Evolution, 2023, **77(9)**, 2068–2080
https://doi.org/10.1093/evolut/qpad118
Advance access publication 2 July 2023
Original Article

Heritability and preadult survivorship costs of ectoparasite resistance in the naturally occurring *Drosophila–Gamasodes* mite system

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Abstract

Our understanding of the evolutionary significance of ectoparasites in natural communities is limited by a paucity of information concerning the mechanisms and heritability of resistance to this ubiquitous group of organisms. Here, we report the results of artificial selection for increasing ectoparasite resistance in replicate lines of *Drosophila melanogaster* derived from a field-fresh population. Resistance, as ability to avoid infestation by naturally co-occurring *Gamasodes queenslandicus* mites, increased significantly in response to selection and realized heritability (*SE*) was estimated to be 0.11 (0.0090). Deployment of energetically expensive bursts of flight from the substrate was a main mechanism of host resistance that responded to selection, aligning with previously documented metabolic costs of fly behavioral defenses. Host body size, which affects parasitism rate in some fly–mite systems, was not shifted by selection. In contrast, resistant lines expressed significant reductions in larva-to-adult survivorship with increasing toxic (ammonia) stress, identifying an environmentally modulated preadult cost of resistance. Flies selected for resistance to *G. queenslandicus* were also more resistant to a different mite, *Macrocheles subbadius*, suggesting that we documented genetic variation and a pleiotropic cost of broad-spectrum behavioral immunity against ectoparasites. The results demonstrate significant evolutionary potential of resistance to an ecologically important class of parasites.

Key words: ectoparasite resistance, avoidance behavior, heritability, costs, ammonia stress, *Drosophila melanogaster*

Introduction

Parasites are ubiquitous in the environment (Price, 1980; Schmid-Hempel, 2021), and by definition, they damage host fitness (Goater et al., 2014). As a consequence, parasites can represent potent agents of natural selection, capable of driving the (co-)evolution of host defensive adaptations and altering host population genetic structure (Little, 2002; Sheldon & Verhulst, 1996). Acquiring estimates of genetic variation for host defensive traits is a key to predicting the evolutionary and ecological consequences of parasite-mediated selection (Endler, 1986; Henter & Via, 1995; Sorci et al., 1997; Wakelin, 1978).

There exists a large body of empirical evidence showing that heritable variation for resistance is indeed a common feature of natural populations of plant, animal, and microbial species (Burdon, 1987; Carius et al., 2001; Duffy & Sivars-Becker, 2007; Foster et al., 2007; Hufbauer & Via, 1999; Little, 2002; Lively & Dybdahl, 2000; Schmid-Hempel, 2021; Sorci et al., 1997). However, the existence of widespread heritability for resistance may also be considered paradoxical, as selection for increasing resistance acting over past generations should have promoted depletion of genetic variation at resistance-conferring loci (Barton & Turelli, 1989; Stearns, 1992). Several mechanisms for the maintenance of standing genetic variation for parasite resistance have been proposed, including frequency-dependent selection (Hamilton & Zuk, 1982; Lively & Dybdahl, 2000; Brown & Tellier, 2011), overdominance (Doherty & Zinkernagel, 1975; Brown & Tellier, 2011), and costs of resistance under heterogeneous parasite pressure (Parker, 1990; Rigby et al., 2002).

Costs of resistance, defined as decrements in fitness of resistant hosts compared with susceptible forms in the absence of parasitism (Parker, 1992, Mitchell-Olds & Bradley, 1996, Rigby et al., 2002), have been found in a wide range of organisms and shown theoretically to result in genetic polymorphisms at relevant loci (Anderson and May, 1982; Gillespie, 1975). Yet, whereas the costs hypothesis has received empirical support, costs of resistance have not always been found, fueling debate over their general importance (Bergelson & Purrington, 1996; Coustau & Chevillon, 2000; Rigby et al., 2002; Simms & Rausher, 1987). For instance, in *Arabidopsis thaliana*, transgenic plants carrying specific resistance alleles conferring protection against a pathogenic bacterium, *Pseudomonas syringae*, experienced on average a 9% decrease in seed production relative to susceptible genotypes, providing evidence for a pleiotropic cost of resistance in this case (Tian et al., 2003). In *Escherichia coli*, mutations conferring resistance to phage also imposed pleiotropic costs, although mutants were shown to vary greatly in their fitness effects across genotypes, indicating that costs may be modified by genetic background (Lenski, 1988). In contrast, in pea aphids, *Acyrthosiphon pisum*, Ferrari et al. (2001) failed to

Received January 3, 2023; revisions received June 6, 2023; accepted June 30, 2023
Associate Editor: Robert Unckless; Handling Editor: Tracey Chapman

detect costs (as decreased fecundity) associated with resistance to two parasitoid wasps and a fungal pathogen, despite finding ample genotypic (clonal) variation for host resistance (and see Henter & Via, 1995, p. 436, for a further discussion of potential costs in *Acyrthosiphon* aphids).

One possible reason for such inconsistency is that environmental variation influences the expression of costs, although such exogenous effects on cost expression also have been variable, and even contradictory in some cases (Boege et al., 2007; Boots, 2011; Cipollini et al., 2014; Osier & Lindroth, 2006; Purrington, 2000; Sandland & Minchella, 2003). It may also be that expression of costs depends on the stage of the host life cycle, possibly reflecting shifting patterns of host resource allocation to different fitness functions through development (Boege & Marquis, 2005; Sandland & Minchella, 2003). For example, in birdsfoot trefoil, *Lotus corniculatus*, costs of producing defensive cyanogenic glycosides are highest during episodes of peak reproductive effort (Briggs & Schultz, 1990). A lack of evidence for costs may thus only indicate that the appropriate environmental conditions or ontogenetic stage were not examined (Lochmiller & Deerenberg, 2000; Sandland & Minchella, 2003).

For animal host–parasite systems, estimates of standing genetic variation for resistance and of associated costs are often derived from studies of endoparasites—parasites that invade the host and grow, proliferate, and/or reproduce within the body (Coustau & Chevillon, 2000). Indeed, we know a great deal about the mechanisms and magnitude of genetic variation and of costs for resistance to parasitoids attacking insects (Carton & Nappi, 1997, 2001; Carton et al., 2005, 2008; Fellowes & Godfray, 2000; Henter & Via, 1995; Kim-Jo et al., 2019; Kraaijeveld & Godfray, 1997; Salt, 1970). In *D. melanogaster*, for example, when flies were artificially selected for increased resistance to parasitoid wasps (as encapsulation ability), replicate fly lines readily responded to selection, demonstrating significant heritable genetic variation for parasitoid resistance. Resistant fly lines in the absence of parasitism had significantly reduced competitive ability, although this negative effect was evident only at heightened levels of larval competition (density) (Fellows et al., 1998; Kraaijeveld & Godfray, 1997), supporting the hypothesis that costs of resistance may be modulated by environmental factors (e.g., crowding, nutritional stress).

In contrast to animal host–endoparasite systems, less information is available about the mechanisms and genetic bases of ectoparasite resistance (Gibson & Amoroso, 2022). Yet, ectoparasites, which attack the body surface of their hosts, are a diverse and ecologically important group of organisms (Behnke, 1990; Clayton et al., 2010; Hopla et al., 1994), and the fitness costs they impose on major host life-history traits can be pronounced (Fitze et al., 2004; Forbes & Baker, 1991; Lehmann, 1993; Møller, 1990a; b; Polak, 1996; Polak & Markow, 1995). Host physiological impairment is a common outcome of heavy ectoparasite burdens through resource extraction, leading to anemia and significant weight loss, nutritional and reproductive impairment, and elevated risk of death (Lehmann, 1993; Møller et al., 1990; Nelson et al., 1975). Ectoparasites may additionally serve as vectors of transmissible parasitic disease in plants and animals, including humans (Behnke, 1990; Jaenike et al., 2007; Marshall, 1981; Moran et al., 2008). Thus, in addition to their evolutionary significance, ectoparasites are of enormous ecological, veterinary, and medical importance.

The varied adaptations animals have evolved in response to ectoparasite activity are legion (Clayton et al., 2010; Hart, 1990; Hart & Hart, 2018; Moore, 2002; Rigby et al., 2002; Thieltges & Poulin, 2008). At the outset, behavioral defenses may serve to prevent contact and colonization of the host body, so-called "first-line" forms of defense (Leung et al., 2001; Poirotte et al., 2017; Schmid-Hempel & Ebert, 2003; Thieltges & Poulin, 2008). In addition to detection and direct avoidance mechanisms, first-line defenses may also involve avoiding certain habitats (Girard et al., 2021), feeding activities (Moore, 2002), nesting sites (Møller, 1990b), food patches (Anderson & McMullan, 2018), and conspecifics including potential mates (Borgia & Collis, 1989; Kavaliers et al., 2003; Read, 1990; Stephenson et al., 2018). After contact is made, hosts may counter parasite establishment and reduce parasite burden by deploying brisk reflex movements directed at the parasites, self-grooming (e.g., foot scratching and rubbing), being groomed by others in the social group (allogrooming), and by self-medicating (DeJoseph et al., 2002; Hart, 1994; Huffman, 1997; Kupfer & Fessler, 2018; Ramanantsalama et al., 2018). Once established, ectoparasite feeding also generally elicits pronounced behavioral and immunological responses that may limit parasite feeding and development, and speed rates of detachment (Owen et al., 2010; Wakelin, 1996; Wikel & Alarcon-Chaidez, 2001). Thus, host defenses against ectoparasites span a range of pre- and postattachment mechanisms, all of which are in theory subject to selection pressure and may incur relevant costs (Sheldon & Verhulst, 1996).

The present study focuses on the host *D. melanogaster* and its naturally co-occurring mite, *Gamasodes queenslandicus* (Acari: Parasitidae). *Gamasodes* mites, which breach fly integument and consume host tissue while attached to their hosts (M. Polak and H. Spitz, unpublished manuscript; see Polak, 1996), are generalist parasites recovered from a number of *Drosophila* species and other insects in Australia and Asia (Halliday et al., 2005; Yao et al., 2020, M. Polak, personal observation). The "behavioral immunity repertoire" of flies encompasses locomotor movements away from mites, running, and reflexive jumps and bursts of flight from the substrate, which are behaviors that require significant power output (Bimbard et al., 2013) and are generally known to be metabolically expensive (Benoit et al., 2020; Harrison & Roberts, 2000; Zabala et al., 2009). When a mite does succeed to grasp a fly, typically a tarsus, flies respond by vigorously prying and pushing at the mite and tarsal flicking to dislodge it (Greene, 2010; Polak, 2003).

We employed artificial selection for increased preattachment host defenses and calculated realized heritability of behavioral immunity to mites separately for male and female flies. Postselection, a wing removal experiment was conducted to evaluate the importance of flight-related behavior in mediating the observed response to selection. Next, adult body size and larva-to-adult survivorship were contrasted between selected and control lines reared under conditions of increasing concentrations of ammonia (an ecologically relevant environmental toxin, Borash et al., 1998, 2000), to evaluate whether any correlated evolutionary shifts occurred in these traits (Kraaijeveld & Godfray, 1997; Luong & Polak, 2007a). Host body size is an important fitness-related trait (Flatt, 2020; Partridge et al., 1987) and mediates mite attachment in some drosophilid species (Campbell & Luong, 2016; Horn et al., 2020). Finally, we tested for cross-resistance in

the selected lines to a different mite, *Macrocheles subbadius* Berlese (Acari: Macrochelidae), a cosmopolitan species known to parasitize drosophilid flies and also other insects (Polak, 1996; Polak & Markow, 1995); the aim here was to evaluate whether resistance to our focal mite could be generalized to another naturally occurring ectoparasite, and hence, whether the defensive traits we studied could have broader ecological significance.

Materials and Methods

Fly and mite laboratory populations

A laboratory base population of *D. melanogaster* Meigen was established with approximately 150 field-caught females and an equal number of males collected in February 2017 at two field sites 12 km apart (16° 5'4.59"S, 145° 27'46.40"E; 16°12'50.19"S, 145°24'16.99"E) at Cape Tribulation, Queensland, Australia. Adult flies were collected directly from fruit substrates at both locations and combined to form a single outbred base population. Flies were returned to the laboratory and cultured on standard cornmeal-agar food medium under controlled light and temperature conditions (12 hr light [24 °C]:12 hr dark [22 °C]) within an environmental chamber. Over this initial time frame, the base population was cultured for four generations in half-pint glass culture bottles prior to the onset of selection. The mass culture was expanded to 30 bottles, and after the fourth generation of culture, six lines were created each with 300 females sampled from across the 30 bottles and an equal number of males similarly harvested, and cultured for two additional generations in a common incubator. Of these 6 lines, 3 lines were randomly chosen to serve as the selected lines. Each selected line was randomly paired with a control line. Thus, a total of six generations of laboratory culture elapsed before the onset of artificial selection. There were three lines per selection treatment (i.e., selected vs. control).

Gamasodes queenslandicus Halliday and Walter mites were harvested directly from the bodies of flies collected at both sites and placed into culture medium to establish a large laboratory population maintained in an environmental chamber under 12 h light (23 °C) and 12 h dark (26 °C) conditions. The culture medium consisted of a rich organic mixture of wheat bran, wood shavings, inactive yeast, and bacteriophagic nematodes as a food source for the mites (modified after Polak, 1996).

Artificial selection for increased ectoparasite resistance

Artificial selection on *D. melanogaster* for increased resistance to *G. queenslandicus* mites was applied for 16 generations in three replicate fly lines, each independently derived from the base population, described above. The protocol used to select for increased ectoparasite resistance is described in detail elsewhere (Polak, 2003). Briefly, at each generation of selection, only male flies were exposed to mites in infestation chambers, comprised of 500-ml glass jars lined with gypsum plaster and containing ≈ 50–80 ml of mite culture medium with mites. Jars were sealed with breathable mesh to allow ample air exchange. For each selected line, 90 male flies were placed into each of four infestation chambers; 360 flies of each selection line were thus exposed to mites every generation. Flies interact with freely moving mites in these chambers on the surface of the medium, and parasitism occurs as in the field, with mites approaching, contacting and attaching to flies from the substrate. Flies avoid contact with mites using a suite of distinctive evasive maneuvers and grooming to rid themselves of mites that have made contact (see Introduction). To apply selection, flies and mites were allowed to interact for 6–12 h in chambers. Using an aspirator, flies were recovered from a given chamber when it was estimated that approximately 1/2–2/3 of the flies acquired mites. Recovered flies were sorted under a stereomicroscope while anesthetized with a light stream of humidified CO_2, and flies with attached mites or mite-induced scars were discarded. Selection was applied by seeding each new generation of a given selection line with the unparasitized fraction of male flies. The proportions of selected flies (median, range) at each generation that were unparasitized after exposure are as follows: Line 1: 0.439, 0.292–0.79; Line 2: 0.444, 0.217–0.627; Line 3: 0.446, 0.314–0.57. Despite these differences in proportions selected, it was assumed that the unparasitized fraction of males recovered from chambers at each generation was a random sample of the class desired for selection, and thus, that the mean of the recovered group was representative of this class (Polak 2003, p. 76). Consequently, we made the assumption that selection intensity was constant across the 16 generations of artificial selection. Females were not selected, and were chosen randomly from within their respective lines and paired (as virgins) with selected males to seed each new generation. The number of females used was 120 per line; 30 virgin females in each of 4 culture bottles per line. Selected males were distributed approximately equally across the four bottles per line.

Each control line was seeded each new generation with the same number of male and female flies as its associated selected line. Male flies of a given control line were unselected flies, randomly chosen from flies that had been loaded into infestation chambers with mite culture medium but without mites: thus, control male flies were exposed to chamber conditions, but not to mites. Males were placed together with virgin control females taken from within their respective line and cultured. Control and selected lines were cultured in parallel in the same incubator. Selection lines are referred to as S1–S3 and control lines as C1–C3.

Response to selection and realized heritability

To track response to selection, resistance assays were conducted at 6 time points over the course of the 16 generations of selection, following Polak (2003) and Luong and Polak (2007b). A given assay involved measuring probability of ectoparasitism in each of the selected lines relative to their counterpart control (unselected) lines within infestation chambers. Multiple chambers per selected line were used in a given assay per sex (the sexes were assayed in separate chambers). For example, after generation 2 of selection, each line was assayed in 6 chambers (3 for males and 3 for females). For a given pair of lines (e.g., S1/C1), groups of selected and control flies equal in number were aspirated into a chamber (total number of flies in each chamber ranged from 50 to 70 flies); note that exposing selected and control flies to mites in a common chamber represented a more sensitive assay compared to exposing selected and control flies in different chamber. Selected and control flies were distinguished by minute wing clips to the tip of one of the wings (≤3%–5% of the wing); clips do not affect susceptibility to mites (Benoit et al., 2020; Polak, 2003). Clips were administered to flies either to

the right or left wing under light CO_2 anesthesia. The wing receiving a clip was alternated between the groups across replicate chambers. Flies were given a minimum of 24 hr to recover from the clipping procedure prior to loading them into infestation chambers.

Flies and mites were allowed to interact in chambers for 6–12 hr, until approximately 50% of flies were infested. Flies were recovered from chambers, scored for the presence of mites and scars, and identified as to their group of origin by their wing clips. Prevalence of parasitism in each group was calculated as the total number of infested plus scarred flies divided by the total number in each group that had been loaded into the chamber. Resistance was modeled as a threshold trait, with an expected underlying continuous variable called the liability, influenced by both genetic and environmental factors (Falconer & 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 selected and control categories (Falconer & Mackay, 1996, p. 301), and the difference in mean liability (in *SD* units) between the categories was used to estimate genetic improvement made by selection. Because we assumed that selection intensity was constant across generations (see above), realized heritability was calculated from the slope of the regression of divergence on generation number (Muir, 1986, p. 382). Because selection was applied to males only, the realized heritability for each selected line was calculated as twice the slope (Roff, 1997, p. 40). Heritabilities were estimated using data from the first 16 generations of selection. Whether mean heritability was significantly different from zero for males and females separately, and when combined between the sexes, was tested using a *t*-test and a one-tailed *p*-value. A two-tailed *p*-value was used to compare mean heritabilities between the sexes. After the 16th generation, selection was applied every two to three generations to maintain divergence.

Wing removal experiment

To address the importance of flight-related behavior in mediating response to selection, we tested the consequences of wing removal for resistance divergence between selected and unselected (control) flies, at two (consecutive) generations after selection. The wings of each fly were removed by cutting each wing off at its base with ultra-fine surgical scissors. Wingless selected and unselected flies were exposed to mites in common infestation chambers, as above; replicate chambers were used for each pair of lines. To distinguish selected and unselected groups, we clipped the scutellar bristles of one of the two groups of flies. Whether the scutellar bristles were clipped was alternated between the groups across replicate chambers. Wingless flies were allowed to recover post-surgery for at least 24 hr and exposed to *Gamasodes* mites. Flies were removed from a given chamber when 40%–60% of flies were estimated to be infested. Recovered flies were scored for the presence/absence of parasitism under CO_2 anesthesia. Assays comparing selected and unselected flies with *intact* wings were conducted in parallel to verify divergence in resistance between these groups. In these assays, the wings of both groups were contacted with the scissors while under CO_2 anesthesia but the wings were not removed. As for the wingless flies, selected and unselected flies were distinguished by scutellar bristle clips. Prevalence of parasitism was the response variable, analyzed using generalized linear models in JMP Pro (vers. 15.0.0). The model used a binomial error structure and a logit link function, where the number of infested flies was the numerator and the total number of flies exposed per group was treated as the binomial denominator. Generation and selection treatment were factors in each model. Models were fitted separately by sex and wing treatment. The objective was to assess degree of divergence between selected and unselected groups among flies with or without wings, by sex.

Body size and preadult survivorship

The effects of selection for resistance on host body size, estimated as thorax length (Partridge & Fowler, 1992; Robertson & Reeve, 1952), was tested at two time points. The first was immediately after generation 16, the generation at which sustained artificial selection was terminated and realized heritability estimates calculated. The second time point was after generation 21 of selection. At this second time point, the design was expanded to incorporate exposure to ammonia as a source of toxic stress (see below), to test for a possible interaction between selection treatment and environmental stress on body size. At the first time point (i.e., after the 16th generation of selection), virgin females were paired with an equal number of males from within their respective line and allowed to lay eggs on grape juice-agar petri dishes (10 cm diameter). Dishes with eggs were incubated at 25 °C for 24 hr, after which exactly 60 first-instar larvae were transferred with blunt dissection probes to food vials containing 6.0 ml standard cornmeal-agar medium. There were two replicate vials for each of the six lines (i.e., the three selected and three control lines). Food vials with larvae were returned to incubator conditions, and flies were allowed to develop to adulthood. Ample pupation substrates (as rolled-up sterile tissue paper) were provided to avoid larval drowning. On emergence, adult flies of both sexes were harvested, counted, allowed to harden, and preserved in 70% ethanol for later thorax length measurement. Thorax lengths of both sexes, taken as the linear distance from the frontal edge of the thorax to the tip of the scutellum, were measured using an ocular micrometer of a stereomicroscope in order to estimate body sizes (Robertson & Reeve, 1952). At the second time point (i.e., after 21 generations of selection), first-instar larvae were harvested as above and transferred to food vials with 6.0 ml of standard cornmeal medium, containing one of four concentrations of ammonium chloride (NH_4Cl, Sigma): 0 g/L (0 M), 15 g/L (0.28 M), 20 g/L (0.374 M), and 25 g/L (0.47 M), bracketing the concentration range shown in previous work to impair homeostasis and to harm the expression of fly life-history traits, including larva-to-adult viability (Borash et al., 1998, 2000). There were four replicate vials per concentration. As above, all emerging adults were counted and preserved in 70% ethanol. Thorax lengths of a randomly selected subset of five male flies from each replicate vial were measured and averaged. Thorax lengths of males from the 25 g/L ammonia vials were not measured because there were too few flies that emerged from this treatment. The effect of selection treatment on thorax length at both time points was evaluated with a restricted maximum likelihood (REML) mixed model, where replicate line was nested within selection treatment and treated as a random effect. Survivorship data were on a binomial scale and analyzed using a generalized linear mixed model, where the number of adult flies that emerged from a given vial was the numerator and the total number

of flies that seeded each vial (i.e., 60) the denominator. Line was treated as a random effect and was nested within selection treatment. The model was implemented in SAS (vers. 9.4) with the GLIMMIX procedure, which uses the binomial distribution as the response distribution, allows random nested effects, returns confidence limits on request, and reports *F* statistics for tests of significance.

Cross-resistance to *M. subbadius*

To test for cross-resistance to *M. subbadius* Berlese mites in the lines selected against *Gamasodes* mites, resistance assays were conducted as described above, except that the mites to which flies were exposed in experimental infestation chambers were *M. subbadius*. Cross-resistance assays were conducted following the 16 generations of artificial selection and 2 generations of mass culture without selection. Resistance to *Gamasodes* was assayed in parallel, so that degree of resistance to the two mite species could be directly compared. The mass culture of *M. subbadius* was established in the laboratory with mites harvested from flies (*D. nigrospiracula*) collected at necrotic saguaro cacti in Arizona (USA), at two sites: 33°20′43.05″N, 111°25′21.47″W; 33°21′42.52″N, 111°23′43.31″W. Mites were recovered directly from the bodies of field-caught *D. nigrospiracula* of both sexes under CO_2 anesthesia, and cultured (Polak, 2003). Data were analyzed using separate generalized linear models with the GLIMMIX procedure in SAS (vers. 9.4), as above, to test for difference between each resistant line against its paired control. Confidence intervals were obtained using JMP Pro (vers. 15.0.0). As a follow-up analysis, a model was constructed to examine the interaction between mite species and selection treatment. In this model, the factors were mite species, selection treatment, line, sex, and the mite species-by-selection treatment interaction term of interest. The model was implemented in SAS (vers. 9.4).

Results

Artificial selection for increased resistance to *Gamasodes* mites applied for 16 generations in replicate lines derived from a field-fresh Australian population of *D. melanogaster* resulted in significant evolutionary responses. Divergence between selected and unselected lines increased steadily over the course of selection, and responses were congruent between replicate selection lines for both males (Figure 1A) and females (Figure 1B). To compare responses (slopes) between the sexes, we conducted a paired *t*-test, which yielded a nonsignificant result ($t = 0.113$, $df = 2$, two-tailed $p = .92$), indicating that males and females responded to a similar degree to artificial selection, which is of particular interest since selection was applied to males only. Table 1 provides slopes of response on generation number and heritability estimates separately by sex. The grand mean (*SE*) realized heritability, calculated across the sexes, was 0.107 (0.0090), which differed significantly from zero ($p < .0001$, Table 1). Thus, the host population we sampled at Cape Tribulation, Australia, harbors significant additive genetic variation for resistance to *Gamasodes* mites. The notably steady and progressive evolutionary response suggests multifactorial inheritance, and the observation that responses were similar in magnitude between the sexes, despite selection having been applied to males only, implies principal effects of autosomal factors.

Among flies with experimentally removed wings, the effect of selection treatment on probability of parasitism was not significant for either males (Table 2A) or females (Table 2B). Indeed, among the wingless flies, selected lines exhibited very similar levels of resistance compared to unselected flies, indicating that the wing removal treatment essentially eliminated the defensive advantage of the selected lines (Figure 2). Resistance assays conducted in parallel using intact flies confirmed the significant separation in resistance between selected and control lines for both sexes, as expected (Table 2; Figure 2). We note that only levels of resistance between control and selected lines within wing removal treatments can be compared meaningfully in this figure, as control and resistance flies were the groups that were exposed together within common infestation chambers. Thus, comparisons, say, between wingless and intact flies of either sex, are not meaningful because differences between them reflect other factors

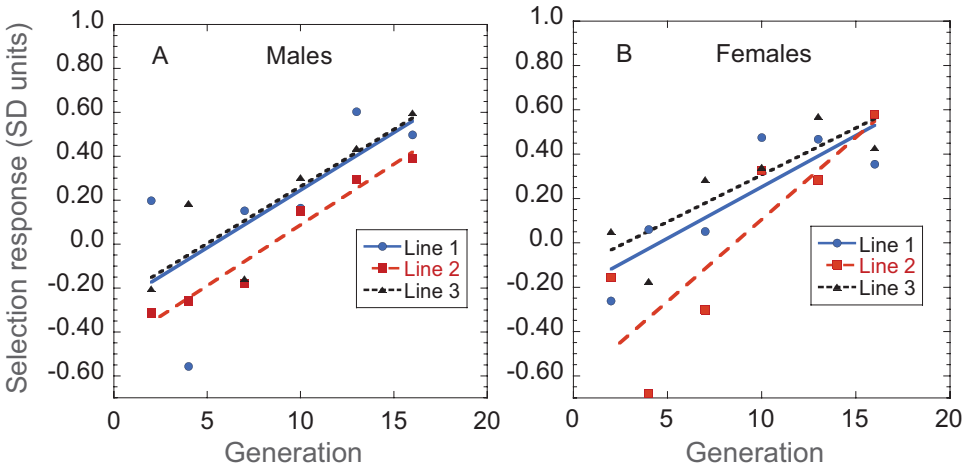

Figure 1. Evolutionary response of ectoparasite resistance in *SD* units across 16 generations of artificial selection in *D. melanogaster*, separately by males (A) and females (B). Selection was applied to male flies only, and response was tracked in both sexes. Table 1 provides realized heritability estimates, and tests of statistical significance.

Table 1. Realized heritability estimates of resistance in *D. melanogaster* flies to *G. queenslandicus* mites. Estimates are reported separately by sex and replicate selection line.

Sex	Line	Slope (*SE*)	*P*	h^2
Male	1	0.0524 (0.0274)	.0643	0.105
	2	0.0553 (0.00566)	.0003	0.111
	3	0.0519 (0.0151)	.0131	0.104
$\bar{x}$ (SE)				0.106 (0.00219)[a]
Female	1	0.0464 (0.0144)	.0160	0.0928
	2	0.0740 (0.0232)	.0168	0.148
	3	0.0423 (0.0136)	.0179	0.0846
$\bar{x}$ (SE)				0.108 (0.0199)[b]

Note.
[a] $t = 48.71$, $df = 2$, $p = .0002$.
[b] $t = 5.45$, $df = 2$, $p = .016$; grand mean ($\bar{x}$ (*SE*)) of h^2 estimates ($n = 6$), 0.107 (0.00897), $t = 11.98$, $df = 5$, $p < .0001$. In all tests, t evaluates H_o: $\bar{x} \leq 0$.

Table 2. Results of generalized linear models on ectoparasite resistance (proportion flies uninfested) by wing removal treatment, for (A) males and (B) females. For each sex, the effect of selection treatment on infestation probability was lost among wingless flies. Resistance assays conducted in parallel using intact flies exhibited significant selection treatment effects, confirming the significant divergence between selected and unselected flies produced by artificial selection (Figure 1).

Effect	*df*	χ^2	*p*
(A) Males	Wings removed		
Generation	1	7.487	.0062
Selection treatment	1	0.506	.48
Generation × Selection trt.	1	0.104	.75
	Wings intact		
Generation	1	9.352	.0022
Selection treatment	1	14.130	<.001
Generation × Selection trt.	1	0.387	.53
(B) Females	Wings removed		
Generation	1	0.521	.47
Selection treatment	1	0.00270	.96
Generation × Selection trt.	1	0.284	.59
	Wings intact		
Generation	1	0.930	.93
Selection treatment	1	13.071	<.001
Generation × Selection trt.	1	0.519	.47

affecting prevalence such as variation across chambers in mite density and the length of time flies were exposed to mites. The key finding of the present experiment that the difference in resistance between control and selected lines disappeared after wing removal identifies flight-related behaviors as a key component of ectoparasite resistance in our study system.

Flies cultured under controlled larval densities after 16 generations of selection did not show a significant difference in thorax length between selected and control lines, either for males or for females (Table 3A; Figure 3). In a second iteration of this experiment (after 21 generations of selection), we evaluated both the effects of selection and ammonia treatments on adult (male) body size and egg-to-adult survivorship. Here again we detected no significant effects of selection or ammonia treatments on body size or of an interaction between these factors (Table 3B). In contrast, there was a strong overall effect of ammonia treatment on egg-to-adult survivorship (Table 4), which decreased sharply with increasing ammonia concentration, confirming earlier work by others (Borash et al. 1998, 2000). No significant effect of selection treatment on this trait was found (Table 4). Importantly, we detected a significant interaction between selection treatment and ammonia concentration on egg-to-adult survivorship (Table 4): Whereas selected and control lines were very similar for this trait at 0 g/L ammonia, selected lines expressed consistent reductions in survivorship relative to unselected lines across ammonia concentrations, and the contrast was significant at highest concentration (25 g/L), indicated by nonoverlapping 95% confidence limits (Figure 4). These results suggest an environmentally modulated preadult survivorship cost of evolved ectoparasite resistance.

Flies selected for increased resistance to *G. queenslandicus* for 16 generations also had evolved cross-resistance to *M. subbadius* as a pleotropic effect (Table 5; Figure 5). Indeed, significant cross-resistance to *M. subbadius* was evident in all three replicate selection lines (Figure 5), indicating a robust result. Yet, some degree of specificity of resistance to *G. queenslandicus* mites could be discerned, as selected host lines consistently showed relatively better resistance to *Gamasodes* than to *Macrocheles*: Selected lines 1–3 were 42.0%, 49.4%, and 34.4% more resistant to *Gamasodes* relative to controls, and 26.6%, 32.4%, and 25.8% more resistant to *Macrocheles*, respectively. Indeed, a statistical model confirmed this difference between mite species, indicated by a significant mite species-by-selection treatment interaction ($F_{1,267} = 3.97$, $p = .0473$). Thus, the results suggest that the defensive mechanisms that evolved under the selection regimen we imposed work more effectively against the particular ectoparasite that was used as agent of selection relative to a novel but similar parasite.

Discussion

Heritability, the additive component of quantitative genetic variation, is a necessary condition for evolutionary response to natural selection of a given trait (Endler, 1986; Falconer & Mackay, 1996). Here, we have documented significant heritability of ectoparasite resistance in a field-derived population of *D. melanogaster*, demonstrating additive genetic variation for this trait; realized heritability (h^2) was estimated to be 0.107, and values were consistent between the sexes, being

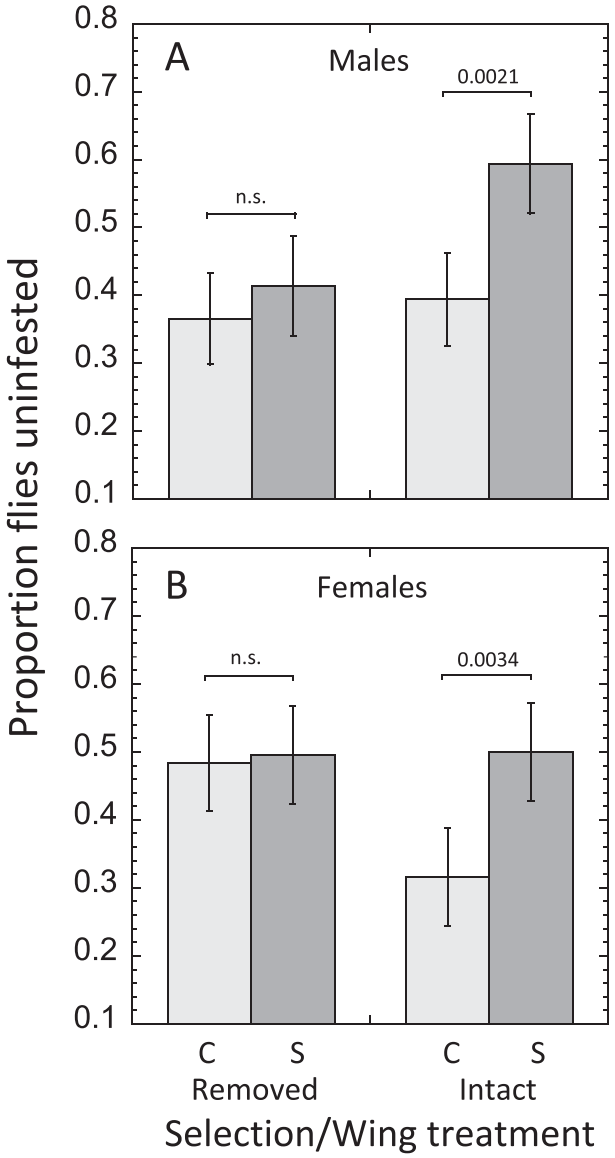

Figure 2. Results of the wing removal experiment for male (A) and female (B) flies. When wings were removed, control (C) and selected (S) flies exhibited similar levels of resistance to mites. In contrast, for flies with intact wings, selected flies were more resistant than controls. Error bars represent 95% CIs. Note that using this figure to directly compare levels of resistance between wingless and intact flies, or between males and females, is not meaningful because flies in these groups were exposed to mites in different infestation chambers. The meaningful contrasts are only between C and S categories, separately by sex and wing treatment.

0.106 for males and 0.108 for females (Table 1). Importantly, the resistance to *Gamasodes* that we increased evolutionarily conferred cross-resistance to a different mite, *M. subbadius* (Figure 5), suggesting that we uncovered standing genetic variation for broad-spectrum defensive ability against ectoparasites (sensu Fellowes et al., 1999; Schmid-Hempel & Ebert, 2003). The defensive abilities we targeted could very well have functional significance in an even broader ecological context, such as in interactions with predators like ants (specifically green tree ants, *Oecophylla*, which are a major class of natural enemy of flies observed at our field site), against which adult flies have been observed to deploy similar evasive maneuvers as against mites (M. Polak, personal observation).

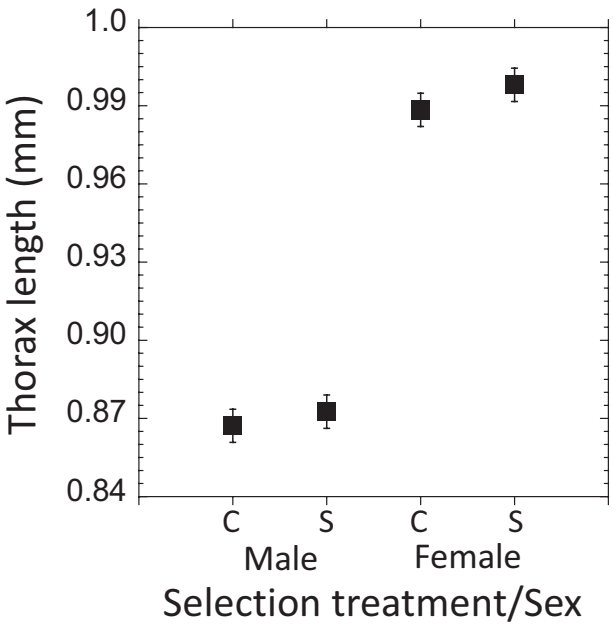

Figure 3. Mean (± 1 SE) thorax length for control and selected flies, by sex, following 16 generations of artificial selection for increased resistance against *Gamasodes* mites.

The heritability estimates we report tend to be lower compared to desert-endemic *D. nigrospiracula*, for which realized h^2 of resistance to *M. subbadius* mites ranged from 0.12 to 0.15 (Luong & Polak, 2007b; Polak, 2003), as well as for other animal hosts (Boulinier et al., 1997; Buzatto et al., 2019; Mazé-Guilmo et al., 2014; Møller, 1990a; Yáñez et al., 2014). One possible explanation at least for the difference between the two *Drosophila*-mite systems is that *Gamasodes* mites generate a more potent selection pressure than *Macrocheles*, which could have progressively depleted genetic variation in *D. melanogaster* over past generations. Moreover, the *Drosophila–Gamasodes* system we studied occurs in tropical Queensland (Australia), and predatory ants, as mentioned, are a notable source of mortality in the fly population. Sustained pressure by multiple natural enemies on the same or overlapping set of defensive traits could have contributed to the depletion of host genetic variation for defensive ability.

An alternative explanation emphasizes that *Gamasodes* mites are a faster-moving and more aggressive species, and notably more efficient at overwhelming host behavioral defenses than *M. subbadius*. The lower h^2 of resistance to *Gamasodes* could therefore also be a result of this higher parasite mobility, which would have the effect of reducing the number of mite-fly interactions leading to attachment, as flies would stand less of a chance of avoiding a very quick-moving and aggressive mite compared to a relatively slow mite. Reducing the number of such interactive events leading to parasitism would erode the relationship between host intrinsic factors and parasitism (effectively increasing the stochastic variance in attachment rate), and reduce heritability (Hoffmann, 1999). A parallel argument has been made to explain variation in heritability of resistance to different ectoparasites attacking tropical cattle, where parameter estimates for numbers of ticks and buffalo flies were 0.34 and 0.06, respectively (Mackinnon et al., 1991). It was suggested that the sharply reduced h^2 for fly counts could have been

Table 3. Results of REML mixed models on body size in *D. melanogaster* following (A) 16 and (B) 21 generations of artificial selection for increasing resistance to *G. queenslandicus* mites.

Fixed effect	Numerator *df*	Denominator *df*	*F*	*p*
(A) Factors in this experiment are sex, selection treatment (selected and control), and line				
Sex	1	232	583.460	<.0001
Selection treatment	1	4	0.973	.340
Sex × Selection treatment	1	232	0.137	.712
(B) Factors here include selection treatment (i.e., selected and control), ammonia treatment (0, 15, and 20 g/L in larval food), and line. Only males were measured in this experiment.				
Selection treatment	1	4	2.536	.186
Ammonia treatment	2	62	2.402	.099
Selection × ammonia treatment	2	62	1.229	.300

Note. Line (nested within treatment and treated as a random effect) variance component: 4.44×10^{-5} (*SE*, 5.91×10^{-5}), $p = .452$. Line (nested within treatment and treated as a random effect) variance component: 7.90×10^{-5} (*SE*, 1.41×10^{-4}), $p = .574$.

Table 4. Results of a generalized linear mixed model on preadult (larva-to-adult) survivorship in lines of *D. melanogaster* selected for resistance to *G. queenslandicus* mites. Lines were reared at varying ammonium chloride concentrations (0, 15, 20, and 25 g/L) in the larval food. Line was treated as a random effect and nested within selection treatment.

Effect	Numerator *df*	Denominator *df*	*F*	*p*
Selection treatment	1	4	2.52	.187
Ammonia treatment	3	131	639.03	<.0001
Selection x Ammonia trt.	3	131	4.86	.0031

Note. Line parameter estimate: 0.0335 (*SE*, 0.0266).

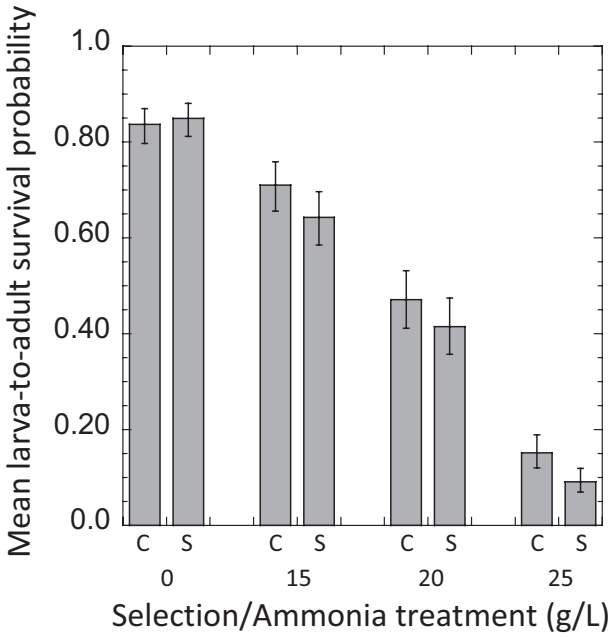

Figure 4. Probability of larva-to-adult survivorship across artificial selection and ammonia treatments. Survivorship of selected lines was significantly reduced in the presence of ammonia in the larval food substrate, reflected in a significant selection treatment-by-ammonia interaction ($p = .0031$; Table 4). Error bars are 95% confidence limits.

due to the high mobility of the attacking flies (Mackinnon et al., 1991).

Among flies whose wings were experimentally removed, selected flies lost their resistance advantage relative to their unselected counterparts (Figure 2). We could observe that wingless flies of both groups were able to deploy substrate-borne locomotor maneuvers such as running and jumping, but as a consequence of the manipulation, they could not deploy takeoff flights when approached or touched. The loss of resistance we observed indicates that the sensorimotor responses to mites we selected for require or are integrated with flight, most likely as microbursts from the substrate. An important aspect of this experiment is that both selected and unselected flies were wingless when they were exposed to mites. This design feature controlled for the possibility that the wing removal treatment altered fly attractiveness to mites, as wingless flies could have been perceived to be aberrant; mite choice in relation to host phenotype has been shown in other fly–mite systems (Campbell & Luong, 2016; Horn et al., 2020; Perez-Leanos et al., 2017). Thus, even if wing removal altered host attractiveness to mites, any such effect presumably would have affected selected and unselected flies similarly, minimizing confounding effects.

The discovery that flight-related behavior was central to the evolutionary response aligns well with our previous physiological analyses. These analyses showed that flies that interacted with mites but that succeeded to avoid parasitism (i.e., the resistant class that we used for selection) experienced loss of body condition, as decreased lipid and glycogen stores (Benoit et al., 2020). These findings are consistent with the fact that insect flight generally is energetically expensive and associated with metabolic expenditures several times that for resting and even for running (Harrison & Roberts, 2000; Zabala et al., 2009). Within the experimental chambers we used for selection, fly responses to mite pressure including bursts of flight are frequent and sustained, likely accounting for these observed negative consequences for host nutrient reserves.

Previous work also has shown that flies that interacted with mites exhibited differential regulation of multiple genes associated with carbohydrate and lipid homeostasis (Benoit et al. 2020). These results identified potential candidate (metabolic) genes that may have underpinned the response to selection

Table 5. Results of cross-resistance assays, showing outcomes of generalized linear models on ectoparasite resistance to both *Gamasodes* (A) and *Macrocheles* (B) mites. Resistance assays were conducted following 16 generations of artificial selection for resistance to *G. queenslandicus*. Results indicate that selection lines consistently had superior resistance to *G. queenslandicus*, as expected, and also to *M. subbadius*, indicating cross-resistance to a novel mite. Each *F* statistic tests for a difference in resistance between the indicated selected line and its paired control.

Line	Numerator *df*	Denominator *df*	*F*	*p*
(A) Resistance to *Gamasodes*				
1	1	66	35.24	<.0001
2	1	66	57.87	<.0001
3	1	66	35.17	<.0001
(B) Cross-resistance to *Macrocheles*				
1	1	22	6.65	.0171
2	1	22	10.43	.0039
3	1	20	9.10	.0068

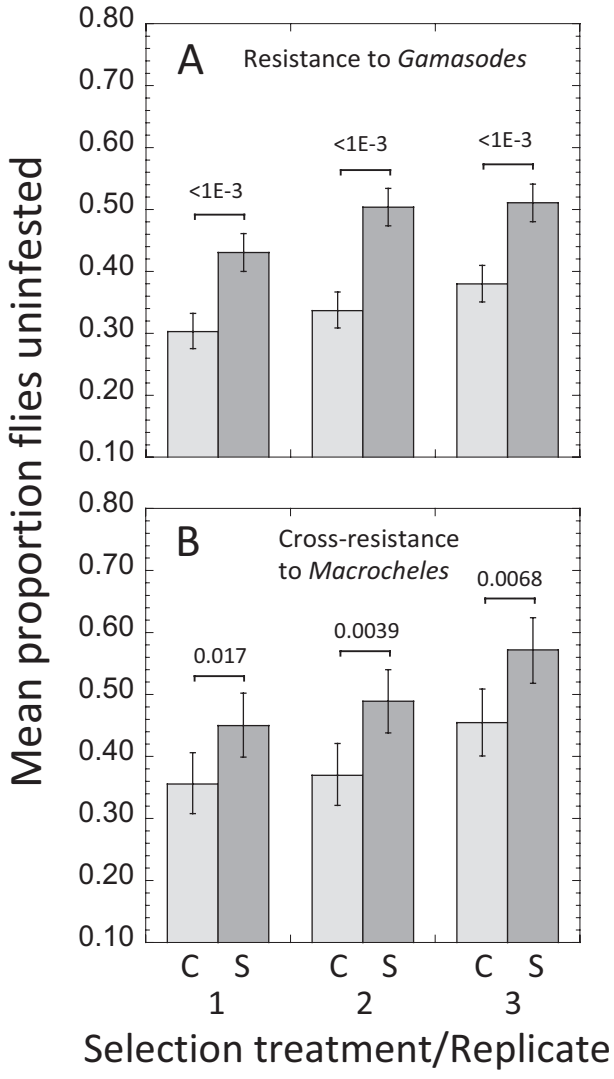

Figure 5. Resistance and cross-resistance in *D. melanogaster* after 16 generations of selection for increasing resistance to *G. queenslandicus* mites. Data are presented for control (C) and selected (S) host lines for each of the 3 replicate selection experiments. Relative to controls, selected lines were strongly more resistant to *G. queenslandicus* (A), confirming the significant response to selection (Fig. 1). Selected lines were also significantly cross-resistant to *M. subbadius* in all cases (B). Error bars are 95% CIs. Exact levels of statistical significance are from generalized linear modeling.

documented here; indeed, there is ample evidence for genetic variation for metabolic genes related to flight parameters in *Drosophila* (Laurie-Ahlberg et al., 1985; Merritt et al., 2006). Neural factors underlying the escape response could also have been involved, for example, by affecting reaction times or fly "irritability," as changes in transcript levels of several neurogenesis-affiliated genes were likewise noted (Benoit et al., 2020). Interestingly, select antimicrobial genes were increased, which may represent host anticipatory immune response to secondary infection by pathogenic microorganisms (Kraaijeveld & Wertheim, 2009; Lemaitre & Hoffmann, 2007), should first-line defenses falter or fail.

In our study, we also tested for correlated responses to selection in both adult body size and preadult survivorship. We found that thorax length did not differ significantly between selected and control lines, either for males or for females. From these results we conclude that the selection program did not significantly alter host body size in the present experiment (cf., Luong and Polak, 2007a). Given that body size can be a determinant of parasitism in other fly–mite systems (Campbell & Luong, 2016; Horn et al., 2020; Perez-Leanos et al., 2017), these results are noteworthy because they underscore the importance of flight-related performance *per se* as a major mechanistic determinant of the resistance phenotype we targeted in the selection work.

In contrast, egg-to-adult survivorship exhibited a complex correlated (indirect) response, such that the strength of the response was dependent on ammonia exposure, an environmental stress factor. In the absence of ammonia, selected and control lines were essentially identical in regard to larva-to-adult survivorship, but in the presence of ammonia, resistant lines exhibited reduced survivorship relative to controls across all concentrations, and significantly so at highest concentration (Figure 4). These results suggest an environmentally modulated cost of resistance and offer support for the more general prediction that trade-offs (genetic correlations) often should be sensitive to environmental conditions (Sandland & Minchella, 2003; Sgrò & Hoffmann, 2004).

The genetic causes underlying the trade-off we observed could have been the result of pleiotropy or linkage disequilibrium (Bell, 2008, p. 167). It is also conceivable that the reduction in larval-to-adult survivorship was influenced by differential inbreeding effects (between selected and control lines) if, for example, the genomic regions in close proximity (in linkage disequilibrium) to selected loci harbored

deleterious recessives (Hartl & Clark, 1997). Our selection lines, however, were independently derived from a large outbred population (where recombination would be expected to be maintaining linkage equilibrium), meant to establish some likelihood that any *consistent* trade-offs between adult resistance and other fitness traits would be the result of antagonistic pleiotropy arising from the resistance alleles themselves, and less likely due to drift or inbreeding, although this design feature would not have excluded effects of tight linkage (Conner, 2002; Lande, 1984; Zhong et al., 2005).

It is worth noting that a similar pattern of expression of this particular preadult cost has been documented in *D. nigrospiracula* selected for resistance to *M. subbadius* mites, a system endemic to the North American Sonoran Desert. In *D. nigrospiracula*, the larva-to-adult survivorship cost was also magnified under increasing stress, but in this case, the stress factor was increasing larval density (Luong & Polak, 2007a; see Kraaijeveld & Godfray 1997, for a similar result involving larval resistance to parasitoid attack). Interestingly, the cost documented in *D. nigrospiracula* could therefore reflect exposure to the same environmental factor studied here, as ammonia, a byproduct of metabolism, builds up in larval substrates from larval excreta as larval densities rise (Borash et al., 1998, 2000).

Collectively, the results emerging from these host–parasite systems identify a potentially general cost of ectoparasite resistance expressed at the preadult stage of the host life cycle, and suggest that other important preadult costs are likely to exist, such as in the form of compromised parasitoid resistance. Given that artificial selection was routinely and expressly applied to field-fresh host populations across the different studies, this growing body of work supports the conclusion that ectoparasite resistance in flies is an ecological important trait with significant evolutionary potential maintained in part by an environmentally modulated developmental cost of resistance.

Funding

The research was supported by National Science Foundation USA grant DEB-1654417 to M.P. and J.B.B.

Data availability

Raw data are deposited and freely accessible on Mendeley Data (DOI: 10.17632/74vn7ffcr5.1).

Author contributions

M.P. designed the research, acquired funding for the research, contributed to conducting the research, analyzed data, wrote the manuscript; J.B. designed the research, conducted the research, analyzed data, contributed to editing the manuscript; J.B.B. designed the research, acquired funding for the research, contributed to editing the manuscript; H.S. contributed to conducting the research, data collection and organization.

Conflict of interest: The authors declare no conflict of interest.

Acknowledgments

We thank Rachel Black for her excellent assistance in media preparation.

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