Microbiome-mediated resilience and cross-generational consequences in male *Drosophila* exposed to combined environmental stressors

Komal Maggu*, Abhishek Meena, Alessio N. De Nardo, Benjamin Eggs, Sonja H. Sbilordo, Rawaa Al Toma Sho & Stefan Lüpold*

Department of Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

Correspondence: komal024maggu@gmail.com, stefan.luepold@uzh.ch

Abstract

Environmental stressors like heat extremes and pesticide exposure can significantly threaten insect reproduction, yet the interplay of these stressors and the potential mitigating role of the gut microbiome remain poorly understood, particularly across generations. This study investigated the interactive effects of acute heat stress and sublethal imidacloprid exposure on male *Drosophila melanogaster* reproductive success and the subsequent fitness of their offspring. We manipulated the gut microbiome of male flies (germ-free or colonized with one or five bacterial species) and subjected them to individual or combined stress. Our findings reveal that combined stress synergistically impairs male fitness traits, an effect partially buffered by higher microbiome diversity. We further demonstrate cross-generational consequences, with paternal stress exposure also impacting offspring fitness. Our results highlight the crucial role of the gut microbiome in mediating resilience to environmental stress

and underscore the importance of considering multi-stressor and intergenerational effects in ecological risk assessments.

Keywords- Microbiota, Thermal fertility, Pesticide exposure, Inter-generational, fruit fly

Introduction

Coping with the increasing prevalence of anthropogenic stressors is a central challenge for organisms under the ongoing global change (Navarro-Ortega et al., 2015; Piggott, Niyogi, et al., 2015; Piggott, Townsend, et al., 2015; Przeslawski et al., 2015; Segner et al., 2014). Among these stressors, climate change and agricultural pesticides are key drivers of ecosystem-wide biodiversity loss (Beketov et al., 2013; Heino et al., 2009; Stehle & Schulz, 2015), with their interaction posing unique risks. Rising temperatures are projected to intensify pesticide use in agriculture as warmer climates favor pest outbreaks (Deutsch et al., 2018), while simultaneously altering how organisms, particularly ectotherms, respond to chemical exposure (Fournier-Level et al., 2016; Hooper et al., 2013a; Noyes et al., 2009). For ectotherms, which cannot regulate internal temperatures (Deutsch et al., 2008; Paaijmans et al., 2013), warming elevates metabolic rates and feeding activity (and so potentially pesticide uptake), forcing energetic trade-offs between detoxification and essential functions like growth or reproduction (Jutfelt et al., 2021; Pörtner et al., 2017).

While the individual effects of temperature and pesticides are well-documented (Dougherty et al., 2024; Wan et al., 2025), their combined impacts remain poorly resolved. Studies report additive (Arambourou & Stoks, 2015), antagonistic (Meng et al., 2022), or synergistic (Wang et al., 2019) interactions, with synergism being most concerning for non-target species as combined harm exceeds the sum of individual detrimental effects (Folt et al., 1999; Siviter et al., 2021; Verheyen & Stoks, 2019, 2023). Such synergisms may arise through physiological

trade-offs, for example if a diversion of resources away from detoxification by heat stress (Dai et al., 2023) is combined with a depletion of antioxidants needed for thermal resilience through pesticide exposure (A. P. N. Silva et al., 2025). Such dual depletion might disproportionately damage gonads and gametes that are particularly prone to oxidative stress due to their high cell proliferation, metabolic rate and generation of reactive oxygen species (Simmons et al. 2018). By eroding reproductive resilience, synergisms threaten to accelerate biodiversity loss in ways single stressors would not (Shahid et al., 2024).

Beyond direct physiological impacts, host-associated microbial communities (microbiomes) are increasingly recognized as mediators for environmental stress tolerance (Feldhaar, 2011; Osborne et al., 2023; Sepulveda & Moeller, 2020b; Shokal et al., 2016). Commensal microbes can enhance host thermal performance (Renoz et al., 2019; Sepulveda & Moeller, 2020a), either as obligate or facultative symbionts (Dunbar et al., 2007; Gruntenko et al., 2017; Montllor et al., 2002; J. A. Russell & Moran, 2006; Zhang et al., 2008). Similarly, a healthy microbiome can bolster insect resistance to insecticides through direct and indirect pathways. For example, symbionts like *Citrobacter* in the oriental fruit fly *Bactrocera dorsalis*, or *Burkholderia* in the bean bug *Riptortus pedestris*, directly metabolize pesticides using encoded degradation enzymes, whereas others, such as *Arsenophonus* in *Nilaparvata lugens* or *Lactobacillus* in *Drosophila melanogaster*, can induce the host's own detoxification gene expression (Cheng et al., 2017; Jones et al., 2015; Kikuchi et al., 2012; Pang et al., 2018). The gut microbiome thus contributes to insect survival and fitness by providing nutrients or mitigating biotic and abiotic stresses (Hector et al., 2022; Perry et al., 2008). However, this protective capacity of the gut microbiota is context-dependent; stressor-induced dysbiosis can impair host fitness, creating feedback loops that may exacerbate vulnerability (Akbar et al., 2022; Evariste et al., 2019; Khosravi & Mazmanian, 2013; Kumar & Kumar, 2019; R. J. Russell et al., 2011; Sepulveda & Moeller, 2020a; Swelam et al., 2022). Despite progress in

studying individual stressors, how microbiomes modulate *combined* effects remains unknown. This gap is critical, as microbiome disruption by one stressor could amplify susceptibility to another, potentially explaining observed synergisms in some species (Karimzadeh et al., 2020; Mao et al., 2019; Zhang et al., 2008; Zhang, Cai, et al., 2021).

A parallel knowledge gap concerns stress effects on male reproduction, as much research on environmental stress in invertebrates focuses on acute toxicity or sterility thresholds (de Franca et al., 2017; Desneux et al., 2007; B. S. Walsh, Parratt, Hoffmann, et al., 2019; Zwoinska et al., 2020), overlooking the significant fitness consequences of *sublethal* exposures. Sublethal effects on female reproductive function are relatively well characterized, but the corresponding consequences for males have only recently come into focus and revealed that male fertility can be far more vulnerable than female fecundity, at least to heat stress (Iossa, 2019; Sales et al., 2018b; Zwoinska et al., 2020). Heat and pesticides have been shown to impair spermatogenesis, sperm DNA integrity, and ejaculate quality more broadly (Aulsebrook, Bertram, Martin, Aulsebrook, Brodin, Evans, Hall, O'Bryan, Pask, & Tyler, 2020; Dougherty et al., 2024; B. S. Walsh, Parratt, Atkinson, et al., 2019). Reductions in ejaculate quality are particularly impactful in promiscuous species, in which the competitive fertilization success depends on the relative ejaculate quantity and quality among rival males (Fitzpatrick & Lüpold, 2014; Parker & Pizzari, 2010; Pizzari & Parker, 2009; Simmons & Fitzpatrick, 2012).

Damage to sperm also has the potential of extending the effects of environmental stress across generations through epigenetic modifications or sperm-borne small RNAs (Bonduriansky et al., 2012; Morgan & Watkins, 2019), with profound consequences for population stability and evolutionary trajectories (Aulsebrook, Bertram, Martin, Aulsebrook, Brodin, Evans, Hall, O'Bryan, Pask, Tyler, et al., 2020; Crean & Immler, 2021). However, although parental exposure to combined thermal and chemical stress has been shown to reduce offspring

99 performance in some species (DeCourten & Brander, 2017; Fathy, 2024), other studies find no
100 clear effects (Massot et al., 2021; Meng et al., 2022) or even reduced offspring vulnerability
101 (Tran et al., 2018), indicating interspecific variation potentially due to differences in stressor
102 intensity (including the thermal sensitivity of pesticides themselves), species-specific
103 acclimation, or compensatory maternal provisioning. Understanding how environmental
104 stressors impact not only directly exposed individuals but also subsequent generations is crucial
105 for predicting long-term ecological consequences and evolutionary adaptation potential.

106 To address these knowledge gaps, we investigated the interactive effects of acute heat stress
107 and sublethal pesticide exposure on the reproductive fitness of male *D. melanogaster*, with a
108 focus on microbiome mediation and cross-generational consequences. We combined sublethal
109 acute heat stress (4 hours at 36°C), previously shown to negatively affect testicular function
110 and the fertilizing capacity of sperm (Meena, De Nardo, et al., 2024; Meena, Maggu, et al.,
111 2024), with exposure to imidacloprid, a widely used systemic insecticide targeting nicotinic
112 acetylcholine receptors (nAChRs) in the central nervous system (Ihara et al., 2017). Sublethal
113 imidacloprid impacts have been documented in diverse insects, affecting enzyme activities,
114 biological performance, and gene expression (He et al., 2011; Kang et al., 2018; W. Li et al.,
115 2018), and ultimately impairing fitness (De França et al., 2017; He et al., 2011; Xiao et al.,
116 2016; Zhang, Xu, et al., 2021; Zhou et al., 2017). To isolate the role of the microbiome, we
117 compared males that were germ-free (axenic) with males associated with defined bacterial
118 communities: either mono-association of two common gut bacteria (*Acetobacter tropicalis* or
119 *Lactobacillus brevis*) or a more complex, yet defined, five-species consortium combining
120 *Acetobacter* and *Lactobacillus* species that are commonly found in *Drosophila* (Broderick &
121 Lemaitre, 2012; Gould et al., 2018; Newell & Douglas, 2014; Ridley et al., 2013). This
122 gnotobiotic approach (i.e., using defined microbiotas) enabled precise assessment of microbial
123 contributions to stress tolerance. Furthermore, we assessed the cross-generational

124 consequences of paternal stress exposure by examining offspring fitness, explicitly testing
125 whether the father's microbiome status influences these cross-generational effects. Our design
126 minimized bacterial transmission to offspring, focusing the analysis on direct paternal effects
127 related to parental condition (Marshall et al., 2010; Moore et al., 2019; Mousseau et al., 2009).

128 Based on the evidence outlined above, we tested the following five predictions: (1) Combined
129 sublethal exposure to heat stress and imidacloprid will synergistically reduce male reproductive
130 fitness more than the individual effects of each stressor. (2) The presence of gut microbes will
131 partially mitigate the negative impacts of individual and combined stressors on male
132 reproductive fitness compared to germ-free males. (3) Different bacterial compositions (mono-
133 associations vs. defined complex community) will vary in their protective capacity, with the
134 more complex community offering greater resilience than either mono-association. (4) Paternal
135 exposure to individual and combined stressors will negatively impact offspring fitness (cross-
136 generational effects), with synergistic interactions between stressors potentially amplifying this
137 fitness loss. (5) The composition of the paternal microbiome will influence the magnitude of
138 these cross-generational effects.

139 Materials and Methods

140 Experimental populations

141 Throughout this study, we used an outbred *D. melanogaster* population (LH_m wild-type strain),
142 maintained under constant conditions of 25°C, 60% humidity, and a 12h:12h light:dark cycle.
143 First, we generated axenic (germ-free) focal flies by collecting eggs (within six hours of
144 oviposition) from four large population containers supplied with Petri dishes containing grape-
145 juice agar medium (300 mL water, 670 mL grape juice, 22g agar, 20g sugar, 19g yeast, 20 mL
146 nipagin per litre) supplemented with a smear of yeast paste. Eggs were washed off the plates

147 into a strainer, pooled, and dechorionated by immersion in 3.5% bleach for 2 minutes, followed
148 by a rinse in 70% ethanol and three rinses in sterile water.

149 Next, eggs were aseptically transferred to sterile glass vials containing autoclaved standard fly
150 food (75g glucose, 100g fresh yeast, 55g corn, 10g flour, and 15mL nipagin antimicrobial agent
151 per litre food media) supplemented with antibiotics [a cocktail of Ampicillin (50mg/mL),
152 Kanamycin (50mg/mL), Erythromycin (10mg/mL), Tetracycline (10 mg/mL)]. Vials were then
153 sealed with sterile plugs and incubated under standard laboratory conditions until adult
154 eclosion. All steps of vial preparation were conducted in a laminar flow chamber to avoid
155 contamination.

156 Upon eclosion, axenic status was confirmed using two methods on a subsample of flies. First,
157 homogenated flies were plated on Lysogeny Broth (LB) and de Man, Rogosa and Sharpe
158 (MRS) agar plates, incubated, and checked for bacterial colony growth. Second, genomic DNA
159 was extracted from pools of 3-5 putatively germ-free and conventional flies (positive control),
160 respectively, using Quick-DNA midiprep plus kit following the instructions provided by the
161 manufacturer. Universal primers (27F and 1492R) were used as forward and reverse primer,
162 respectively. To prepare the PCR master mix, 10µL of nuclease-free water, 0.5µL of forward
163 and reverse primer, 1µL of template DNA and 13µL Taq green master mix were mixed in a
164 sterile eppendorf tube. PCR conditions were: initial denaturation at 95°C for 90s, followed by
165 35 cycles of denaturation at 95°C for 30s, annealing at 55°C for 30s and extension at 72°C for
166 90s, and final extension at 72°C for 5 minutes). PCR products were visualized on a 1% agarose
167 gel under ultraviolet light to confirm the absence of bacterial DNA amplification in axenic
168 samples and its presence in controls.

169 **Bacterial cultures and gnotobiotic associations**

170 Axenic males were associated with defined compositions of microbial species, all originally
171 isolated from the gut of wild-type flies (Obadia et al., 2017): *Acetobacter tropicalis* (*At*),
172 and *Lactobacillus brevis* (*Lb*) for mono-associations based on their contrasting effects on male
173 reproductive investments in our previous studies (Maggu et al. in prep.). For comparison, we
174 also included axenic males and males inoculated with a defined five-species community
175 consisting of common and abundant *Drosophila* gut bacteria (*A. tropicalis*, *A. orientalis, A.*
176 *pasteurianus, L. plantarum, L. brevis*), resulting in four distinct microbial treatments.

177 Bacterial strains were cultured overnight in an incubator at 30°C: *Acetobacter* in MRS +
178 mannitol medium with shaking, and *Lactobacillus* in MRS broth under stationary conditions.
179 The following morning, cultures were checked for growth before measuring their optical
180 densities (OD) at 600 nm and centrifuging at 7000 rpm for 5 minutes. Bacterial pellets were
181 then resuspended in sterile phosphate-buffered saline (PBS) to a final concentration of 10^8
182 cells/mL, following established protocols (Koyle et al., 2016; Newell & Douglas, 2014).

183 To inoculate vials, 50µL of bacterial suspension (approx. 5×10^6 CFUs) was added to sterile
184 food vials, with equal representation of bacterial strains in the 5-species consortia. This
185 bacterial density is comparable to previous reports for conventionally reared flies (Gould et al.,
186 2018; Newell & Douglas, 2014). Inoculated food vials were then allowed to air-dry in a laminar
187 flow cell before introducing ten 1-day-old axenic males to each to generate gnotobiotic flies.
188 Males were transferred to freshly inoculated vials with the same bacterial treatment every 48h
189 for a total of four days to ensure stable colonization. Successful colonization with the intended
190 bacteria was confirmed by plating homogenates of males from each treatment and checking for
191 characteristic colony morphologies (see *Bacterial abundance of males*).

192 **Stress treatments**

193 Following the 4-day colonization period, males were transferred to vials containing sterile food
194 and exposed to pesticide and heat stress in a fully factorial design. Specifically, half of the
195 males from each microbial group were subjected to food supplemented with imidacloprid,
196 whilst the remaining half were maintained on uncontaminated standard food. We used a
197 concentration of 1 ppm (parts per million) of imidacloprid as concentrations of 0.04 to 20 ppm
198 have been used effectively in studies on larval or adult *D. melanogaster*, depending on the
199 exposure regime (Charpentier et al., 2014a; Frantzios et al., 2008; Martelli et al., 2020). These
200 concentrations are low enough to avoid immediate lethality but sufficient to induce sublethal
201 effects on behavior, reproduction, and physiology. Males were exposed to imidacloprid for an
202 acute window of 18 h, following an additional thermal treatment of 4-hour in a hot water bath.
203 To this end, half of the males from each microbiome and pesticide treatment were subjected to
204 acute heat stress by transferring them to vials containing only sterile 1% agar (to prevent
205 dehydration) and placing these vials in a water at 36°C for 4 hours. The remaining males
206 (thermal controls) were treated identically but at benign 24.5°C. All males were then allowed
207 to recover overnight in standard food vials under standard laboratory conditions.

208 **Hatching success and fertility**

209 To quantify the fitness consequences of heat and pesticide exposure and their potential
210 mitigation by the microbiome on the morning after the stress treatments, experimental males
211 were individually mated to standard females of the same population. All females had been
212 collected as virgins (within 7 hours of eclosion) and reared under axenic conditions along with
213 the experimental males to minimize confounding contributions of female microbes to the
214 measured outcomes. After mating ended, females were allowed to lay eggs for 24 h, and
215 another 30 h later (allowing time for embryonic development), oviposition vials were frozen
216 for later quantification of the proportion of eggs hatching as a proxy of fertilization success,

217 and the total number of larvae estimating male-mediated fecundity. This assay was performed
218 in two blocks, each with 25 males per treatment combination, totaling 800 experimental males
219 (4 bacterial treatments × 2 pesticide treatments × 2 temperature treatments × 2 blocks × 25
220 males).

221 **Sperm competitiveness**

222 To assess male sperm competitiveness, the above experimental design was repeated, but this
223 time using standard females that had mated once with standard competitor males two days
224 before the focal mating. These standard competitors were of the same strain but genetically
225 modified to ubiquitously express green fluorescent protein (GFP), permitting paternity
226 assignment against the focal wild-type males (Lüpold et al., 2012, 2020). Pairs were monitored
227 for remating for up to 8 h. Remated females were then isolated in fresh vials for oviposition,
228 while pairs not mating were separated and given another 8-hour mating opportunity on the
229 following day. Females still not remating were excluded. Offspring paternity (focal vs.
230 competitor) was assessed via GFP fluorescence to calculate P_2 as the proportion of offspring
231 sired by the second (focal) male. The treatment design of this assay was identical to the non-
232 competitive experiment above, but this time with 30 trials per treatment combination in each
233 of the two experimental blocks (i.e., 960 trials in total).

234 **Bacterial abundance of males**

235 To investigate the impact of stress treatments on the bacterial load, colony forming units
236 (CFUs) were compared between ten random males from each experimental treatment, surface-
237 sterilized with 70% ethanol, and individually homogenized in 1.5 mL microcentrifuge tubes
238 containing sterile PBS using a pestle. Homogenates were serially diluted to different dilutions
239 at a final volume of 100 μL, then plated on MRS media for quantification of CFUs at

240 appropriate dilutions (approx. 100 CFUs/plate). For the control groups with both *Acetobacter*
241 and *Lactobacillus* species, MRS agar was supplemented with 10 µg/mL ampicillin to avoid the
242 growth of *Lactobacillus,* whilst replicate plates were incubated in a CO_2 atmosphere to exclude
243 the growth of *Acetobacter*. The presence of only target bacterial species on plates was
244 confirmed based on colony morphologies, with *A. tropicalis* producing small tan-brown
245 colonies and *L. brevis* producing small yellowish colonies. CFUs per fly were calculated as:
246 CFU = C × D/P × V, where C = number of colonies counted, D = dilution, P = volume plated,
247 V = volume of fly homogenate (Koyle et al., 2016). For control males, with all the five bacterial
248 species, bacterial colonies were identified by their morphology and total CFUs per fly was
249 obtained by adding CFUs from all the five bacterial species.

250 **Cross-generational effects**

251 To assess cross-generational impacts of paternal (F_0) stress exposure, we quantified hatching
252 success and fecundity in both male and female offspring (F_1) of our experimental males. Within
253 each experimental block, up to 20 freshly hatched larvae from each of ten randomly selected
254 vials per parental treatment combination (i.e., 16 combinations) were transferred to rearing
255 vials containing sterilized standard fly food. This immediate transfer minimized the potential
256 acquisition of parental microbes via consumption of the egg chorion or contaminated food.
257 These larvae developed under standard laboratory conditions.

258 Upon eclosion, 4-5 virgin F_1 male and female offspring were collected from each rearing vial,
259 separated by sex. At 2-3 days of age, three males and three females from each lineage were
260 individually mated with a virgin standard fly of the opposite sex reared under axenic conditions
261 (to avoid potential confounding effects of their microbiota). Following copulation, hatching
262 success and fecundity were measured for these F_1 flies as in their parents (see above). These
263 offspring themselves were not subjected to any experimental treatment, thus assessing solely

264 the cross-generational effects. This experiment was carried out in two independent blocks of
265 480 offspring per sex from 160 F_0 males (i.e. total of 1,920 mating trials).

266 **Statistical analysis**

267 All statistical analyses were performed in R version 4.4.0 (R core team, 2024), using
268 generalized linear mixed-effects models (GLMMs) and generalized linear models (GLMs) to
269 assess the male responses to heat stress and/or pesticide exposure under different microbiome
270 conditions. Each model included microbiome composition (4-level factor) along with the
271 binary thermal and pesticide treatments with all two- and three-way interactions as fixed
272 effects, and experimental block as a fixed or random effect. Pairwise comparisons were
273 performed using the emmeans package (Searle et al., 1980) (Estimated Marginal Means) to
274 assess the significance of differences between levels of each factor.

275 Hatching success (i.e., proportion of all eggs that hatched) was analyzed in a quasibinomial
276 GLMM (as the binomial distribution resulted in under-dispersion), with block as a fixed effect.
277 Male-mediated female fecundity (i.e., total number of eggs laid) was modeled using a truncated
278 Poisson model with a zero-inflation component as implemented in the package glmmTMB
279 (Brooks et al., 2017), also including block as a fixed effect. Finally, for sperm competitiveness
280 (P_2), we fitted a GLMM (glmmTMB) with a Tweedie distribution and a log link function to
281 account for skewness and under-dispersion in the data. Experimental block and male ID were
282 included as random effects.

283 The impact of the different experimental treatments was also analyzed for the total bacterial
284 abundances (CFUs) and, separately, for compositional shifts in the five-species consortia. For
285 the former, we used a linear mixed-effects model with block as a random effect. For
286 microbiome community composition in the five-species consortium, we quantified the species-

287 specific abundances per sample, then applied a Hellinger transformation to these count data
288 and computed Bray-Curtis distances using the *vegdist* function in the package vegan (Oksanen
289 et al., 2023). We assessed treatment effects (temperature, pesticide, and their interaction) in a
290 permutational multivariate analysis of variance (PERMANOVA) using *adonis2* in the same
291 package. To visualize compositional shifts, we further ran non-metric multidimensional scaling
292 (NMDS) (*metaMDS*) on the Bray-Curtis dissimilarities and fitted species vectors onto the
293 NMDS ordination with *envfit*, which indicates the direction and strength of each species-
294 specific association with the ordination space. We also compared the relative abundances at
295 the genus level, using a binomial GLMM with block as a random effect and accounting for
296 overdispersion by including an additional observation-level random effect.

297 Finally, the effects of paternal (F_0) treatments on offspring (F_1) fitness were quantified
298 separately for male and female progeny using GLMMs. Each model included F_0 treatments
299 and their interactions as fixed effects (as above), with experimental block and sire ID as random
300 effects. GLMMs for hatching success included a zero-inflation component (for females), whilst
301 those for hatching success used a Tweedie distribution to account for under-dispersion.

302 We investigated the performance of all models using the DHARMa package (Florian, 2016).

303 **Results**

304 **Hatching success**

305 To assess the combined effects of heat stress, pesticide exposure, and microbiome composition
306 on male reproductive function, we first investigated fertilization success, estimated as the
307 proportion of eggs hatched out of all eggs laid by females. A GLM revealed a significant three-
308 way interaction between microbiome composition, temperature, and pesticide presence (N =
309 650, $\chi^2 = 16.07$, df = 3, p = 0.0011; Fig. 1, Supplementary Tables S1, S2), highlighting that the

310 combined effects of these environmental stressors depended on the microbial composition of
311 the focal males. Overall, males associated with all five bacterial strains exhibited the highest
312 hatching success, followed by *At*- and *Lb*-associated males, with axenic males being most
313 severely affected. These general patterns were also confirmed by the significant two-way
314 interactions of temperature × microbiome ($\chi^2 = 12.13$, df = 3, $p = 0.007$) and temperature ×
315 pesticide ($\chi^2 = 43.32$, df = 1, $p < 0.0001$), as well as by all main effects (all $\chi^2 \geq 520.76$, df ≥ 3,
316 $p \leq 0.001$; Supplementary Tables S1, S2).

317 **Male-mediated fecundity**

318 Next, we investigated male-mediated fecundity, quantified as the total number of larvae
319 produced per female. In contrast to hatching success, the three-way interaction was not
320 significant ($\chi^2 = 0.319$, df = 3, $p = 0.956$; Supplementary Table S3, S4) for this metric and was
321 removed from the final model. The simplified model revealed significant two-way interactions
322 between microbiome and temperature (N = 650, $\chi^2 = 7.85$, df = 3, $p = 0.049$) and between
323 temperature and pesticide ($\chi^2 = 5.41$, df = 1, $p = 0.020$; Supplementary Tables S3, S4). All main
324 effects were again significant (all $\chi^2 \geq 5.41$, $p \leq 0.001$). The relative differences in fecundity
325 between males with different microbiome statuses largely mirrored the patterns observed for
326 hatching success (Fig. 2).

327 **Sperm competitiveness**

328 Since sperm competitive ability is a crucial determinant of male reproductive fitness in
329 polygamous species and there are reports of adverse effects of increased temperatures and
330 pesticide exposure on sperm quality and function (Chirault et al., 2015; Meena, De Nardo, et
331 al., 2024; D. R. Moreira et al., 2022; S. Moreira et al., 2021; Roy et al., 2017; Sales et al.,
332 2018a, 2024), we evaluated the combined effects of our treatments on the proportion of

333 progeny sired by focal males relative to their standard competitors (i.e., P_2). Although the three-
334 way interaction was not statistically significant (N = 331, χ^2 = 1.003, df = 3, p = 0.800), all
335 two-way interactions were (Supplementary Tables S5, S6): temperature × microbiome (χ^2 =
336 22.92, df = 3, p < 0.001), microbiome × pesticide (χ^2 = 11.89, df = 3, p < 0.001), and
337 temperature × pesticide (χ^2 = 27.33, df = 1, p < 0.001). Similarly, all main effects on P_2 were
338 significant (all $\chi^2 \geq 472.33$, $p \leq 0.001$). These results indicate strong interdependent effects of
339 the treatments on sperm competitiveness, with the relative performance across different
340 microbiome groups again showing similar patterns to those observed for hatching success and
341 fecundity (Fig. 3).

342 **Bacterial abundance in males in response to heat stress and pesticide exposure**

343 We also analyzed the effect of heat stress and pesticide exposure and their interactions on the
344 growth and viability of commensal bacteria inside their host under the different stress
345 treatments by quantifying their colony forming units in homogenates of focal males
346 immediately after mating. For total CFU counts, we found a significant three-way interaction
347 (χ^2 = 5.06, df = 2, p = 0.008; Supplementary Tables S7, S8), suggesting that the bacterial strains
348 differed in their accumulation patterns in adult flies depending on the combined effects of
349 environmental stress factors. Additionally, we documented significant two-way interactions
350 and main effects of all three factors (all $\chi^2 \geq 42.31$, $p \leq 0.001$), except for the two-way
351 interaction between the two stressors (χ^2 = 2.36, df = 2, p = 0.127). Among these and relative
352 to the control microbiome, higher temperature had a stronger negative effect on the total
353 number of CFUs than pesticide exposure (Fig. 4; Supplementary Tables S7, S8).

354 A PERMANOVA controlling for block effects ($F_{1,35}$ = 0.20, p = 0.874, Supplementary Table
355 S9) revealed that temperature was also the dominant driver of variation in the relative

356 abundances of the five bacterial species constituting the consortium, explaining 26.3% of the
357 variation in community composition ($F_{1,35}$ = 13.88, p = 0.0001; Supplementary Table S9). In
358 contrast, the main effect of pesticide was not statistically significant (2.2% of variation, $F_{1,35}$ =
359 1.16, p = 0.314), and the temperature × pesticide interaction showed a marginal effect (4.8%
360 of variation, $F_{1,35}$ = 2.55, p = 0.068). NMDS based on Bray-Curtis dissimilarities provided a
361 visual representation of community shifts across treatments. The NMDS ordination (Fig. 5)
362 indicated that the first axis (NMDS1) predominantly tracked the temperature gradient, while
363 the second axis (NMDS2) aligned more, but only weakly, with pesticide exposure. Overlaying
364 species vectors revealed notable differences in species associations (all $R^2 \geq 0.54$, $p \leq 0.0001$),
365 in that both *Lactobacillus* species showed positive (≥ 0.68), and all *Acetobacter* exhibited
366 negative (≤ -0.38), loadings on NMDS1 (Supplementary Table S10), suggesting an increase in
367 the relative abundance of *Acetobacter* in the high-temperature regimes. By contrast, loadings
368 on NMDS2 were negative for *A. tropicalis* (-0.92) and *L. brevis* (-0.44) but positive for all
369 remaining species (≥ 0.56). This axis tended to be biased toward negative values in the absence
370 of imidacloprid, but more ambiguous in its presence.

371 Finally, in a binomial GLMM focusing only on the relative abundances of acetic acid versus
372 lactic acid bacteria, where the three *Acetobacter* species should theoretically constitute 60% of
373 the total CFUs among the five species, temperature and pesticide presence interacted
374 significantly (N = 40, z = 2.15, p = 0.032). Specifically, the relative representation of
375 *Acetobacter* was increased to around 66% under heat stress (z = 2.84, p = 0.013), whereas
376 pesticide exposure reduced its representation (z = -2.32, p = 0.021), although only at the benign
377 temperature (Fig. 6).

378 **Cross-generational effects on offspring fitness**

379 Finally, we investigated whether the effects of paternal stress exposure carried over to the next
380 generation by measuring the hatching success and fecundity of F_1 offspring produced by F_0
381 males from each treatment group.

382 We found a significant interactive effect between paternal temperature and pesticide treatments
383 on the hatching success of both male ($N = 726$, $\chi^2 = 19.39$, $df = 1$, $p < 0.001$; Supplementary
384 Tables S11,12) and female offspring ($N = 750$, $\chi^2 = 10.084$, $df = 1$, $p < 0.001$; Supplementary
385 Tables S13,14). This indicates that the combined effects of heat and pesticide stress
386 experienced by fathers carried over to the next generation. However, the composition of the
387 paternal gut microbiome did not significantly influence offspring hatching success ($\chi^2 \leq 4.76$,
388 $df = 3$, $p \geq 0.19$). The interactive effect of paternal temperature and pesticide exposure was not
389 significant for the fecundity induced by sons ($\chi^2 = 0.36$, $df = 1$, $p = 0.55$; Supplementary Tables
390 S15,16). In contrast, the interaction between these two paternal stressors showed significant
391 effect on fecundity of F_1 daughters ($N = 750$, $\chi^2 = 3.990$, $df = 1$, $p = 0.006$; Supplementary
392 Tables S17,18). There was also a non-significant trend for a main effect of paternal microbiome
393 composition on offspring fecundity in daughters ($\chi^2 = 7.26$, $df = 3$, $p = 0.06$; Supplementary
394 Tables S17,18).

395 **Discussion**

396 We investigated the role of the gut microbiome in modulating male reproductive success in *D.*
397 *melanogaster* under individual and combined exposure to acute heat stress and the pesticide
398 imidacloprid. Our results confirm and extend previous work that demonstrated the detrimental
399 effects of sublethal temperatures (Canal Domenech & Fricke, 2022; David et al., 2005; Fasolo
400 & Krebs, 2004; Gandara & Drummond-Barbosa, 2022, 2023; Meena, De Nardo, et al., 2024;
401 Pilakouta & Baillet, 2022; Sales et al., 2024; B. S. Walsh, Parratt, Atkinson, et al., 2019) or
402 imidacloprid (Charpentier et al., 2014b; Xiao et al., 2016; Zhang, Xu, et al., 2021; Zhou et al.,

403 2017) on different reproductive functions in diverse organisms. However, our study adds a
404 crucial layer of complexity by revealing both the combined effects of these stressors and the
405 critical role of the microbiome in mediating the response of stressed males. Specifically, our
406 findings highlight the importance of the gut microbiome in mitigating fitness losses under
407 stress. *Acetobacter* generally offered stronger protection than *Lactobacillus*, while the complex
408 microbial community provided the highest benefits. Furthermore, we show that paternal stress
409 exposure imposes fitness costs on the next generation, although these cross-generational effects
410 were not significantly mediated by the paternal microbiome in our experimental design aimed
411 at minimizing direct microbial transmission.

412 **Interactive effects of combined stressors on male reproduction**

413 Our study revealed that initial exposure to imidacloprid followed by heat shock reduced male
414 reproductive functions more than either stressor alone. This observation aligns with previous
415 studies indicating complex, often order-dependent interactions between thermal stress and
416 pesticide toxicity (Ahmed et al., 2023; Deng et al., 2016; Meng et al., 2022; Ricupero et al.,
417 2020; Y.-C. Wang, Chang, Bai, Zhang, Iqbal, Lu, Gong, et al., 2021). For example, heat stress
418 has been shown to increase the tolerance to subsequent insecticide exposure in diverse insect
419 pests, such as the serpentine leaf miner, *Liriomyza trifoli* (Y.-C. Wang, Chang, Bai, Zhang,
420 Iqbal, Lu, Hu, et al., 2021a); diamond back moth, *Plutella xylostella* (Gu et al., 2010); wheat
421 aphid, *Sitobion avenae* (Cao et al., 2021), or brown planthopper, *Nilaparvata lugens* (Ge et al.,
422 2013). The order of stress treatments, however, has had an opposing effect in other insects
423 (Delnat et al., 2019; Y.-C. Wang, Chang, Bai, Zhang, Iqbal, Lu, Gong, et al., 2021). In *L. trifoli*,
424 for example, where both orders in stress exposure were examined, elevated temperatures
425 improved survival under exposure to the insecticide abamectin, whereas the reverse stress

426 sequence reduced survival (Y.-C. Wang, Chang, Bai, Zhang, Iqbal, Lu, Gong, et al., 2021),
427 indicating the importance of stress sequence in cross-protection between multiple stressors.

428 Pre-exposure to insecticides can also decrease heat stress tolerance, suggesting that warmer
429 temperatures may exacerbate insecticide toxicity depending on the exposure sequence.
430 Increased imidacloprid toxicity at higher temperatures has been documented in mayflies
431 (Macaulay et al., 2020), earthworms *Eisenia andrei* and collembolans *Folsomia candida*
432 (Bandeira et al., 2020). In the terrestrial ectotherm *Hypogastrura viatica*, sublethal
433 concentrations of imidacloprid became lethal on increasing the temperatures resulting in
434 reduced survival, growth and egg production (A. S. J. Silva et al., 2023). Our study corroborates
435 these findings, with decreased male reproductive success when pesticide exposure preceded
436 heat stress. These multi-stressor studies highlight the complex interplay between temperature
437 and toxicity, which can be positive or negative depending on the mode of action of the
438 insecticide, the route of exposure, and the insect species. Therefore, the sequence of exposure
439 must be carefully considered when evaluating combined stressor effects on insect performance.

440 The mechanisms underlying these sequence-dependent interactions likely involve intricate
441 physiological adjustments. For example, exposure to one stressor might compromise pathways
442 needed to cope with the subsequent stressor (lack of cross-tolerance) or trigger shared signaling
443 pathways that amplify susceptibility (negative cross-talk). Resource allocation trade-offs are
444 also probable (Sokolova, 2013), in that coping with pesticide toxicity may deplete energy
445 reserves or specific molecules (e.g., antioxidants, chaperones) needed to subsequently mount
446 an effective heat stress response. Generally, stressful environments may alter resource
447 allocation among essential processes like growth, reproduction, or immune defense to optimize
448 fitness outcomes (Kooijman, 2000). As such, elevated temperature may increase somatic

449 maintenance costs (Verberk et al., 2016), potentially reducing resources available for defense
450 and repair (Hallman & Brooks, 2015).

451 While the precise mechanisms underlying increased toxicity at high temperatures remain
452 unclear, they may overlap with those explaining the increased toxicity of organophosphate
453 pesticides at higher mean temperatures (Noyes et al., 2009; Noyes & Lema, 2015a). Increased
454 temperatures can enhance the activity of detoxification enzymes like cytochrome P450s (Y.-C.
455 Wang, Chang, Bai, Zhang, Iqbal, Lu, Hu, et al., 2021b), accelerating the breakdown of
456 pesticides like pyrethroids (Ahmad, 2007). Increased uptake of chemicals at higher
457 temperatures (Holmstrup et al., 2010), possibly due to higher basal metabolism (Hooper et al.,
458 2013b), increased membrane permeability (Noyes & Lema, 2015b), and or smaller body size
459 (Gardner et al., 2011) has also been observed. Stressful conditions can promote the
460 accumulation of reactive oxygen species (ROS) due to reduced respiration and increased water
461 loss, thereby negatively affecting development and reproduction (Dampc et al., 2021). For
462 example, low doses of imidacloprid have been shown to cause ROS formation and metabolic
463 impairment in *D. melanogaster* (Martelli et al., 2020). Both pesticide tolerance (Bagchi et al.,
464 1996) and heat stress (Neven, 2000) are also associated with overexpression of heat-shock
465 proteins like Hsp70 and Hsp90. In the honeybee, for example, these two Hsps followed similar
466 expression patterns in response to both thermal stress and imidacloprid exposure (Manzi et al.,
467 2020). This thermal stress-mediated chaperone activation could also be a relevant mechanism
468 in *D. melanogaster* males exposed to imidacloprid, for cellular protection against combined
469 stressors. If so, the observed fitness loss following heat and pesticide stress might at least in
470 part be attributable to the costs associated with enhanced Hsp expression (Hoekstra et al., 2013;
471 S. Li et al., 2015), which may become overwhelming under combined stress.

472 **Microbiome as a modulator of stress resilience**

473 A key finding of our study was the significant role of the gut microbiome in mitigating the
474 detrimental reproductive consequences of heat and pesticide stress. Across all measured fitness
475 components (hatching success, fecundity, sperm competitiveness), axenic males consistently
476 performed worse than males colonized with bacteria, highlighting the baseline importance of
477 the microbiome for reproductive function even under benign conditions, and its crucial role
478 under stress. The reduced fitness of axenic males under combined stress suggests an impaired
479 ability to metabolize and excrete imidacloprid, potentially leading to higher internal toxin loads
480 and thus increased vulnerability to subsequent heat stress.

481 Interestingly, the degree of protection conferred depended on the microbial composition. While
482 both *Lactobacillus brevis* and *Acetobacter tropicalis* mono-associations offered benefits over
483 the axenic state, the 5-species community consistently provided the greatest resilience,
484 resulting in the highest reproductive fitness across most conditions. This underscores the
485 potential importance of microbial diversity and inter-species interactions for robust host
486 function (Coyte et al., 2015; Freilich et al., 2011; Levy & Borenstein, 2013; Magnúsdóttir et
487 al., 2017; Noecker et al., 2019; Zelezniak et al., 2015). Mono-associations might lack the
488 metabolic versatility or synergistic cross-feeding capabilities present in more complex
489 consortia, limiting their ability to buffer the host against multiple stressors.

490 Our results partially contrast with Daisley et al. (2017), who reported that *Lactobacillus*
491 *plantarum* (Lp39) supplementation rescued *D. melanogaster* from harmful imidacloprid
492 effects, accompanied by increased *Acetobacter* and *Lactobacillus* abundance. We did not
493 observe such a strong rescue with *L. brevis*, nor an increased abundance of either bacterial
494 genus. This discrepancy could stem from strain-specific functions, similar to opposing effects
495 on chlorpyrifos (CP) toxicity between *L. plantarum* ISO and *Lactobacillus ATCC* in *D.*
496 *melanogaster* (Daisley et al., 2017). Alternatively, differences in experimental protocols could

497 also contribute to the contrasting results between our and Daisley's studies (e.g., our acute 18h
498 imidacloprid exposure versus Daisley et al.'s (2017) continued exposure).

499 We also observed shifts in the relative abundance of *Acetobacter* and *Lactobacillus* within the
500 host, aligning with studies showing heat-induced microbiome disruptions (Jaramillo &
501 Castañeda, 2021; Kokou et al., 2018; Moghadam et al., 2018a). Apparently, in mono-
502 associations, *Lactobacillus* was more abundant and less affected by treatments than
503 *Acetobacter*, however, the latter seemed to be overrepresented in 5-species consortium relative
504 to the expected proportion. Generally, we found a higher proportional representation of
505 *Acetobacter* following acute heat stress, consistent with prior work reporting increased
506 *Acetobacter* (but fewer *Leuconostoc* bacteria) in heat- compared to cold-acclimated flies
507 (Moghadam et al., 2018b). While this previous study exposed individuals to thermal treatments
508 throughout development, our findings demonstrate that such compositional shifts can occur
509 rapidly (within hours).

510 The mechanisms by which the microbiome enhances stress tolerance likely involve multiple
511 pathways. Beyond direct detoxification (Cheng et al., 2017; Jones et al., 2015; Kikuchi et al.,
512 2012; Pang et al., 2018), gut microbes significantly impact host nutrition and metabolism
513 (Douglas, 2009; Ridley et al., 2012), which are intrinsically linked to thermal tolerance in
514 ectotherms (Henry et al., 2020; Moeller et al., 2020; Moghadam et al., 2018a; Semsar-
515 Kazerouni et al., 2020). It has been shown in *D. melanogaster* that the gut microbiota influences
516 nutrient allocation, including levels of the thermo-protectant trehalose (Overgaard et al., 2007;
517 Ridley et al., 2012; Schwasinger-Schmidt et al., 2012). They can also modulate host immune
518 responses and potentially Hsp expression in the gut epithelium (Arnal & Lalles, 2016; Liu et
519 al., 2014). Given the critical role of Hsps in mitigating thermal stress in *D. melanogaster*
520 (Calabria et al., 2012; Sørensen et al., 2003), and their rapid induction after heat exposure

521 (Hoekstra et al., 2013), the microbiota may enhance heat tolerance in conventional and mono-
522 associated flies by promoting higher Hsp levels. Disrupting the microbiome can alter these
523 processes. For instance, heat-induced reductions in the abundance of the bacterial
524 symbiont *Buchnera aphidicola* in pea aphids lowered heat resistance, with fitness costs for the
525 host due to a deficiency of *Buchnera*-derived essential amino acids (Dunbar et al.,
526 2007). Similarly, temperature-driven microbiome shifts have been reported in mice (Chevalier
527 et al., 2015) and *Nilaparvata lugens*, with the disrupted microbiome assembly in the latter case
528 being harmful by increasing imidacloprid susceptibility (Zhang, Cai, et al., 2021).

529 **Cross-generational impacts of parental stress**

530 We found clear evidence for cross-generational fitness costs of paternal stress exposure, with
531 reduced hatching success and fecundity in offspring of fathers exposed to heat and/or pesticide
532 stress. Cross-generational effects can either increase (Pölkki et al., 2012; Schultz et al., 2016)
533 or decrease (Brausch & Salice, 2011; Kim et al., 2014; Reátegui-Zirena et al., 2017) offspring
534 vulnerability to stressors compared to the parental generation. Our results are consistent with
535 studies reporting detrimental cross- or transgenerational effects of paternal heat stress (Ferrer
536 et al., 2013; Guillaume et al., 2016; Shama et al., 2014; M. R. Walsh et al., 2014) or toxicant
537 exposure (Bhandari et al., 2015; Kimberly & Salice, 2015; Schultz et al., 2016; Yu & Liao,
538 2016), possibly through epigenetic processes (Schultz et al., 2016). The observed synergism
539 between paternal heat and pesticide exposure in offspring fitness underscores the potential for
540 long-lasting consequences of multi-stressor events.

541 Offspring from stressed fathers exhibited reduced fecundity and hatching success and did not
542 demonstrate increased tolerance to the same stressors, indicating that paternal exposure to
543 warming and/or pesticide does not buffer against these environmental stressors. These results
544 contrast with a growing number of studies suggesting that cross-generational effects can buffer

545 offspring against rapid environmental change (Donelson et al., 2018; Munday, 2014), with
546 parental heat exposure even being considered as a strategy to enhance species resilience
547 (Chakravarti et al., 2017). Instead, our findings align with previous work revealing negative
548 effects of heat and pesticide stress on offspring survival (Tran et al., 2018) and support the
549 view that cross-generational effects of stress are often non-adaptive or costly for offspring
550 (Uller et al., 2013), highlighting the potential for cumulative fitness declines across generations
551 in populations facing recurrent multi-stressor events.

552 Intriguingly, the paternal microbiome did not significantly mediate these cross-generational
553 effects in our study. This could be attributable to our experimental design, which deliberately
554 minimized vertical transmission or environmental acquisition of microbes by using axenic
555 mothers and transferring these and their offspring to sterile food vials immediately after mating
556 or hatching, respectively. This allowed us to isolate direct paternal effects (potentially
557 epigenetic or related to gamete quality) based on the sire's microbial status. However, we
558 cannot rule out a role for the paternal microbiome if transmission occurs naturally (e.g., during
559 copulation) (Guilhot et al., 2025), which then triggers differential maternal effects or microbial
560 deposition on the chorion of eggs (direct paternal transmission of microbes to their offspring
561 seems rather unlikely as males have little control over female oviposition). Future studies
562 explicitly allowing or manipulating vertical transmission would be needed to explore this.

563 **Conclusions and broader implications**

564 Our study demonstrates that acute heat stress and sublethal exposure to pesticides (e.g.,
565 imidacloprid) interact, often synergistically, to negatively impact male reproductive fitness in
566 *D. melanogaster*, with consequences extending to offspring viability and fecundity. Crucially,
567 we show that the gut microbiome is a key determinant of resilience, offering significant

protection against these stressors, with benefits enhanced by microbial complexity. However, the microbiome itself is vulnerable to disruption by these same stressors.

Our findings underscore the critical need to consider multi-stressor interactions and the role of host-associated microbes when assessing the ecological risks posed by climate change and agrochemical use. Predicting population responses requires moving beyond single-stressor studies to understand these complex interdependencies. The observed negative cross-generational effects further suggest that transient stress events could have prolonged demographic consequences. From a conservation perspective, these results highlight the potential vulnerability of insect populations, particularly those with depleted or altered microbiomes, to the combined pressures of warming climates and continued pesticide application. Future research should explore these interactions across diverse species, environments, and stressor combinations to improve ecological forecasting and develop strategies for mitigating biodiversity loss in the Anthropocene.

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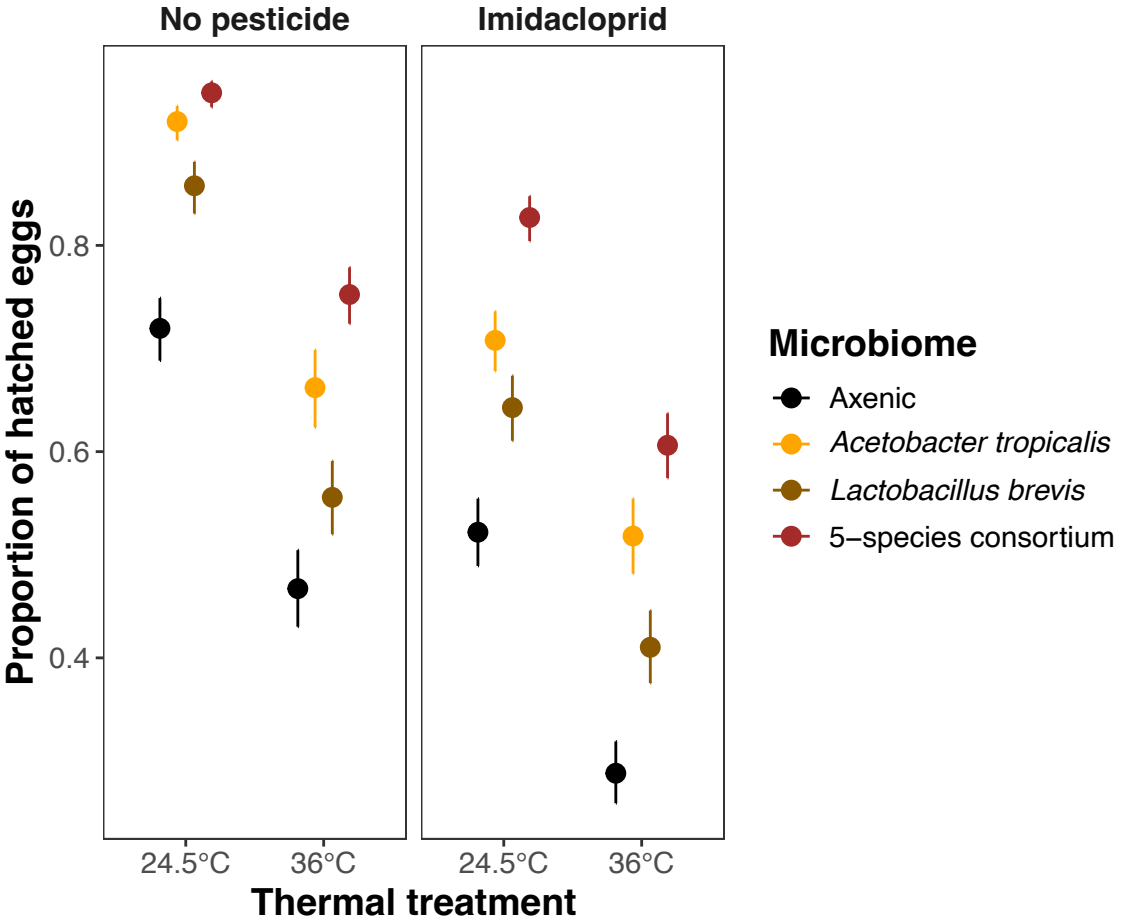
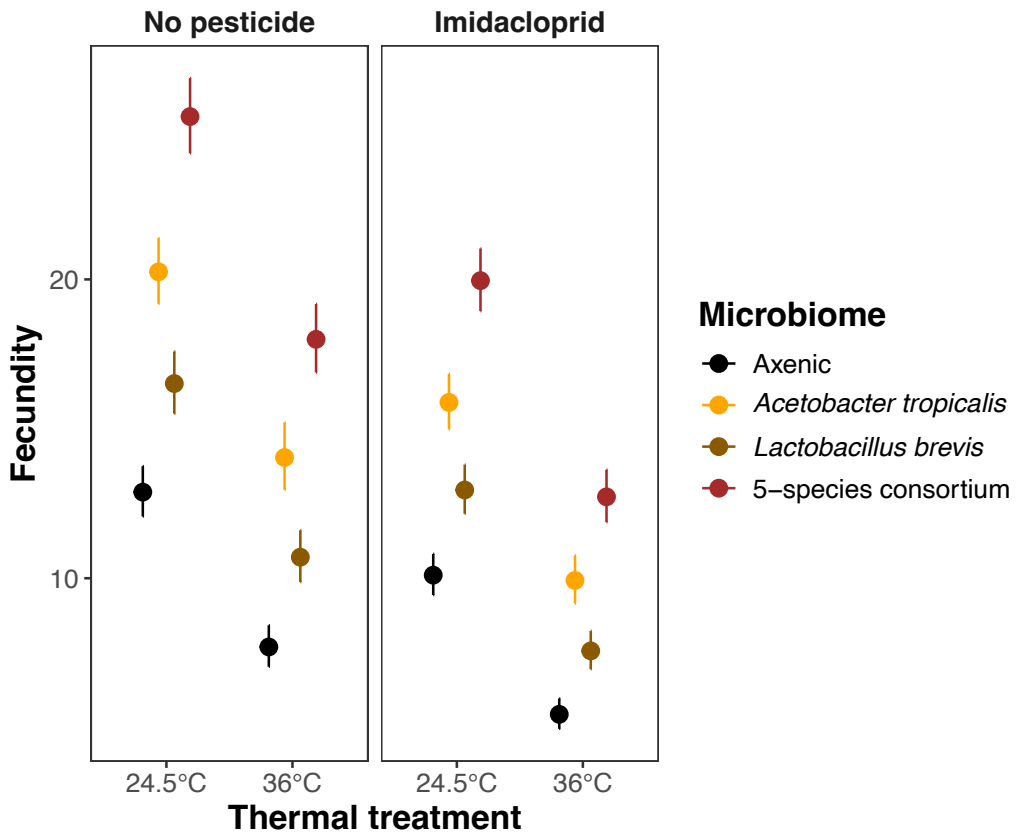


Figure 1 Proportion of eggs fertilized by males under varying conditions of thermal treatments (low: 24.5°C; high: 36°C), microbiome type (Axenic, *Acetobacter tropicalis*, *Lactobacillus brevis*, Control), and pesticide exposure (present and absent). Each colour represents a different microbiome treatment and two different panels correspond to presence/absence of pesticide treatment with two temperature treatments within each panel. Each circle represents the mean of all the replicates in that treatment combination with error bars corresponding to 95% confidence intervals.



1179

1180 **Figure 2** Mean fecundity (±95% CI) of females mated to males exposed to different
1181 combinations of two thermal treatments (low: 24.5°C; high: 36°C), microbiome type (Axenic,
1182 *Acetobacter tropicalis*, *Lactobacillus brevis*, and Control), and pesticide exposure (No
1183 pesticide, Imidacloprid). Each circle represents the mean of replicates per treatment group. The
1184 left and right panels correspond to pesticide absence and presence, respectively, with
1185 temperature conditions shown on the x-axis. Colours represent different microbiome
1186 treatments.

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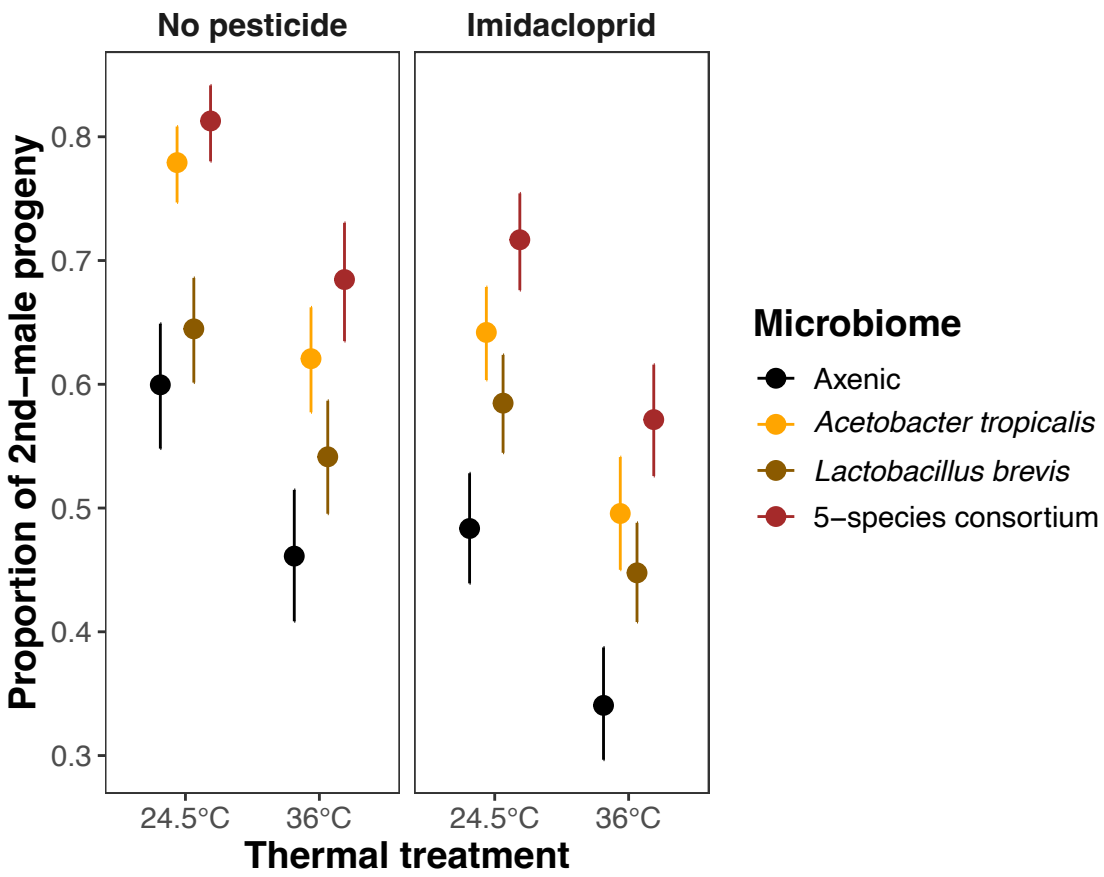

Figure 3 Proportion of progenies sired by the focal males (2nd in order to mate with the female) under varying conditions of thermal treatments (low: 24.5°C; high: 36°C), microbiome type (Axenic, *Acetobacter tropicalis*, *Lactobacillus brevis*, Control), and pesticide exposure (present and absent). Each colour represents a different microbiome treatment and two different panels correspond to presence/absence of pesticide treatment with two temperature conditions (low/high) within each panel. Each circle represents the mean of all replicates in that treatment combination with error bars corresponding to 95% confidence intervals.

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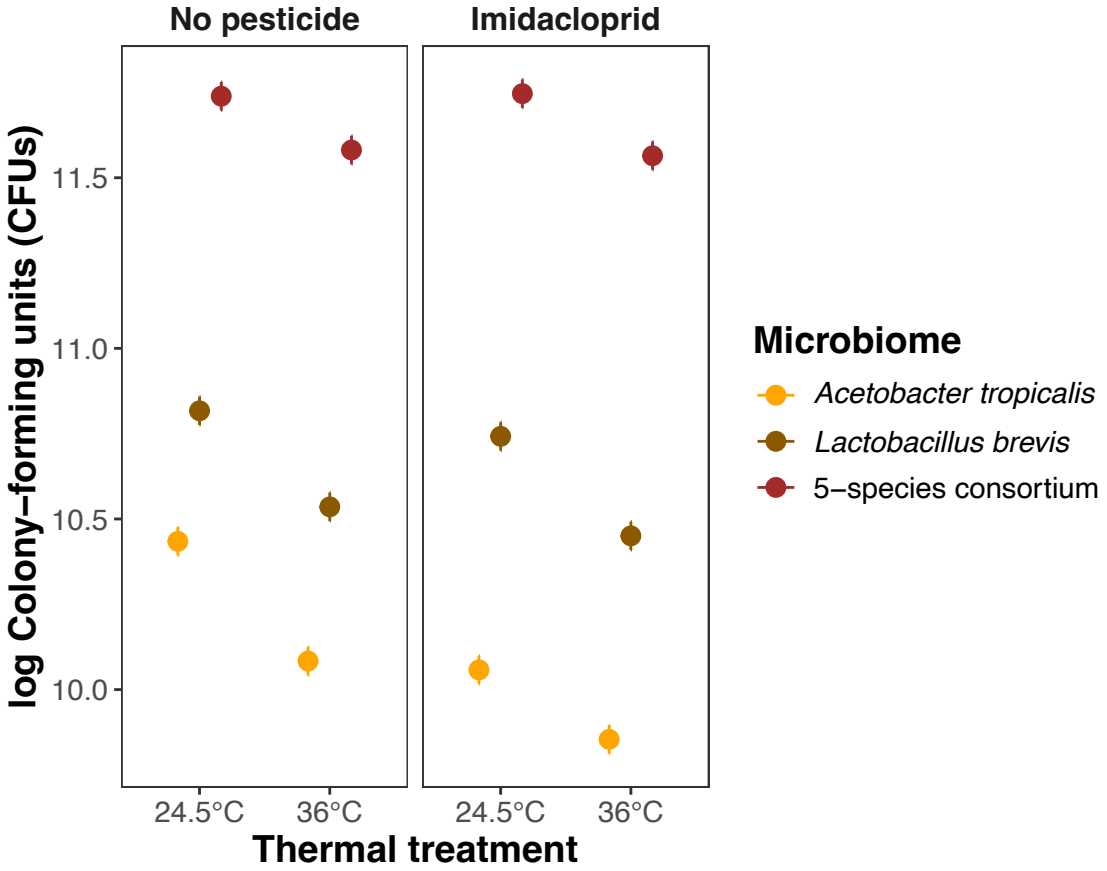

1203

1204 **Figure 4** Bacterial abundance/colony forming units of different microbial treatment

1205 (*Acetobacter tropicalis*, *Lactobacillus brevis*, Control) in response to different conditions of

1206 thermal treatments (low: 24.5°C; high: 36°C), and pesticide exposure (present and absent).

1207 Each colour represents a different microbiome treatment and two different panels correspond

1208 to presence/absence of pesticide treatment with two temperature conditions (low/high) within

1209 each panel. Each circle represents the mean of all the replicates in that treatment combination

1210 with error bars corresponding to 95% confidence intervals.

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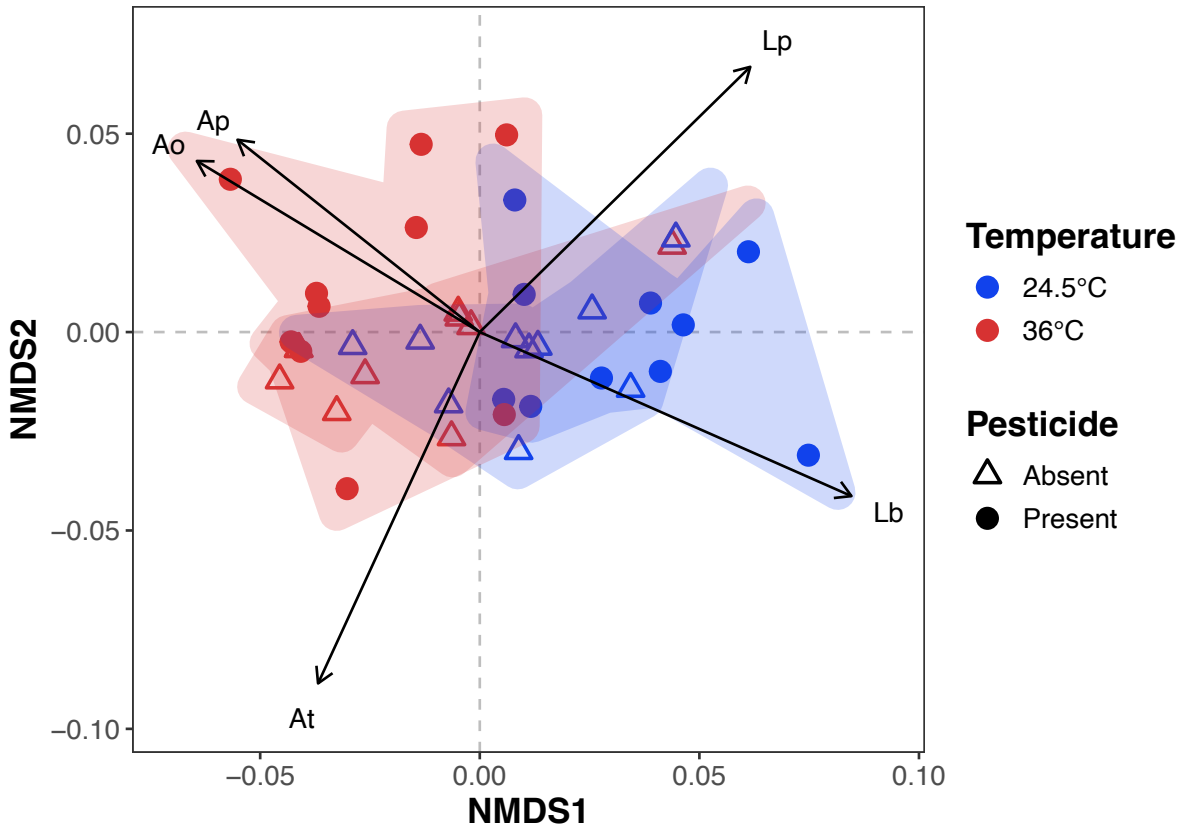
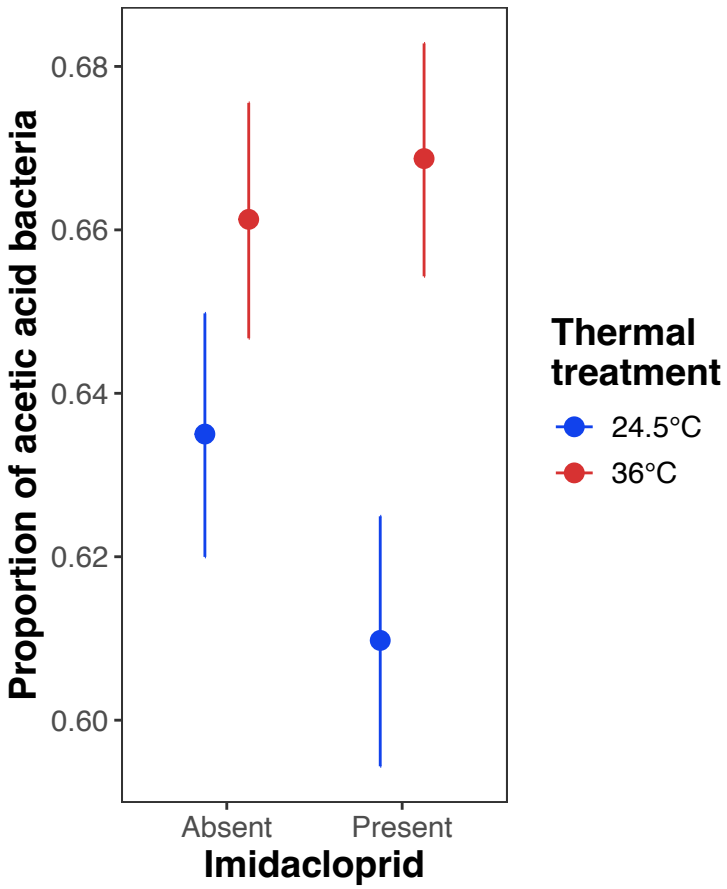


Figure 5. Non-metric multidimensional scaling (NMDS) ordination (Bray-Curtis distance, k = 4, stress = 0.046) of microbial community composition across temperature and pesticide treatments. Each point represents one male, colored by temperature treatment (red = Low, blue = High) and shaped by pesticide exposure (circle = Absent, triangle = Present). Shaded convex hulls denote 95% confidence areas for each temperature × pesticide group. Arrows represent the direction and strength of species (Hellinger-transformed) most strongly correlated with the ordination space, as identified by envfit. Species abbreviations: Ao = *Acetobacter orientalis*, Ap = *A. pasteuriensis*, At = *A. tropicalis*, Lb = *Lactobacillus brevis*, Lp = *L. plantarum*.



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1227 **Figure 6:** Proportion of acetic acid bacteria in the five-species consortium in response to heat

1228 and pesticide stress. Each circle represents the mean of all replicates in that treatment

1229 combination with error bars corresponding to 95% confidence intervals.

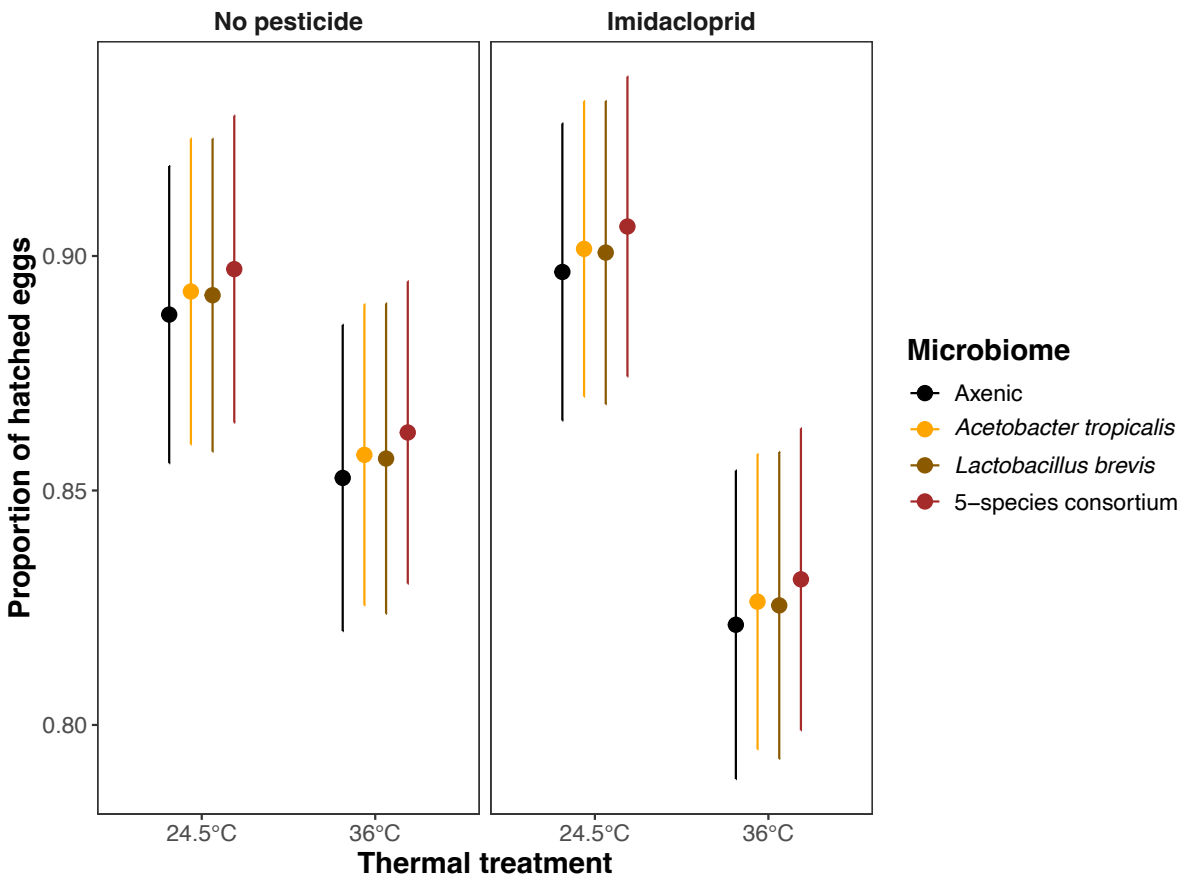

Figure 7. Proportion of eggs hatched out of total number of eggs fertilized by male offspring produced by fathers under different conditions of thermal treatments (low: 24.5°C; high: 36°C), microbiome type (Axenic, *Acetobacter tropicalis*, *Lactobacillus brevis*, Control), and pesticide presence (present and absent). Each colour represents a different F_0 microbiome treatment and two different panels correspond to presence/absence of F_0 pesticide treatment with two F_0 temperature conditions(low/high) within each panel. Each circle represents the mean of all the replicates in that treatment combination with error bars corresponding to 95% confidence intervals.

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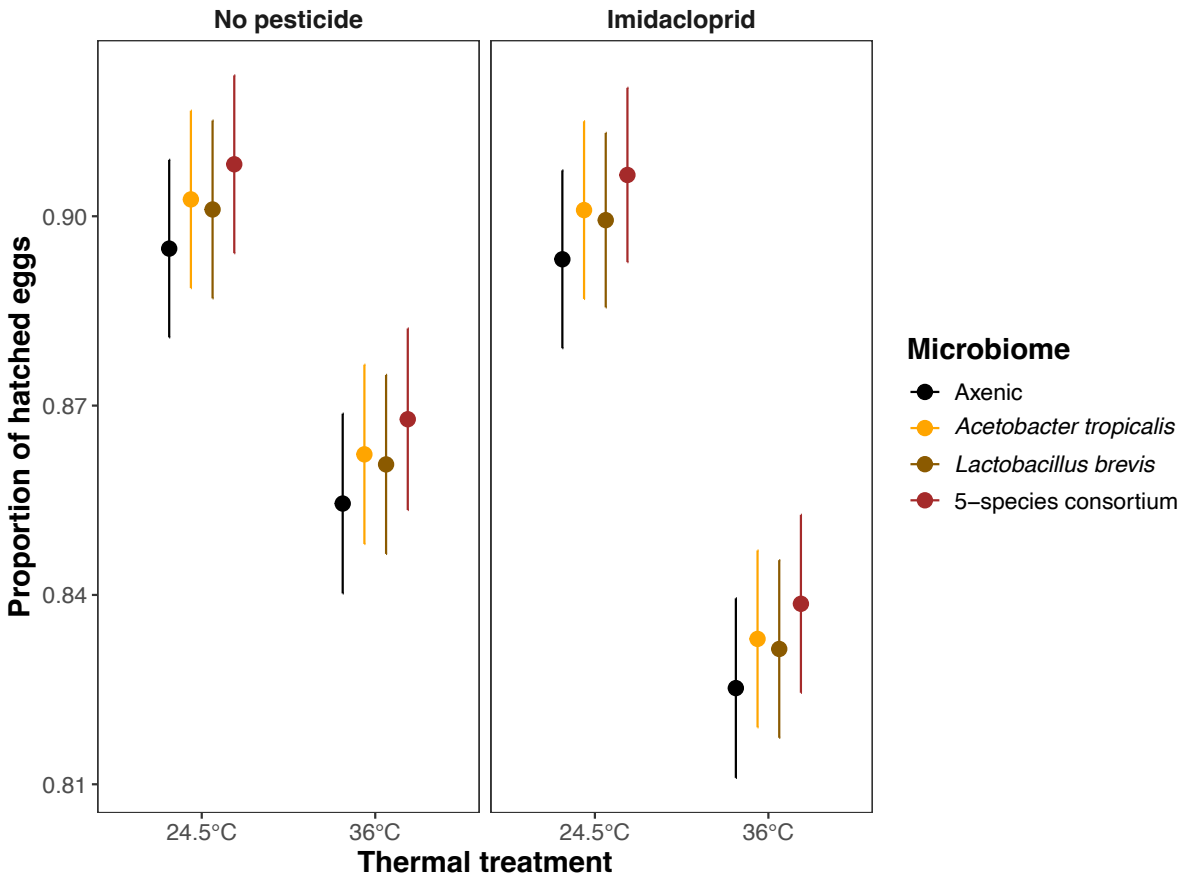

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1248 **Figure** 8. Proportion of hatched eggs out of total number of eggs laid by female offsprings
1249 produced by fathers under varying conditions of thermal treatments (low: 24.5°C; high: 36°C),
1250 microbiome type (Axenic, *Acetobacter tropicalis*, *Lactobacillus brevis*, Control), and pesticide
1251 presence (present and absent). Each colour represents a different F_0 microbiome treatment and
1252 two different panels correspond to presence/absence of F_0 pesticide treatment with two F_0
1253 temperature conditions(low/high) within each panel. Each circle represents the mean of all the

1254 replicates in that treatment combination with error bars corresponding to 95% confidence
1255 intervals.

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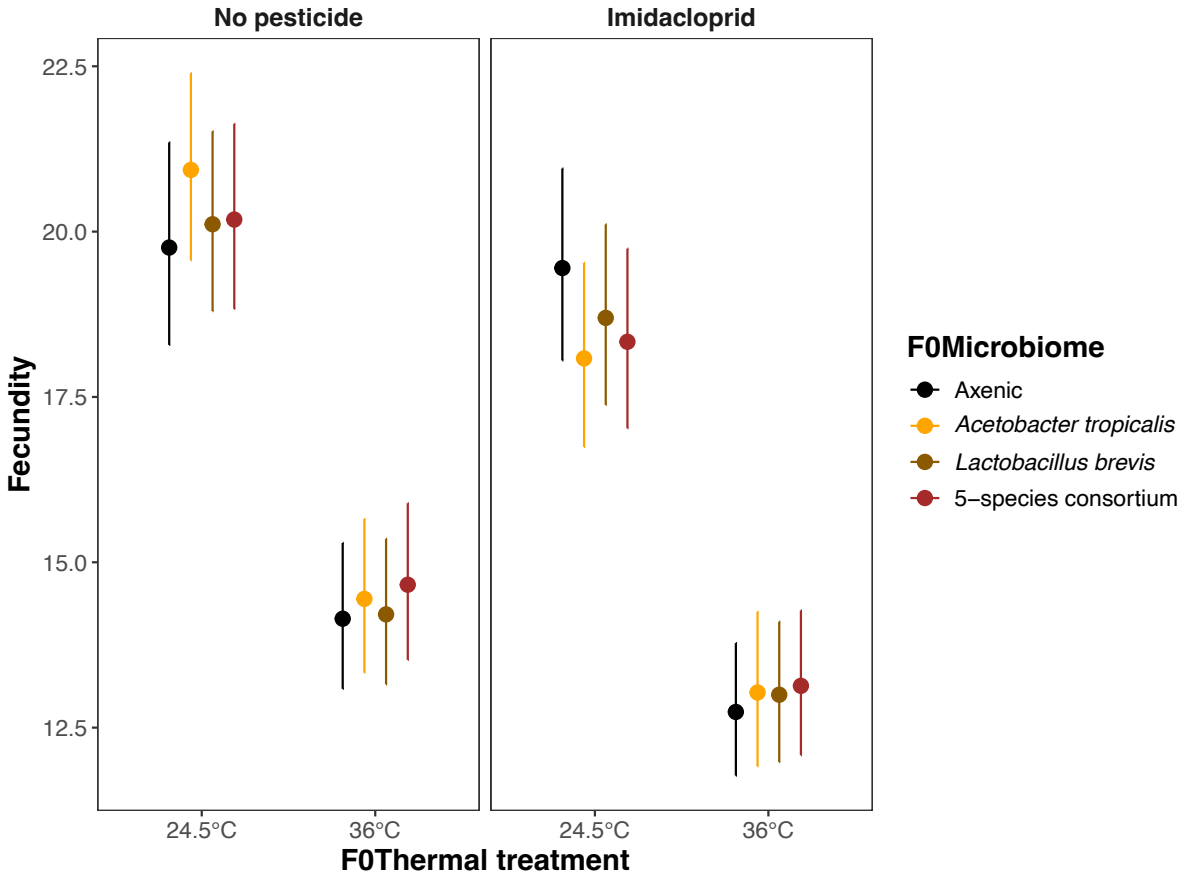
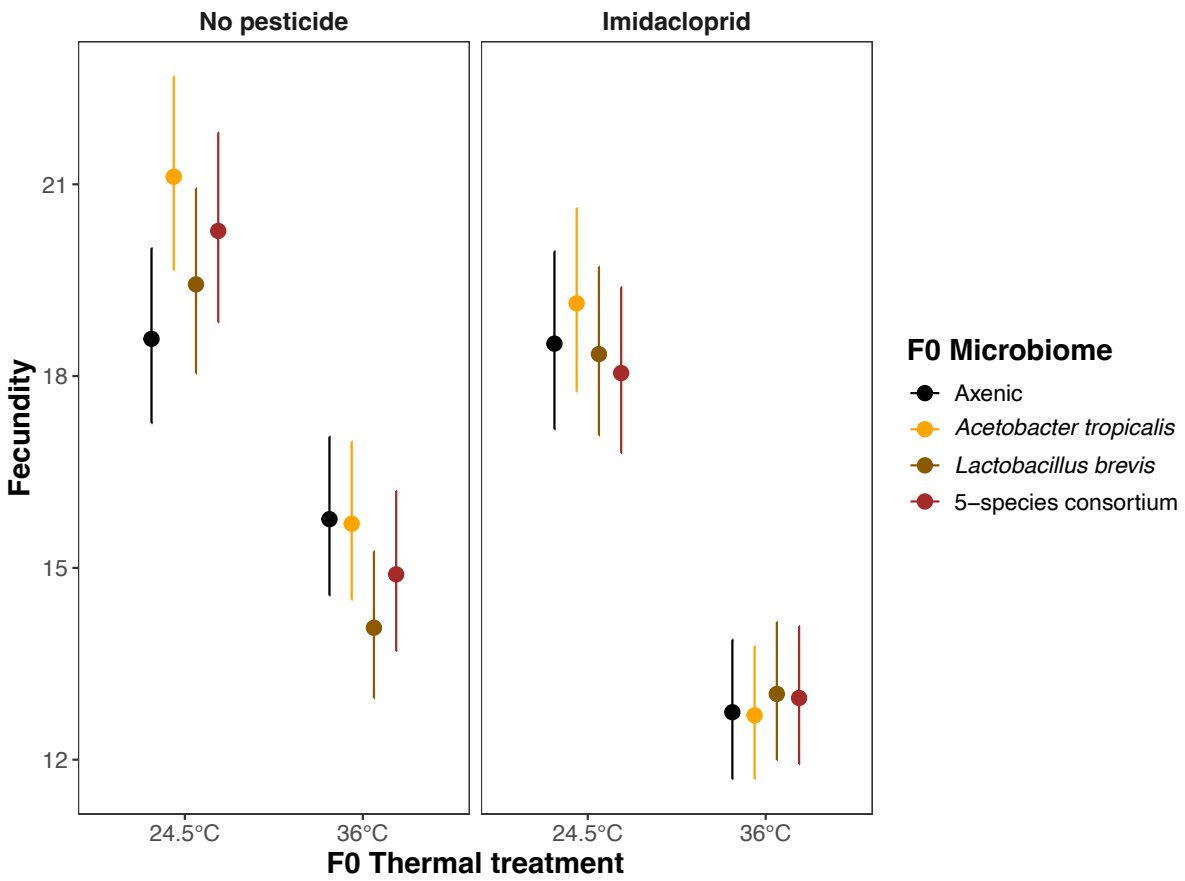


1257

1258 **Figure 9.** Total number of fertilised eggs laid by females mated to male offsprings produced
1259 by fathers under varying conditions of thermal treatments (low: 24.5°C; high: 36°C),
1260 microbiome type (Axenic, *Acetobacter tropicalis*, *Lactobacillus brevis*, Control), and pesticide
1261 presence (present and absent). Each colour represents a different F_0 microbiome treatment and
1262 two different panels correspond to presence/absence of F_0 pesticide treatment with two F_0
1263 temperature conditions (low/high) within each panel. Each circle represents the mean of all the
1264 replicates in that treatment combination with error bars corresponding to 95% confidence
1265 intervals.



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1267 **Figure 10.** Total number of fertilised eggs laid by female offsprings produced by fathers
1268 under varying conditions of thermal treatments (low: 24.5°C; high: 36°C), microbiome type
1269 (Axenic, *Acetobacter tropicalis*, *Lactobacillus brevis*, Control), and pesticide presence
1270 (present and absent). Each colour represents a different F_0 microbiome treatment and two
1271 different panels correspond to presence/absence of F_0 pesticide treatment with two F_0
1272 temperature conditions (low/high) within each panel. Each circle represents the mean of all
1273 the replicates in that treatment combination with error bars corresponding to 95% confidence
1274 intervals.