

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

3

4 **Running title: Social effects on fly microbiomes**

5

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28

29 **Abstract**

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

51

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

53

54

## 55 **Introduction**

56 Social environments have multiple effects on individual health, including immune responses  
57 (Cole, 2014; Leech, Evison, Armitage, Sait, & Bretman, 2019), ageing and ultimately lifespan  
58 (Flintham et al., 2018; Hawkley & Capitanio, 2015; Leech, Sait, & Bretman, 2017). Indeed,  
59 meta-analyses show that adverse social environments are a health risk factor on a par with  
60 obesity and smoking (Holt-Lunstad, Smith, & Layton, 2010). Effects of social environments  
61 are complex. They are seen in animals not usually thought of as social, there are marked  
62 sex differences, and hence what constitutes a stressful social environment is not  
63 straightforward (Flintham et al., 2018; Leech et al., 2019; Leech et al., 2017). For example,  
64 periods of social isolation can be beneficial even in gregarious species (Bailey & Moore,  
65 2018). The mechanisms that translate social information into these effects are unclear, but it  
66 has been suggested that the microbiome (the community of microorganisms living  
67 symbiotically with a host) plays a role (Flintham et al., 2018).

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

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

83         So far, the influence of host social interactions on microbiome composition has been  
84 investigated largely in mammals. Similarities in microbiomes driven by cohabitation, social  
85 group membership or social networks seen in ring-tailed lemurs (*Lemur catta*) (Bennett et  
86 al., 2016), wild baboons (*Simia hamadryas*) (Tung et al., 2015) and humans (Song et al.,  
87 2013) likely represent extrinsic effects of social environments. However, recent evidence  
88 demonstrates that the social environment has effects on microbiome beyond impacting  
89 transmission. In mice, social stress alters gut immune gene expression and their gut  
90 microbial community (Galley et al., 2014) and faecal transfers from mice stressed through  
91 isolation recapitulates isolation behaviours in non-isolated mice (Gacias et al., 2016). Far  
92 less is known about effects in invertebrates, though progress has been made in colony-living  
93 bees. Microbiota derived from faeces of nestmates protect bumble bees from parasitic  
94 infection (Koch and Schmid-Hempel 2011), and isolated queen honey bees show a larger  
95 and more diverse microbiome compared to those held with workers (Powell, Eiri, Moran, &  
96 Rangel, 2018). To broaden our understanding of these effects, we used an invertebrate  
97 model system in which simple experimental manipulations of social contact alter ageing and  
98 lifespan.

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

108 same-sex partners (Leech et al., 2019). Males use the presence of other males as a cue of  
109 potential sperm competition, making sophisticated adjustments to their reproductive  
110 behaviour and ejaculate (Bretman, Fricke, & Chapman, 2009; Hopkins et al., 2019). During  
111 development, larval density can alter growth rates and adult body size, and the prior  
112 presence of adults on food substrates can increase larval survival (Wertheim, Marchais, Vet,  
113 & Dicke, 2002). This could occur via the provision of beneficial bacterial species from the  
114 adults to the larvae. Furthermore, the flies could also gain an advantage from the  
115 competitive inhibition of potentially pathogenic bacterial or fungal species (Wertheim,  
116 Marchais, Vet and Dicke, 2002). In addition, when food resources are not limiting, both  
117 higher density and the presence of adult males (cues of future sperm competition) stimulate  
118 males to develop larger accessory glands (Bretman, Fricke, Westmancoat, & Chapman,  
119 2016), and males raised at lower density are better at learning when adult (McDowall,  
120 Rouse, Sait, & Bretman, 2019).

121

122         The fly microbiome affects a range of traits including development (Shin et al., 2011),  
123 metabolism (Wong, Dobson, & Douglas, 2014), immune responses (Blum, Fischer, Miles, &  
124 Handelsman, 2013) and longevity (Guo et al., 2014). The fly microbiome is relatively simple  
125 (Broderick & Lemaitre, 2012) and its composition changes across life stages and ages  
126 (Wong, Ng, & Douglas, 2011), though it is seeded primarily at hatching when larvae eat the  
127 faeces-covered chorion (Bakula 1969). Bacterial species richness increases in the gut during  
128 larval stages, then decreases significantly during metamorphosis (Wong et al., 2011). This  
129 decrease is associated with a large increase in host immune factors, such as antimicrobial  
130 peptides (Broderick, 2016). However, there is the possibility that bacteria could be  
131 transmitted from pupal to adult stages after being maintained in the larval midgut (Broderick  
132 and Lemaitre, 2012). Though transmission through metamorphosis has been observed for  
133 the bacterium *Enterococcus mundtii* in *Galleria mellonella* wax moths (Johnston and Rolff,  
134 2015), as well as the pathogenic *Providencia rettgeri* in *Drosophila* (Duneau and Lazarro,

135 2018), more work is required to establish whether this is indeed a mode of transmission  
136 between life stages. Differences in microbiome community are driven by the environment,  
137 for example wild-caught versus laboratory rearing, or maintenance on different food sources  
138 (Staubach, Baines, Kunzel, Bik, & Petrov, 2013). Larvae gain gut microbes through ingestion  
139 of their egg casing and from their food, and this environmental replenishment continues  
140 during adulthood (Blum et al., 2013), so extrinsic effects of the social environment are likely.

141         Beyond transmission effects, there is potential for intrinsic effects of social  
142 environments acting through the microbiota-gut-brain axis. Fly gene expression is socially  
143 sensitive, including immune, stress and lifespan related genes (Leech et al., 2019;  
144 Mohorianu et al., 2017). The host IMD pathway, primarily induced by Gram negative  
145 bacteria, is one mechanism used by hosts to control the microbiome (Iatsenko, I., Boquete,  
146 J. P., & Lemaitre, 2018). Mutants for the IMD pathway component PGRP-SD show a  
147 proliferation of *Lactobacillus plantarum* in the fly gut and a reduced lifespan (Iatsenko et al.,  
148 2018). Epigenetic regulation of the fly immune IMD pathway alters the microbiome and fly  
149 social behaviour (Chen et al., 2019). Similarly, opportunistic pathogenic bacteria can  
150 activate DUOX-dependent immune responses via uracil release, whilst many commensals  
151 do not (Lee et al., 2013). Likewise, the JAK-STAT pathway is involved in immune and stress  
152 responses in flies, and its activation can result in metaplasia, gut pH changes and dysbiosis  
153 (Li, Qi & Jasper, 2016). Further, this pathway is induced upon oral infection with *Erwinia*  
154 *carotovora* (Buchon, Broderick, Poidevin, Pradervand & Lemaitre 2009), suggesting it is  
155 another important factor in maintaining gut-microbiota homeostasis. Thus, disruption of  
156 immune pathways could have significant consequences on microbiome composition.  
157 Changes in the activation status of stress pathways could induce dysbiotic states, for  
158 example, the higher microbial loads observed in ageing flies (Clark et al., 2015). Ultimately,  
159 such dysbiosis influences ageing and lifespan. Maintaining flies axenically alters lifespan,  
160 though can both lengthen (e.g. Yamada, Deshpande, Bruce, Mak & Ja 2015; Galenza,  
161 Hutchinson, Campbell, Hazes, & Foley 2016; Téfit & Leulier 2017) or shorten lifespan

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

168

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

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

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

200

201

## 202 **Materials and Methods**

### 203 **Fly stocks and maintenance**

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

210

### 211 **Larval social environment**

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



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

221

## 222 **Adult social environment**

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

233

## 234 **16s rRNA sequencing and bioinformatics**

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

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

244

#### 245 **Microbiome statistical analysis**

246 All statistical analysis was conducted using R v3.3.2 (R Core Team, 2017) using the  
247 phyloseq (McMurdie & Holmes, 2013), vegan (Oksanen et al., 2012), ggplot2 (Wickham,  
248 2009), DESeq2 (Love, Huber, & Anders, 2014), and lme4 (Bates, Machler, Bolker, & Walker,  
249 2015) packages. Prior to analysis 18 contaminant Operational Taxonomic Units (OTUs)  
250 present in the negative controls were removed (Iulia, Bianca, Cornelia, & Octavian, 2013).  
251 One female pupal sample from the larval density treatment was identified as an extreme  
252 outlier (Grubb's test  $p < 0.05$ ) in number of OTUs (suggestive of contamination) and hence  
253 was removed from all subsequent analysis. Sequences were rarefied in order to normalise  
254 library sizes. For larval density, the data were rarefied to 20,140 sequences, and for adult  
255 presence to 22,718. For adult social environment groups, all were rarefied to 10,840  
256 sequences.

257         Alpha diversity was estimated using the Chao1 species richness indicator (Chao,  
258 1984). Predictors of alpha diversity were analysed using GLM with social environment, sex  
259 and life-stage/age as fixed factors. Models were simplified from the full model using Analysis  
260 of Deviance (AOD). We visualised differences in bacterial community structure among  
261 samples (beta diversity) using Non-metric Multidimensional Scaling (NMDS) plots of Bray-  
262 Curtis distances. We used PERMANOVA (with 1000 permutations) to examine the effects of  
263 social environment, sex and life stage/age on bacterial beta diversity. Our data conformed to  
264 a negative binomial distribution so we used DESeq2 (Love et al., 2014) to identify OTUs that  
265 differed significantly in relative abundance between groups. Where differentially-abundant  
266 OTUs were classified only to genus level, we cross-referenced the sequence in the

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

277

#### 278 **Effects of larval social environment on lifespan**

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

285

#### 286 **Effects of adult social environment on survival post-oral infection**

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

293 for death every 24 h for one week. We also confirmed that any patterns seen were not driven  
294 by a difference in amount of food eaten using a CAFÉ (CApillary FEeder) assay (Ja et al.,  
295 2007) (see SI). This assay allows for the measurement of food consumed in a given period  
296 through the changes in its level in a glass capillary used for food provision. Since post-  
297 infection lifespan data was limited to one week, a chi squared test was used to determine if  
298 the number of flies that died differed by sex and social environment.

299

## 300 **Results**

### 301 **The presence of adults, but not rearing density, during larval development alters**

#### 302 ***Drosophila* microbiomes**

303 In pupae, being raised in the presence of adults increased species richness measured as  
304 alpha diversity (effect of adult presence  $F_{1,77} = 4.648$ ,  $p = 0.034$ ; effect of pupal vs 1-day old  
305 adult stage  $F_{1,78} = 31.39$ ,  $p < 0.001$ ; Figure 1A). There was no effect of sex (Table S1). This  
306 is echoed in community structure (beta diversity) where we detected an interaction between  
307 life stage (specifically, pupal vs 1-day old adult) and adult presence (PERMANOVA  $F_{1,79} =$   
308  $7.20$ ,  $p < 0.001$ ). Distinct separation occurred in the bacterial communities of adult presence  
309 and absence groups in the pupal stage (Figure 1B), but not in the 1-day-old adults (Figure  
310 1C). Again, there was no effect of sex (Table S2). *Lactobacillus plantarum*, *L. brevis* and  
311 *Corynebacterium* sp. in particular exhibited differential relative abundances dependent on life  
312 stage and the presence of adults (Table S3). A complete OTU table (to the genus level) for  
313 both pupal and adult life stages can be found in the data archive.

314

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

318 35.37,  $p < 0.001$ ; Figure 1D) irrespective of density or sex (Table S4). Likewise, community  
319 structure (beta diversity) shows distinction between pupae and 1-day old adult flies ( $F_{1,78} =$   
320 4.52,  $p < 0.001$ ; Figure 1E), but this was not affected by density or sex (Table S5). Pupae  
321 showed increases in *Staphylococcus* sp., *Lactococcus* subsp. *lactis* and *Lactobacillus* sp.  
322 compared to adults (Table S6).

323

#### 324 **Adult social environment alters microbiome composition**

325 We found that the effect of group housing on the microbiome of adult flies was dependent on  
326 age and sex. In 11-day-old flies, bacterial species richness (alpha diversity) was unaffected  
327 by social environment and sex (Table S7; Fig 2A). Likewise, community structure (beta  
328 diversity) was unaffected by social environment, but males and females had distinct  
329 communities (Table S8; Fig 2B). However, in 49-day-old flies, bacterial richness was  
330 significantly affected by social environment ( $F_{1,46} = 8.699$ ,  $p = 0.0007$ ) with co-aged groups  
331 having higher richness compared to single flies or those in mixed-age groups (Table S7; Fig  
332 2C). Community structure was driven by an interaction between social environment and sex  
333 ( $F_{1,47} = 12.920$ ,  $p < 0.0001$ ; Fig 2D), consistent with the hypothesis that intrinsic factors (e.g.  
334 host stress/immune responses) are at play. To understand this interaction further, we split  
335 the data by sex and found that in males there was a highly significant effect of social  
336 environment on community structure ( $F_{1,22} = 14.054$ ,  $p < 0.0001$ ), but not in females ( $F_{1,22} =$   
337 2.188,  $p = 0.099$ ).

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

343

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

345 We hypothesised that the socially-driven effects of microbiome alteration in early life (and  
346 subsequent predicted functional pathway changes, Figure S2; Table S11) were likely to have  
347 lasting effects in adulthood and chose to examine lifespan as an easily tested gross  
348 phenotype. We found that the presence of adults reduced lifespan (Fig 3A, Cox PH  $X^2_1 =$   
349 6.545,  $p = 0.011$ ), whereas larval density had no effect (Fig 3B, Cox PH  $X^2_1 = 1.266$ ,  $p =$   
350 0.261), though in both experiments females lived longer than males (Adult presence Cox PH  
351  $X^2_1 = 109.27$ ,  $p < 0.001$ ; Larval density Cox PH  $X^2_1 = 107.56$ ,  $p < 0.001$ ). This echoes findings in  
352 adult social environments, where treatments showing differences in lifespan (same-sex  
353 contact reducing lifespan more in males) also show alterations in their microbial community.

354

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

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

375

## 376 **Discussion**

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

381

### 382 **The presence of adults during development alters the microbiome of pupae**

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

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

405

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



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

431

432         Regardless of sex or social manipulation, we found that pupae had a greater species  
433 richness than young adults, in line with results observed by Wong et al. (2011). This is  
434 perhaps unsurprising given that pupae undergo large modifications before eclosion,  
435 including expression of antimicrobial peptide genes (Tryselius, Samakovlis, Kimbrell, &  
436 Hultmark, 1992), which may regulate the bacterial community (Broderick & Lemaitre, 2012),  
437 decreasing the number of bacterial taxa observed (Wong et al., 2011). We found increased  
438 relative abundance of *Lactobacillus plantarum* in pupae reared with adults. This bacterium  
439 has been shown to affect larval growth through the TOR kinase nutrient signalling pathway  
440 under poor nutrient conditions (Storelli et al., 2011). A second *Lactobacillaceae*, *L. brevis*,  
441 was also elevated in the adult presence pupae. Under dysbiotic conditions, this bacterium  
442 can induce inflammation in the gut (Lee et al., 2013). Additional functional analyses (Fig S2;  
443 Table S11) suggests that in pupae, adult presence could potentially increase the differential  
444 enrichment of the *FoxO* and longevity pathways, but decrease the enrichment of the  
445 apoptosis pathway, though caution must be taken given the inferred nature of this analysis.  
446 Further investigation is required, but it is possible that if these alter developmental  
447 trajectories (e.g. through *FoxO* activity (Mirth et al., 2014)) they could have long lasting  
448 effects even though microbial community alteration itself did not carry-over into adulthood.  
449 Changes in nutrient storage (Ridley et al., 2012) or immune activity (Iatsenko et al., 2018)  
450 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

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

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

505 be that the effects of social stress only become apparent as the flies senesce and gene  
506 expression becomes less tightly controlled, allowing unchecked proliferation of gut bacteria  
507 that impacts gut homeostasis (Clark et al., 2015; Guo et al., 2014). Furthermore, activation  
508 of the JAK/Stat signalling pathway in ageing flies has been found to induce metaplasia, and  
509 ultimately lead to dysbiosis of the microbiota and dysplasia of the gut (Li et al., 2016). Such  
510 a cumulative rather than acute effect of social contact would again be suggestive of intrinsic  
511 effects of the social environment acting through the microbiota-gut-brain axis. A recent  
512 synopsis of the role that the insulin signalling pathway plays in relation to dietary restriction  
513 and longevity suggests that it has evolved as a general mediator of adaptive plasticity in  
514 response to a wide range of stimuli, not just those relating to diet (Regan, Froy, Walling,  
515 Moatt, & Nussey, 2020). The results of our functional analysis (S4; Tables S12-17), which  
516 implicated the insulin signalling pathway and its key transcriptional regulator, FoxO, appear  
517 to be in concordance with this idea; our results indicate that social environment is one such  
518 external factor, and that it may be acting via changes in the microbiome. Changes in the gut-  
519 brain axis associated with ageing are wide-ranging, and treatment with pro- and pre-biotic  
520 formulations have been shown to have distinct consequences on longevity, metabolism,  
521 inflammatory activity and oxidative stress in flies, suggesting that changes in the microbiome  
522 can have broad effects with age (Westfall, Lomis, & Prakash, 2018). Given the multiple  
523 studies that link the microbiome with ageing in flies (e.g. Brummel et al., 2004, Guo et al  
524 2014, Sannino et al., 2018, Lee et al 2019, Matthews et al., 2020) this could prove an  
525 excellent model for examining potential causal relationships between the environment,  
526 microbiome and host health.

527

528

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536

537 **Data accessibility:** Sequencing data has been submitted to the NCBI Sequence Read  
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540

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- 819

820 **Figure legends**

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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.

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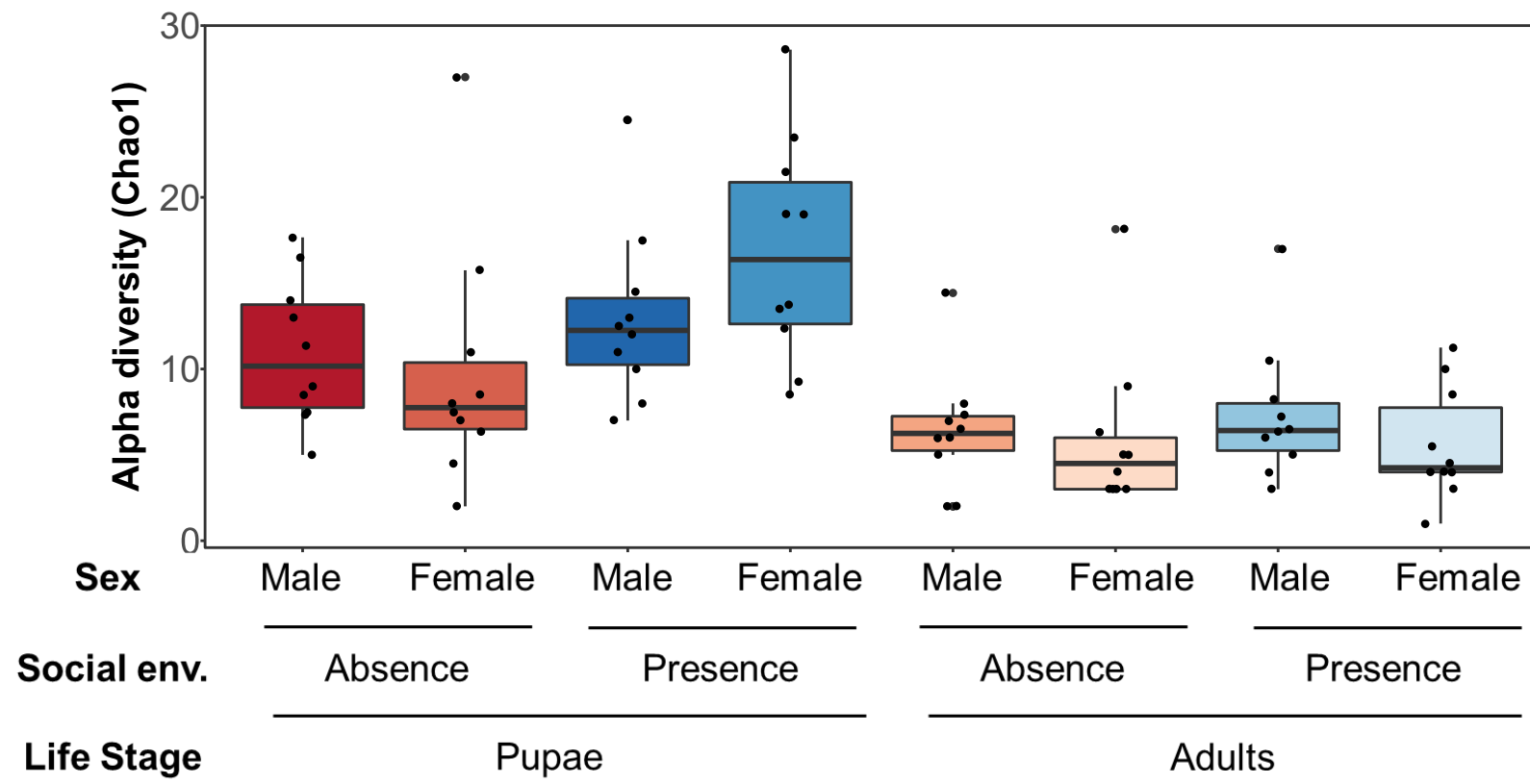
834 **Figure 2 Group housing affects the microbiome of older adult flies.** Flies were housed  
835 singly or in same-sex groups and were harvested at 11 days (A-B) or 49 days (C-D) post  
836 eclosion. For 49-day old flies, groups were either “Co-aged” with the focal fly or were 1-7  
837 days old (“Mixed”). 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).

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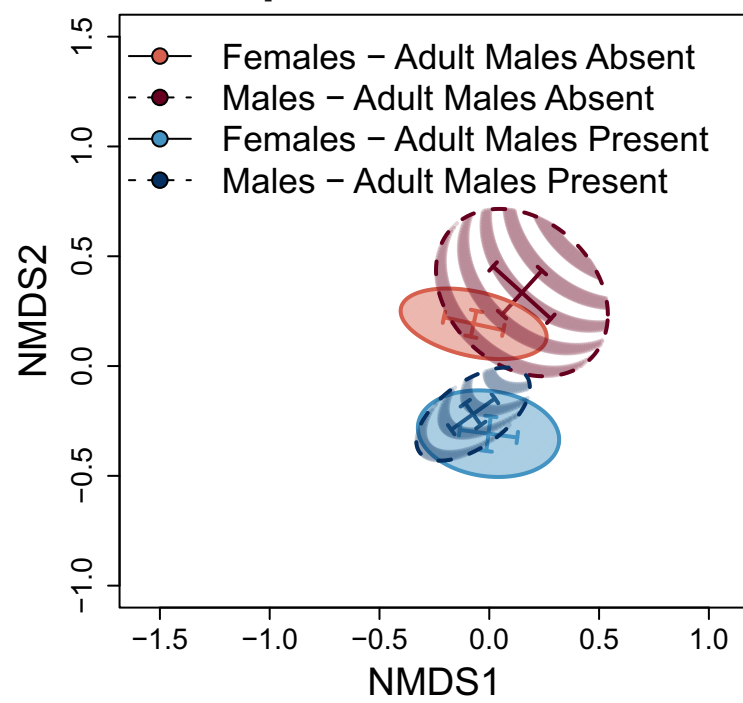
842 **Figure 3 Larval social environment alters adult lifespan.** Lifespan of male and female  
843 flies raised (A) in the absence or presence of adults and (B) at low or high density.

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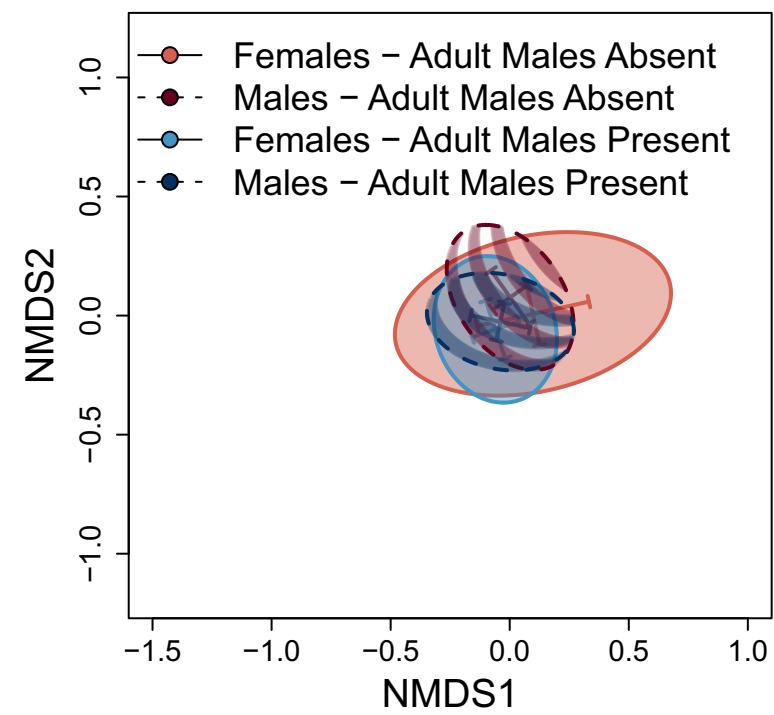
### A - Presence of Adult Males



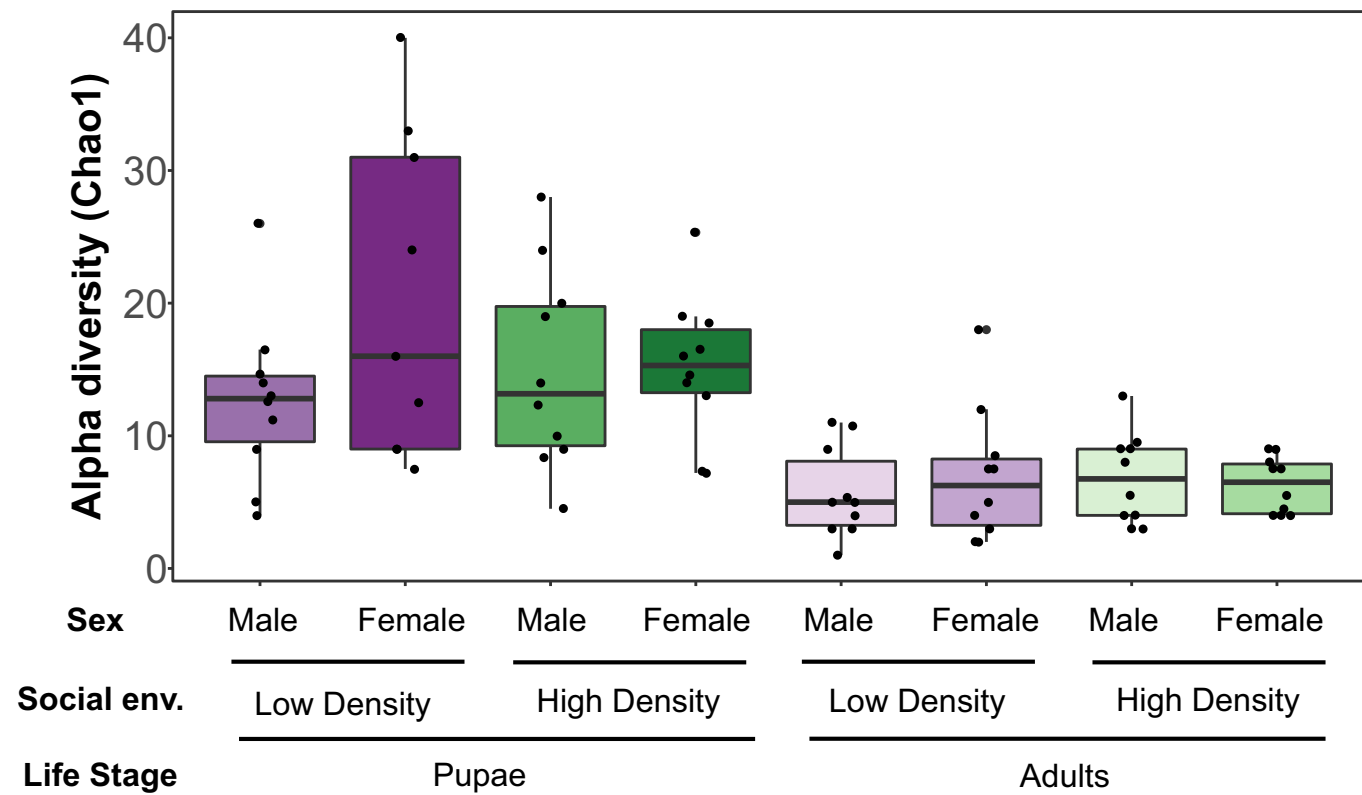
### B - Pupae



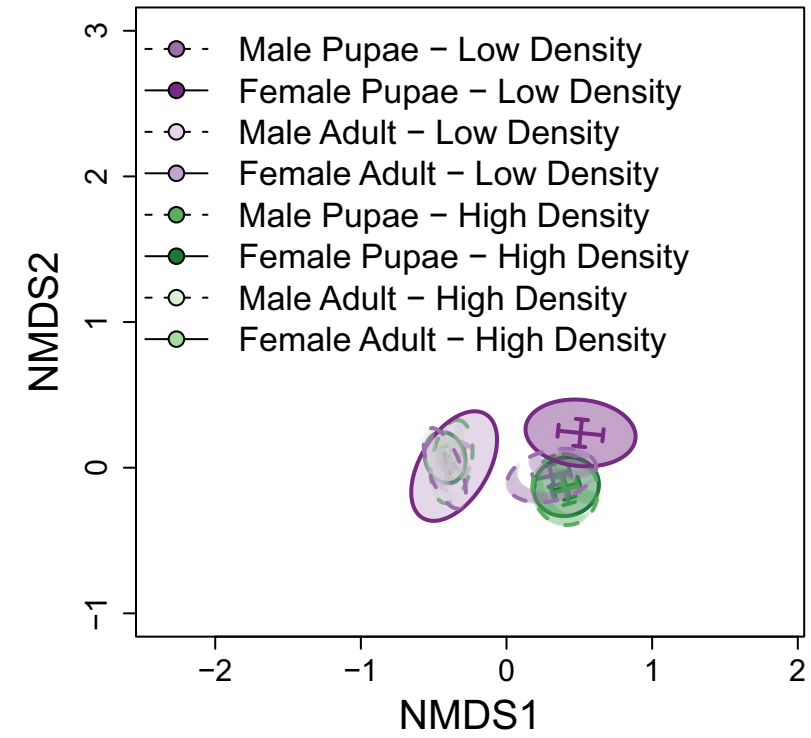
### C - 1 Day Old Adults

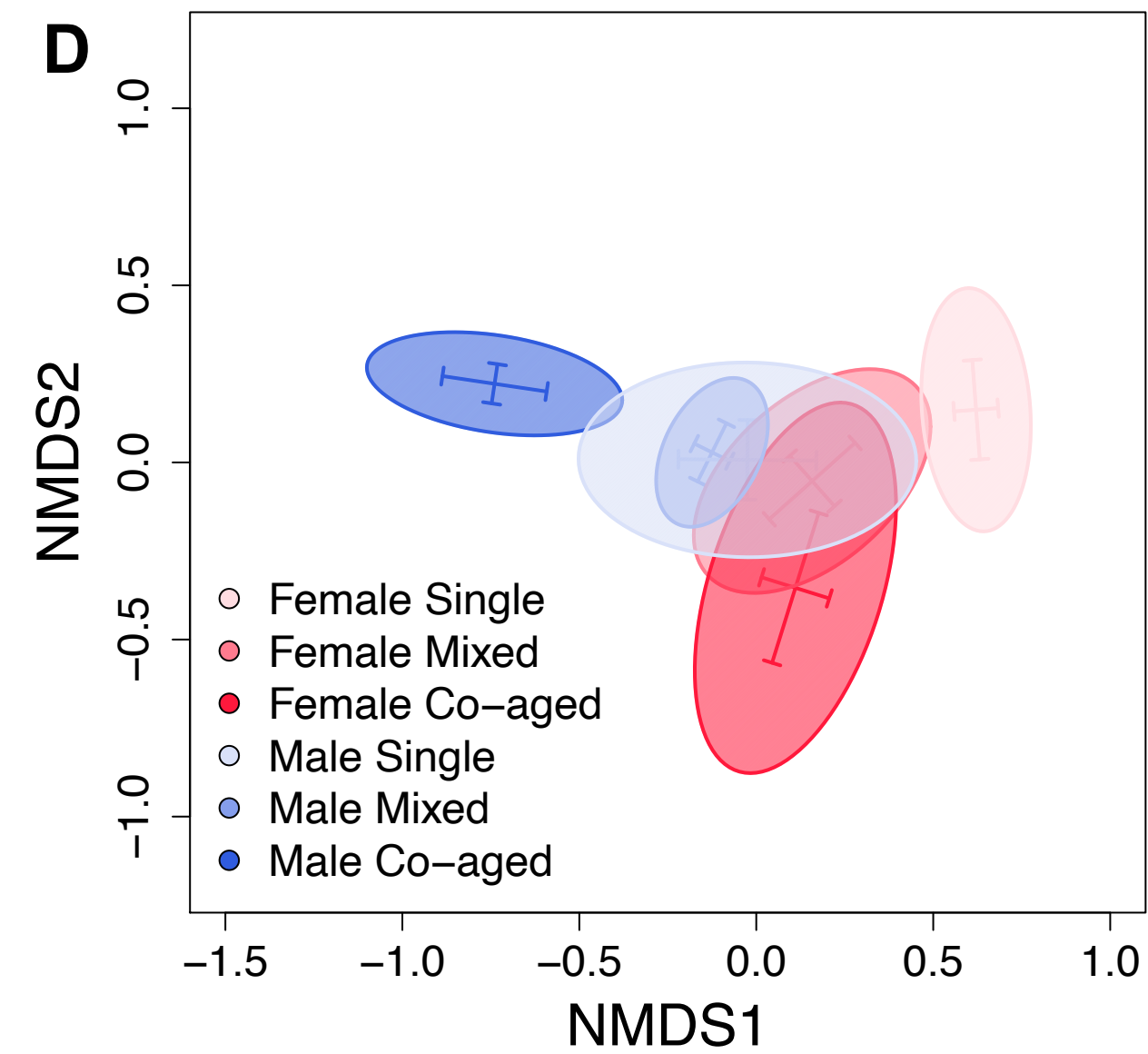
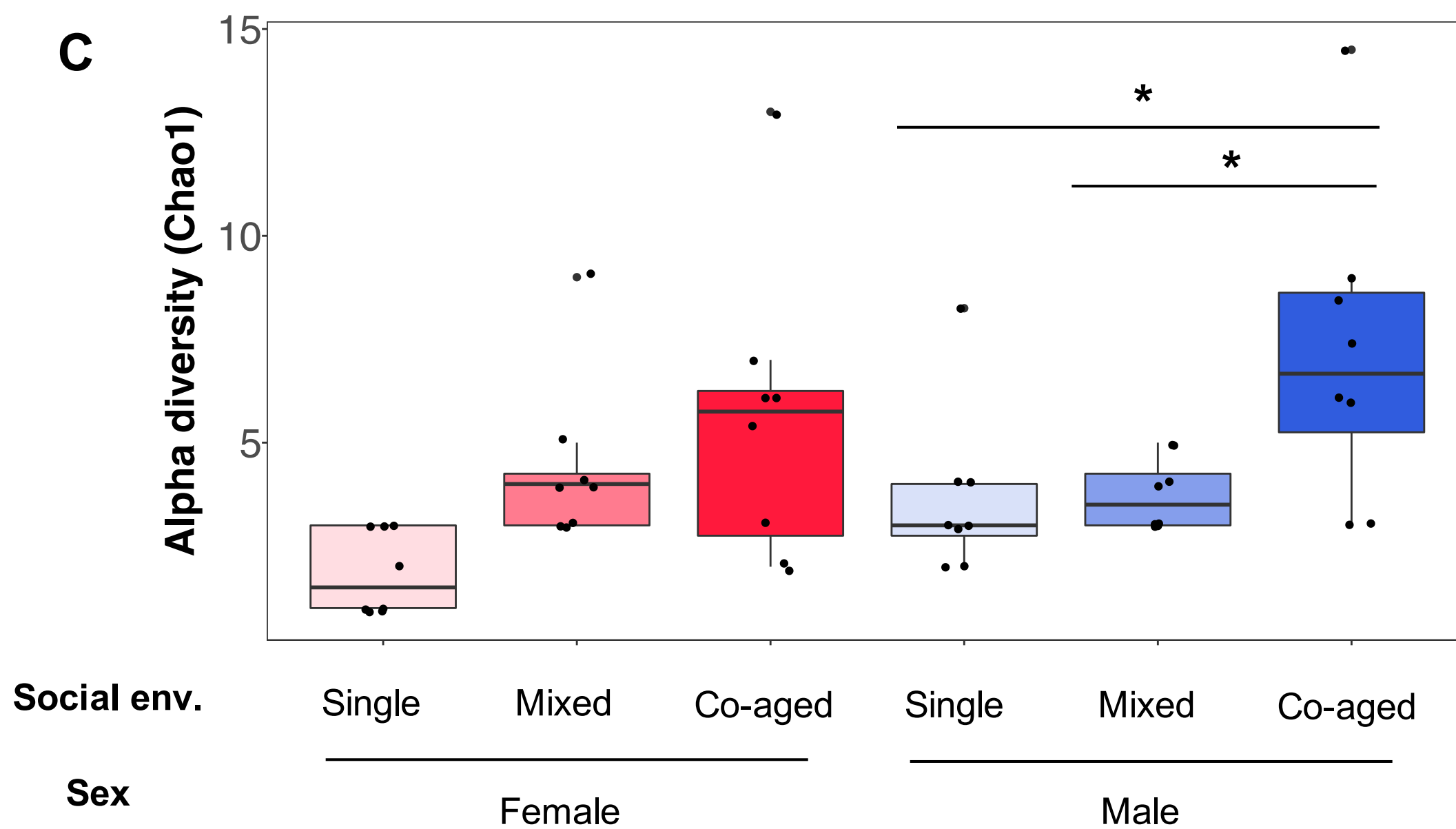
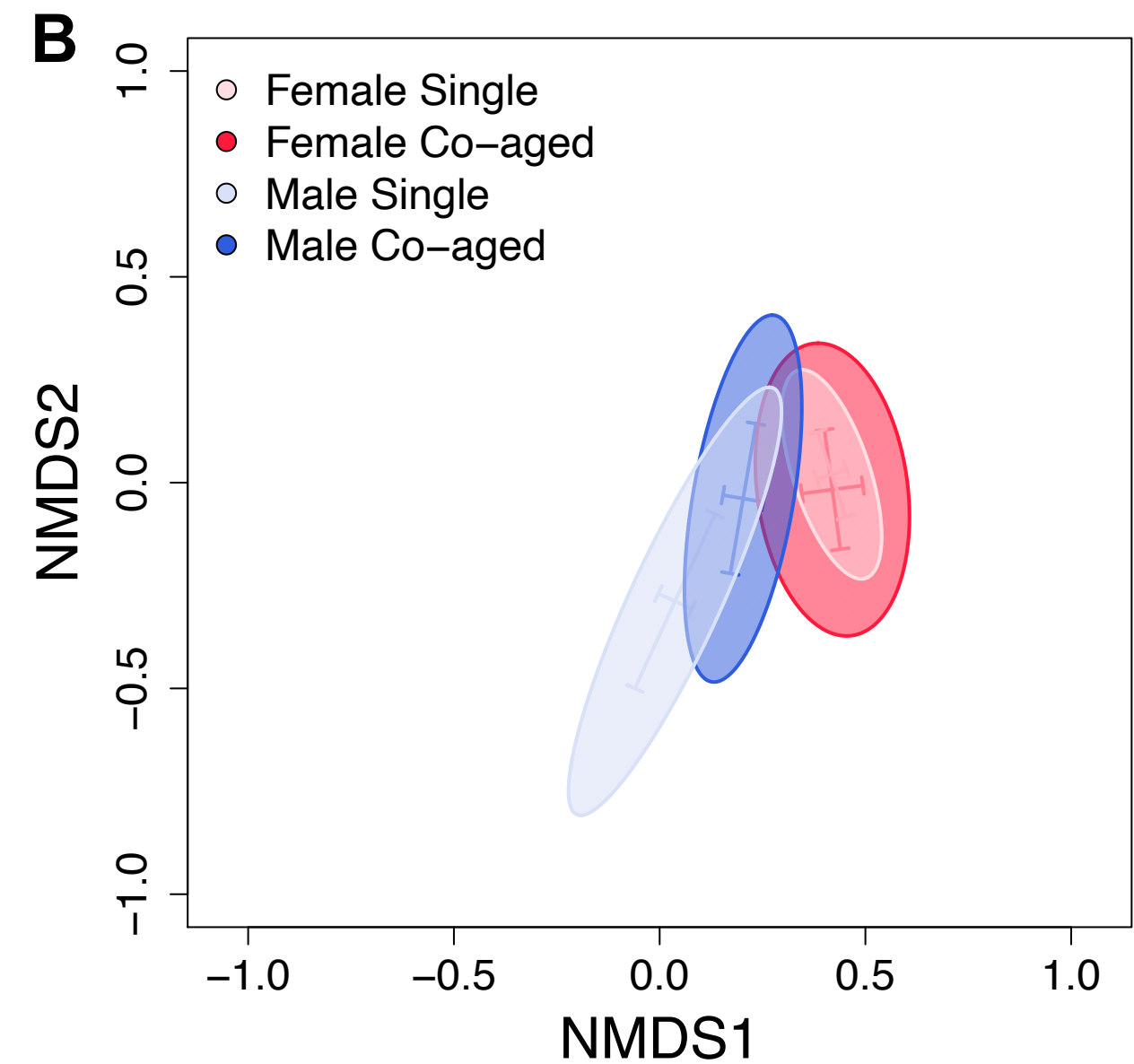
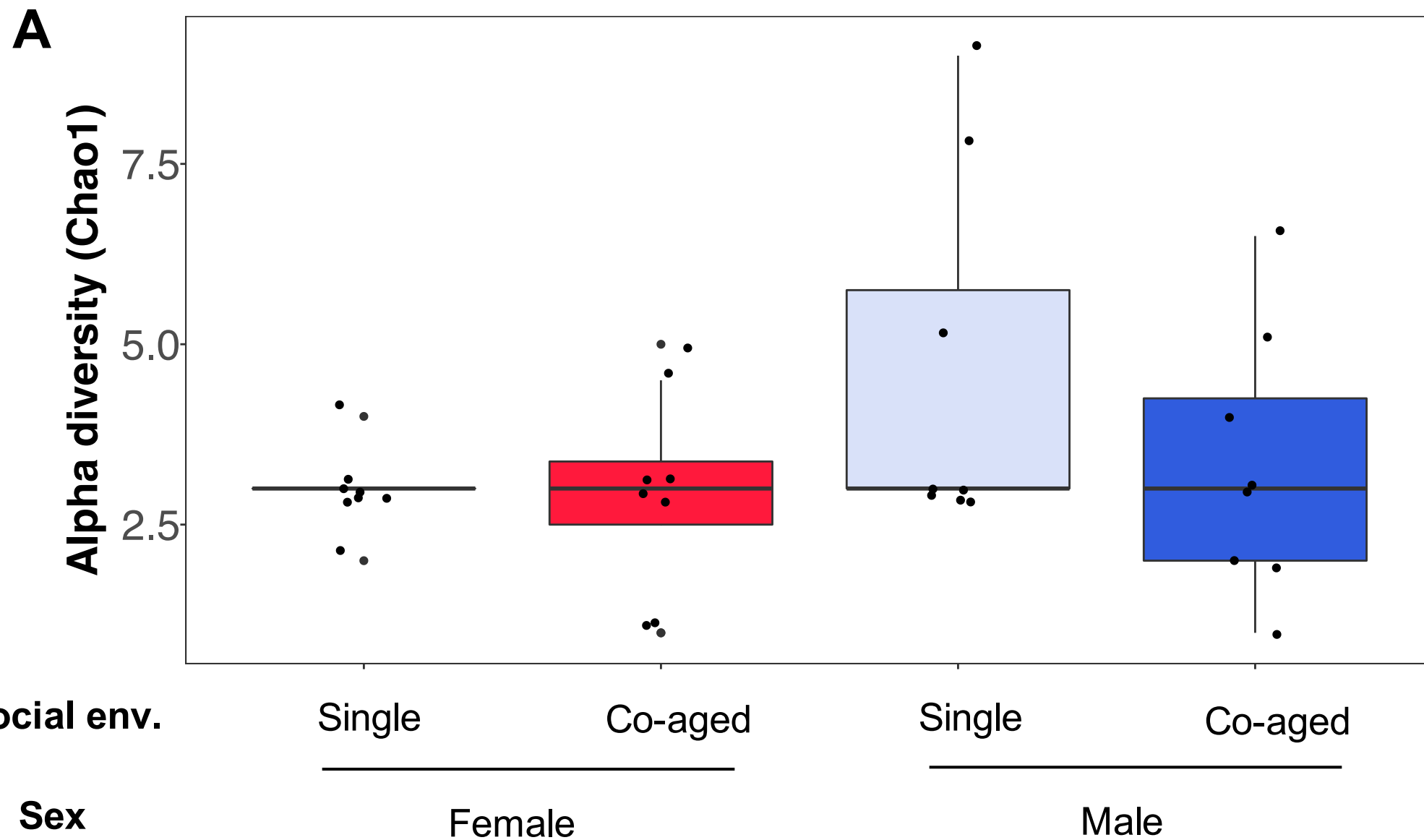


### D - Larval Density



### E - Larval Density

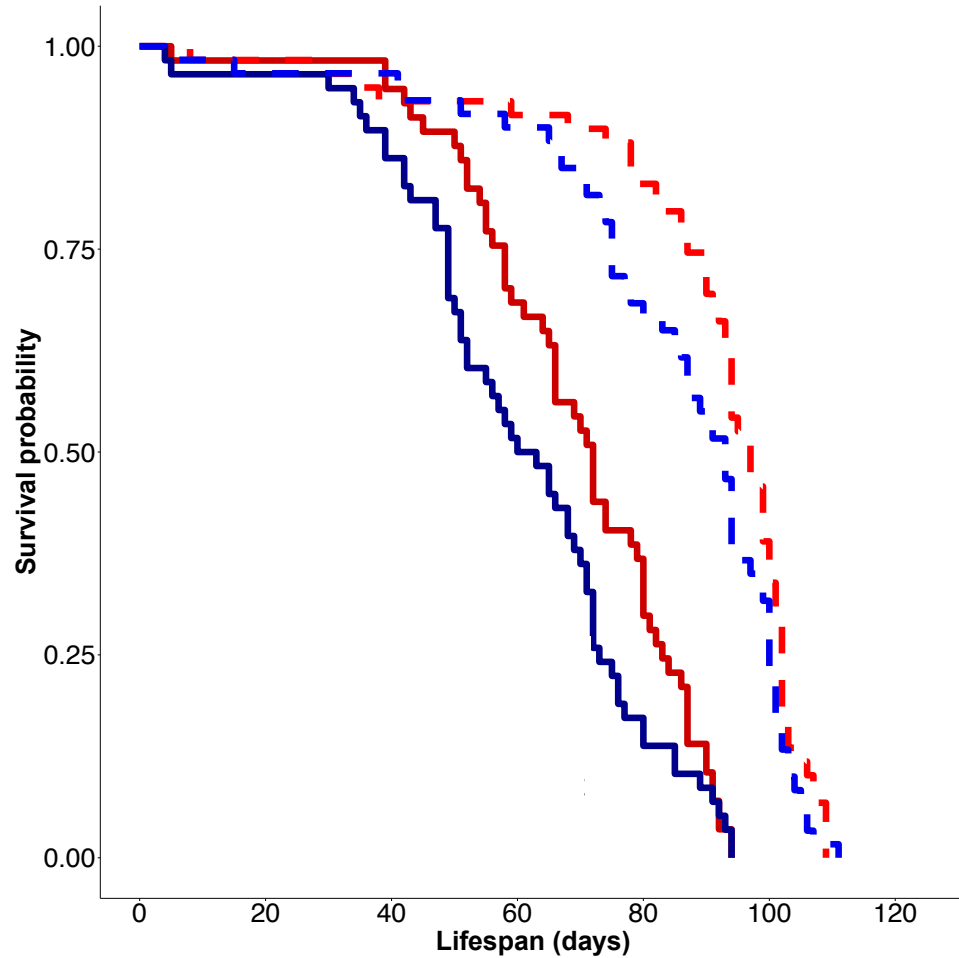




**A**

Larval Social Environment

- Males – Adult Males Absent
- - Females – Adult Males Absent
- Males – Adult Males Present
- - Females – Adult Males Present

**B**

Larval Social Environment

- Males – Low Density
- - Females – Low Density
- Males – High Density
- - Females – High Density

