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Leech, T orcid.org/0000-0002-9504-0739, McDowall, L orcid.org/0000-0001-8425-5702, Hopkins, KP orcid.org/0000-0002-3251-7093 et al. (3 more authors) (2021) Social environment drives sex and age-specific variation in Drosophila melanogaster microbiome composition and predicted function. Molecular Ecology, 30 (22). pp. 5831-5843. ISSN 0962-1083

https://doi.org/10.1111/mec.16149

***Title*: Social environment drives sex and age-specific variation in *Drosophila melanogaster* microbiome composition and predicted function.**

Running title: Social effects on fly microbiomes

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Abstract

Social environments influence multiple traits of individuals including immunity, stress and ageing, often in sex-specific ways. The composition of the microbiome (the assemblage of symbiotic microorganisms within a host) is determined by environmental factors and the host's immune, endocrine and neural systems. The social environment could alter host microbiomes extrinsically by affecting transmission between individuals, likely promoting homogeneity in the microbiome of social partners. Alternatively, intrinsic effects arising from interactions between the microbiome and host physiology (the microbiota-gut-brain axis) could translate social stress into dysbiotic microbiomes, with consequences for host health. We investigated how manipulating social environments during larval and adult life-stages altered the microbiome composition of *Drosophila melanogaster* fruit flies. We used social contexts that particularly alter the development and lifespan of males, predicting that any intrinsic social effects on the microbiome would therefore be sex-specific. The presence of adult males during the larval stage significantly altered the microbiome of pupae of both sexes. In adults, same-sex grouping increased bacterial diversity in both sexes. Importantly, the microbiome community structure of males was more sensitive to social contact at older ages, an effect partially mitigated by housing focal males with young rather than co-aged groups. Functional analyses suggest that these microbiome changes impact ageing and immune responses. This is consistent with the hypothesis that the substantial effects of the social environment on individual health are mediated through intrinsic effects on the microbiome, and provides a model for understanding the mechanistic basis of the microbiota-gut-brain axis.

Key Words: microbiota-gut-brain axis, infection, ageing, development, stress

Introduction

Social environments have multiple effects on individual health, including immune responses (Cole, 2014; Leech, Evison, Armitage, Sait, & Bretman, 2019), ageing and ultimately lifespan (Flintham et al., 2018; Hawkley & Capitanio, 2015; Leech, Sait, & Bretman, 2017). Indeed, meta-analyses show that adverse social environments are a health risk factor on a par with obesity and smoking (Holt-Lunstad, Smith, & Layton, 2010). Effects of social environments are complex. They are seen in animals not usually thought of as social, there are marked sex differences, and hence what constitutes a stressful social environment is not straightforward (Flintham et al., 2018; Leech et al., 2019; Leech et al., 2017). For example, periods of social isolation can be beneficial even in gregarious species (Bailey & Moore, 2018). The mechanisms that translate social information into these effects are unclear, but it has been suggested that the microbiome (the community of microorganisms living symbiotically with a host) plays a role (Flintham et al., 2018).

Social impacts on microbiomes are expected given that close contact aids horizontal transmission of microbes (Kulkarni & Heeb, 2007; Lax et al., 2014; Sarkar et al., 2020) and social partners will often have similar diets, a key driver of microbiome composition (David et al., 2014). Such extrinsic processes would lead to greater homogeneity in the microbiome of social partners, or a “social microbiome” meta-community (Sarkar et al., 2020), but would not necessarily have any fitness consequences for the host. However, there is a great deal of interaction between the microbiome and host immune pathways, hormones and neurotransmitters known as the ‘microbiota-gut-brain axis’ (Carabotti, Scirocco, Maselli, & Severi, 2015). Hence, host social environments that impact stress and immune responses (Cole, 2014; Leech et al., 2019; Mohorianu et al., 2017) could indirectly alter the microbiome in an intrinsic manner. This could have profound consequences for host health given the microbiome’s influence on development and behaviour (Hsiao et al., 2013), susceptibility to pathogens (Knutie, Wilkinson, Kohl, & Rohr, 2017), ageing (Clark et al., 2015; Guo, Karpac,

Tran, & Jasper, 2014) and fitness trade-offs (Gould et al., 2018). Therefore, social stress that drives dysbiosis could mediate the effects of social environments on lifespan.

So far, the influence of host social interactions on microbiome composition has been investigated largely in mammals. Similarities in microbiomes driven by cohabitation, social group membership or social networks seen in ring-tailed lemurs (*Lemur catta*) (Bennett et al., 2016), wild baboons (*Simia hamadryas*) (Tung et al., 2015) and humans (Song et al., 2013) likely represent extrinsic effects of social environments. However, recent evidence demonstrates that the social environment has effects on microbiome beyond impacting transmission. In mice, social stress alters gut immune gene expression and their gut microbial community (Galley et al., 2014) and faecal transfers from mice stressed through isolation recapitulates isolation behaviours in non-isolated mice (Gacias et al., 2016). Far less is known about effects in invertebrates, though progress has been made in colony-living bees. Microbiota derived from faeces of nestmates protect bumble bees from parasitic infection (Koch and Schmid-Hempel 2011), and isolated queen honey bees show a larger and more diverse microbiome compared to those held with workers (Powell, Eiri, Moran, & Rangel, 2018). To broaden our understanding of these effects, we used an invertebrate model system in which simple experimental manipulations of social contact alter ageing and lifespan.

Work in *Drosophila melanogaster* fruit flies has demonstrated multiple effects of social environments on individual behaviour and physiology. We focus on social conditions to which males are particularly sensitive, therefore extrinsic effects of the social environment should affect both sexes equally, but intrinsic effects would be seen to a greater extent in males (Fig S1). In adults, same-sex social contact has sex-specific impacts on actuarial (lifespan) and functional senescence (Bretman, Westmancoat, Gage, & Chapman, 2013; Flintham et al., 2018; Leech et al., 2017). Male lifespan is reduced disproportionately by the presence of same-sex cohabitants, especially when given an immune challenge (Leech et al., 2017), but both sexes can survive longer post-infection with certain bacteria if held with

same-sex partners (Leech et al., 2019). Males use the presence of other males as a cue of potential sperm competition, making sophisticated adjustments to their reproductive behaviour and ejaculate (Bretman, Fricke, & Chapman, 2009; Hopkins et al., 2019). During development, larval density can alter growth rates and adult body size, and the prior presence of adults on food substrates can increase larval survival (Wertheim, Marchais, Vet, & Dicke, 2002). This could occur via the provision of beneficial bacterial species from the adults to the larvae. Furthermore, the flies could also gain an advantage from the competitive inhibition of potentially pathogenic bacterial or fungal species (Wertheim, Marchais, Vet and Dicke, 2002). In addition, when food resources are not limiting, both higher density and the presence of adult males (cues of future sperm competition) stimulate males to develop larger accessory glands (Bretman, Fricke, Westmancoat, & Chapman, 2016), and males raised at lower density are better at learning when adult (McDowall, Rouse, Sait, & Bretman, 2019).

The fly microbiome affects a range of traits including development (Shin et al., 2011), metabolism (Wong, Dobson, & Douglas, 2014), immune responses (Blum, Fischer, Miles, & Handelsman, 2013) and longevity (Guo et al., 2014). The fly microbiome is relatively simple (Broderick & Lemaitre, 2012) and its composition changes across life stages and ages (Wong, Ng, & Douglas, 2011), though it is seeded primarily at hatching when larvae eat the faeces-covered chorion (Bakula 1969). Bacterial species richness increases in the gut during larval stages, then decreases significantly during metamorphosis (Wong et al., 2011). This decrease is associated with a large increase in host immune factors, such as antimicrobial peptides (Broderick, 2016). However, there is the possibility that bacteria could be transmitted from pupal to adult stages after being maintained in the larval midgut (Broderick and Lemaitre, 2012). Though transmission through metamorphosis has been observed for the bacterium *Enterococcus mundtii* in *Galleria mellonella* wax moths (Johnston and Rolff, 2015), as well as the pathogenic *Providencia rettgeri* in *Drosophila* (Duneau and Lazarro,

2018), more work is required to establish whether this is indeed a mode of transmission between life stages. Differences in microbiome community are driven by the environment, for example wild-caught versus laboratory rearing, or maintenance on different food sources (Staubach, Baines, Kunzel, Bik, & Petrov, 2013). Larvae gain gut microbes through ingestion of their egg casing and from their food, and this environmental replenishment continues during adulthood (Blum et al., 2013), so extrinsic effects of the social environment are likely.

Beyond transmission effects, there is potential for intrinsic effects of social environments acting through the microbiota-gut-brain axis. Fly gene expression is socially sensitive, including immune, stress and lifespan related genes (Leech et al., 2019; Mohorianu et al., 2017). The host IMD pathway, primarily induced by Gram negative bacteria, is one mechanism used by hosts to control the microbiome (Iatsenko, I., Boquete, J. P., & Lemaitre, 2018). Mutants for the IMD pathway component PGRP-SD show a proliferation of *Lactobacillus plantarum* in the fly gut and a reduced lifespan (Iatsenko et al., 2018). Epigenetic regulation of the fly immune IMD pathway alters the microbiome and fly social behaviour (Chen et al., 2019). Similarly, opportunistic pathogenic bacteria can activate DUOX-dependent immune responses via uracil release, whilst many commensals do not (Lee et al., 2013). Likewise, the JAK-STAT pathway is involved in immune and stress responses in flies, and its activation can result in metaplasia, gut pH changes and dysbiosis (Li, Qi & Jasper, 2016). Further, this pathway is induced upon oral infection with *Erwinia carotovora* (Buchon, Broderick, Poidevin, Pradervand & Lemaitre 2009), suggesting it is another important factor in maintaining gut-microbiota homeostasis. Thus, disruption of immune pathways could have significant consequences on microbiome composition. Changes in the activation status of stress pathways could induce dysbiotic states, for example, the higher microbial loads observed in ageing flies (Clark et al., 2015). Ultimately, such dysbiosis influences ageing and lifespan. Maintaining flies axenically alters lifespan, though can both lengthen (e.g. Yamada, Deshpande, Bruce, Mak & Ja 2015; Galenza, Hutchinson, Campbell, Hazes, & Foley 2016; Téfit & Leulier 2017) or shorten lifespan

(Brummel, Ching, Seroude, Simon & Benzer, 2004), depending on experimental procedures such as egg bleaching (Lee, Lee, Lee, Lee & Min 2019). The impact of the microbiota on fly lifespan has been connected to methionine metabolism, vitamin B and glucose (Matthews et al., 2020), and the provision of thiamine to its host (Sannino et al., 2018). Overall, this suggests that the microbiome can have important effects on host ageing, and potentially mediate the effects of stressful environments on host health.

We captured the bacterial component of the microbiome using 16S rRNA gene sequencing, but for brevity hereafter we refer to this as the microbiome. We examined the effect of larval rearing density or presence of adult males, conditions that alter development (Bretman et al., 2016; McDowall et al., 2019; Wertheim et al., 2002), on the microbiome of pupae and one day old adults. As the *D. melanogaster* microbiome is dependent on regular replenishment from ingesting bacteria from the environment, potentially from excreta from other flies (Blum et al., 2013), we expected that larvae developing in high densities or kept with adults would show greater species richness and changes in microbiome composition during the pupal stage i.e. extrinsic factors. We also investigated whether these would carry over into early adulthood. For example, compared to axenic flies, bacteria-associated individuals show increased larval growth rates and differences in carbohydrate stores as adults (Ridley et al., 2012), suggesting that the microbiome could have distinct long-lasting consequences in *D. melanogaster*. Indeed, despite the changes associated with metamorphosis, part of the larval midgut is preserved throughout the transition, providing a potential opportunity for retention of bacterial species (Broderick and Lemaitre, 2012).

In adults we compared socially isolated flies to those kept in co-aged same sex groups, conditions that alter lifespan in a sex-specific manner (Flintham et al., 2018; Leech et al., 2017). In addition, we investigated the effect of the age of the cohabitants by housing an ageing focal fly with a group of consistently young flies, as the effect of social contact on ageing in males can be altered by the age of the partner flies, likely due to modulation of

oxidative stress resistance (Ruan & Wu, 2008). We predict that the social environment could influence microbiome composition through direct transfer between individuals (extrinsic) or through effects on the host immune/ stress responses (intrinsic factors). We expect that where intrinsic factors exert significant effects, these would differ between ages and sexes, such that signatures of dysbiosis (e.g. an increase in uracil producing bacteria) would reflect sex differences we have previously observed. Specifically, we expect greater changes in males in response to increased larval density and the presence of adults during development, and in adult males housed with co-ageing males. In light of our findings, we tested the effect of larval social environments on lifespan, and because of the importance of microbiomes in combatting infections (Blum et al., 2013), the ability of adult flies to survive an oral infection with a pathogenic bacterium.

Materials and Methods

Fly stocks and maintenance

Drosophila melanogaster wild type (strain Dahomey) were raised on standard sugar-yeast agar medium (Bass et al., 2008; full recipe in SI). Flies for all experiments were maintained at a constant 25ºC and 50% humidity with 12h:12h light:dark cycle. Experimental larvae were raised at a density of 100 larvae (unless otherwise stated) per 7ml vial supplemented with a live yeast. Upon eclosion, virgin adult flies were sexed under ice anaesthesia and transferred to the relevant social environment.

Larval social environment

Larval density treatments consisted of 20 (low) or 200 (high) larvae per vial on a concentrated medium (recipe in SI) to prevent food becoming a limiting factor at high density

(Bretman et al., 2016; McDowall et al., 2019). Adult presence/ absence groups were raised at 100 larvae per vial. The adult presence treatment had 20 adult males added to the vial, removed the day before eclosion of the experimental adult flies. Pupae were also collected the day before eclosion, and sexed by the presence of sex combs on male legs. Adults were collected within 8 hours of eclosion, and transferred singly to a vial containing fresh food for approximately 24 hours before freezing at -80°C. Each individual originated from a separate larval vial.

Adult social environment

Adult males and females were kept alone or in same-sex groups consisting of one focal fly and nine cohabitants. All focal flies were given a small wing-clip so that those in groups could be identified. Focal flies were sacrificed at either 11 days old, an age at which flies are sexually mature (Ruhmann et al., 2016) and at which we have observed cognitive effects of social partners (Rouse et al., 2020) but before differences in survival are observed (Leech et al., 2017), or 49 days old, when the senescent effects of social environment become apparent (Leech et al., 2017). For old flies, to assess the effect of co-ageing within groups, cohabitants were either the same age as the focal fly, or were changed weekly by mouth aspiration for adults that had eclosed the day before (i.e. constantly aged 1-7 days). Food was changed weekly.

16s rRNA sequencing and bioinformatics

For sequencing, each biological replicate was a pool of 8 flies (n = 10 per social environment for larval experiments and n= 8 per social environment for adult experiments). DNA was extracted using the Mobio PowerSoil® DNA Isolation Kit and quality checked using NanoDrop (ND-1000) before being sequenced using paired end 250bp v2 chemistry on an Illumina MiSeq (see SI). Post-sequencing bioinformatics were conducted using mothur

(version 38.2) (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013; Schloss et al., 2009). Detailed information on library preparation, sequencing and bioinformatic protocols are provided in Supplementary Information. The average library size was ~40k reads per sample after passing quality control. OTU tables are given in the data archive.

Microbiome statistical analysis

All statistical analysis was conducted using R v3.3.2 (R Core Team, 2017) using the phyloseq (McMurdie & Holmes, 2013), vegan (Oksanen et al., 2012), ggplot2 (Wickham, 2009), DESeq2 (Love, Huber, & Anders, 2014), and lme4 (Bates, Machler, Bolker, & Walker, 2015) packages. Prior to analysis 18 contaminant Operational Taxanomic Units (OTUs) present in the negative controls were removed (Iulia, Bianca, Cornelia, & Octavian, 2013). One female pupal sample from the larval density treatment was identified as an extreme outlier (Grubb's test $p < 0.05$) in number of OTUs (suggestive of contamination) and hence was removed from all subsequent analysis. Sequences were rarefied in order to normalise library sizes. For larval density, the data were rarefied to 20,140 sequences, and for adult presence to 22,718. For adult social environment groups, all were rarefied to 10,840 sequences.

Alpha diversity was estimated using the Chao1 species richness indicator (Chao, 1984). Predictors of alpha diversity were analysed using GLM with social environment, sex and life-stage/age as fixed factors. Models were simplified from the full model using Analysis of Deviance (AOD). We visualised differences in bacterial community structure among samples (beta diversity) using Non-metric Multidimensional Scaling (NMDS) plots of Bray-Curtis distances. We used PERMANOVA (with 1000 permutations) to examine the effects of social environment, sex and life stage/age on bacterial beta diversity. Our data conformed to a negative binomial distribution so we used DESeq2 (Love et al., 2014) to identify OTUs that differed significantly in relative abundance between groups. Where differentially-abundant OTUs were classified only to genus level, we cross-referenced the sequence in the

GreenGenes database (DeSantis et al., 2006) using BLAST to identify to species level where possible. We identified differentially enriched functional pathways using Piphillin (Iwai et al., 2016) (for further information see Supplementary Information) and made eight targeted pairwise comparisons between treatments based on where we observed effects on the microbial community, again using DESeq2 (Love et al., 2014). For larval treatments, this was the presence versus absence of adults, measured in pupae or 1-day old adults. For adult treatments, this was females versus males when either held singly or in co-ageing groups, and for males only, 11-day versus 49-day old flies when either held singly or in co-aged groups, and singly versus grouped males either when the group was co-aged or mixed ages.

Effects of larval social environment on lifespan

To examine adult lifespan, a further 60 flies per treatment group were collected from larval social environments as they eclosed, and kept in single sex groups of 10 on fresh yeast-sugar medium. Each day, the number of mortalities was recorded and then these were removed. Surviving flies were transferred weekly onto fresh food. Differences in lifespan were analysed using a Cox Proportional Hazards model, with sex and social treatment as factors.

Effects of adult social environment on survival post-oral infection

Males and females were raised singly or with a same sex partner to 50 days old (food and non-focal flies were changed weekly as above) before being starved for 3 h and then infected with *Pseudomonas fluorescens* via feeding with a bacteria/sucrose/yeast solution (see SI). Pairs were used in this experiment, rather than groups of 10, since previous work had shown a single partner is enough to elicit socially-driven changes in both immune responses (Leech et al., 2019) and ageing patterns (Leech et al., 2017). Flies were checked

for death every 24 h for one week. We also confirmed that any patterns seen were not driven by a difference in amount of food eaten using a CAFÉ (CApillary FEeder) assay (Ja et al., 2007) (see SI). This assay allows for the measurement of food consumed in a given period through the changes in its level in a glass capillary used for food provision. Since post-infection lifespan data was limited to one week, a chi squared test was used to determine if the number of flies that died differed by sex and social environment.

Results

The presence of adults, but not rearing density, during larval development alters *Drosophila* microbiomes

In pupae, being raised in the presence of adults increased species richness measured as alpha diversity (effect of adult presence $F_{1, 77} = 4.648$, $p = 0.034$; effect of pupal vs 1-day old adult stage $F_{1, 78} = 31.39$, $p < 0.001$; Figure 1A). There was no effect of sex (Table S1). This is echoed in community structure (beta diversity) where we detected an interaction between life stage (specifically, pupal vs 1-day old adult) and adult presence (PERMANOVA $F_{1, 79} = 7.20$, $p < 0.001$). Distinct separation occurred in the bacterial communities of adult presence and absence groups in the pupal stage (Figure 1B), but not in the 1-day-old adults (Figure 1C). Again, there was no effect of sex (Table S2). *Lactobacillus plantarum, L. brevis* and *Corynebacterium* sp. in particular exhibited differential relative abundances dependent on life stage and the presence of adults (Table S3). A complete OTU table (to the genus level) for both pupal and adult life stages can be found in the data archive.

There were no effects of larval density on microbiome composition, but again we observed differences between pupae and 1-day old adults. Pupae generally displayed a greater species richness (alpha diversity) than their 1-day-old adult counterparts ($F_{1, 77} =$

35.37, p <0.001; Figure 1D) irrespective of density or sex (Table S4). Likewise, community structure (beta diversity) shows distinction between pupae and 1-day old adult flies ($F_{1, 78}$ = 4.52, p <0.001; Figure 1E), but this was not affected by density or sex (Table S5). Pupae showed increases in *Staphylococcus* sp., *Lactococcus* subsp. *lactis* and *Lactobacillus* sp. compared to adults (Table S6).

Adult social environment alters microbiome composition

We found that the effect of group housing on the microbiome of adult flies was dependent on age and sex. In 11-day-old flies, bacterial species richness (alpha diversity) was unaffected by social environment and sex (Table S7; Fig 2A). Likewise, community structure (beta diversity) was unaffected by social environment, but males and females had distinct communities (Table S8; Fig 2B). However, in 49-day-old flies, bacterial richness was significantly affected by social environment ($F_{1, 46}$ = 8.699, p = 0.0007) with co-aged groups having higher richness compared to single flies or those in mixed-age groups (Table S7; Fig 2C). Community structure was driven by an interaction between social environment and sex ($F_{1, 47}$ = 12.920, p < 0.0001; Fig 2D), consistent with the hypothesis that intrinsic factors (e.g. host stress/immune responses) are at play. To understand this interaction further, we split the data by sex and found that in males there was a highly significant effect of social environment on community structure ($F_{1, 22}$ = 14.054, p < 0.0001), but not in females ($F_{1, 22}$ = 2.188, p = 0.099).

There was no significant effect of social environment on relative levels of individual bacterial species, though there were effects of sex and age. Females have significantly lower levels of *Lactobacillus plantarum* and *L. brevis* compared to males (Table S9). Effects of age were only observed in males (Table S10) with young flies having significantly less *L. plantarum* and *L. brevis* than old flies.

Socially-driven changes in microbiomes likely affect host ageing and immunity

We hypothesised that the socially-driven effects of microbiome alteration in early life (and subsequent predicted functional pathway changes, Figure S2; Table S11) were likely to have lasting effects in adulthood and chose to examine lifespan as an easily tested gross phenotype. We found that the presence of adults reduced lifespan (Fig 3A, Cox PH X^2_1 = 6.545, p = 0.011), whereas larval density had no effect (Fig 3B, Cox PH X^2_1 = 1.266, p = 0.261), though in both experiments females lived longer than males (Adult presence Cox PH X^2_1 109.27, p<0.001; Larval density Cox PH X^2_1 = 107.56, p<0.001). This echoes findings in adult social environments, where treatments showing differences in lifespan (same-sex contact reducing lifespan more in males) also show alterations in their microbial community.

To attempt to understand possible functional consequences of changes observed in the adult flies from different social conditions, we examined immune responses by carrying out an oral infection assay, as a healthy microbiome, and in particular the presence of *L. plantarum,* can protect against infections (Blum et al., 2013). We have previously shown that social contact can increase survival after infection (Leech et al., 2019), however our mode of infection was injection, which therefore bypassed the gut microbiome. We predicted that if social contact caused dysbiosis then we would find post-infection survival reduced if the infection was orally acquired. Indeed, we found that isolated males had greater survival after oral infection with *Pseudomonas fluorescens* than grouped males (X^2_1 = 8.294, p = 0.004; Figure S3A), but there was no social effect in females (X^2_1 = 0.699, p = 0.403), mirroring the patterns in the microbial community. However, we could not link this to alterations of particular bacterial species, i.e. differences in relative abundance of the protective *L. plantarum*. We tested whether this could be driven by males ingesting more of the pathogen. Paired males did not eat more than those held singly so it is unlikely that fewer survived because they consumed more infected food (X^2_1 = 14.312, p = 0.852; Figure S3B). We also found that paired females ate more than single females (X^2_1 = 25.375, p = 0.044), and this

social effect on appetite deserves further investigation. In combination with our predicted gene function analysis (Figure S4; Tables S12-17) this indicates that changes in the microbiome could explain why males are susceptible to the immunological and longevity costs of same-sex social contact.

Discussion

We found that the fly microbiome was sensitive to the social environment in a sex, age and life-stage dependent manner, and that these changes could have functional effects on fly immunity and lifespan, in line with the idea that it may be a mediator of social effects on health.

The presence of adults during development alters the microbiome of pupae

Our prediction that more complex social environments would impact microbiome composition was only borne out for the manipulation of adult presence that displayed an increased microbial diversity in pupae. No difference in microbial diversity was detected for larval density. Similarly, Henry, Tarapacki, and Colinet (2020) found no difference in gut microbiota in larvae reared at different densities, despite changes in substrate microbial communities. We chose these social manipulations as they signal future sperm competition to males, hence induce differences in male development and are potentially stressful for males (Bretman et al., 2016; McDowall et al., 2019). In particular, males reared in the presence of adults or from high larval densities develop larger accessory glands (Bretman et al., 2016). However, their effects on development are not identical, suggesting that they convey different social information. We previously found differences in the learning abilities of males in a sexual-context learning assay in those from a low larval density compared to those from a higher density, but not in those reared with adult males present (McDowall et al., 2019). Given these differences, it is perhaps unsurprising that their effect on the

microbiome is likewise not the same. We measured the microbiome at the end of development when flies could be sexed, before and after metamorphosis (pupae and 1-day old adults). This adult age is in line with our previous work (McDowall et al., 2019) and was also designed to capture information about early life stage effects in the young adult flies, whilst reducing potential confounding effects of the adult conditions. The lack of sex differences in the microbiome at this stage suggests that the underlying mechanism is not associated with the (potentially costly) alterations in development of males to signals of future mating competition (Bretman et al., 2016).

There is still much discussion on the relative contributions and effects of horizontal versus vertical transmission (Fine, 1975; Ebert, 2013). It is generally assumed that horizontal transfer is increased under higher host density conditions, yet vertical transmission is expected to increase under conditions that promote host fecundity, usually lower densities (Ebert, 2013). Whilst some symbionts use only one mode of transmission, for example, *Buchnera aphidicola* is vertically transmitted in aphids (Chong et al., 2019), many are predicted to employ both forms of transmission (Ebert, 2013), yet measuring the relative contribution remains difficult and can be confounded by the complexity of diverse microbiome systems. For example, there is no evidence for similarity amongst the microbiota of sponge siblings, suggesting poor vertical transmission from parents to offspring in these species, despite predicted fitness benefits of efficient vertical transmission between the generations (Bjork et al., 2019). Indeed, whilst both modes are individually predicted to lead to evolutionary changes in traits of combined host and microbiome (the 'holobiont') (Roughgarden et al., 2020), modelling has also suggested that mixed mode transmission (horizontal and vertical) can lead to persistent and higher frequency associations between hosts and bacteria (Leftwich et al., 2020). In our study, we cannot rule out that there was an effect of horizontal microbial transfer from the adults, especially as the presence of adult females improves larval survival partly through inoculating the substrate with yeasts that are

an important component of larval diet (Wertheim et al., 2002). Indeed, adult flies will seed new environments with their associated microbiome (Tefit et al., 2018), providing a distinct mechanism for transmission between generations. Furthermore, as our pupae were not surface sterilised, it is also possible that bacteria deposited by the adults in the adult presence conditions onto the pupal case could be a contributory factor. Interestingly, this also has the potential to be another source of microbiome seeding in newly eclosed adult flies.

Regardless of sex or social manipulation, we found that pupae had a greater species richness than young adults, in line with results observed by Wong et al. (2011). This is perhaps unsurprising given that pupae undergo large modifications before eclosion, including expression of antimicrobial peptide genes (Tryselius, Samakovlis, Kimbrell, & Hultmark, 1992), which may regulate the bacterial community (Broderick & Lemaitre, 2012), decreasing the number of bacterial taxa observed (Wong et al., 2011). We found increased relative abundance of *Lactobacillus plantarum* in pupae reared with adults. This bacterium has been shown to affect larval growth through the TOR kinase nutrient signalling pathway under poor nutrient conditions (Storelli et al., 2011). A second *Lactobacillaceae, L. brevis*, was also elevated in the adult presence pupae. Under dysbiotic conditions, this bacterium can induce inflammation in the gut (Lee et al., 2013). Additional functional analyses (Fig S2; Table S11) suggests that in pupae, adult presence could potentially increase the differential enrichment of the *FoxO* and longevity pathways, but decrease the enrichment of the apoptosis pathway, though caution must be taken given the inferred nature of this analysis. Further investigation is required, but it is possible that if these alter developmental trajectories (e.g. through *FoxO* activity (Mirth et al., 2014)) they could have long lasting effects even though microbial community alteration itself did not carry-over into adulthood. Changes in nutrient storage (Ridley et al., 2012) or immune activity (Iatsenko et al., 2018) are potential mechanisms for effects transmitted beyond metamorphosis. We found that,

451 remarkably, lifespan of both sexes was reduced by the presence of adult males during the
452 larval stage. The lack of difference in lifespan between the larval density groups suggests
453 this is not related to the increased investment in production of male reproductive tissues that
454 is found both in males reared at high larval density or with adult males present during larval
455 development (Bretman et al., 2016). We have previously suggested that these manipulations
456 convey different social information (McDowall et al., 2019), and the effects we see here may
457 be part of that. Nevertheless, we remain cautious and acknowledge that the effects of the
458 presence of adults could be mediated by mechanisms unrelated to the microbiome. Further
459 investigation is required to determine any causal links.

460

461 **Adult social environment alters microbiome composition**

462 The sex specific patterns we observe on microbiome composition in response to social
463 environment indicates that extrinsic factors, such as shared diet or direct bacterial transfer,
464 are unlikely to be solely responsible for the patterns we observe, as these ought to affect
465 males and females equally. Likewise, whilst we did not measure bacterial titres, one would
466 expect these to change to the same extent in males and females if it is simply a function of
467 social contact. Previous work has shown that sex differences in the microbiome become
468 apparent in older adult flies (Wong et al., 2011) and the effect of the microbiome on fly
469 metabolism is sex-specific (Wong et al., 2014). Furthermore, there are sex-specific
470 differences in gut function and morphology during ageing (Regan et al., 2016). The social
471 manipulation we used causes sex differences in lifespan, suggesting that it is more stressful
472 for males than females, or prompts differential investment in physiological processes
473 underlying lifespan-reproduction trade-offs (Flintham et al., 2018; Leech et al., 2017). There
474 is increasing evidence for a reciprocal relationship between host stress responses and the
475 microbiome (Foster, Rinaman, & Cryan, 2017), and one direct source of social stress is
476 aggressive interactions. In mice, aggression between males affects colonic mucosa-
477 associated bacterial communities, reducing the relative abundance of key genera including

Lactobacillus (Galley et al., 2014). In *D. melanogaster,* males are more aggressive to each other than females, however we have previously been unable to relate levels of aggression to sex-specific patterns in senescence (Bretman et al., 2013; Leech et al., 2017). Males respond to sexually competitive environments by increasing mating duration and therefore reproductive fitness (Bretman et al., 2009), but this comes at the cost of lifespan and successful later-life mating attempts (Bretman et al., 2013). If investment in reproduction trades-off with immunosenescence, the result could be quicker ageing and more severe microbial dysbiosis in grouped males. However, neither of these scenarios explain why the effect of grouping on male microbiomes can be ameliorated by housing with young males. There is some evidence that the age of social companions has differential effects on ageing profiles. Males carrying a mutation in the antioxidant enzyme *Sod* have extended lifespan if housed with young males, perhaps because young social partners increased the activity of the focal flies (Ruan & Wu, 2008). Whether this increased activity drives the extension of lifespan, or is a symptom of a less stressful social context, and how this relates to the fly microbiome, remains unclear. However, we are cautious about drawing further conclusions as, due to logistical reasons, our mixed-age treatment were novel to the focal fly whereas the co-aged groups were not. Further tests are required to distinguish fully between the effect of social partner age and social familiarity and to investigate the possibility that young flies seed the environment with a “healthy” microbiome.

The effects of same-sex social contact on male behaviour, ejaculate and gene expression can be observed on a timescale of hours to a few days (Hopkins et al., 2019; Mohorianu et al., 2017; Rouse & Bretman, 2016). However, we observed no effect on the microbiome of young flies, but rather only at older ages, in line with declines in functions, such as mating success (Bretman et al., 2013) and climbing ability (Leech et al., 2017). In *D. melanogaster*, microbial abundance increases with age (Guo et al., 2014), with all bacterial taxa increasing significantly and having major impacts on microbial community structure (Clark et al., 2015). One explanation for the lack of observed differences in young flies may

be that the effects of social stress only become apparent as the flies senesce and gene expression becomes less tightly controlled, allowing unchecked proliferation of gut bacteria that impacts gut homeostasis (Clark et al., 2015; Guo et al., 2014). Furthermore, activation of the JAK/Stat signalling pathway in ageing flies has been found to induce metaplasia, and ultimately lead to dysbiosis of the microbiota and dysplasia of the gut (Li et al., 2016). Such a cumulative rather than acute effect of social contact would again be suggestive of intrinsic effects of the social environment acting through the microbiota-gut-brain axis. A recent synopsis of the role that the insulin signalling pathway plays in relation to dietary restriction and longevity suggests that it has evolved as a general mediator of adaptive plasticity in response to a wide range of stimuli, not just those relating to diet (Regan, Froy, Walling, Moatt, & Nussey, 2020). The results of our functional analysis (S4; Tables S12-17), which implicated the insulin signalling pathway and its key transcriptional regulator, FoxO, appear to be in concordance with this idea; our results indicate that social environment is one such external factor, and that it may be acting via changes in the microbiome. Changes in the gut-brain axis associated with ageing are wide-ranging, and treatment with pro- and pre-biotic formulations have been shown to have distinct consequences on longevity, metabolism, inflammatory activity and oxidative stress in flies, suggesting that changes in the microbiome can have broad effects with age (Westfall, Lomis, & Prakash, 2018). Given the multiple studies that link the microbiome with ageing in flies (e.g. Brummel et al., 2004, Guo et al 2014, Sannino et al., 2018, Lee et al 2019, Matthews et al., 2020) this could prove an excellent model for examining potential causal relationships between the environment, microbiome and host health.

Acknowledgments and author contributions: Thanks to Zahra Nikakhtari and James Rouse for help with fly work. This work was supported by a University of Leeds 110 Anniversary PhD scholarship to TL, a Boothman, Reynolds and Smithells Scholarship to LM, a ZSL Mission Opportunity Fund grant and Royal Society Research grant (RG130550) to XAH and a University of Leeds Academic fellowship to AB. AB, SMS, LM, TL and XH designed the research; LM, TL, KH and AB performed the research. LM, TL and XH analysed the data. The manuscript was written by all authors.

Data accessibility: Sequencing data has been submitted to the NCBI Sequence Read Archive (PRJNA565891, PRJNA565929, PRJNA565132), and all other data are freely accessible from the Leeds Research Data Repository (https://doi.org/10.5518/985).

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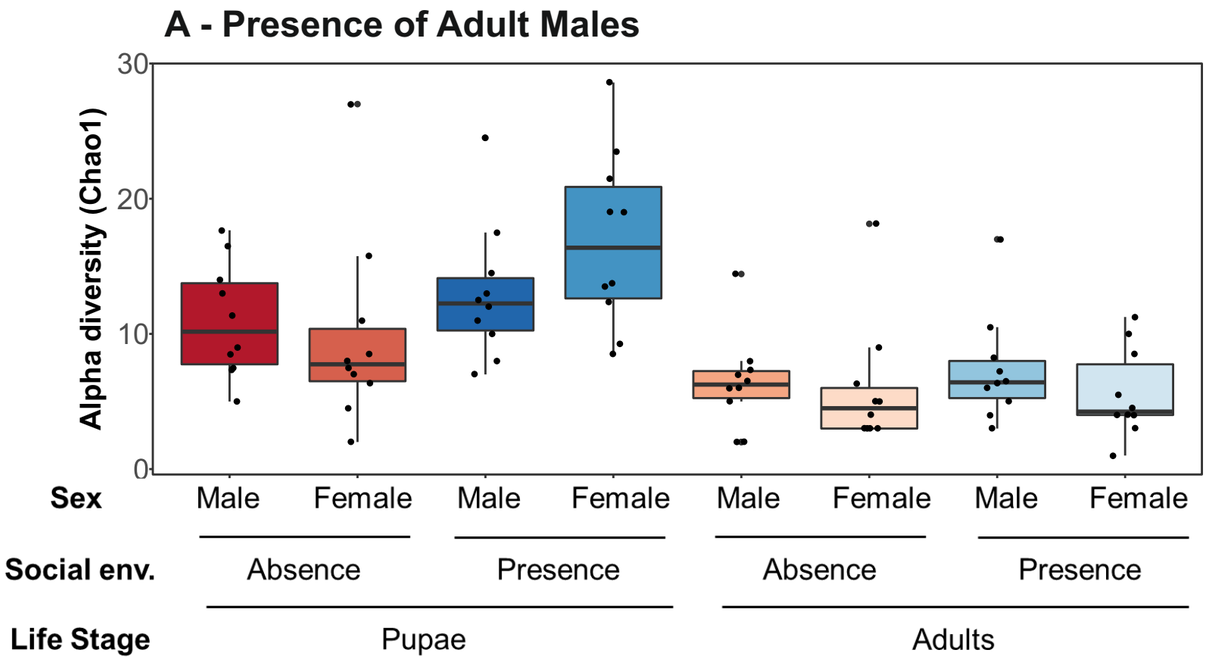
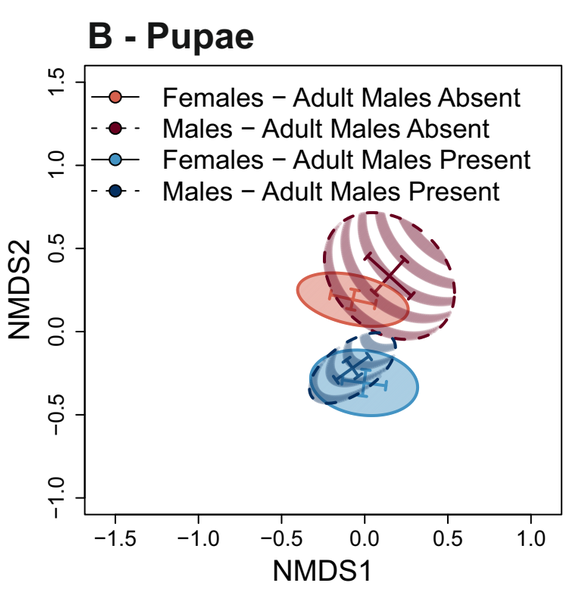
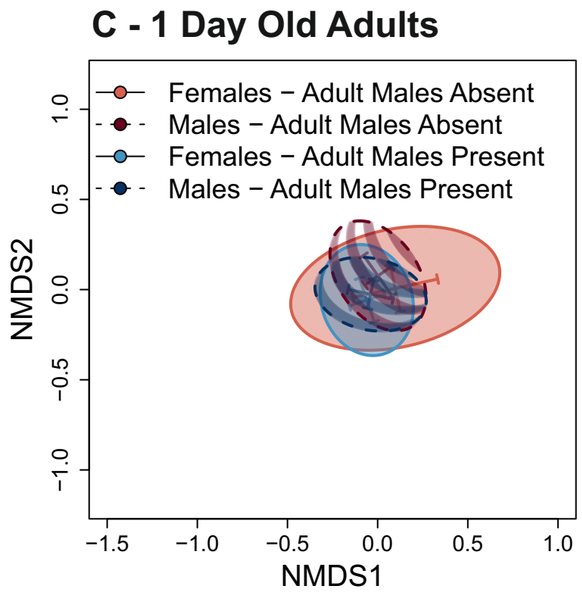
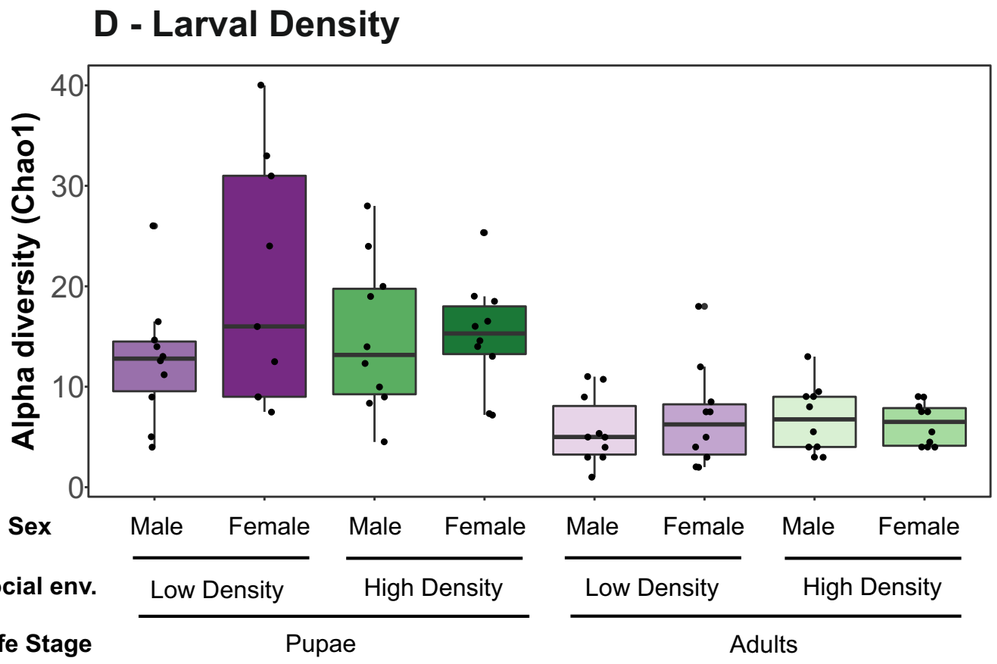
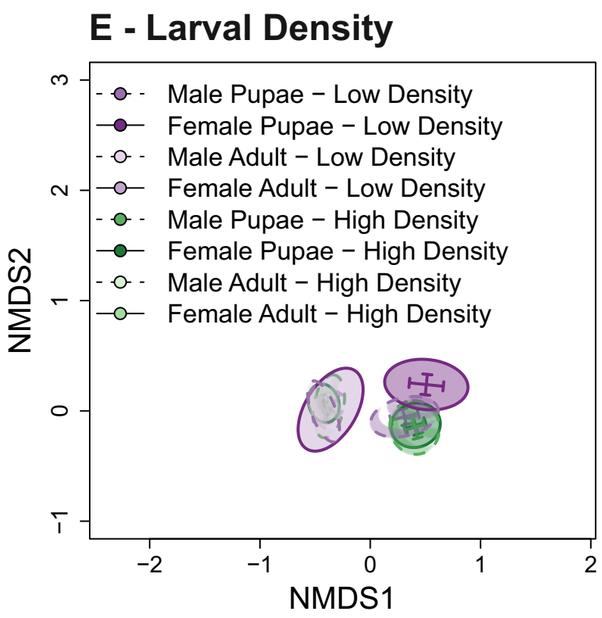
820 **Figure legends**
821
822
823 **Figure 1 The presence of adults, but not larval density, during larval development**
824 **alters fly microbiomes**. A-C) Larvae were reared in the “Absence” or “Presence” of adult
825 male flies or D-E) were reared low (20) or high (200) density. Flies were sampled as “Pupae”
826 or 1-day-old “Adults”, with males and females analysed separately. Microbiome composition
827 was measured as (A and D) species richness (alpha diversity using the Chao1) and
828 community structure (beta diversity visualised as NMDS plots using Bray-Curtis Dissimilarity
829 Index with 95% confidence ellipses and S.E. bars, with dashed lines for males; solid lines for
830 females) for pupae (B) and 1-day old adults (C) separately for those raised in the presence
831 or absence of adults, or E) all larval density groups together.
832
833
834 **Figure 2 Group housing affects the microbiome of older adult flies.** Flies were housed
835 singly or in same-sex groups and were harvested at 11 days (A-B) or 49 days (C-D) post
836 eclosion. For 49-day old flies, groups were either “Co-aged” with the focal fly or were 1-7
837 days old (“Mixed”). Microbiome composition was measured as (A and C) species richness
838 (alpha diversity using the Chao1) and (B and D) community structure (beta diversity
839 visualised as NMDS plots using Bray-Curtis Dissimilarity Index with 95% confidence ellipses
840 and S.E. bars).
841
842 **Figure 3 Larval social environment alters adult lifespan.** Lifespan of male and female
843 flies raised (A) in the absence or presence of adults and (B) at low or high density.
844


A - Presence of Adult Males
Alpha diversity (Chao1)
30
20
10
0
Sex
Male
Female
Male
Female
Male
Female
Male
Female
Social env.
Absence
Presence
Absence
Presence
Life Stage
Pupae
Adults

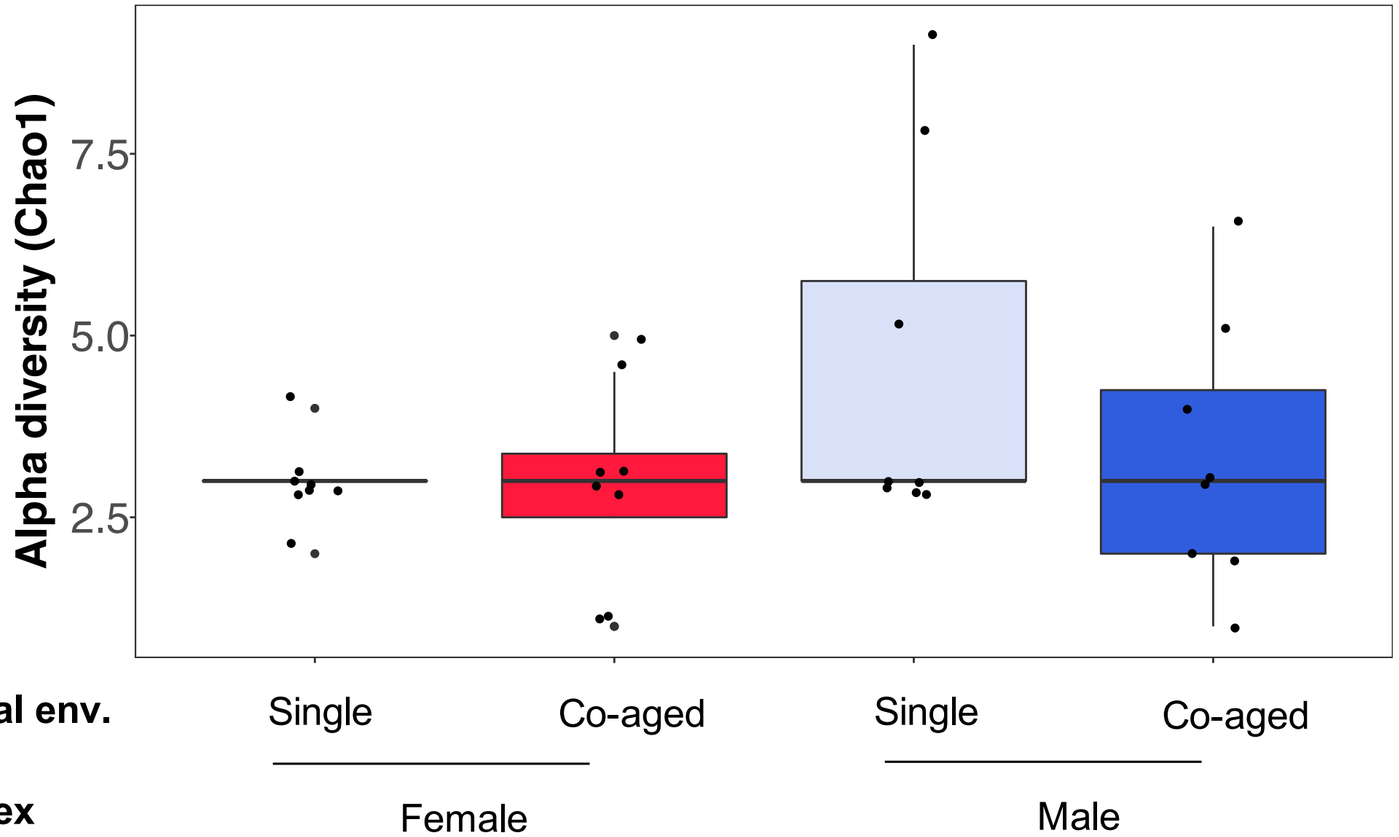

B - Pupae
Females − Adult Males Absent
Males − Adult Males Absent
Females − Adult Males Present
Males − Adult Males Present
NMDS2
1.5
1.0
0.5
0.0
−0.5
−1.0
−1.5
−1.0
−0.5
0.0
0.5
1.0
NMDS1


C - 1 Day Old Adults
Females − Adult Males Absent
Males − Adult Males Absent
Females − Adult Males Present
Males − Adult Males Present
NMDS2
1.0
0.5
0.0
−0.5
−1.0
−1.5
−1.0
−0.5
0.0
0.5
1.0
NMDS1

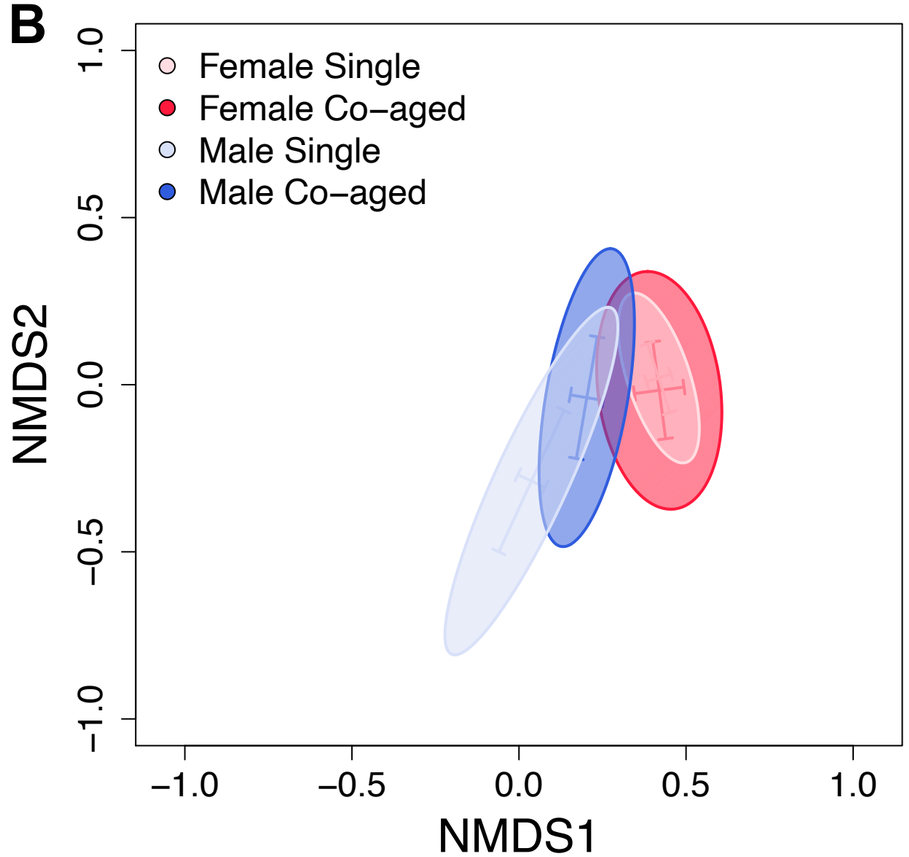

D - Larval Density
Alpha diversity (Chao1)
40
30
20
10
0
Sex
Male
Female
Male
Female
Male
Female
Male
Female
Social env.
Low Density
High Density
Low Density
High Density
Life Stage
Pupae
Adults


E - Larval Density
Male Pupae − Low Density
Female Pupae − Low Density
Male Adult − Low Density
Female Adult − Low Density
Male Pupae − High Density
Female Pupae − High Density
Male Adult − High Density
Female Adult − High Density
NMDS2
3
2
1
0
−1
−2
−1
0
1
2
NMDS1

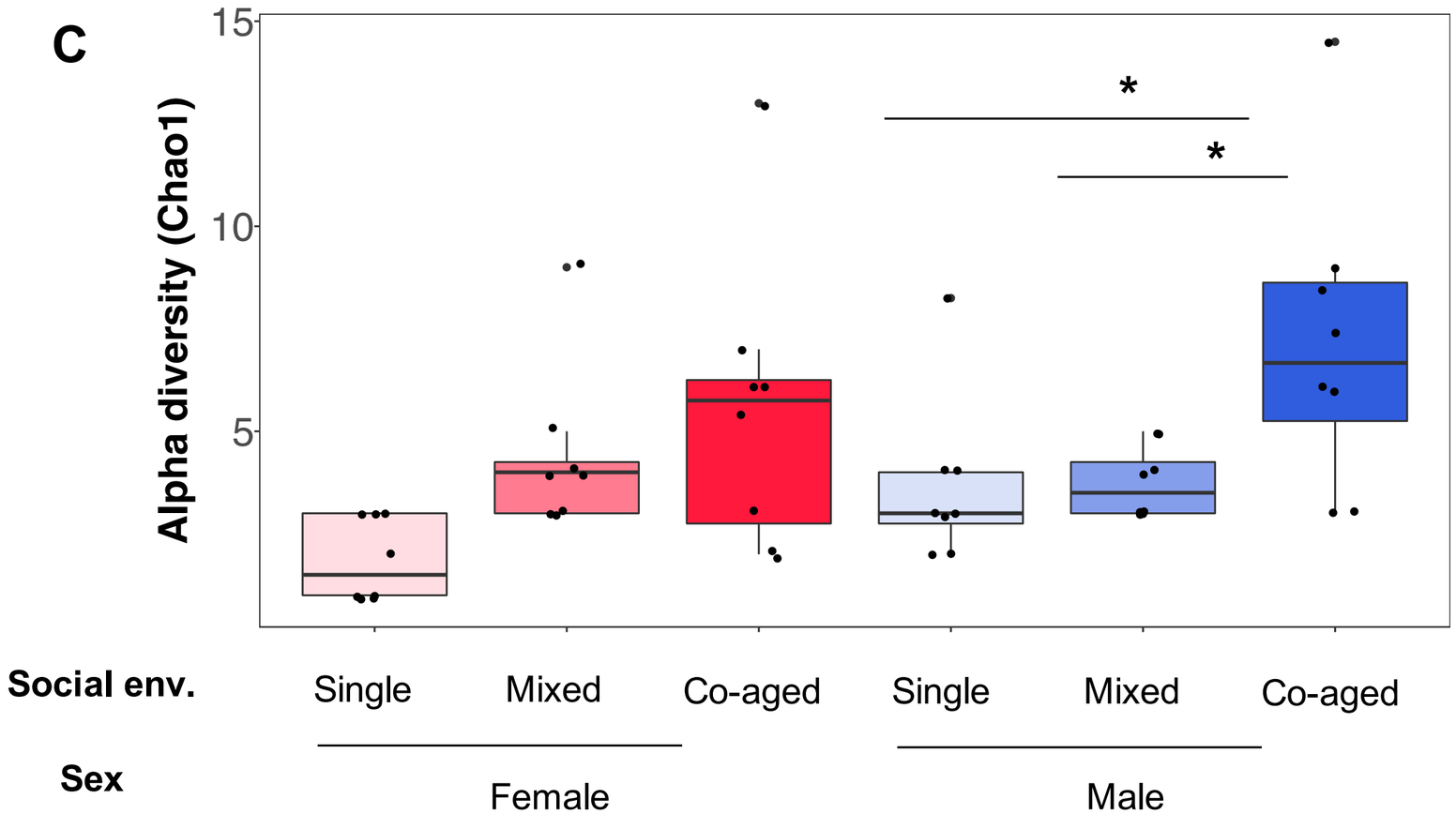
A


Alpha diversity (Chao1)
7.5
5.0
2.5
Social env.
Single
Co-aged
Single
Co-aged
Sex
Female
Male

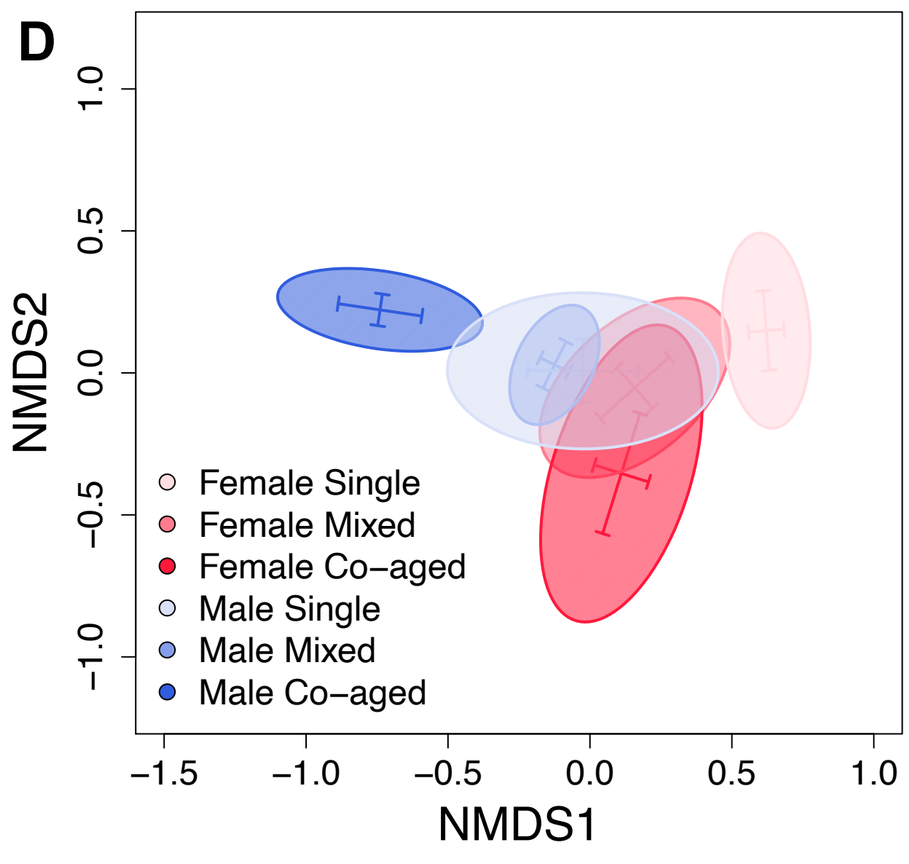
B

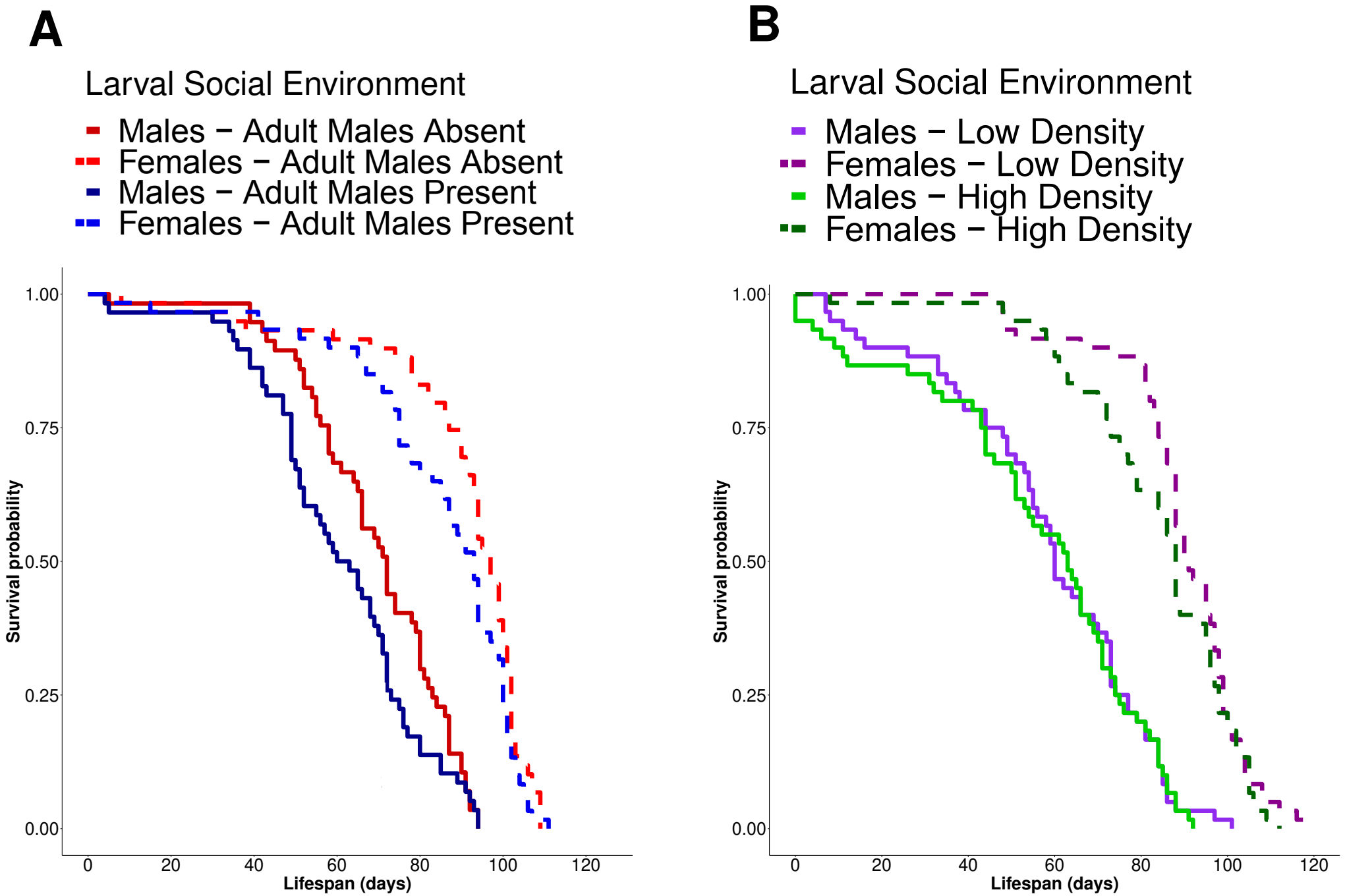

Female Single
Female Co–aged
Male Single
Male Co–aged
NMDS2
1.0
0.5
0.0
−0.5
−1.0
−1.0
−0.5
0.0
0.5
1.0
NMDS1

C


Alpha diversity (Chao1)
15
10
5
*
*
Social env.
Single
Mixed
Co-aged
Single
Mixed
Co-aged
Sex
Female
Male

D


Female Single
Female Mixed
Female Co–aged
Male Single
Male Mixed
Male Co–aged
NMDS2
1.0
0.5
0.0
−0.5
−1.0
−1.5
−1.0
−0.5
0.0
0.5
1.0
NMDS1


A
Larval Social Environment
Males − Adult Males Absent
Females − Adult Males Absent
Males − Adult Males Present
Females − Adult Males Present
Survival probability
1.00
0.75
0.50
0.25
0.00
0
20
40
60
80
100
120
Lifespan (days)
B
Larval Social Environment
Males − Low Density
Females − Low Density
Males − High Density
Females − High Density
Survival probability
1.00
0.75
0.50
0.25
0.00
0
20
40
60
80
100
120
Lifespan (days)