Male gut microbiome mediates post-mating sexual selection in *Drosophila*

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ABSTRACT

In polyandrous systems, male reproductive success is critically influenced by sperm competition, a process often mediated by ejaculate characteristics that are sensitive to male condition. While condition dependence is a cornerstone of sexual selection theory, the role of the gut microbiome in modulating male reproductive fitness under sperm competition remains unexplored. Here, we investigated the impact of varying gut microbial compositions on sperm competitive ability in *Drosophila melanogaster*. By inoculating germ-free (axenic) males with all 32 full-factorial combinations of five common commensal bacteria, we demonstrated that the presence and diversity of the gut microbiota significantly enhance male sperm offense ability. Specifically, males associated with a more diverse microbiota exhibited superior competitive fertilization success, accompanied by increased sperm transfer, higher sperm viability, and a greater proportion of sperm stored by females. Predictive models further revealed complex, non-additive interactions among bacterial species in their effects on sperm competitiveness. These findings suggest that the gut microbiome acts as a critical modulator

of male reproductive fitness, challenging traditional models of sexual selection that overlook contributions of non-pathogenic bacteria to condition-dependent traits.

INTRODUCTION

In polyandrous species, where females mate with multiple males, sperm competition often determines fertilization success based on ejaculate size and quality (1, 2). However, producing large, high-quality ejaculates is costly by diverting resources from other fitness traits (3–8). The dependence of trait expression on resource availability and allocation between growth, maintenance, and reproduction is central to sexual selection theory, linking phenotypic quality to underlying genetic variation: traits that reliably indicate a male's ability to acquire and allocate limited resources are hypothesized to signal heritable fitness benefits to mates or rivals (9, 10). This 'good genes' framework assumes that male condition is shaped by genetic variation in resource acquisition, allocation efficiency, or stress tolerance (11, 12). However, mounting evidence suggests that the gut microbiome, a dynamic microbial consortium, may decouple condition from host genetics by enhancing nutrient extraction, metabolic regulation, and hormone synthesis (7–9). If microbial communities independently modulate resource budgets, they could fundamentally alter the heritability of condition-dependent traits, challenging a cornerstone of sexual selection theory.

The gut microbiome directly influences host physiology, including nutrient assimilation (13–15) and energy allocation (16–17). For example, *Acetobacter* and *Lactobacillus* species in *Drosophila melanogaster* differentially regulate host metabolism, with *Acetobacter*-dominated microbiomes enhancing nutrient absorption and fecundity in females (18, 19). Conversely, disruptions to the gut microbiome can alter host metabolism, gene expression, or epigenetic modifications through changes in metabolite, enzyme, and hormone signalling (20–23), which can impair reproductive functions (23–25) or cause pathologies (26–28). While these effects

are well-documented (29, 30), the role of the gut microbiome in male reproductive performance, particularly in the context of post-mating sexual selection, remains entirely unexplored.

In *D. melanogaster*, a model system for host-microbe interactions, the gut microbiome is simple, tractable (31, 32), and critical for female reproductive traits (33, 34). For example, microbial composition shapes female fecundity, lifespan, and offspring development (18, 19). Such knowledge, however, is lacking for males. Limited work in mammals links gut dysbiosis to impaired testicular development (35) and spermatogenesis (36), while seminal microbes alter sperm motility in humans (37, 38). Yet, nothing is known about how gut microbes influence male fertilization success, let alone under sperm competition.

Here, we tested the hypothesis that the gut microbiome modulates male reproductive fitness under competitive fertilization by enhancing condition-dependent ejaculate traits. Using *D. melanogaster* males inoculated with all 32 combinations of five gut bacteria (*Acetobacter orientalis*, *A. tropicalis*, *A. pasteurianus*, *Lactobacillus brevis*, and *L. plantarum*; (19)), we quantified competitive fertilization success (P_2) and its mechanistic underpinnings: sperm viability, transfer, and storage. We predicted that (i) males with diverse microbiomes outperform axenic or low-diversity males due to improved nutrient acquisition (39, 40), (ii), synergistic interactions (e.g., *Acetobacter* dominance) amplify competitive advantages (19), and (iii) microbiome-driven gains in sperm viability and transfer efficiency underpin competitive fertilization success (41). By resolving how microbial communities shape sperm competition, this study bridges microbial ecology with sexual selection theory, offering novel insights into the evolution of condition-dependent traits.

74 **Results**

75 **Microbial diversity enhances sperm competitiveness**

76 To test if gut microbes influence male reproductive fitness under post-mating sexual selection,
77 we compared the competitive fertilization success of male *D. melanogaster*, inoculated in all
78 possible combinations between our five strains of gut bacteria, each against either an axenic
79 (germ-free) or a conventional male (i.e., associated with an intact microbiome). These standard
80 competitor males, which mated three days before our wild-type focal males, ubiquitously
81 expressed green fluorescent protein (GFP) to facilitate paternity assignment (42, 43).

82 In competition against axenic rivals, we found experimental males harbouring any single
83 bacterial species (except *L. brevis*) or consortium to outperform males without a microbiome,
84 with second-male paternity (P_2) increasing with species richness (t_{30} = 2.18, P = 0.037; Fig.
85 1A). Being associated with all five species translated into a 27% gain in P_2 compared to the
86 axenic state (mean ± s.d. = 0.85 ± 0.05 vs. 0.67 ± 0.05, respectively). Further, *Acetobacter*-
87 dominated communities conferred greater benefits than those biased toward *Lactobacillus* (Fig.
88 2A). These patterns persisted when focal males competed against rivals with intact microbiotas
89 (increase with richness: t_{30} = 2.44, P = 0.021; relative gain for 5-species association: 20% (0.72
90 ± 0.08 vs. 0.60 ± 0.03)), confirming that microbial benefits are not contingent on competitors
91 being axenic. The contrasting impacts of *Acetobacter* and *Lactobacillus* on P_2 were further
92 supported by bootstrapped correlations (Fig. 3A) showing P_2 varied with the relative abundance
93 (based on CFU counts) of each species across different microbial communities, not just with
94 their presence or absence (Fig. 3A).

95

96 **Microbial interactions shape competitive outcomes**

97 Mixed-effects models accounting for experimental block revealed that microbial interactions,
98 rather than additive effects, drove variation in P_2. Even though the five-way interaction between

99 bacterial strains was not statistically significant in competitions against either axenic ($N = 646$,
100 $t = 0.58$, $P = 0.561$) or conventional rivals ($N = 624$, $t = -0.86$, $P = 0.388$), eight and six of the
101 10 pairwise interactions, respectively, were statistically significant (all $t \leq -2.00$, $P \leq 0.046$),
102 with another two trends among the three-way interactions ($t \geq 1.69$, $P \leq 0.092$). Further, when
103 removing the non-significant five-way interaction, the four-way interaction of *At* × *Ap* × *Lb* ×
104 *Lp* ($t = 2.13$, $P = 0.034$) was also indicative of a more complex contribution of microbial
105 composition in this scenario.

106 Predictive modelling showed that averaging mono-association effects between bacterial
107 strains poorly forecasted outcomes for multi-species communities (e.g., mean of *At*, *Ap*, and
108 *Lb* to predict the *At-Ap-Lb* combination), with only eight (30.8%) of the 26 possible
109 combinations correctly predicted based on 95% confidence intervals (95%CI) including zero
110 difference in competitive matings against axenic rivals (Fig. 2B). Averaging pairwise
111 combinations to predict three-way species interactions (e.g., *At-Ap*, *At-Lb* and *Ap-Lb* to predict
112 *At-Ap-Lb*) indicated similar accuracy (31.2%: 5 of 16 predicted combinations; Fig. 2B). These
113 predictions by mono- or di-associations were broadly comparable when focal males competed
114 against conventional rather than axenic first males (33.0 and 37.5%, respectively). Combined,
115 these results further support the importance of interactions between bacterial species.

116 Additionally, pairwise interactions were context-dependent, varying conditionally on the
117 remaining ("bystander") species as reflected by interactions trending from positive
118 (synergistic) to negative (antagonistic) between the same two species depending on the
119 configuration of additional species in the microbiota (Fig. 4A). This dependence on bystanders
120 also held for three-way interactions between focal species (Fig. 4B).

121

122

123

124 **Mechanistic basis of microbiome-mediated fitness**

125 To dissect how microbiomes enhance competitive fertilization success, we quantified sperm
126 transfer, sperm viability, and female sperm retention and storage patterns, based on prior
127 knowledge (42–45). Due to the similar results between axenic and conventional competitor
128 males, we here restricted our mechanistic investigation to axenic first males. For visualization
129 of sperm, all assays except sperm viability involved standard and experimental males that,
130 respectively, expressed green or red fluorescent protein in sperm heads (44).

131 While axenic and mono-associated focal males transferred similar sperm quantities,
132 multi-associated males transferred 3–36% more sperm than either of the former categories,
133 with gains scaling with microbial diversity (t_{30} = 5.63, P < 0.0001; Figs. 1B and 2C,D). Sperm
134 viability also increased with diversity: males with all five species had 94.9 ± 2.3% viable sperm
135 compared to 54.5 ± 6.5% in axenic males (t_{30} = 11.54, P < 0.0001; Figs. 1C and 2E, F).
136 Critically, although freshly remated females did not significantly change the timing of ejecting
137 displaced and excess sperm from first (standard) and second (experimental) males,
138 respectively, in response to second-male microbial diversity (t_{30} = 1.10, P = 0.100; Figs. 1D
139 and 2G,H), the sperm retained were increasingly biased toward focal males if these had more
140 diverse microbiomes (t_{30} = 13.10, P < 0.0001; Figs. 1E, 2I,J). Hence, microbiome-enhanced
141 sperm quality or displacement efficiency biased post-mating sperm storage dynamics.
142 Confirming prior work (42, 43, 45, 46), the relative numbers of sperm stored and their viability
143 were significantly correlated with paternity outcomes (Fig. 3B). Further, these phenotypic traits
144 again covaried mostly positively with *Acetobacter* and negatively with *Lactobacillus* when
145 considering relative colony forming units (CFUs) rather than species presence or absence (Fig.
146 3A).

147 Similar to P_2, sperm viability and the relative representation of focal-male sperm in
148 storage after female sperm ejection responded to microbial interactions, with significant five-

149 way interactions in generalized linear mixed models for both sperm viability (N = 320, z = -
150 2.03, P = 0.043) and the proportion of second-male sperm in storage (N = 582, z = 2.11, P =
151 0.035). Predictive models for both variables also indicated considerable underestimation of
152 measured values by both averaged mono- or di-associations (Fig. 2F,J), highlighting the
153 importance of higher-order interactive effects, whereas this dependence was weaker for both
154 the number of sperm transferred and time to sperm ejection. For all stages of competitive
155 fertilization process, the interactive effects between pairs or triplets of microbes were strongly
156 influenced by bystander species in their contribution to phenotypic variation (Fig. 4C-J).
157

158 **Discussion**

159 Our study of *Drosophila melanogaster* establishes the gut microbiome as a significant
160 determinant of male reproductive success competitive fertilization. We demonstrate that
161 microbial diversity and composition, shaped by complex interspecific interactions, markedly
162 influence male competitive fertilization success (P_2). A reproductive advantage is directly
163 linked to improvements in critical ejaculate components, including increased sperm transfer,
164 substantially enhanced sperm viability (e.g., from ~55% in axenic males to ~95% in males with
165 the full 5-species consortium), and a greater proportion of sperm stored by females after mating
166 (Fig. 2). These findings redefine condition-dependent reproductive success by revealing the
167 gut microbiome as a dynamic mediator of sexual selection. This challenges classical
168 frameworks that largely attribute condition dependence to host resource status or stress
169 resilience (9–12) highlighting instead how symbiotic microbial interactions can directly shape
170 ejaculate traits and competitive outcomes, thereby acting as a critical, previously overlooked
171 evolutionary driver in reproductive adaptation (47).
172
173

174 **Linking microbial ecology to ejaculate quality**

175 The pronounced reproductive deficits in axenic males align with established connections
176 between microbiome absence and physiological dysregulation in *Drosophila* (48). While our
177 study did not directly assay host metabolic states, prior work compellingly shows that axenic
178 flies often exhibit hyperlipidemia and impaired insulin signalling – conditions detrimental to
179 overall health and potentially sperm function (46, 49–53). These metabolic disturbances can
180 be rescued or modulated by specific gut bacteria; for example, *Acetobacter* species are known
181 to restore lipid homeostasis, potentially via acetate production (54), whilst certain
182 *Lactobacillus* species can also influence host metabolism, albeit sometimes with trade-offs like
183 increased oxidative stress (55).

184 It is important to emphasize that our experimental design involved inoculating males only
185 after adult eclosion, following axenic development. This approach effectively isolates the
186 microbiome's influence on adult physiological processes critical for ejaculate production,
187 maintenance, and competitive performance. However, this focus on adult function means that
188 our estimates of the total contribution of the gut microbiome to male reproductive success are
189 likely to be conservative. Microbes present during larval and pupal development in insects are
190 known to impact host growth rate, final body size, and the maturation of various tissues (56).
191 Had these microbial partners also influenced the developmental trajectory—potentially
192 affecting body size, resource acquisition capacity, or the formation of reproductive organs—
193 the observed effects on adult competitive ability could have been further amplified or otherwise
194 altered. Disentangling these life-stage specific microbial effects remain an important avenue
195 for future research.

196 Based on our experimental context, we hypothesize that the observed improvements in
197 ejaculate quality and subsequent P_2 gains conferred by species-rich microbial communities
198 stem from their ability to restore or optimize host metabolic homeostasis and nutrient allocation

toward reproduction. The superior performance associated with *Acetobacter*-dominated communities (Fig. 1) lends support to this hypothesis, given their known efficacy in nutrient assimilation and influencing host metabolism (48). These microbes may channel resources more effectively towards energetically costly ejaculate production and maintenance, potentially mitigating damage from metabolic byproducts. However, the significant non-additive effects underscore that the *integrated function* of the microbial consortium, rather than simply the presence of key species, dictates the ultimate physiological impact (Fig. 3), mirroring the metabolic interplay seen in microbial ecology (57). Direct confirmation of these proposed metabolic links—identifying specific microbial metabolites (e.g., acetate or lactate) and tracing their effects on host resource allocation and sperm physiology—awaits future metabolomic and transcriptomic studies.

Evolutionary implications

Our findings prompt a re-evaluation of core tenets in sexual selection theory. By acting as an amplifier of condition-dependent reproductive traits, the microbiome introduces complexity to the "good genes" model, where such traits are assumed to reliably signal host genetic quality (10, 11). Our results suggest that the microbiome functions as a "hidden mediator," bridging host genetics and environmental inputs (58), with at least three key evolutionary implications. First, the microbiome potentially introduces a transmissible component influencing fitness that is sensitive to environmental factors (diet, social context) and operates outside strict Mendelian inheritance (58). If microbial consortia are consistently acquired or partially transmitted (vertically or horizontally), this could contribute to maintaining heritable variation in sexually selected traits, offering a potential factor in resolving the lek paradox (11). Furthermore, the microbiome might act as an "environmental sensor," allowing males to buffer reproductive output against nutritional fluctuations (5), thereby stabilizing their fitness.

224 Second, the context-dependent microbial interactions (Fig. 3) and potential trade-offs
225 (e.g., benefits vs. costs like oxidative stress potentially associated with *L. brevis*) suggest
226 complex coevolutionary dynamics. Selection may favour host mechanisms for managing
227 microbial partners ("screening" or selectively retaining beneficial symbionts), while microbes
228 may adapt to host environments, potentially leading to conflicts or mutualisms influencing
229 reproductive outcomes (59).

230 Third, the influence of the microbiome clearly extends into the female reproductive tract.
231 The finding that females preferentially stored sperm from males with more diverse
232 microbiomes (Figs. 1E and 2I) strongly suggests that microbiome-mediated enhancements to
233 ejaculate quality (sperm function and viability, possibly seminal fluid composition) interact
234 with female physiological processes. This opens a potential new dimension for cryptic female
235 choice (60, 61) with selection acting on microbiome-influenced male phenotypes.

236 The prevalence of synergistic effects also suggests that microbiome complexity and
237 functional integration might themselves be targets of selection, favouring males capable of
238 harbouring stable, high-performing microbial communities. Mechanistically, this could
239 involve selection for host factors that influence microbial colonization, resource availability
240 within the host environment, or the establishment of specific microbial interactions. Males with
241 genotypes promoting a harmonious and efficient microbial ecosystem would likely experience
242 enhanced physiological performance, ultimately translating to greater reproductive success.

243

244 **Towards microbiome-integrated sexual selection**

245 Realizing a comprehensive, microbiome-aware framework for sexual selection necessitates
246 several key avenues of future research. Foremost among these is the need to elucidate the
247 precise functional mechanisms underlying the observed fitness effects. This will require

integrated 'omics' approaches (e.g., metabolomics, transcriptomics) to map the links between microbial communities, their metabolites, and key ejaculate features influencing fertilization success. Alongside understanding the functional basis, it is also critical to determine how these fitness-enhancing microbiotas are maintained within populations. Future studies should therefore quantify the relative importance of different routes of transmission—including vertical passage, coprophagy, social interactions, or seminal transfer—and assess the stability, or heritability in a broad ecological sense, of beneficial microbial consortia across generations and varying environmental conditions.

Furthermore, exploring the role of females in these interactions would be an important avenue for future research. Investigations should examine female biases in both pre- and post-mating choice relative to potential male microbial cues or the enhanced quality of ejaculates produced by microbiome-rich males. A particularly crucial, yet unexplored, aspect is whether the female's own resident microbiome interacts with the incoming ejaculate or associated microbes, potentially influencing sperm storage patterns and biasing competition outcomes independently of male traits.

Finally, the ecological context and broader applicability of our findings remains to be explored. Research should examine how interactions between diet and the microbiome shape male reproductive strategies and sperm competition dynamics across different resource conditions. Assessing the generality of the microbiome-mediated effects on reproductive fitness in other species, particularly those with diverse mating systems, diets, and modes of microbial transmission, will be essential for understanding the widespread evolutionary significance of host-microbiome interactions in sexual selection.

Conclusion

Our research demonstrates that the gut microbiome is an active participant in shaping male reproductive competitiveness. By influencing ejaculate quality and quantity, likely through modulation of host metabolism and resource allocation (6, 62, 63), these microbial symbionts redefine the landscape of condition dependence. Integrating the microbiome into sexual selection theory may fundamentally alter our understanding of how fitness variation is generated, maintained, and acted upon by selection in polyandrous systems and beyond. Our study underscores the importance of considering the hologenome—the host plus its associated microbiota—as the relevant unit of selection when investigating the evolution of complex reproductive traits.

Material and Methods

Experimental populations

Throughout this study, we used *Drosophila melanogaster* of the LH_m strain. These were either derived from an outbred wild-type population or, for standard competitors in the sperm competition assay, from a backcrossed population that had been genetically modified to ubiquitously express green fluorescent protein (GFP), permitting paternity assignment against the focal wild-type males (42, 43). To study biases in female sperm storage, we used focal males whose sperm heads expressed red fluorescent protein (RFP), and our standard competitors with ubiquitous GFP expression, permitting quantification of each competitor's sperm inside the female reproductive tract (42–44). We maintained all fly populations at moderate densities under constant conditions of 25°C, 60% humidity, and a 12h:12h light:dark cycle.

297 **Generating axenic flies**

298 To generate axenic flies, we performed all steps hereafter in a laminar flow chamber to avoid
299 contamination. First, we collected eggs from Petri dishes containing grape-juice agar with a
300 smear of yeast paste that had been placed in four large population cages after a six-hour egg-
301 laying period. After washing these eggs off the plates into a strainer, we dechorionated them
302 with 3.5% bleach for 2 minutes, followed by a rinse in 70% ethanol and three rinses with sterile
303 water. We then transferred these eggs to autoclaved standard fly food supplemented with
304 antibiotics [a cocktail of Ampicillin (50mg/mL), Kanamycin (50mg/mL, Erythromycin
305 (10mg/mL), Tetracycline (10 mg/mL)] in autoclaved glass vials. These vials were then left
306 undisturbed under standard conditions for flies to develop.

307 Upon fly eclosion, we collected both males and females as virgins, separated them by
308 sex and confirmed their axenic state by plating a homogenized subsample of flies on LB and
309 MRS agar followed by 16S PCR. Briefly, we extracted genomic DNA from 3−5 pooled axenic
310 flies per sample using the ZymoBiomics™ DNA Microprep kit following the instructions
311 provided by the manufacturer. We also extracted DNA from flies with an intact microbiome to
312 serve as positive controls. For DNA amplification, we used bacterial universal primers 27F and
313 1492R as forward and reverse primers, respectively. We prepared the PCR master mix using
314 9.5 µl of nuclease free water, 12.5 µl kappa HiFi master mix, 1 µl of forward and reverse primer
315 and 1 µl of template DNA. The PCR cycle included an initial denaturation at 95°C for 90 s,
316 followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, an extension
317 at 72°C for 90 s, and a final extension at 72°C for 5 mins. We ran the PCR products on a 1%
318 agarose gel and visualized them under ultraviolet light after staining with SYBR Safe
319 (Invitrogen) to confirm the absence of bacteria in axenic individuals and the presence of
320 bacteria in controls.

321 We maintained all females and standard competitors as axenic flies throughout the
322 experiment and supplemented axenic focal males with different bacterial cultures to produce
323 gnotobiotic associations as described below.

324

325 **Bacterial cultures and gnotobiotic associations**

326 Throughout this study, we used five bacterial species isolated from the gut of wild-type flies
327 (20): *Acetobacter orientalis*, *A. tropicalis*, *A. pasteurianus*, *Lactobacillus plantarum*, and *L.*
328 *brevis*. We associated our focal flies with all 32 possible combinations among these bacterial
329 strains following the general protocol of Gould et al. (19). To prepare bacterial cultures, we
330 grew them overnight: *Lactobacillus* spp. in MRS (De Man–Rogosa–Sharpe) broth without
331 shaking and *Acetobacter* spp. in MRS + mannitol medium at 30°C on a shaker. The next
332 morning, we checked these bacterial cultures for growth and performed their optical density
333 (OD) measurements at 600 nm. Then, we spun down these different bacterial cultures at 7000
334 rpm for 5 minutes and resuspended the resulting pellets to a concentration of 10^8 cells/mL in
335 sterile phosphate-buffered saline (PBS). Resuspension volume was determined following
336 established protocols (64, 65). Finally, we added 50 µL (5×10^6 colony forming units, CFUs)
337 of bacterial culture to sterile food vials, with equal representation of bacterial strains for
338 combinations with more than one association. This bacterial density was comparable to prior
339 reports for conventional flies (19, 65).

340 After adding the bacterial culture to sterile food vials and air-drying these under in laminar
341 flow hood, we transferred 10 one-day-old axenic virgin males to each vial for inoculation with
342 the added bacterial strains. We moved these males to fresh food vials with same bacterial
343 treatment every alternate day for 5 days. Thereafter, we confirmed gnotobiotic associations by

344 plating together the homogenates of 3-4 adult flies from same treatments and checking their
345 colony morphologies, and by Sanger sequencing of 16S PCR homogenates. For multi-
346 associations, we plated the fly homogenates and picked the distinct types of colonies from a
347 given plate for PCR and performed sequencing to confirm that morphologically similar species
348 corresponds to same species in all the replicates, thus allowing us to distinguish between
349 different bacterial species by looking at their colony appearance. Thereafter, we tested the
350 presence of the expected bacterial species in a particular bacterial combination by plating them
351 and looking at the colony morphologies.

352

353 **Bacterial abundance in different combinations**

354 To determine bacterial abundance in different gnotobiotic associations, we counted the CFUs
355 per fly for each bacterial species across the different gnotobiotic associations. To this end, we
356 washed males with 70% ethanol, individually homogenized them with a pestle in
357 microcentrifuge tubes, and then diluted these homogenates to different dilutions with a final
358 volume of 100 µl to find a dilution with comparable colony counts (approx. 100 CFUs) for
359 visual quantification. In gnotobiotic cultures with both bacterial genera, we transferred an
360 aliquot of well-mixed fly homogenate to two plates, supplementing the MRS media of one
361 plate with 10 µg/ml ampicillin to selectively inhibit the growth of *Lactobacillus*, while
362 incubating the other in a CO_2 atmosphere to prevent the growth of *Acetobacter*. We achieved
363 within-genus differentiation of strains by colony morphologies: *A. orientalis* with flat colonies
364 with ruffled borders, *A. tropicalis* with small tan coloured and highly reflective colonies, and
365 *A. pasteurianus* with large tan colonies. Similarly, *L. brevis* produces brownish yellow colonies
366 and *L. plantarum* milky white colonies (Supplementary Fig. S1). Using 16S PCR amplification
367 followed by Sanger sequencing, we confirmed specificity of these colony properties,

368 demonstrating the robustness of our method based on morphological identifications during
369 experiments. After counting the number of colonies on each plate, we calculated CFU per fly
370 as CFU = C × D × V / P, where C = number of colonies counted, D = dilution factor, V =
371 volume of fly homogenate, and P = volume plated (64).

372 Across all 10 replicate plates combination examined, the expected species were present, and
373 none of the remaining species. Consistent with Gould et al. (19), total CFU counts increased
374 with species richness while the species-specific abundances declined (Supplementary Fig. S2).

375

376 **Sperm competitive ability**

377 To investigate sperm competitiveness of focal males, we quantified their sperm offense ability
378 (P_2) by mating them as the second males with axenic females that had each mated with a an
379 axenic GFP male three days earlier. We monitored the occurrence of copulations for 7-8 hours
380 and transferred remated females to oviposition vials while giving those that did not remate
381 another two 8-hour remating opportunities on the following days. Flies that did not remate by
382 the last opportunity (within these 3 days) were discarded. We allowed all remated females to
383 lay eggs for three days. After all progeny eclosed, we scored them for paternity under a
384 fluorescence stereoscope, based on the presence/absence of the ubiquitous GFP marker in
385 standard competitors. The proportion of second-male progeny indicated the focal-male sperm
386 competitive ability (i.e., sperm offense). We replicated this experiment thrice with 30 replicates
387 per bacterial treatment each time (total N = 2,880 trials).

388

389 **Sperm viability**

390 We quantified sperm viability for 10 males per bacterial treatment (total N = 320 males). Once
391 males were six days old and inoculated, we anaesthetized them on ice and dissected them on a

392 microscopic slide to isolate the seminal vesicles from their reproductive tissue. We then placed
393 one of the seminal vesicles in 10 µl of Ringers solution and punctured it with an insect pin to
394 release the sperm mass for immediate staining with LIVE/DEAD fluorescent dye, following
395 the principles of Tourmente et al. (41). This two-component dye consists of SYBR-14 ('LIVE'
396 component emitting green fluorescence), which is actively incorporated into living cells, and
397 propidium iodide (PI, with red fluorescence) that can only permeate damaged membranes
398 (typical of dead cells). We prepared an aqueous staining solution by combining 2.4 µL of
399 SYBR-14 stock solution (1 mM in DMSO) and 4.5 µl of PI stock solution (2.4 mM in water)
400 in 93.1 µl of Ringer's solution. Then, we added 5 µl of the staining solution to the sperm sample
401 before covering it with a coverslip and incubating it for 5 minutes in the dark. Under an
402 Olympus BX50 fluorescence microscope with a GFP/RFP dual filter, we captured images of
403 randomly chosen areas of each sample to later count at least 200 sperm and calculate the
404 proportion of viable sperm in ImageJ v. 1.34 (66).
405
406 **Visualizing sperm competition within female reproductive tract**
407 Since competitive fertilization events ultimately are the result of both male and female effects
408 (43, 67), we investigated how females bias their sperm storage based on interactions with males
409 supplemented with different microbiome. For this experiment, we again mated axenic virgin
410 females first with an axenic GFP male and, three days later, with a focal male as for sperm
411 competitive ability (above). To visualize sperm of both males, we used focal males expressing
412 RFP in their sperm heads. Immediately after remating, we transferred females to individual
413 wells of cell-culture plates, with a coverslip held in place with dots of rubber cement in two
414 corners. We checked these females every 10 min for up to 6 h for the ejection of the sperm
415 mass containing both displaced first-male and excess second-male sperm (42, 44). The timing
416 and extent of sperm ejection have previously been reported to affect relative numbers of first-

417 and second-male sperm stored by females and, consequently, competitive fertilization success
418 among males (43).

419 Upon female sperm ejection, we recorded the interval from mating to ejection before removing
420 females from the well and freezing them individually in Eppendorf tubes. Thereafter, we
421 dissected these females and counted the green (standard competitors) and red sperm (focal
422 males) within the female reproductive tract (paired spermathecae, seminal receptacle and bursa
423 copulatrix) under a fluorescence microscope. From these counts, we determined S_2, the
424 proportion of all sperm stored by the female that came from the second (experimental) male.
425 We also counted the respective sperm numbers in the ejected sperm mass to estimate, combined
426 with above counts, the total number of sperm transferred by the second male and the number
427 of first-male sperm still residing in storage at the time of remating. This experiment was
428 performed in five blocks with 10 replicates per treatment for each block (total $N = 1{,}600$ trials).

429

430 **Statistical analyses**

431 We estimated the contributions of microbial interactions to variation in reproductive traits
432 using mixed-effects models with the five bacterial species and all two- to five-way interactions
433 as predictors, while accounting for experimental block as a random effect. Models were either
434 linear (for number of sperm transferred, time to sperm ejection) or generalized with a binomial
435 error structure (for proportions of viable sperm or of second-male sperm in storage). For the
436 proportion of second-male offspring, we chose a linear model on logit-transformed P_2 data
437 instead of a binomial model because the latter showed poor performance, also when adjusting
438 for dispersion or residual biases by using betabinomial or Tweedie link functions instead of the
439 default 'binomial' function. Additionally, we quantified the effect of species richness on trait

440 expression in models using the number of bacterial species per treatment as the fixed effect,
441 and block, the treatment group and (where applicable) observation as random effects. Here, the
442 binomial model on P_2 performed well and was preferred.

443 To better understand the importance of species combinations in the microbiome and to isolate
444 emergent effects beyond individual species contributions, we used an epistasis-based
445 framework to elucidate how well the averaged phenotypic effects of single- or pairwise species
446 associations predict higher-order phenotypes (19). For single-species predictions, we compared
447 the measured trait values of males associated with two- to five-species microbiotas to values
448 averaged across mono-associated males that jointly represented the same bacterial
449 combinations. These differences (averaged prediction minus measured trait value) were then
450 plotted with bootstrapped 95% confidence intervals to visually determine if predictions
451 deviated from the measured estimates (i.e., confidence interval excluding zero difference). We
452 then repeated this procedure to predict phenotypes by averaging the estimates of di-associated
453 males (for calculations see (19)).

454 Following the same general principle, we further quantified how the presence or absence of
455 non-focal ("bystander") community members modulates both pairwise and three-way
456 microbial interactions by exhaustively enumerating all possible bystander contexts. We then
457 compared this "contextual" analysis against a "standard" test to reveal how higher-order
458 community background shapes emergent effects on host traits. Following Gould et al.'s (19)
459 nomenclature, the "standard" test calculated the interaction coefficient among all species
460 present in a specific community configuration, whereas the "contextual" tests assessed the
461 interaction between a focal species pair within the context of additional bystander species. We
462 computed interaction coefficients for each focal species pair or triplet across all possible
463 presence/absence combinations of bystander species and plotted them with bootstrapped

standard errors to highlight the varying directions of interactions depending on the bystander combinations.

In both the single/double-species predictions and these standard/contextual tests, we derived the coefficients to calculate the deviations from a mixed-effects models with an appropriate link function for each microbial combination, controlling for block effects. We repeated each model across 1,000 bootstrapped datasets to calculate the standard errors or 95% confidence intervals from the distribution of the calculated coefficient or interaction differentials.

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Figures

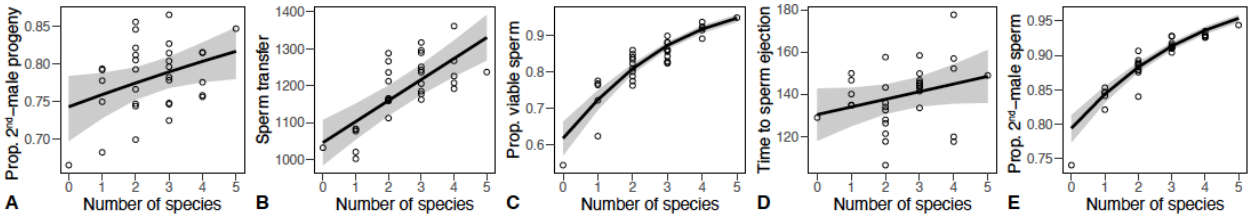

Fig. 1. Associations between the number of bacterial species and the different fitness traits. Each point indicates the mean of all replicates for a given microbiome consortium (N = 32). The lines and5% confidence bands are derived from (generalized) linear mixed-effects models with microbial species composition and block as random effects (for binomial models additionally also an observation-level random effect to address overdispersion).

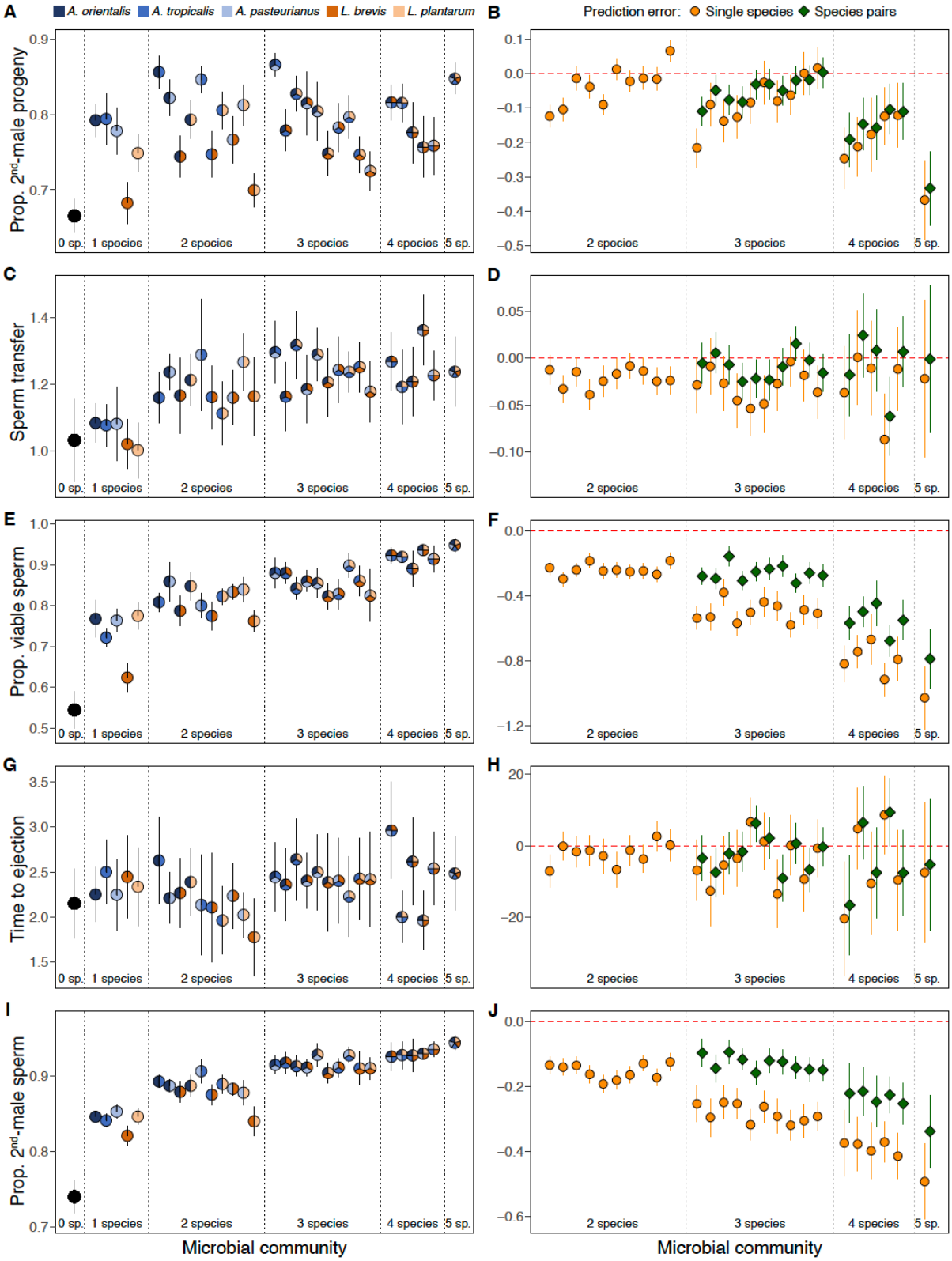

Fig. 2. Variation in male fitness traits relative to their microbial community (left) and error in predicting these values for males associated with multiple species based on single- or pairwise species averages (right). In the left panels, colours indicate the different combinations of

640 *Acetobacter* and *Lactobacillus* species associated with experimental males. The right panels
641 depict the deviations in trait predictions from measured traits by averaging the corresponding
642 coefficients of (generalized) linear mixed-effects models for individual species (orange points)
643 or species pairs (green diamonds) that jointly represented the same species communities. The
644 relevant microbial combinations are plotted in the same order across all panels. Error bars
645 reflect 95% confidence intervals (from raw distributions in left panels, from bootstrapped
646 prediction differentials in right panels).

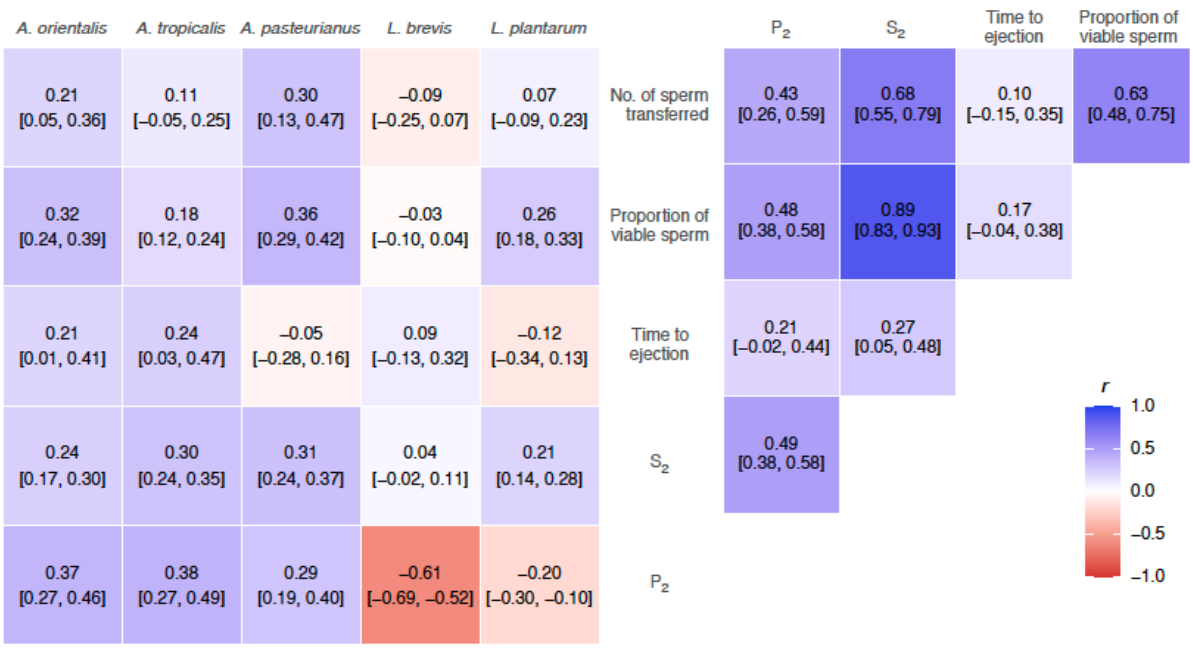

Fig. 3. Correlations between the different phenotypic traits and the relative abundance (based on CFU counts) of each of the five bacterial species (left) and among the traits themselves (right). Values reflect the averaged correlation coefficients with 95% confidence limits of pairwise Pearson correlations across bootstrapped group means (N = 1,000 samples) among males with a given microbiome consortium. Relative abundances and proportional traits (sperm viability, S_2, P_2) were logit-transformed before bootstrapping. Colours indicate the strength and direction of correlations as reflected by the colour-gradient bar.

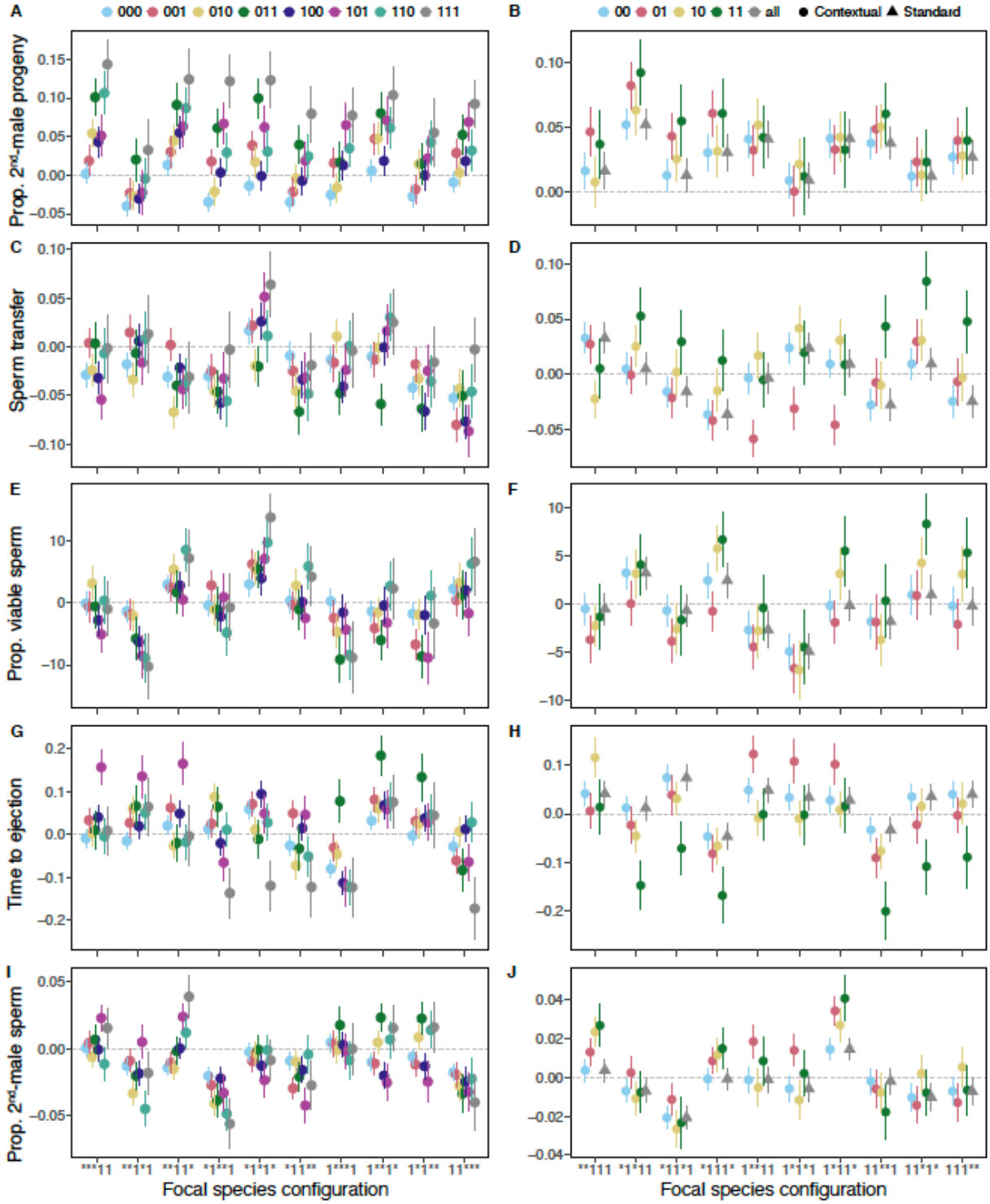

Fig. 4. Interactive effects between bacterial species contingent on bystander species, with two-way interactions between pairs of focal of species in the left panels, and three-way interactions between trios of focal species in the right panels. For each code along the x-axes, 1's indicate the species configuration of the interaction and asterisks denote placeholders for bystander species (order within codes from left to right: *A. orientalis*, *A. tropicalis*, *A. pasteurianus*, *L.*

661 *brevis*, *L. plantarum*). The binary codes in the legends indicate the configuration of bystanders
662 in the order, in which they would fill the placeholders (*) in each focal species configuration
663 (1 = presence, 0 = absence). The right panels distinguish between the “standard” tests (grey
664 triangles; i.e., global interaction across all community members present) and "contextual" tests
665 (coloured points; i.e., separate interactions between a focal species pair or triplet for each
666 bystander combination) for each focal triplet. All coefficients and standard errors were derived
667 from (generalized) linear mixed-effects models across 1,000 bootstrapped datasets.