fitness traits (Williams 1957; Antonovics and Thrall 1994; Mitchell-Olds and Bradley 1996; Partridge 2001). Genetic hitchhiking is less likely than pleiotropy to be a factor here, for the major reason that our fly cultures were initiated from random samples of natural populations; in large outbreeding populations physical linkage decays relatively quickly, and is therefore not expected to be a persistent cause of genetic correlations (Crow and Kimura 1970; Clegg et al. 1980; Mitchell-Olds and Bradley 1996; Conner 2002). Thus, the consistency of

the correlated decrease in fecundity across independent replicate lines supports a role for pleiotropy. However, the possibility still remains that during the selection program itself, detrimental alleles at loci tightly linked to resistance genes increased in frequency, at least in some of our lines, thereby contributing to the observed reduction in fecundity. Although contributions from tight linkage cannot be excluded, the facts that we detected consistent costs despite backcrossing, mass-culturing, and reselection, and that fecundity costs were consistently expressed across experimental time blocks (which were each separated by one to two generations of relaxed selection, i.e., episodes of selection-free recombination), further point to pleiotropy (and see Conner 2002; Zhong et al. 2005).

In contrast, neither male nor female survivorship in *D. nigrospiracula* was significantly affected by the selection treatment, at either temperature; whereas the higher temperature magnified the differences in fecundity between resistant and control lines, it had no such effect on the mean longevity of adults. For either sex, longevity and survivorship were similar for the resistant and control lines, regardless of their reproductive status. So even under these varying conditions of temperature and reproduction, we did not detect correlated responses in life span (and see Kraaijeveld and Godfray 1997; Hurd et al. 2005). Although Yan et al. (1997) did report a relative reduction in longevity among resistant *A. aegypti*, the role of inbreeding depression cannot be entirely excluded because mosquito populations in this study were derived from selective inbreeding.

In our study, the reduction in fecundity concomitant with the evolution of resistance may be driven by a reallocation of a general, limited resource pool (e.g., protein or energy) (Zwaan et al. 1995; Lochmiller and Deerenberg 2000; Rigby et al. 2002). For example, selection for behaviorally mediated resistance may have enhanced traits such as general hyperactivity. Given that behavioral defense against ectoparasitism can be energetically expensive (Luong et al. 2007), costs could result from a pleiotropic reallocation of metabolic reserves (such as fat, Zwaan et al. 1995). For example, Kraaijeveld et al. (2001) suggested that the reduced larval competitive ability among resistant *D. melanogaster* might be due to a shift in the energy investment of the host from feeding efficiency to haemocyte synthesis, which is positively correlated with increased resistance. However, why reproduction in our system would be more sensitive than life span to an energy limitation is unknown. Thus, a different view is that this disparity may reflect differences in *how* resources are allocated to life span versus reproduction (Worley et al. 2003; Fuller et al. 2005). Alternatively, perhaps resistance-conferring allele(s) have narrower effects than on the partitioning of a general resource pool. For example, a specific resource may have been diverted to resistance critical for reproduction but not for survival, or pleiotropic effects may have been exerted on structures, enzymes (e.g., Thomson et al. 2002), or behaviors specifically related to oogenesis or oviposition.

The magnification of the fecundity cost we observed with temperature suggests that resistance levels in nature may track environment conditions, at least loosely. Temperatures experienced by flies outside and inside cactus rot pockets fluctuate greatly seasonally and even daily (Gibbs et al. 2003), and despite behavioral avoidance of extreme microclimates, adult flies are known to experience considerable variation in heat stress at different times of the year. Thus, if costs are magnified during the hottest months, then resistance levels may tend to get dragged downward at these times of the year (and see Stearns et al. 1990; Sgrò and Hoffmann 2004). The mechanisms by which ambient temperature alters the trade-off between resistance and fecundity are unknown, but perhaps the induction of specific protective systems within the body in response to temperature stress exacerbates the antagonistic relationship between them.

In summary, this study demonstrates a context-dependent fitness cost of behavioral defenses, which are ubiquitous in animal hosts (Hart 1997; Rigby et al. 2002). Our results may therefore help identify constraints on the evolution of host defensive systems operating in other species. In the present association, the intensity of ectoparasitism is known to vary in both ecological space and time, and to depend on the quality and age of the cactus necrosis (Polak and Markow 1995). Thus, these fluctuating selection pressures, combined with fecundity costs documented here, may be contributing to the maintenance of additive genetic variation for parasite resistance observed in nature.

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