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Supporting Information

Interactive effects of social environment, age and sex on immune responses in *Drosophila melanogaster*

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General experimental design

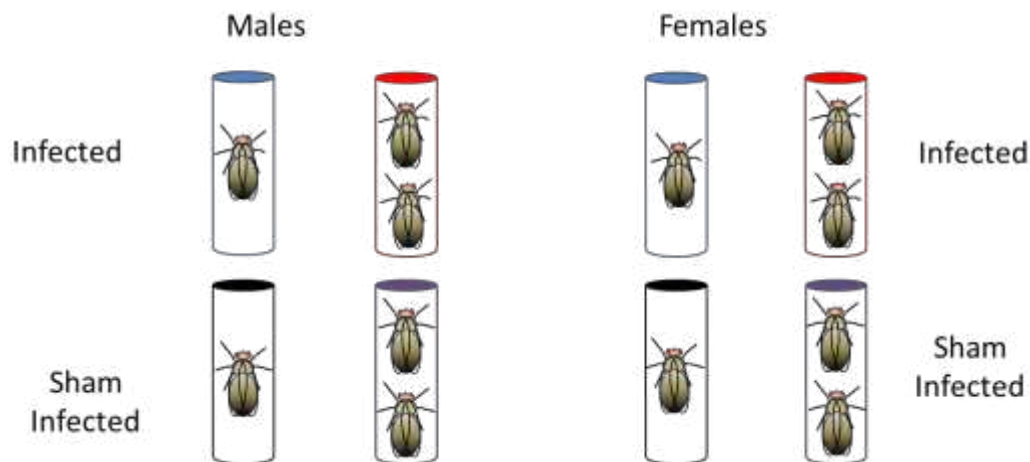


Figure S1 General infection assay experimental design. Males and females maintained either alone or in same-sex pairs for 10 or 52 days, before being injected with one of the three possible bacteria, or a sterile sham injection. Phagocytosis assays kept flies in the same conditions (without infection), as did gene expression studies (without use of young flies).

Flow cytometry gating strategy

Instrument thresholds and software analysis gates were first established based on relevant unlabelled haemocytes (unstained), NucBlue-labelled haemocytes (UV), and pHrodo-bacteria conjugate (green only) control samples. To distinguish between irrelevant autofluorescent debris and NucBlue labelled haemocytes, unstained and UV only controls were compared. To differentiate between haemocytes and non-phagocytosed bacterial particles, UV only and green only control samples were compared. To calculate phagocytic activity the number of UV+ green- (non-phagocytosing haemocytes) was compared to UV+ green+ (phagocytosing haemocytes).

UV labelled haemocytes (NucBlue) were detected in the UV gated (780/60 band-pass) and green labelled phagocytes in the Blue gated (530/30 band-pass) instrument parameters. Data analysis was limited to haemocytes by selection based on the UV fluorescent signal and then displaying the phagocytes associated in a dualparameter logarithmic dot plot of green signal vs. side scatter-area. Phagocytic activity was calculated for each sample by counting non-phagocytic (UV+ green-) and phagocytic (UV+ green+) haemocytes to determine their relative number.

Calculation of differential gene expression

To calculate relative expression, the average C_q of the three technical replicates was first calculated and the relative quantity (ΔC_q) was then calculated using the formula:

$$\text{Relative Quantity}_{\text{sample (GOI)}} = E_{\text{GOI}}^{(C_q(\text{control}) - C_q(\text{sample}))}$$

Where:

- E = Efficiency of primer. This efficiency is calculated with the formula:
(% Efficiency * 0.01) + 1, where 100% efficiency = 2
- C_q (control) = Average C_q for the control sample
- C_q (sample) = Average C_q for any samples with a GOI
- GOI = Gene of interest (one target)

When calculating gene expression changes a constant single sample was used to normalise against, within replicates. For example, to calculate the gene expression change for paired

males, single females and paired females, these were all normalised against single males, within replicate.

Normalised relative quantity was then calculated using Hellemans method which allows for the use of more than one reference gene (Hellemans et al., 2007):

Normalised relative quantity (fold change)

$$= \frac{(E_{GOI})\Delta C_T(GOI \text{ control} - \text{sample})}{r\sqrt{\prod(E_{ref\ gene})\Delta C_T(GOI \text{ ref gene control} - \text{sample})}}$$

The average of the replicates was then taken and Log_2 transformed (Hellemans and Vandesompele, 2011).

Table S1 Sample sizes for infection experiments

Bacteria	Age	Sex	Social Environment	Sample size (n)
P. aeruginosa	Young	Male	Single	35
			Paired	37
		Female	Single	38
			Paired	36
	Old	Male	Single	38
			Paired	37
		Female	Single	34
			Paired	37
P. fluorescens	Young	Male	Single	40
			Paired	38
		Female	Single	32
			Paired	35
	Old	Male	Single	29
			Paired	31
		Female	Single	31
			Paired	33
B. thuringiensis	Young	Male	Single	37
			Paired	39
		Female	Single	36
			Paired	38
	Old	Male	Single	36
			Paired	36
		Female	Single	36
			Paired	38

Table S2 Primer sequences and RT-qPCR calculations

Gene	Forward Primer	Reverse Primer	Amplicon size (bp)	Primer efficiency (%)
Act5c	GTGGATACTCCTCCCGACAC	GCAGCAACTTCTTCGTCACA	150	91.3
E1f	GTCTGGAGGCAATGTGCTTT	AATATGATGTCGCCCTGGTT	97	106.6
Dro	GCCCGCCTAAAGATGTGTG	CGTGTGTTTATTGCTTACTGTTTGC	118	91.1
eater	GGCAATAATAACCACCATGC	TAAAGCTCAGGCTCGAATGA	130	101.2
vir 1	GAAGAACGCCAACCACTT	CACCAAGCGGACCTTAAAGA	109	96.6
Tot A	GCTTCAGCGTTCCAAAAAGT	AGAGGACTAATCAGCAGCAGTG	83	98.1
Tot M	TTCGAGTTTGAAAGCCAAGC	AGCATTTACCTTTCCCAGCA	96	103.8
Foxo	AGGCGCAGCCGATAGACGAATTTA	TGCTGTTGACCAGGTTTCGTGTTGA	156	95.6

Table S3 Parameter estimates for post infection lifespan analysed by GLM. Males and females were kept alone or in same sex pairs until being infected at 10 days (young) or 52 days (old) post eclosion, and these were analysed separately using social treatment and sex as fixed factors. Factors were removed from the maximal model using AOD, and parameter estimates from the minimal model are shown. Where no factors were significant the full model is shown.

bacteria	Age	Minimal model	Estimate	SE	t	p
Pseudomonas aeruginosa	Young	Social treatment*Sex	0.016	0.028	0.583	0.561
		Social treatment	-0.026	0.044	-0.589	0.557
		Sex	-0.030	0.044	-0.684	0.495
	Old	Social treatment	0.120	0.054	2.216	0.028
		Sex	0.112	0.054	2.065	0.040
Pseudomonas fluorescens	Young	Sex	0.063	0.012	3.213	0.002
	Old	Sex	0.203	0.083	2.430	0.017
Bacillus thuringiensis	Young	Social treatment*Sex	-0.128	0.128	-0.994	0.322
		Social treatment	0.111	0.206	0.538	0.592
		Sex	0.275	0.200	1.374	0.172
	Old	Sex	0.249	0.089	3.045	0.003

Table S4 Parameter estimates for phagocytosis results analysed by GLM. The full model contained the factors sex and social environment (social treatment) and their interaction as explanatory variables for phagocytotic index (PI) and total haemocytes. Models were simplified using AOD and the parameter estimates from the minimal model are presented. Where no factors were significant the full model is shown.

Data	Response variable	Minimal model	Estimate	SE	t	p	
All	PI	Sex	-0.250	0.107	-2.337	0.021	
		Total haemocytes	Sex*Age*Social treatment	0.016	0.233	0.067	0.947
			Social treatment*Age	0.146	0.862	0.17	0.865
			Social treatment*Sex	0.737	0.895	0.824	0.412
			Age*Sex	1.030	0.872	1.181	0.24
			Sex	-1.951	1.428	-1.367	0.174
			Age	-0.840	1.333	-0.63	0.53
			Social treatment	-0.593	1.353	-0.438	0.662
Males only	PI	Social treatment*Age	0.003	0.303	0.011	0.992	
		Age	0.098	0.466	0.211	0.834	
		Social treatment	0.017	0.470	0.037	0.971	
	Total haemocytes	Social treatment*Age	-0.222	0.334	-0.665	0.509	
		Age	0.190	0.513	0.37	0.713	
		Social treatment	0.145	0.517	0.28	0.78	
Females only	PI	Social treatment	-0.414	0.147	-2.82	0.006	
	Total haemocytes	Social treatment*Age	-0.591	0.451	-1.309	0.196	
		Age	1.220	0.719	1.697	0.095	
		Social treatment	0.882	0.748	1.178	0.243	

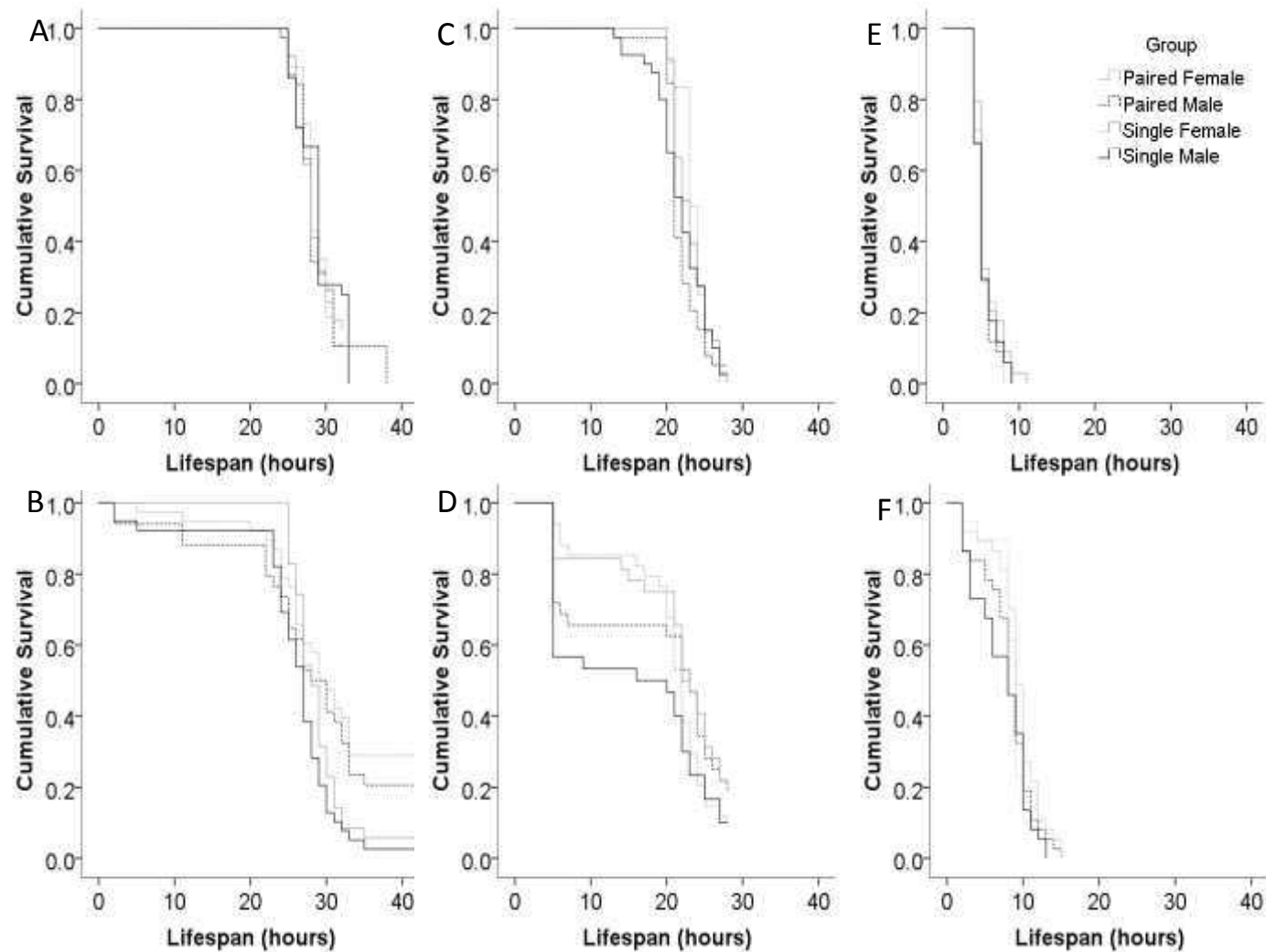


Figure S2 Kaplan Meier curves showing cumulative survival post infection. Males are shown in black and females in grey, whilst dotted lines denote paired groups and solid lines are single flies. The top row (panels A, C and E) show young flies and the bottom row (panels B, D and F) show old flies. Panels in the same column were infected with the same bacteria – A and B were infected with *P. aeruginosa*, C and D were infected with *P. fluorescens* and E and F were infected with *B. thuringiensis*.

Table S5 Parameter estimates from GLMs for gene expression of immunity and age-related genes. The full model contained the factors sex and social environment (social treatment) and their interaction as explanatory variables for log2 normalised expression. Models were simplified using AOD and the parameter estimates from the minimal model are presented.

Gene	Minimal model	Estimate	SE	t	p
Dro	Sex	-2.855	0.301	-9.499	<0.0001
vir-1	Sex	-2.241	0.226	-9.912	<0.0001
eater	Sex	-1.450	0.193	-7.496	<0.0001
TotA	Sex	-1.561	0.267	-5.852	<0.0001
	Social treatment	-0.815	0.267	-3.055	0.006
TotM	Sex*Social treatment	-1.830	0.450	-4.066	0.001
Foxo	Sex	-0.625	0.167	-3.743	0.001

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