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

**Running title: Sociality alters ageing and immunity in *Drosophila***

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

Running title: Social contact alters immunity in *Drosophila*

**Abstract**

Social environments have been shown to have multiple effects on individual immune responses. For example, increased social contact might signal greater infection risk and prompt a prophylactic upregulation of immunity. This differential investment of resources may in part explain why social environments affect ageing and lifespan. Our previous work using *Drosophila melanogaster* showed that single-sex social contact reduced lifespan for both sexes. Here, we assess how social interactions (isolation or contact) affect susceptibility to infection, phagocytotic activity and expression of a subset of immune and stress related genes in young and old flies of both sexes. Social contact had a neutral, or even improved, effect on post-infection lifespan in older flies and reduced the expression of stress response genes in females, however it reduced phagocytotic activity. Overall the effects of social environment were complex and largely subtle, and do not indicate a consistent effect. Together, these findings indicate that social contact in *D. melanogaster* does not have a predictable impact on immune responses and does not simply trade-off immune investment with lifespan.

**Keywords:** gene expression, infection, phagocytosis, senescence, stress, sex differences

## Introduction

Social environments have multifaceted effects on individual physiology and behaviour, even in animals not classically thought of as “social” (Bailey & Moore, 2018, Cacioppo et al., 2011). Social isolation is often viewed as negative (Hawkley & Capitanio, 2015) though this is being challenged (Bailey & Moore, 2018). For example, social contact can be risky because it is necessary to the transmission of communicable diseases (Godfrey et al., 2009). Accordingly, some animals use the presence or density of conspecifics as a signal of increased infection risk and so prime their immune responses in preparation (Barnes & Siva-Jothy, 2000, Wilson et al., 2002, Zhong et al., 2013). Part of this preparation involves altering gene expression and multiple studies have shown that immune gene regulation is sensitive to social environment (Ellis & Carney, 2011, Mohorianu, Bretman et al., 2017).

However, the relationship between social contact and immune response is not clear cut. Both social isolation and contact can be stressful and have been linked with disorders of the immune system (Cohen et al., 1997, Bartolomucci, 2007) and immunosenescence (Epel et al., 2004). What constitutes social stress (isolation or contact) is dependent on the species and context. Isolation disrupts T cell function in mice (*Mus musculus*) (Clausing et al., 1994) and reduces haemolytic activity in prairie voles (*Microtus ochrogaster*) (Scotti et al., 2015). Social contact reverses the effects of immunosenescence in honey bees (*Apis mellifera*) (Amdam et al., 2005). In humans, social stress impacts immune function via neuroendocrine systems (McEwen, 2012) and social isolation causes upregulation of inflammatory pathways which can lead to heart disease (Cole et al., 2007). Increased social competition in high density environments can also have significant deleterious effects. For example, aggressive encounters common to high density groups negatively influence immunity in house finches (*Haemorrhous mexicanus*) (Hawley et al., 2006). Therefore, social upregulation of immune activity would be beneficial in the event of contracting an infectious disease, but can be driven out of control by stressful social environments and could be costly to maintain. Such factors could help to explain the widely observed social impacts on

ageing (Steptoe et al., 2013), especially as ageing and immunity share common mechanistic pathways (Carnes et al., 2015). However, a further consideration is that responses of males and females are likely to differ between social environments, for example because male-male aggression is generally responsive to population density (Knell, 2009) whereas for females interactions with males are stressful and ultimately increase their rate of senescence (Bretman & Fricke, 2019).

Given this complex picture, we sought to investigate the role of simple social contact or isolation on immune responses using *Drosophila melanogaster* fruit flies. Our previous work in *D. melanogaster* showed a negative impact of same-sex social contact on lifespan for both sexes, though more strongly for males. This effect on lifespan was exacerbated by a generic immune stressor (wounding) for males only (Leech et al., 2017) but was not explained by male-male aggression or increased activity (Leech et al., 2017). An alternative explanation is that this reflects a sex-specific trade-off in investment between reproduction and immune responses, as males use contact with another male as a cue of future sperm competition and alter both their mating behaviour and ejaculate content accordingly (Bretman et al., 2013). Furthermore, exposure to a rival male causes enrichment of immune genes (Mohorianu, Bretman et al., 2017). Here, we tested the effect of social contact on various aspects of immune responses in *D. melanogaster*. We hypothesised that sex differences in ageing patterns under different social contexts are driven by a trade-off between investment in reproductive or immune traits in males, or that competitive social stress is more extreme for males. In either scenario males exposed to social contact would show poorer immune responses or faster immunosenescence than would females. We tested these ideas by measuring post-infection lifespan and *in vitro* phagocytic response to a bacterial challenge in young and aged flies that were isolated or kept in same-sex pairs. We predicted that post-infection lifespan and phagocytic response for paired flies would be reduced for both sexes, but to a greater extent in males. We subsequently examined expression of six immunity and stress response-related genes (Table 1), hypothesising that

paired flies would express higher levels of these genes, indicating a greater level of immunosenescence.

## **Materials and Methods**

### **Fly stocks and experimental design**

*Drosophila melanogaster* wild type (strain Dahomey) were raised on standard sugar-yeast agar (SYA) (Bass et al., 2007). Flies were kept at 25°C and 50% humidity with a 12:12 light:dark cycle. Experimental larvae were raised at a density of 100 larvae per 7ml SYA vial supplemented with a live yeast. Upon eclosion, virgin adult flies were sexed under ice anaesthesia and transferred to the relevant social environment, either singly or in same-sex pairs (Supporting Information Figure S1). In paired treatments, non-focal flies were identified with a wing-clip and were changed weekly when all flies were transferred to fresh vials. Focal flies underwent one of two immune phenotype assays, post-infection lifespan and phagocytosis ability, at either 10 days or 52 days post eclosion. For gene expression analyses, young fly treatments were omitted because effects on post-infection lifespan and phagocytosis were only observed in older flies (see Results). Measures of immunity (post-infection lifespan, phagocytosis and gene expression) were designed to capture information at an organismal, humoral and genetic level, but other aspects of immunity we have not measured may also be playing a part.

### **Post-infection lifespan**

To assess how social contact altered fly gross responses to infection, we measured their lifespan after infection with one of three bacteria known to be pathogenic to *D. melanogaster*, *Pseudomonas aeruginosa* (Apidianakis & Rahme, 2009) or *P. fluorescens* (Olcott et al., 2010) (Gram-negative), or *Bacillus thuringiensis* (Vallet-Gely et al., 2008) (Gram-positive). Bacteria were grown from frozen glycerol stocks in LB medium, *P.*

*aeruginosa* (strain PAO1) and *B. thuringiensis* (DSMZ 2046) for 24 hours at 37°C with 200rpm shaking, *P. fluorescens* (DSMZ 50090) for 48 hours at 25°C without shaking. Cultures were serially diluted 1 in 10 in sterile 10mM MgSO<sub>4</sub>. To standardise bacterial dose, flies were injected in the thorax using a pulled glass needle and a microinjector (Nanoliter 2010) with 9.2nl of 10<sup>-2</sup> *P. aeruginosa*, 13.8nl of 10<sup>-2</sup> *P. fluorescens* and 13.8nl of 10<sup>-1</sup> *B. thuringiensis* (the equivalent of between 120-220 CFU's) or a sterile sham solution (10mM MgSO<sub>4</sub>). Sample sizes for infection experiments ranged from 29-40 (Table S1). Flies were then checked hourly until all flies in at least one treatment had died (*B. thuringiensis* and *P. fluorescens*) or until no flies had died for 3 consecutive checks (*P. aeruginosa*).

### **Phagocytosis activity**

Phagocytic activity was investigated because previous work showed around 40 genes involved in phagocytosis to be differentially expressed under different social conditions (Mohorianu, Bretman et al., 2017). Haemolymph was extracted from flies surface-sterilised using ethanol and was pooled from 3 flies per biological replicate (n = 8 per treatment). pHrodo Green STP ester was used to label 50mg of freeze-dried *Pseudomonas aeruginosa* PAO1. 0.33mg of pHrodo-conjugated bacteria was sonicated in 50µl of Hanks Balanced Salt Solution for 45 minutes, before 5µl pHrodo-labelled bacteria/buffer mixture was added to the extracted haemolymph and incubated in the dark for 1 hour at 25°C. Cells were fixed with 150µl 0.5% paraformaldehyde solution and 40µl NucBlue solution was added to stain nuclei to aid identification of intact haemocytes. Fixed and stained samples were counted using a BD LSRII flow cytometer using the low flow rate for 3 minutes. We measured both the total haemocyte count and a Phagocytotic Index (phagocytotic haemocytes/ total haemocytes).

### **Differential expression of immunity and stress-related genes**

As we were limited by resources, we assessed expression of six genes, chosen to represent a range of immunological or stress response mechanisms which are under the control of discrete regulatory networks, and based on previous research which revealed them to be socially sensitive in males exposed to a rival (Mohorianu, Bretman et al., 2017) (Table 1). At 52 days old, focal flies from each group were individually flash frozen in liquid nitrogen and stored at -70°C. For each replicate, 12 flies were pooled, into seven biological replicates per treatment, amplified by RT-qPCR in three technical replicates. RNA was extracted using the Zymo research Directzol RNA miniprep, including a DNase treatment. The quantity and purity of RNA was measured using a NanoDrop and the integrity was checked by gel electrophoresis. RNA was standardised to 700ng/µl and cDNA was generated using the Thermo Scientific first strand synthesis cDNA kit. Primers were designed for *Dro*, *vir-1*, *eater*, *TotA*, *TotM* and *Foxo* and reference genes *Actin5c* and *EF1* using primer3plus (Untergasser et al., 2007) and where possible, were designed to span intron/exon boundaries (Table S2). RT-qPCR was carried out on the Biorad C1000 touch using the Kicqstart SYBR Green Readymix (Sigma). Final primer concentrations were 300nM, cycle conditions were as follows: 95°C for 3 minutes, 95°C for 10s, 60°C for 30s, repeated 39 times before a final step at 65°C for 3 minutes. Relative expression was calculated using Hellemans et al's (2007) method which takes account of multiple reference genes and primer efficiencies, more details can be found in the Supporting information.

**Table 1 Immunity, stress and ageing related genes chosen to be measured for differential expression.** Genes were first identified as socially sensitive in previous work (Mohorianu, Bretman et al., 2017) and were chosen to represent an assortment of underlying regulatory pathways.

Gene name	Short name	FlyBase ID	Function	Detail	Prediction	References
Drosocin	<i>Dro</i>	FBgn0010388	Immunity - antibacterial	An antimicrobial peptide under regulatory control of the Imd pathway which responds to both Gram-positive and Gram-negative bacteria.	Higher expression in males relative to females. Higher expression in paired flies compared to those kept singly.	(Lemaitre and Hoffmann, 2007)
virus-induced RNA 1	<i>vir-1</i>	FBgn0043841	Immunity - antiviral	Largely under the control of the JAK-STAT pathway. It is induced almost exclusively by viral infection.	As above	(Dostert et al., 2005)
eater	<i>eater</i>	FBgn0243514	Immunity - phagocytosis	A transmembrane EGF-like receptor expressed on haemocytes and pro-haemocytes and binds to Gram-positive and Gram-negative bacteria.	As above	(Kocks et al., 2005)
Turandot A	<i>TotA</i>	FBgn0028396	Stress response	Encodes humoral factors that are induced under a number of stressful conditions including heat shock, UV irradiation, oxidative stress and bacterial infection.	Higher expression in males relative to females. Higher expression in paired males compared to those kept singly, lower expression for paired	(Ekengren and Hultmark, 2001)

					females in accordance with functional senescence in our previous work.	
Turandot M	<i>TotM</i>	FBgn0031701	Stress response	As above. <i>TotM</i> has also been shown to aid the tolerance of fungal infection and to be socially sensitive.	As above	(Zhong et al., 2013)
forkhead box, subgroup O	<i>Foxo</i>	FBgn0038197	Ageing/immunity	A well-characterised transcription factor involved in regulation of the insulin signalling pathway. It has been implicated in a variety of physiological functions including regulation of longevity, ageing and immunity.	Higher expression in males relative to females. Higher expression in paired flies compared to those kept singly.	(Hwangbo et al., 2004; Guo et al., 2014)

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## Statistical analysis

Analyses were conducted using R (i386 3.2.0) and SPSSv21. All data were analysed using generalised linear models (GLMs) with appropriate error structures (quasi Poisson for post-infection lifespan and total haemocyte count, quasi-binomial for phagocytotic index, Gaussian for normalised gene expression). The full models were simplified by removing a term and comparing the model with and without that term using Analysis of Deviance (AOD). For the post-infection lifespan, as young and old flies were assayed in independent experiments, these were analysed separately. Sham infected flies were removed from the analysis as only one died over the course of the experiment. Hence for post-infection lifespan (in hours), the full model contained only the fixed factors of sex and social treatment and their interaction. Post infection lifespan could not be analysed using Cox Proportional Hazards as the data violated the proportional hazards assumptions of the model, but survival curves are given for illustration (Figure S2). For phagocytosis experiments (response variables total haemocyte count and phagocytotic index), since young and old flies were from the same cohort, the full model including age, sex and social treatment as fixed factors was first used. To explore the sex differences identified in this analysis the data was then separated by sex and explored with models using age and social treatment as factors. Gene expression was normalised (see Supporting information) and then  $\log_2$  transformed (Hellemans & Vandesompele, 2011). Expression for each gene was then analysed using sex and social treatment as fixed factors. As multiple genes were analysed for each replicate, the Bonferroni-Holm method (Holm, 1979) was used to correct for multiple testing.

## Results

### Post-infection lifespan

Social environment and sex did not affect survivorship in aged flies *before* they were infected (social environment;  $F_{1,21} = 0.888$ ,  $p = 0.357$ ; sex;  $F_{1,23} = 0.428$ ,  $p = 0.520$ ). In young flies

infected with *P. aeruginosa* there was no effect of pairing or sex (Table 2 and S3; Figures 1A and S2A). In old flies, pairing increased post-infection lifespan and females survived for longer after infection (Table 2 and S3; Figure 1B and S2B). For flies infected with *P. fluorescens*, pairing had no effect on lifespan after infection at either age, but females survived longer when both young (Table 2 and S3; Figures 1C-D and S2C-D). For flies infected with *B. thuringiensis* there were no effects of pairing at either age, or of sex for young flies (Table 2 and S3; Figures 1E-F and S2E-F). In old flies, females again had a significantly longer post-infection lifespan (Table 2 and S3; Figures 1F and S2F).

**Table 2 Post infection lifespan analysed using GLMs and AOD.** 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 it is the AOD statistics that are presented. Factors significantly affecting the explanatory power of the model are shown in bold.

<b>Bacteria</b>	<b>Age</b>	<b>Explanatory variable</b>	<b>df</b>	<b>F</b>	<b>p</b>
<i>Pseudomonas aeruginosa</i>	Young	Social treatment	1, 147	0.013	0.911
		Sex	1, 149	0.169	0.681
		Social treatment*Sex	1, 147	0.340	0.561
	Old	Social treatment	1, 145	4.916	<b>0.028</b>
		Sex	1, 147	4.553	<b>0.035</b>
		Social treatment*Sex	1, 143	0.093	0.761
<i>Pseudomonas fluorescens</i>	Young	Social treatment	1, 145	0.343	0.559
		Sex	1, 147	10.316	<b>&lt;0.001</b>
		Social treatment*Sex	1, 145	0.137	0.712
	Old	Social treatment	1, 125	0.631	0.429
		Sex	1, 127	5.949	<b>0.016</b>
		Social treatment*Sex	1, 125	1.795	0.183
<i>Bacillus thuringiensis</i>	Young	Social treatment	1, 152	0.046	0.828
		Sex	1, 150	2.314	0.130
		Social treatment*Sex	1, 150	0.143	0.706
	Old	Social treatment	1, 147	0.044	0.835
		Sex	1, 149	6.562	<b>0.011</b>
		Social treatment*Sex	1, 147	1.709	0.193

**Phagocytosis activity**

There was no effect of age, sex or treatment on total haemocyte counts (Tables 3 and S4; Figures 2A and B). For phagocytotic index (PI) there was a non-significant trend for a pairing by sex interaction and when this was removed this left a significant effect of sex (Tables 3 and S4), males had a slightly higher PI (Figures 2C and D). When analysing each sex separately, pairing reduced PI for females at both ages but there were no effects in males (Table 3 and S4; Figures 1D and E).

**Table 3 Phagocytosis analysed by GLM and AOD.** Males and females were kept alone or in same sex pairs and either aged to 10 or 52 days. Haemolymph was then extracted and phagocytosis of *Pseudomonas aeruginosa* was subsequently analysed *in vitro* using flow cytometry. Two response variables were examined – total number of haemocytes and phagocytic index (proportion of phagocytic haemocytes out of total haemocyte counts). Initially the full model contained the fixed factors sex, age and social treatment, and subsequently the data were split by sex for further analysis. Factors were removed from the maximal model using AOD, and it is the AOD statistics that are presented. Factors significantly affecting the model are shown in bold.

Dataset	Response Variable	Explanatory Variable	df	F	p
All	PI	Sex	1, 127	5.484	<b>0.021</b>
		Age	1, 125	1.037	0.311
		Social treatment	1, 125	2.849	0.094
		Social treatment*Sex	1, 123	3.733	0.056
		Sex*Age*Social treatment	1, 121	0.270	0.604
	Total haemocytes	Sex	1, 125	0.656	0.420
		Age	1, 125	0.368	0.545
		Social treatment	1, 127	0.746	0.389
		Sex*Age*Social treatment	1, 121	0.438	0.509
		Males only	PI	Age	1, 63
Social treatment	1, 61			0.021	0.884
Social treatment*Age	1, 61			<0.0001	0.992
Total haemocytes	Age		1, 62	0.650	0.423
	Social treatment		1, 62	1.200	0.278
	Social treatment*Age		1, 60	0.443	0.508
Females only	PI	Age	1, 63	0.965	0.330
		Social treatment	1, 61	8.021	<b>0.006</b>
		Social treatment*Age	1, 61	0.533	0.468
	Total haemocytes	Age	1, 62	2.117	0.151
		Social treatment	1, 62	0.053	0.819
		Social treatment*Age	1, 60	1.732	0.193

### Differential gene expression

Social environment had no effect on expression of the immunity and aging genes, *Dro*, *vir-1*, *eater* and *Foxo*, but expression of these genes was significantly lower in females than in males (Tables 4 and S5; Figures 3A, B, C and F). In contrast, both sex and social environment affected expression of the stress induced humoral peptides *TotA* and *TotM*. For *TotA* there was no significant interaction between sex and social environment (Tables 4 and S5; Figure 3D), but expression was significantly lower in females (Tables 4 and S5; Figure 3D), and was also lower in paired flies (Tables 4 and S5; Figure 3D). For *TotM*, there was a significant interaction between sex and treatment (Tables 4 and S5; Figure 3E) – paired females had lower gene expression, whereas paired and single males had similar expression.

**Table 4 Gene expression analysed by GLM and AOD.** Log2 normalised expression for each gene was analysed separately, with sex and treatment as fixed factors. Factors were removed from the maximal model using AOD, and it is the AOD statistics that are presented. Factors significantly affecting the model are shown in bold. P values were corrected for multiple testing using the Holm-Bonferroni method.

<b>Gene</b>	<b>Explanatory Variable</b>	<b>df</b>	<b>F</b>	<b>P</b>
<i>Dro</i>	Sex	1, 25	90.233	<b>&lt;0.0001</b>
	Social treatment	1, 23	0.134	0.365
	Sex*Social treatment	1, 23	1.896	0.717
<i>vir-1</i>	Sex	1, 26	98.25	<b>&lt;0.0001</b>
	Social treatment	1, 24	0.824	0.442
	Sex*Social treatment	1, 24	1.583	0.442
<i>eater</i>	Sex	1, 25	56.184	<b>&lt;0.0001</b>
	Social treatment	1, 23	2.647	0.235
	Sex*Social treatment	1, 23	2.474	0.13
<i>TotA</i>	Sex	1, 24	34.243	<b>&lt;0.0001</b>
	Social treatment	1, 23	9.336	<b>0.011</b>
	Sex*Social treatment	1, 23	3.058	0.094
<i>TotM</i>	Sex	1, 24	15.758	<b>0.002</b>
	Social treatment	1, 22	6.908	<b>0.002</b>
	Sex*Social treatment	1, 22	16.535	<b>0.015</b>
<i>Foxo</i>	Sex	1, 27	14.013	<b>0.001</b>
	Social treatment	1, 25	0.4539	0.507
	Sex*Social treatment	1, 25	0.0419	0.840

## Discussion

Based on previous work (Bretman et al., 2013, Leech et al., 2017) we hypothesised that social contact would act as a stressor, especially in males, and consequently that infected flies would be less-able to mount an effective immune response. This was not borne out; paired flies either performed the same or better than single flies in terms of post-infection lifespan. Indeed, where paired flies had increased post-infection lifespan this was at older ages. This pattern was not explained by immune responsiveness measured via phagocytic activity, as pairing reduced phagocytosis of *P. aeruginosa* in females only. Likewise, the pattern of expression of some immunity and stress genes did not explain infection responses, but did highlight sex differences in expression of all genes tested. The two *Turandot* genes tested are down-regulated in paired females but not males, further evidence that same-sex contact is less stressful for females.

Post-infection lifespan was improved for flies held in same-sex pairs, but only when infected with *P. aeruginosa*, the least virulent of the three bacteria we used (Figure S2). As virulence of the infecting bacteria increases, so too does the ability of the bacteria to overwhelm the host immune system (Schmid-Hempel & Frank, 2007). Therefore, the benefit provided by being in pairs may only be apparent with less virulent infections. It is not clear whether this represents a prophylactic response to increased infection risk as signalled by the presence of conspecifics as seen in other animals (Wilson et al., 2001). Investment in immunity may not be universal across all defences since there are trade-offs between different immune traits (Cotter et al., 2004), and the senescence of individual immune traits does not occur at the same rate (Reavey et al., 2015). This could explain why post-infection lifespan, gene expression and phagocytic responsiveness do not show a consistent pattern in response to social contact. We are cautious in making comparisons across all three traits since we did not measure gene expression in younger flies. Our intention was to explore mechanisms underlying the post-infection lifespan differences which were only apparent at older ages, but this approach does make interpretation more difficult. The sex differences in

post-infection lifespan for older flies were consistent across all three bacteria tested. This suggests that the pattern of deterioration in the immune system in *D. melanogaster* differs between sexes, which is in line with previous studies that report the severity of infection is generally worse for males than females (Ramsden et al., 2008) and that the phagocytic response senesces differently for males and females (Mackenzie et al., 2011). It should be noted, however, that the aspects of immunity we have chosen to measure do not capture the complexity of the immune system in totality and therefore other, unmeasured, aspects of immunity may be playing a significant role.

In contrast to the response in post-infection lifespan, social contact resulted in a significantly lower proportion of phagocytosing cells for females at both ages. Haemocyte differentiation and ROS activity (and therefore phagocytosis) under stressful conditions is increased (Shim, 2015), further supporting the idea that paired females are less stressed than paired males. Our results do not support a decline in phagocytic responsiveness with age as found in some previous studies (Horn et al., 2014, Mackenzie et al., 2011), perhaps because our own study looked at phagocytosis *ex vivo* as compared to *in vivo*. However, our data do agree that generally males demonstrate higher phagocytic activity than females (Mackenzie et al., 2011), despite males' poorer post-infection performance. Further work is needed to understand whether males are more stressed regardless of social environment or that females do not need to invest as much in standing immunity to still outperform males.

Social environment affected expression of stress response genes *TotA* and *TotM* (Ekengren & Hultmark, 2001), a pattern consistent with our hypothesis and previous work that being in pairs is less stressful than social isolation for females (Leech et al., 2017). *TotM* and *TotC* are upregulated in females exposed to courtship song (Zhong et al., 2013).

Together with our work, this shows that the expression of the *Turandot* family of genes is socially sensitive in both intra- and intersexual contexts. As up-regulation of immune genes with age is a hallmark of immunosenescence (Zerofsky et al., 2005), paired females expressing less of these stress genes may be a sign of slowed immunosenescence

compared to those kept singly. Likewise in our previous study, isolated females showed faster senescence in climbing activity than paired females (Leech et al., 2017). Social environment did not affect the expression of any of the directly immune-related genes we measured. However, there was a consistent pattern of higher expression in males. As previous work has shown an upregulation of most of these genes in 5-7 day old flies with 2-50 hours exposure to other males (Mohorianu, Bretman et al., 2017), the lack of a plastic response in gene expression in males indicates that senescence has reduced their ability to respond to environmental cues appropriately. Ageing is characterised by a decline in regulatory control of antimicrobial peptide genes (Zerofsky et al., 2005) so the lower expression of *Drosocin* in females as compared to males may explain why females in general have an improved post-infection lifespan at older ages. Reduced transcription of *eater* causes lower phagocytic activity (Horn et al., 2014) and dysregulation with age results in higher expression levels of immune genes (Zerofsky et al., 2005). Therefore, since the lower relative expression of *eater* in paired females translates into less phagocytosis without trade-offs in post-infection lifespan, this could also strengthen the argument that social contact delays immunosenescence in females.

In conclusion, our results show a complex relationship between social environment and immunity in flies. Social contact either improved lifespan after bacterial infection or did not affect it at all, in contrast to other types of immune elicitor such as injury (Leech et al., 2017), evidence that the evolutionary consequences of social contact or isolation are not identical across traits (Bailey & Moore, 2018). Likewise there was not a consistent pattern in phagocytic responsiveness or expression of the genes measured here. Social contact reduces phagocytic activity in females, but this does not have detrimental effects on lifespan following infection. The differential gene expression we observed highlights certain immune pathways are more socially-responsive than others (notably stress response genes in females) and that males succumb to immune dysregulation quicker than females, regardless of social environment. Overall our data support the idea that same-sex pairing in fruit flies

slows immunosenescence of some traits in females, in contrast to males, who are generally more negatively affected by same-sex social contact. The route of infection would be an interesting avenue of future research, as orally ingested pathogens are likely to have different effects given their interaction with the microbiome, which itself is altered by social environments (Archie & Tung, 2015). In this context expression of PGRP-SC2 would be interesting to evaluate, given its role in regulating the microbiome and promoting the lifespan extension (Guo et al., 2014). Subsequent work should also test whether pathogens that are more likely to be socially transmitted (e.g. viruses) stimulate greater responses in the host than those less reliant on social contact (e.g. fungi), as suggested by the transcriptomic profiles of socially isolated humans (Cole et al., 2011).

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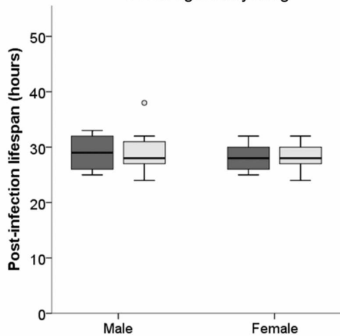
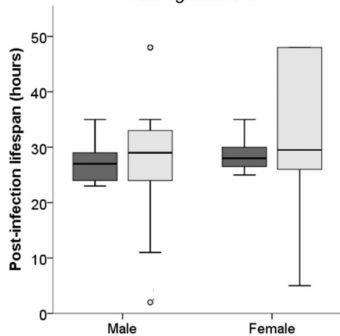
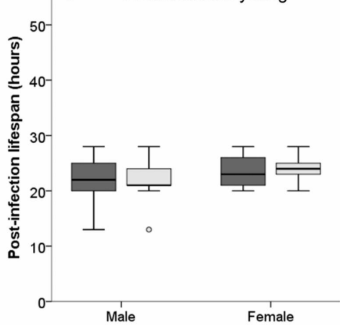
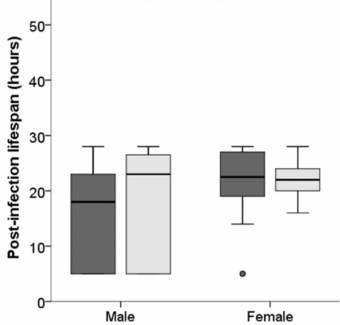
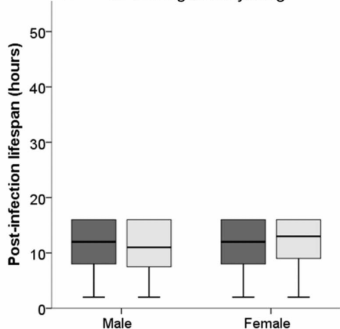
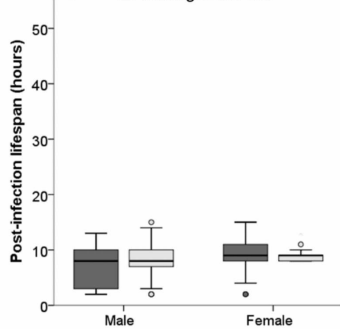
Turandot M promotes immunity against sexually transmitted fungal infections.  
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## Figure legends

**Figure 1** Median lifespan post infection for flies kept singly (dark grey) or in same-sex pairs (light grey). Flies were infected when young (A, C and E) or old ( B, D and F), with standardised doses of either *P. aeruginosa* (A and B), *P. fluorescens* (C and D) or *B. thuringiensis* (E and F). Whiskers represent maximum and minimum non-outlier values. Circles indicate outliers ( $Q1/Q3 \pm 1.5 \times IQ$  range).

**Figure 2** Haemocyte counts (A, B) and Phagocytic Index (C, D) for young (A, C) and old (B, D) flies that were held singly (dark grey) or in same-sex pairs (pale grey). PI is the number of phagocytosing haemocytes/ total haemocyte count. Whiskers represent maximum and minimum non-outlier values. Circles indicate outliers ( $Q1/Q3 \pm 1.5 \times IQ$  range). Eight biological replicates per treatment were conducted, each pooled from 3 flies.

**Figure 3** Differential expression (mean  $\pm$  S.E.M.) of six immune or stress- related genes in 52 day old flies kept singly (dark grey) or in same-sex pairs (light grey). (A) *Dro*, the antimicrobial peptide, (B) the anti-viral gene *vir-1*, (C) the phagocytosis receptor *eater*, (D) the stress induced humoral peptide *TotA*, (E) the stress induced humoral peptide *TotM* and (F) *Foxo*, the transcriptional activator. Each biological replicate consisted of a pool of 12 flies, with seven biological replicates in total.

**A** *P. aeruginosa* young**B** *P. aeruginosa* old**C** *P. fluorescens* young**D** *P. fluorescens* old**E** *B. thuringiensis* young**F** *B. thuringiensis* old**Sex****Sex**

