Genetic variance and phenotypic selection on pathogen-linked oviposition choice in Drosophila

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Short title: Selection on pathogen avoidance during oviposition

Abstract

Pathogen-avoidance behaviour is assumed to be adaptive, yet its phenotypic variability and genetic heritability are rarely quantified. In species lacking post-oviposition care, avoiding potentially infectious egg-laying substrates would improve offspring survival and should therefore be under strong selection. We used two-choice oviposition assays to quantify the phenotypic and genetic variance in, and the fitness consequences of, oviposition preference in *Drosophila melanogaster* when exposed to egg-laying substrates containing the pathogenic bacterium *Pseudomonas aeruginosa* PA14. Oviposition preference varied substantially in an outbred population and was bimodal: many females laid all eggs on the bacterial substrate or all on the clean substrate. Across all choice assays, more eggs and more viable offspring were produced on the bacterial surface, with no overall difference in egg-to-adult viability between substrates. Among extreme phenotypes, females laying exclusively on bacteria laid more eggs but had lower egg-to-adult viability than those laying exclusively on clean food, resulting in similar numbers of adult offspring. The broad-sense heritability of oviposition preference, measured in 24 inbred fly lines, was moderate (H^2 = 0.30). Selection analyses indicated opposing selection acting on oviposition preference via different fitness components, with balancing acting via egg number and positive directional selection for avoidance acting via egg-to-adult viability. Such opposing effects may reflect a trade-off females face between avoiding high density oviposition sites, while also avoiding potential pathogenic effects on egg development.

Key-words: Pathogen avoidance, Oviposition site selection, Drosophila melanogaster, *Pseudomonas aeruginosa* (PA14), Phenotypic selection, Heritability

Background

Organisms routinely face hazards that impose large fitness costs (Schmid-Hempel 2021; Gibson and Amoroso 2022). Parental care can mitigate these costs, for example when mothers choose environments that enhance offspring survival (Royle et al. 2012). A key component of environmental quality is pathogen exposure risk. Although immune defences can reduce the costs of infection, infection itself often causes morbidity and mortality, and even successful immune responses are metabolically costly, and often divert resources required for reproduction and competitive performance (Schwenke et al. 2016; Nystrand and Dowling 2020; Foo et al. 2023). Avoiding exposure altogether is therefore preferable, via behaviours that reduce contact with infectious substrates or conspecifics(Amoroso et al. 2025). Pathogen avoidance behaviours are taxonomically widespread, and especially well-described in insects, spanning direct avoidance of infectious stages, infected conspecifics, and contaminated substrates or environments (de Roode and Lefèvre 2012; Vale et al. 2018; Amoroso et al. 2025).

Pathogen avoidance is especially important during oviposition decisions (Vale and Duffy 2026). In many insect species, sessile eggs make maternal oviposition site selection a major determinant of offspring environment and infection risk (Lefèvre et al. 2012; Kacsoh et al. 2013; Siva-Jothy et al. 2018). Under the preference-performance, mother-knows-best, or optimal oviposition hypothesis, selection should therefore favour females that lay eggs on substrates that maximise offspring survival and thus maternal fitness, (Jaenike 1978, 1982).

Oviposition site selection (OSS) is a widely used model of decision-making in insects (Yang et al. 2008; Vijayan et al. 2022; Vale and Duffy 2026). In *D. melanogaster*, females explore and evaluate potential sites prior to egg laying, and the sociochemical cues guiding OSS are well characterised (Aranha and Vasconcelos 2018; Cury et al. 2019; Milutinović and Schmitt 2022). Infection avoidance during oviposition occurs in response to diverse pathogens and cues, including visual detection of parasitoid wasps (Kacsoh et al. 2013) and olfactory detection of bacteria and fungi (Stensmyr et al. 2012; Kurz et al. 2017). OSS in response to abiotic substrates (e.g. ethanol, plant phenolics) is also phenotypically variable within populations (Courtney and Chen 1988; van Delden and Kamping 1990; Possidente et al. 1999; Mery and Kawecki 2002; Vale and Duffy 2026).

Despite extensive work on cues and sensory mechanisms, the extent of phenotypic and genetic variation in pathogen avoidance during OSS, and its capacity to respond to selection, is largely unknown. This matters because pathogen avoidance is widespread (Lopes et al. 2022; Poirotte and Charpentier 2022; Gibson and Amoroso 2022) and often assumed to be adaptive (Curtis et al. 2011; Curtis 2014). Yet, key prerequisites for adaptation - heritable genetic variance and fitness covariation (Kingsolver and Pfennig 2007; Walsh and Lynch 2018) - are rarely measured in the context of pathogen avoidance during OSS (Gibson and Amoroso 2022; Amoroso et al. 2024; Monteith et al. 2024; Vale and Duffy 2026).

In the present work, we quantify phenotypic and genetic variance in, and fitness consequences of, pathogen avoidance during oviposition site selection. We employ both a large, outbred population of *D. melanogaster* and a panel of inbred lines from the Drosophila Genetic Reference Panel (DGRP) (Mackay et al. 2012), exposed to the generalist bacterium *Pseudomonas aeruginosa*, a standard model pathogen that causes substantial mortality following oral and systemic infection (Apidianakis and Rahme 2009; Gupta et al. 2017; Chen et al. 2024). In two-choice oviposition assays, mated females chose between a clean food substrate and an otherwise identical substrate bearing a lawn of *P. aeruginosa*. We define the oviposition preference index (OI) as the preference for the clean over the bacterial substrate, ranging from −1 (all eggs on the bacterial substrate) to 1 (all eggs on the clean substrate), with 0 indicating no preference. In addition, we measured the broad-sense heritability of OI in a panel of inbred fly lines.

We predicted that females would preferentially avoid the bacterial substrate and that eggs laid on it would have reduced survival. We further hypothesised that stabilising selection would be acting on OI, with an intermediate optimum balancing the benefits of pathogen avoidance against the potential costs of intense larval competition if all eggs are laid on the clean substrate (Jaenike 1978; Scheirs 2002). To test these hypotheses, we allowed the eggs laid on each substrate to develop and we measured egg-to-adult viability and adult offspring number. We then asked how OI and key these components of fitness (number of eggs laid, egg-to-adult viability, total adult offspring and offspring sex ratio) are distributed when females choose between clean and bacterial substrates. Following Robertson's Secondary Theorem of Natural Selection, the expected change in mean trait value equals the covariance between the trait and standardised fitness (Queller 2017; Walsh and Lynch 2018); by measuring variation in OI and its association with three fitness components, we use this framework to estimate directional and quadratic selection on OI and we then infer the form and strength of selection acting on this pathogen-linked OSS (directional, disruptive, or stabilising)(Kingsolver and Pfennig 2007).

Methods

Fly stocks

We studied an outbred *Drosophila melanogaster* population (Ashworth Outcrossed, AOx) created by crossing 113 DGRP lines and then randomly outbred for at least 151 generations (at the time of the experiment) (Monteith et al. 2019, 2024; Savola et al. 2021). To generate mated focal females, 20 vials (sugar-cornmeal medium; Table S1) were set up with 10 females and 5 males. After 24 h adults were transferred to fresh vials for a further 24 h, then removed. Females emerging from eggs laid in these vials were used in choice assays. Thirty-six hours before trial, each focal female was placed with two males and yeast in a food vial to encourage mating. All flies were 2–5 days old, maintained at 25°C (±2°C) under 12:12 h light:dark and reared on sugar–cornmeal medium (Table S1).

For heritability estimates, 24 randomly chosen DGRP lines (Mackay et al., 2012) were maintained at 18°C (±2°C), 12:12 h light:dark for two weeks. For each line, 10 females and 5 males were placed in a food vial and transferred to new vials every 3-4 days. Females aged 1-4 days were assayed. Thirty-six hours before each oviposition assay, each female was placed with two males from her line and yeast. Assay conditions were 25°C (±2°C), 12:12 h light:dark.

Bacterial culturing

All culturing was performed in a microbiological safety cabinet (MSC). Luria-Bertani (LB) broth (20 ml) was dispensed into two 50 ml tubes. One tube was inoculated with frozen *Pseudomonas aeruginosa* PA14 (−70°C stock) using a sterile loop; the other served as an uninoculated LB control. Tubes were loosely capped to allow aeration and taped. Cultures were incubated at 37°C with shaking at 120 rpm for 18 h to obtain an overnight culture.

Choice chamber and oviposition assay

Two plastic caps (Bijou vial caps) were filled with sugar-cornmeal medium (Table S1). One cap was overlaid with 100 µl of the PA14 overnight culture and the other with 100 µl of sterile LB (clean control). After drying in the MSC, caps were fixed 45 mm apart on opposite sides of a 90 mm lidded, Petri dish using BluTack to form a two-choice chamber. Control chambers contained two clean caps. A single mated female was very lightly anaesthetised with CO_2 and placed centrally in the chamber. Dishes were sealed with tape and incubated for 24-28 h at 25°C (±2°C), 12:12 h light:dark. Start times were standardised (10:00-12:00), and the position of sites relative to the incubator walls was alternated across assays to distribute positional effects. Females were then removed from the assay chambers under CO_2 anaesthesia and the number of eggs on each substrate were counted under a dissecting microscope.

Oviposition index and fitness components

The oviposition index (OI) was defined as OI = (C − B)/(C + B), where C and B are eggs laid on the **C**lean and **B**acterial substrates, respectively. OI ranges from −1 (all eggs on bacteria) to 1 (all eggs on clean). For each female we recorded: total eggs laid; egg-to-adult viability (proportion of eggs eclosing as adults per site); total adult offspring. Given known sex differences in susceptibility to *P. aeruginosa* in adult *D. melanogaster* (Vincent and Sharp 2014; Gupta et al. 2017), we also tested whether the sex ratio of surviving offspring differed between substrates. To measure fly development, each cap was taped onto a standard vial containing fly medium and incubated at 25°C (±2°C), 12:12 h light:dark, until adult emergence (13–14 days). Adults from both sites were counted to calculate egg viability, total adult offspring and sex ratio.

Sample sizes and exclusions

Altogether, we assayed 130 single females: 100 in treatment chambers (clean versus bacterial substrate) and 30 in control chambers (two clean substrates). To avoid undue leverage of very small clutches, females laying fewer than 10 eggs were excluded from all analyses, yielding a final sample size of 87 treatment and 27 control females. For 15 treatment and 2 control females, ≤6 adults escaped; these were included in egg viability but excluded from sex ratio. Within-female contrasts between substrates (egg number, viability, offspring number, sex ratio) were thus based on paired data from the retained treatment females. We also analysed the subset of assays where females laid exclusively on one substrate (where OI = −1 or 1); sample sizes for these contrasts are given with the model outputs (Table 1, Models 5–8). For the heritability assay, 24 DGRP lines, with ten females per line, were tested using the same protocol described above (240 choice assays).

Phenotypic selection and heritability

We quantified phenotypic selection acting pathogen-related egg-laying choice by regressing the mean-standardised fitness components (egg number, viability, offspring number, sex ratio) on the oviposition index (OI) to estimate both linear (β) and quadratic (γ) selection gradients (Kingsolver and Pfennig 2007; Queller 2017; Walsh and Lynch 2018). Standardised fitness for each component was calculated by dividing the individual value by the corresponding population mean (W), such that values >1 indicate above-average performance. Broad-sense heritability (H^2) of OI was estimated using ten females from each of 24 randomly selected DGRP lines. We calculated $H^2 = V_G/(V_G + V_E)$, where V_G is the among-line variance in OI. Because OI was modelled as a binomial trait, its environmental variance V_E is the sum of the residual variance V_R and the variance of the logistic distribution $\frac{\pi^2}{3}$ (Nakagawa and Schielzeth 2010; Mackay and Huang 2018). The within-line (environmental) variance V_E is therefore calculated as $V_R+\frac{\pi^2}{3}$

Statistical analysis

Analyses were conducted in R 4.3.3 (R Core Team, 2023) using ggplot2 (Wickham 2009), lme4 (Bates et al. 2015) and lmerTest (Kuznetsova et al. 2017). Linear mixed models (LMMs) were used for egg number, adult offspring number, OI and relative fitness components when residuals were approximately normal; generalised linear mixed models (GLMMs) were used otherwise. Experimental block was fitted as a random effect in all mixed models. For within-individual comparisons across substrates (egg number, viability, offspring number, sex ratio), fly identity was also fitted as a random effect. Offspring counts per site were analysed with Poisson GLMMs; egg-to-adult viability and sex ratio with binomial GLMMs using cbind(successes, failures) in order to analyse the number of eggs, rather than the ratio. We compared bacterial versus clean substrates, treatment versus control conditions, within-control differences, and the two extreme OI classes (−1 versus 1). For heritability, a binomial GLMM with cbind(eggs on clean, eggs on bacteria) as the response and DGRP line as a random effect was fitted; among-line and residual variances were extracted to compute H^2 as described above.

Results

Oviposition decisions indicate a preference for bacterial substrates

Within control chambers (where females chose between two clean sites), there was no difference between sites in egg number, egg-to-adult viability, adult offspring number, or sex ratio (Figures S1-2; Table S2). In females given a choice between a clean and a bacterial surface (n = 87; **Figure 1A-E**), egg number (mean = 42.68, variance = 342.85) and offspring sex ratio (mean = 0.46, variance = 0.03) were approximately normal, whereas egg-to-adult viability was right-skewed with high values common (mean = 0.66, variance = 0.11). Adult offspring number showed a relatively uniform distribution (mean = 28, variance = 335.48).

In these choice assays, the oviposition index (OI) was strongly bimodal (**Figure 1A**), with peaks at −1 and 1 (mean = −0.20, variance = 0.774). Egg-laying preference was skewed towards the bacterial substrate, which resulted in improved reproductive output (**Figure 2A-D**): females laid more eggs on the bacterial than the clean surface (**Figure 2A**; Table 1, Model 1), and more adults emerged from the bacterial surface (**Figure 2C**; Table 1, Model 3). Egg-to-adult viability (**Figure 2B**; Table 1, Model 2) and offspring sex ratio (**Figure 2D**; Table 1, Model 4) did not differ significantly between clean and bacterial surfaces.

Restricting the analysis to assays showing extreme preference phenotypes (OI = −1 versus OI = 1, **Figure 2E-H**) revealed that females laying exclusively on bacteria produced more eggs (**Figure 2E**; Table 1, Model 5) but had lower egg-to-adult viability (**Figure 2F**; Table 1, Model 6) than females laying exclusively on clean substrate. The two extreme groups did not differ significantly in total adult offspring (Figure 2G; Table 1, Model 7) or sex ratio (Figure 2H; Table 1, Model 8).

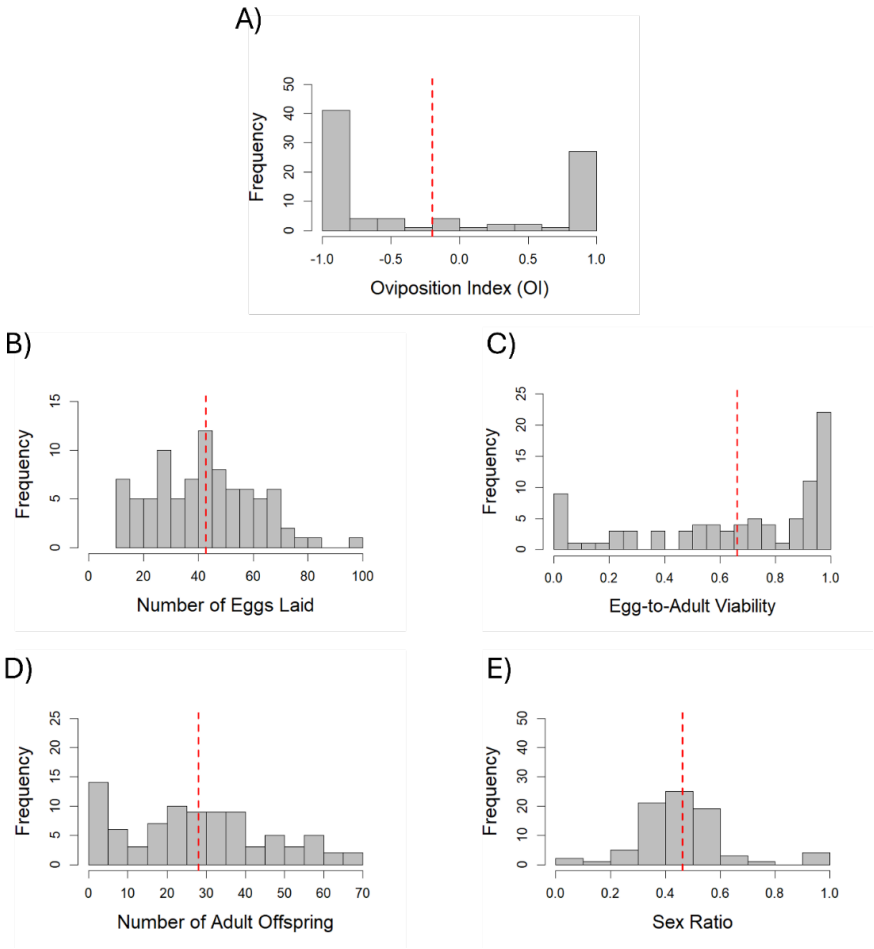

Figure 1. Distribution of traits in the outbred population. Red dashed line represents the mean value for oviposition index (A), number of eggs laid (B), egg-to-adult viability (C), adult offspring number (D), and sex ratio (E).

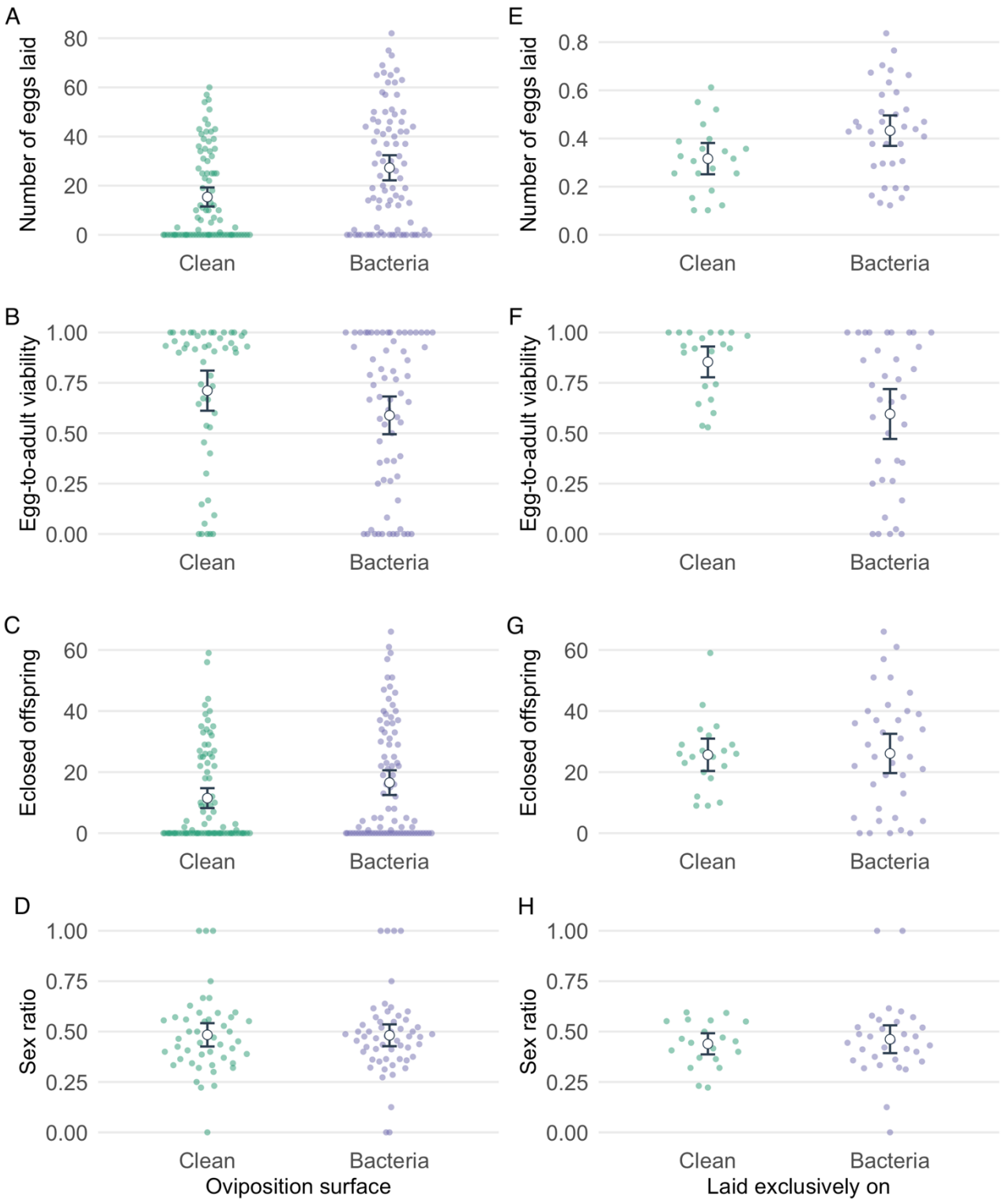

Figure 2. The number of eggs laid, egg-to-adult viability (calculated as the proportion of eggs laid that eclosed as adults), number of eclosed offspring and the sex ratio (calculated as the proportion of the surviving adult male offspring) between oviposition surfaces (A-D) and (E-H) in assays where flies laid exclusively on either the bacterial substrate (OI=-1) or the clean substrate (OI=1). Dots are data from individual flies, overlaid with the mean value; error bars describe the standard error. See Table 1 for model outputs.

Pathogen-related oviposition choice is broadly heritable

We then repeated the same two-choice oviposition assay on a panel of 24 inbred fly lines form the DGRP panel (Figure 3). Notably, almost all lines showed a preference for bacterial egg-laying substrate, and mean oviposition index across all 24 lines was negative (-0.51, variance=0.419) which was consistent with the attraction to bacterial substrates measured in the outbred population. Broad-sense heritability of OI was estimated to be non-zero and moderate at H^2 = 0.298 (95% CI: 0.052–0.35) based on estimates of among- and between line variance measured on 24 DGRP lines, with ten females per line (Figure 3; Table 2).

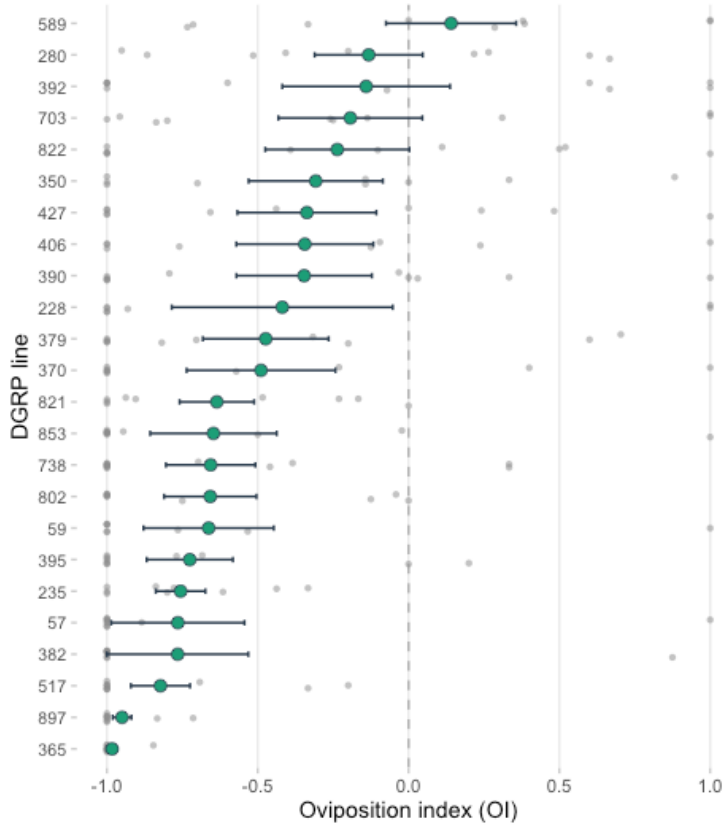

Figure 3. Oviposition Index showing egg-laying preference for either the bacterial substrate (OI=-1) or the clean substrate (OI=1) measured in ten females in each of 24 DGRP lines. Green dots represent the line mean ± standard error; grey dots are OI of individual flies.

Selection on oviposition decisions is driven by opposing effects of different fitness components

To estimate the fitness impact of flies' egg-laying decisions, we considered three distinct measures of fitness (number of eggs laid; egg-to-adult viability; number of eclosed offspring). This analysis showed that distinct fitness components impose different forms selection on pathogen-related oviposition decisions. Standardised egg number showed a nonlinear association with OI, with a

negative quadratic term consistent with stabilising selection, peaking at OI = −0.15 with a maximum standardised egg number = 1.28 (quadratic selection gradient γ = −0.37; **Figure 4A**; Table 3, Model 9). Standardised egg-to-adult viability, however, increased linearly with OI (β = 0.14), with highest standardised viability (1.48) measured in flies avoiding bacterial substrates at OI = 1 and lowest (1.17) at those preferring bacterial substates at OI = −1 (Figure 4B; Table 3, Model 10). For the third measure of fitness, there was no detectable relationship between OI and standardised adult offspring number (Figure 4C; Table 3, Model 11).

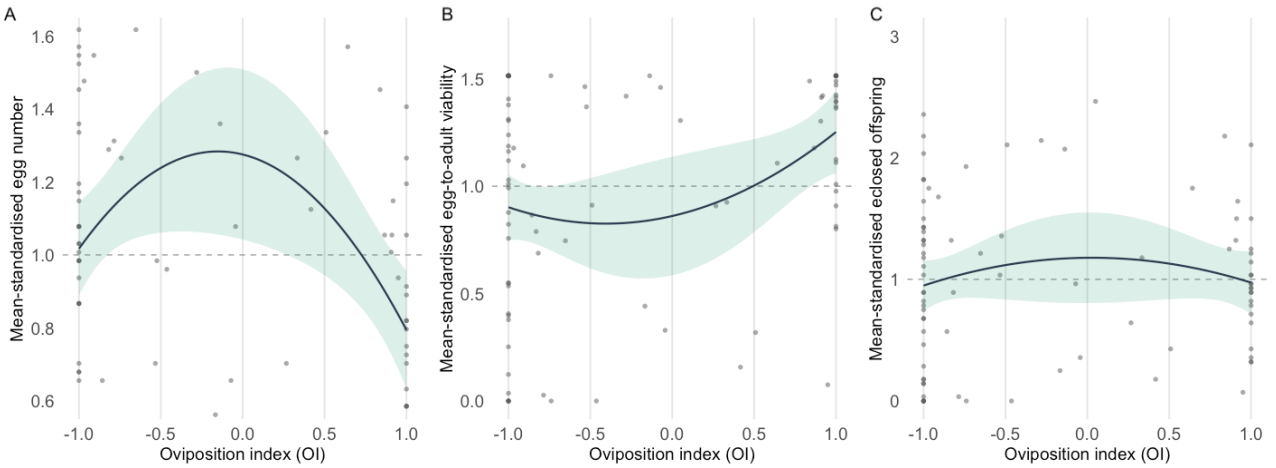

Figure 4. Oviposition index (OI) regressed on mean-standardised fitness components: egg number (A), egg-to-adult viability (B) and adult offspring number (C). Grey dots are data for individual female flies. Black line indicates the predicted polynomial model for each response variable, with the 95% confidence interval shown as green shading. See Table 3 for linear and quadratic selection coefficients.

Table 1: Models 1-8 output.

Model	Response (y)	Type of model	Predictor (x)	Intercept	Estimate	P value
1	Egg number	LMM	Egg-laying substrate	27.28	-11.87	0.00030*
2	Egg-to-adult viability	GLMM	Egg-laying substrate	0.94	0.74	0.096
3	Adult offspring number	GLMM	Egg-laying substrate	17.26	-5.07	<2e-16*
4	Sex ratio	GLMM	Egg-laying substrate	-0.64	0.20	0.63
5	Egg number	LMM	Oviposition Index	36.95	-5.86	0.014*
6	Egg-to-adult viability	GLMM	Oviposition Index	8.08	7.47	<2e-16*
7	Adult offspring number	LMM	Oviposition Index	26.80	-0.81	0.70
8	Sex ratio	GLMM	Oviposition Index	-0.75	-0.10	0.74

Table 2: Heritability analysis.

Number of lines	24
Number of females per line	10
Mean oi	-0.51
Oi standard error	0.043
V_g(Genetic variance)	1.346
V_e (Environmental variance) = $v_r+\frac{\pi^2}{3}$	3.166
Phenotypic variance	4.512
Broad-sense heritability ($\mathbf{H^2}$)	0.298
95% CI	0.052, 0.35

Table 3: Models 9-11 output.

Model	Response (y)	Type of test	Predictor (x)	Intercept	Estimate	P value
9	Relative egg number fitness	LMM	Oviposition Index	1.28	-0.11	0.031*
			$(\text{Oviposition Index})^2$		-0.37	0.0076*
10	Relative egg-to-adult viability fitness	LMM	Oviposition Index	1.08	0.14	0.0095*
			$(\text{Oviposition Index})^2$		0.16	0.26
11	Relative offspring number fitness	LMM	Oviposition Index	1.28	-0.02	0.77
			$(\text{Oviposition Index})^2$		-0.28	0.17

Discussion

Our aim was to quantify phenotypic variation, selection and heritability of pathogen avoidance during oviposition in *Drosophila melanogaster* exposed to *P. aeruginosa*. We found extensive phenotypic variation in oviposition preference: the oviposition index (OI) measured in an outbred population was bimodal with many females laying exclusively on one surface. On average, females laid more eggs on the bacterial substrate, yet egg-to-adult viability did not differ between substrates overall. Among extreme egg-laying phenotypes, where choice was unimodal, females laying exclusively on bacterial substrates produced more eggs but these had lower viability than those laid exclusively on clean food, yielding similar numbers of adult offspring. Selection analyses indicated stabilising selection on egg number (peak near OI = −0.15), positive directional selection on egg-to-adult viability (higher at OI = 1), and no detectable association between OI and total adult offspring. Broad-sense heritability of OI was moderate (H^2 = 0.298), implying the potential for evolutionary response.

Distribution of OI and oviposition performance

Evidence of *Drosophila* avoiding pathogen-contaminated substrates during oviposition is common, e.g. (Stensmyr et al. 2012; Kacsoh et al. 2013; Kurz et al. 2017). In contrast, we found more eggs laid and, consequently, more adults on the bacterial surface, with no overall difference in mean egg viability. This implies that females detect and respond to bacterial cues by increasing clutch allocation, yet developmental survival per egg is broadly similar across substrates under typical allocations. This preference for bacterial substrates has also been observed during two-choice feeding assays, where flies were found to evaluate bacterial substrates as likely sources of nutrition (Monteith et al. 2024). It is therefore plausible that similar evaluations of the potential benefit of bacterial substrates are made by females during the usual cycle of exploration, evaluation and commitment to an oviposition substrate (Aranha and Vasconcelos 2018; Cury et al. 2019)

We also observed a high frequency of complete preference for the bacterial substrate (OI = −1), alongside complete avoidance (OI = 1). When focusing on these extreme phenotypes, viability was lower on the bacterial substrate. This difference may reflect a direct effect of PA14 on developing larvae, consistent with reports that *Pseudomonas* can impair larval development in flies (Olcott et al. 2010). Alternatively, it may arise through density dependence, because bacterial-preferring females laid all their eggs on the same substrate, and larval crowding is a common cause of delayed development and reduce survival (Klepsatel et al. 2018). Disentangling these explanations would require egg-density controlled assays in which equal numbers of eggs are placed on clean versus PA14 substrates, coupled with longitudinal measures of larval growth, pupation, eclosion and internal bacterial loads (Olcott et al. 2010). Sex-ratio did not vary between substrates, consistent with limited sex-specific developmental sensitivity to PA14 under these conditions, despite known adult sex differences in the susceptibility of Drosophila to this pathogen (Vincent and Sharp 2014; Gupta et al. 2017).

Patterns of phenotypic selection on OI and its components

Selection gradients revealed opposing effects of different fitness components: stabilising selection on egg number (negative quadratic term with a peak near OI = −0.15) and positive directional selection on viability (higher at OI = 1). These components appear to offset each other, as total adult offspring did not covary with OI. Thus, under our assay conditions, differential selection via maternal fecundity and offspring survival may cancel to yield weak net selection on oviposition preference. Moreover, the most common phenotype (complete preference of the bacterial substrate, OI = −1) was not the fittest on either component scale, emphasising that the observed OI distribution is unlikely to be solely shaped by the measured components of fitness.

Several non-exclusive explanations could account for the contrasting forms of selection via different fitness components. First, there could unmeasured effects that could favour bacterial oviposition, such as microbially mediated terminal investment that increase reproductive output (Duffield et al. 2017; Hudson et al. 2020) or immune priming that reduces susceptibility to subsequent infection (Contreras-Garduño et al. 2016; Prakash et al. 2024). Secondly, although bacteria-preferring females laid more eggs while clean-preferring females had higher egg-to-adult viability, these opposing component effects cancel each other, as total adult offspring does not covary detectably with OI (Fig. 4C). Consequently, females that prefer bacteria do not suffer a measurable deficit in overall reproductive output in this environment, even though one fitness component (egg-to-adult viability) is reduced. This pattern is consistent with weak net selection on oviposition choice in our assay.

Heritability and predicted response to selection

The estimated broad-sense heritability of OI (H^2 = 0.298; 95% CI: 0.052–0.35) indicates appreciable, non-zero genetic variance for oviposition preference. This supports the view that the behaviour is evolvable in principle. However, we note that H^2 captures all genetic components (additive, dominance, epistasis) and any persistent line-specific non-genetic effects (e.g. maternal effects or stable microbiome differences). Short-term evolutionary response, however, depends on additive genetic variance (h^2). Thus, our measured H^2 of 0.30 provides an upper bound on h^2 and may slightly overstate the expected response to selection. Further, heritability is environment- and context-dependent. Our estimate was obtained at 25°C in a two-choice assay against a particular PA14 bacterial culture, so we can expect genotype-by-environment (G×E) interactions affecting both the magnitude and even the sign of genetic effects on preference can change across bacterial species and doses, or environmental and social contexts. Therefore, the presence of genetic variance in this assay does not guarantee similar variance in other ecologically relevant settings. Notwithstanding these important caveats, non-zero genetic variance and positive directional selection on egg-to-adult

viability indicates that oviposition preference has the potential to evolve toward greater bacterial avoidance and opens the possibility for longer-term selection experiments to test this prediction.

Conclusion

This study demonstrates substantial phenotypic variation and non-zero heritability for pathogen-related oviposition preference in *D. melanogaster*. We find fitness components acting in opposing directions, showing stabilising selection via fecundity and directional selection via egg-to-adult viability, yielding weak net selection on total offspring under our conditions. The maintenance of extreme preferences, including frequent complete preference for the bacterial substrate, likely reflects weak or context-dependent selection and/or unmeasured benefits of laying eggs on bacterial substrates.

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Data availability

All raw data and analysis code is available at Zenodo; 2026. doi:10.5281/zenodo.19185860 (Duffy and Vale 2026).

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Supplementary material

Table S1: Nutritional content of sugar-cornmeal medium

Protein:Carbohydrate ratio	Protein (%)	Yeast (g)	Sugar (g)	Maize (g)			Agar (g)	Food dye (g)	Nipagin (ml)	dH_20 (l)
				Total	Carbohydrate	Protein				
1:6	14	112.5	562.5	415	290.5	37.8	41.2	3	90	6

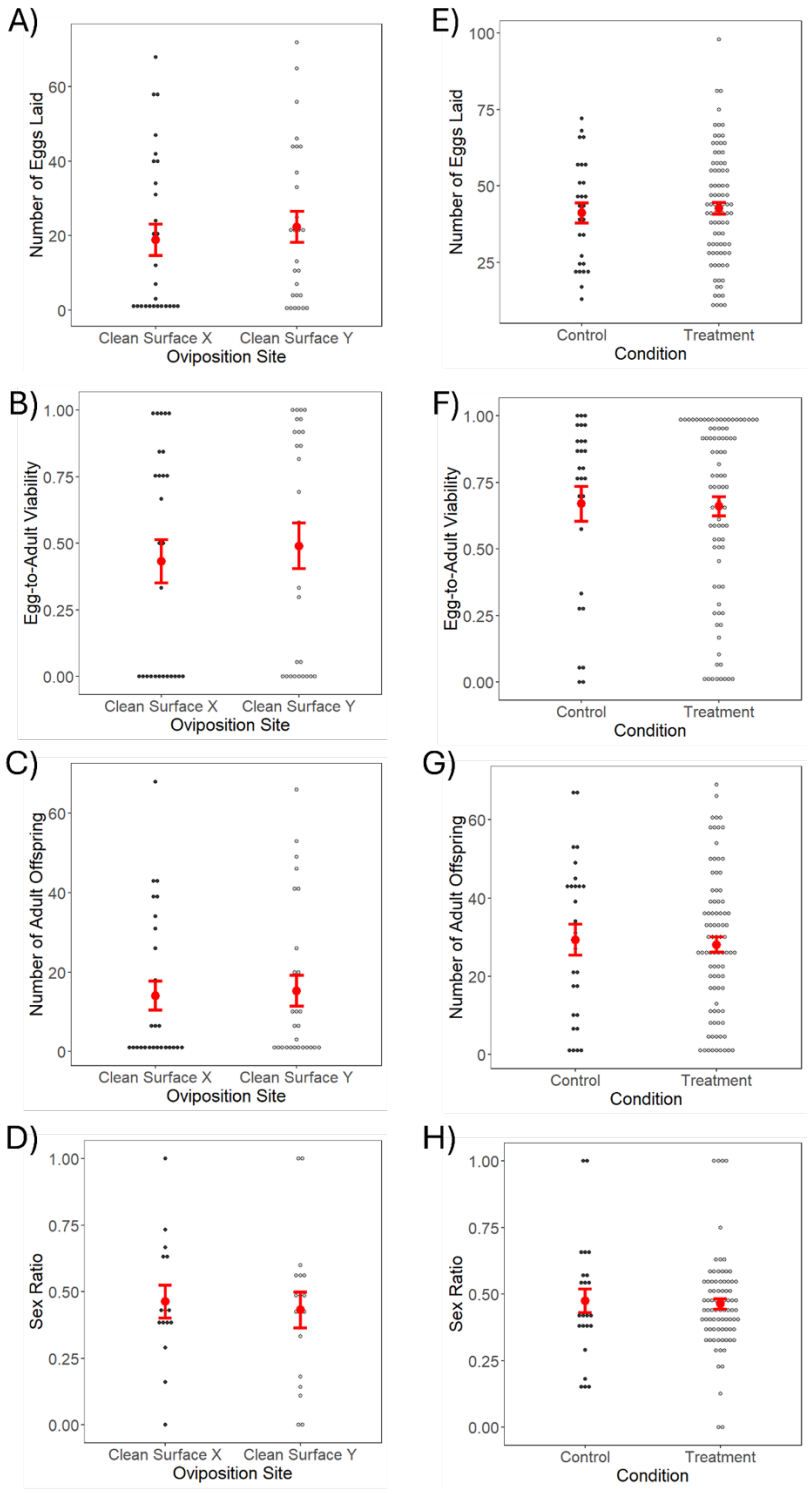

Figure S1 Control assays. Number of eggs laid, egg-to-adult viability, number of adult offspring, and sex ratio between the two conditions (A-D) and within controls between the two clean surfaces (E-G). Red dots represent the mean value as follows: (A) Clean Surface X=18.85, Clean Surface Y=22.33, (B) Clean Surface X=0.43, Clean Surface Y=0.49, (C) Clean Surface X=14.04, Clean Surface Y=15.26, (D) Clean Surface X=0.46, Clean Surface Y=0.43, (E) Control=41.18, Treatment=42.68, (F) Control=0.67, Treatment=0.66, (G)

Control=29.30, Treatment=28.03, (H) Control=0.47, Treatment=0.46. Bars show the standard error. See Table S2 for model outputs.

Table S2. Model S1-S9 output.

Model	Response (y)	Type of test	Predictor (x)	Intercept	Estimate	P value
D1	Egg number	LMM	Condition	41.19	1.48	0.71
D2	Egg-to-adult viability	GLMM	Condition	0.078	1.27	0.12
D3	Adult offspring number	LMM	Condition	23.88	5.21	0.26
D4	Sex ratio	GLMM	Condition	-0.41	-0.29	0.54
D5	Index	LMM	Condition	0.12	-0.31	0.15
D6	Egg number	LMM	Control surface	18.85	3.48	0.56
D7	Egg-to-adult viability	GLMM	Control surface	-0.22	0.30	0.59
D8	Adult offspring number	LMM	Control surface	14.04	1.22	0.82
D9	Sex ratio	GLMM	Control surface	-0.15	-0.13	0.86

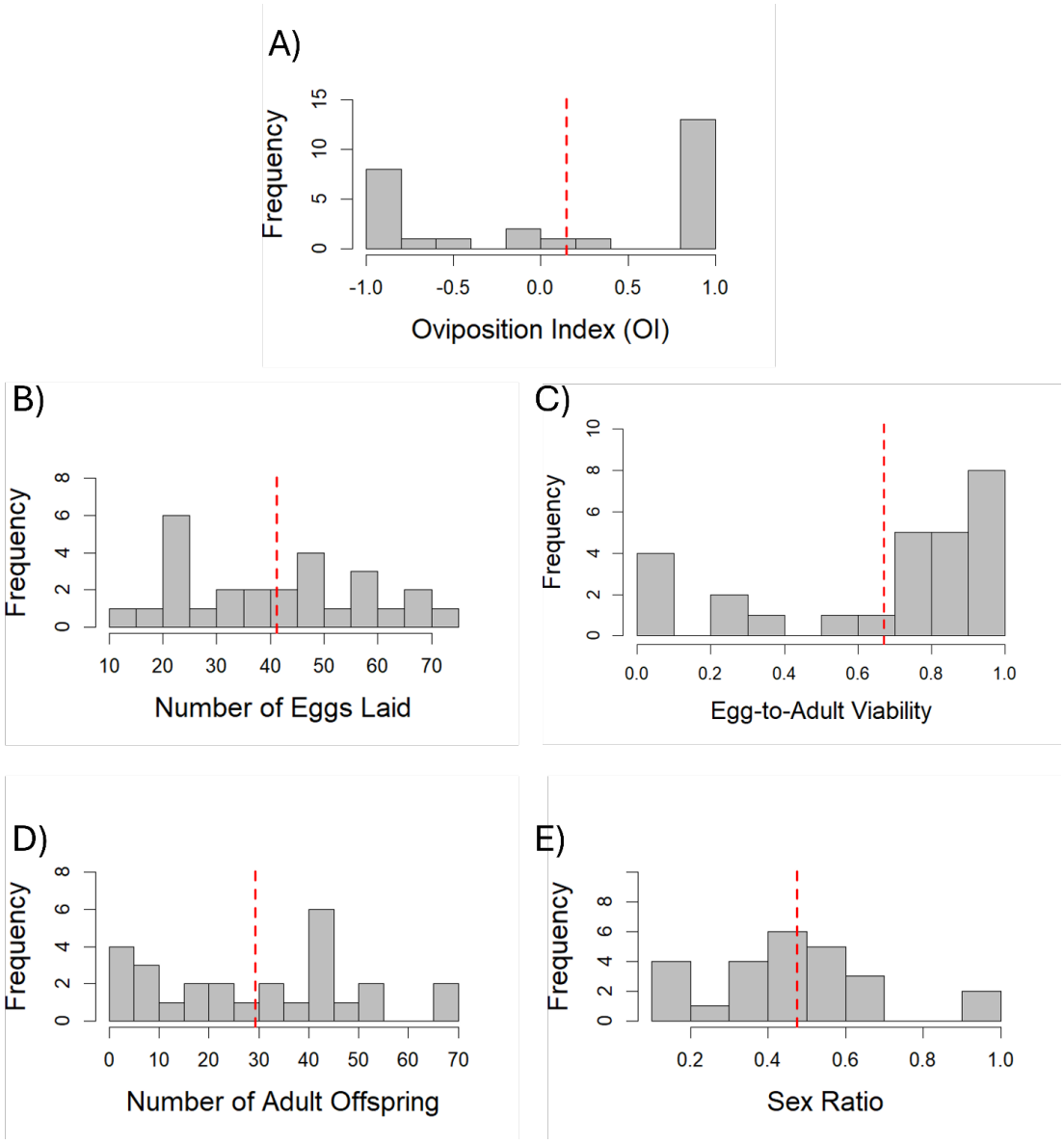

Figure S2. Distribution of traits from the control group. Red dashed line represents the mean value for that trait: 0.15 (A), 41.19 (B), 29.30 (C), 0.67 (D), 0.48 (E). The variance for the traits is as follows: 0.78 (A), 298.08 (B), 0.12 (C), 426.22 (D), 0.049 (E).