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**Speciation by Symbiosis:  
The Microbiome and Behavior**

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10 **ABSTRACT**

11           Species are fundamental units of comparison in biology. The newly discovered  
12 importance and ubiquity of host-associated microorganisms is now stimulating work on the roles  
13 that microbes can play in animal speciation. We previously synthesized the literature and  
14 advanced concepts of speciation by symbiosis with notable attention to hybrid sterility and  
15 lethality. Here, we review recent studies and relevant data on microbes as players in host  
16 behavior and behavioral isolation, emphasizing the patterns seen in these analyses and  
17 highlighting areas worthy of additional exploration. We conclude that the role of microbial  
18 symbionts in behavior and speciation is gaining exciting traction, and the holobiont and  
19 hologenome concepts afford an evolving intellectual framework to promote research and  
20 intellectual exchange between disciplines such as behavior, microbiology, genetics, symbiosis  
21 and speciation. Given the increasing centrality of microbiology in macroscopic life, microbial  
22 symbiosis is arguably the most neglected aspect of animal and plant speciation, and studying it  
23 should yield a better understanding of the origin of species.

24

25 **MINIREVIEW**

26 In 1998, Carl Woese referred to the microbial world as the "sleeping giant" of biology  
27 (1). Almost two decades later, unprecedented attention to our microbial world has turned the  
28 fields of zoology (2) and botany (3) inward - towards an increased awareness and understanding  
29 of individual animals and plants as holobionts (4–6). The term "holobiont" denotes a host plus all  
30 of its microbial symbionts, including inconstant and constant members that are either vertically  
31 or horizontally transmitted or environmentally acquired; it was first coined in 1991 by Lynn  
32 Margulis (reviewed in 5). The ubiquity and importance of microbes in and on holobionts,  
33 including humans, is evident in studies of host development (7), immunity (8), metabolism (9–  
34 12), behavior (13, 14), speciation (15, 16), and numerous other processes. Host-microbe  
35 interactions provide the holobiont with disadvantages (17–19) such as increasing the risk of  
36 cancer (20), and advantages (7, 21–23) such as driving the evolution of resistance to parasites  
37 and pathogens (24–26), and among other things producing signal components (i.e., metabolites)  
38 used to recognize differences in potential mates (27, 28).

39 The newfound importance of diverse microbial communities in and on animals and plants  
40 led to the development of the hologenome theory of evolution (4, 29). The "hologenome" refers  
41 to all of the genomes of the host and its microbial symbionts, and the theory emphasizes that  
42 holobionts are a level of phenotypic selection in which many phenotypes are produced by the  
43 host and microbial members of the holobiont. This developing scientific framework distinguishes  
44 itself by placing importance not only on well-studied primary microbial symbionts and vertical  
45 microbial transmission, but also on the vast diversity of host-associated microbes and horizontal  
46 microbial transmission. The key reason for aligning these different transmission modes and  
47 levels of complexity into an eco-evolutionary framework is that the community-level parameters

48 among host and symbionts in the holobiont (e.g., community heritability, selection and  
49 coinheritance) can be analyzed under a common set of concepts to the parameters that occur in  
50 the nuclear genome (6, 30).

51 As natural selection operates on variation in phenotypes, the hologenome theory's most  
52 significant utility is that it reclassifies the target of "individual" selection for many animals and  
53 plants traits to the holobiont community. This claim is straightforward given the overwhelming  
54 influence of microbes on host traits (31–34). The question going forward is whether the response  
55 to this community-level selection is relevant to the biology of holobionts. In other words, can  
56 host-associated microbial communities be selected such that shifts in the microbial consortia  
57 over multiple generations are a response to selection on holobiont traits? Community selection at  
58 the holobiont level is shaped by genetic variation in the host and microbial species and  
59 covariance between hosts and their microbial consortia, the latter of which can be driven by (i)  
60 inheritance of the microbial community from parents to offspring (35, 36) and/or (ii) community  
61 heritability  $H^2_C$  (30, 37). We recently summarized ten foundational principles of the holobiont  
62 and hologenome concepts, aligned them with pre-existing theories and frameworks in biology,  
63 and discussed critiques and questions to be answered by future research (6).

64 In the context of the widely accepted Biological Species Concept (38, 39), the principles  
65 of holobionts and hologenomes offer an integrated paradigm for the study of the origin of  
66 species. The Biological Species Concept operationally defines species as populations no longer  
67 capable of interbreeding. Reproductive isolation mechanisms that prevent interbreeding between  
68 holobiont populations are either prezygotic (occurring before fertilization) or postzygotic  
69 (occurring after fertilization). In the absence of reproductive isolation and population structure,  
70 unrestricted interbreeding between holobiont populations will homogenize populations of their

71 genetic and microbial differences (6). While postzygotic isolation mechanisms include hybrid  
72 sterility or inviability, prezygotic isolation mechanisms can include biochemical mismatches  
73 between gametes and behavior mismatches between potential partners.

74 Symbionts can cause prezygotic reproductive isolation in two modes: broad-sense and  
75 narrow-sense (40). Broad-sense symbiont-induced reproductive isolation refers to divergence in  
76 host genes that result in a reproductive barrier because of selection on the host to accommodate  
77 microorganisms. In this case, loss or alteration of the symbiont does not have an impact on the  
78 capacity to interbreed; rather host genetic divergence and reproductive isolation evolve in  
79 response to microbial symbiosis and cause isolation regardless of whether the hosts are germ-  
80 free or not. Conversely, narrow-sense symbiont-induced reproductive isolation occurs when  
81 host-microbe or microbe-microbe associations result in a reproductive barrier, namely one that  
82 can be ameliorated or removed via elimination of the microbes. Therefore, narrow-sense  
83 isolation can be experimentally validated if it is reversible under microbe-free rearing conditions  
84 and inducible with the reintroduction of microbes. Isolation barriers that require host and  
85 microbial component underpin hologenomic speciation (6, 16).

86 We recently synthesized the literature and concepts of various speciation mechanisms  
87 related to symbiosis, with notable attention to postzygotic isolation (40–42). While aspects of the  
88 microbiology of prezygotic isolation are less understood, seminal cases exist (43–45) and control  
89 of behavior by symbionts is an emerging area of widespread interest (14, 46, 47). Here we  
90 emphasize the patterns seen in these new and old analyses (Table 1) and highlight important and  
91 tractable questions about the microbiome, behavior, and speciation by symbiosis. For the  
92 purposes of this review, we refer to the microbiome as the community of microorganisms in and  
93 on a host.

94

95

## 96 **SIGNALING & MICROBIOME HOMOGENIZATION**

97         Recognizing signals of species membership (48), gender (49), relatedness (50), and  
98 colony or group membership (51) is relevant to choosing a mate. Visual (48), auditory (49), and  
99 chemosensory signals (52) can each be used to relay this information, with the latter being  
100 particularly influenced by the microbiome in either "microbe-specific" or "microbe-assisted"  
101 ways. Both mechanisms involve the expression of chemosensory cues, but microbe-specific  
102 processes involve bacterial-derived products such as metabolites while microbe-assisted  
103 mechanisms involve bacterial modulation of host-derived odorous products (Fig. 1).

104         The microbiome's capacity to provide identity information for mate recognition may rely  
105 on products being an honest signal of holobiont group membership, requiring that many or all  
106 members of the group (i.e., gender, population or species) contain appropriate microbial  
107 members that express equivalent signal profiles. Holobionts can be colonized by similar  
108 microbes via a number of different mechanisms, spanning behavioral similarities and contact  
109 with shared environmental sources (53, 54), similar ecological niches and diets (55–57), and host  
110 genetic effects (16, 58). Each of these mechanisms may explain a portion of the variation in the  
111 microbial communities of holobionts (40, 42, 59–61).

112         In the context of group living, humans in the same household (54, 62) and chimpanzees  
113 (63) or baboons (53) in the same social group have more similar microbial communities than  
114 non-group members. Among several mammalian species, microbial community composition  
115 covaries with odorous secretions, and similarities are shared based on host age, sex, and  
116 reproductive status allowing for potential signaling and recognition of these traits (27, 64). In

117 hyenas, there is less microbial community variation within species than between them, and clans  
118 have more comparable microbial communities due to the marking and remarking of collective  
119 territory to signal clan ownership (64). In baboons, there is less microbiome variation within  
120 social groups than between them, and baboons involved in communal grooming behaviors share  
121 even more similarities (53). Insect populations such as termites can stabilize their gut  
122 microbiomes by way of trophallaxis, a behavior in which nestmates supply nutrients and  
123 microbes (e.g., cellulolytic microbes) to other colony members through fluids they excrete from  
124 their hindgut (65). However, Tung *et al* appropriately note, "*one of the most important*  
125 *unanswered questions is whether social network-mediated microbiome sharing produces net*  
126 *fitness benefits or costs for hosts*" (53). From the perspective of the origin of species, it will be  
127 similarly important to determine if fitness impacts of the microbiome in turn affect the evolution  
128 of group living and reproductive isolation. On one hand, socially-shared microbiomes could  
129 drive the evolution of population-specific mating signals and ensuing behavioral isolation. On  
130 the other hand, they could fuse incipient species in sympatry that socially share bacterial  
131 communities responsible for mating signals.

132         Similarities in diet can also influence microbiome homogenization, particularly in the  
133 digestive tract. For instance, *Drosophila melanogaster* reared on similar food sources carry  
134 comparable microbial communities (43). Trophically similar ant species also share microbial  
135 species (66). In humans, gut microbiome variation in taxonomy and functions correlates with  
136 dietary variation (67), and alterations in human diet can rapidly and reproducibly change the  
137 structure of the microbiome (68, 69). Seasonal variation in wild howler monkey diet is also  
138 correlated to shifts in the microbiome (70). Mediterranean fruit flies (71) and olive flies (72)  
139 acquire microbes from their food that increase clutch size and oviposition rate of females

140 exposed to diets lacking essential amino acids (71, 72). Intriguingly, male sexual  
141 competitiveness of Mediterranean fruit flies increases up to two-fold with diets enriched with  
142 *Klebsiella ozytoca* versus a conventional diet (73).

143 Host genetics also affects microbial community assembly. In mice, there are 18 candidate  
144 loci for modulation and homeostatic maintenance of Bacteroidetes, Firmicutes, Rikenellaceae,  
145 and Provetellaceae in the gut (58, 74). Moreover, the presence of many rare bacterial groups in  
146 the gills of the Pacific oyster are correlated to genetic relatedness (75). Congruently, genetic  
147 variability in human immune-related pathways are associated with microbial profiles on several  
148 body sites including various locations along the digestive tract (76), and the largest twin cohort  
149 to date examined members of the gut microbiome and found that the bacterial family  
150 Christensenellaceae has the highest heritability ( $h^2 = 0.39$ ), and associates closely with other  
151 heritable gut bacterial families (77). Human genetic background also influences the risk of  
152 developing gastric cancer caused by *Helicobacter pylori*, indicating that incompatibilities  
153 between hosts and symbionts can produce deleterious effects (20). Phylosymbiosis, characterized  
154 by microbial community relationships that reflect host phylogeny (30), has also been reported in  
155 several cases. For instance, closely related *Nasonia* species that diverged roughly 400,000 years  
156 ago share more similar microbial communities than species pairs that diverged a million years  
157 ago (16, 40). Similar phylosymbiotic patterns are observed in hydra (59), ants (60) and primates  
158 (61).

159 The overall complexity inherent in microbial community structures and processes may  
160 be problematic for animal holobionts seeking to interpret a vast array of signaling information.  
161 However, recognition and differentiation of these microbe-induced signals may be possible if a  
162 subset of the microbiome affects the production of the particular signal. Furthermore, it may also



163 be challenging to disentangle social, environmental, and diet effects on microbial assemblages in  
164 natural populations (53). Nonetheless, the important theme among all of these cases is that  
165 microbial community variation often appears to be less within holobiont groups/species than  
166 between them. This pattern, if sustained in natural populations, could facilitate the evolution of  
167 microbe-specific and/or microbe-assisted mating signals that promote recognition within  
168 populations or species and discrimination between them. Once this critical point is passed,  
169 speciation has commenced. There are parallels here with inclusive fitness theory, which posits  
170 that individuals can influence their own reproductive success or the reproductive success of other  
171 individuals with which they share genes (78, 79). If one follows the continuity from genes to  
172 microbial symbionts, then the inclusive fitness framework may also apply to holobionts in which  
173 specific microbial symbionts may influence their reproductive success by increasing the  
174 reproductive success of their hosts through microbe-specific and/or microbe-assisted mating. A  
175 case-by-case analysis of the reliance of the symbiont on the host for transmission (e.g., maternal,  
176 social, environmental transmission) will augment the relevance of this framework.

177

## 178 **MICROBE-ASSISTED MODIFICATION OF MATING SIGNALS**

179 A common, microbe-assisted modification involves manipulation of host signals (Fig.  
180 1A). One seminal study found that *D. melanogaster* acquires more *Lactobacillus* when reared on  
181 starch than on a molasses-cornmeal-yeast mixture (43, 80). The increased *Lactobacillus*  
182 colonization correlates with an upregulation of 7,11-heptacosadiene, a cuticular hydrocarbon sex  
183 pheromone in the female fly, resulting in an ability to distinguish fly holobionts raised in the  
184 starch environment from those reared on the molasses-cornmeal-yeast substrate (43, 81). This  
185 microbe-assisted positive assortative mating is reproducible, reversible, and maintained for

186 several dozen generations after diet homogenization (43, 82). Moreover, this diet-dependent  
187 homogamy appears to be directly mediated by different gut bacteria, as inoculation of germ-free  
188 flies with *Lactobacillus* causes a significant increase in mating between flies reared on the  
189 different diets (43). Replication of these experiments found that inbred strains specifically  
190 followed this mating pattern (82). Moreover, another *D. melanogaster* study involving male mate  
191 choice and antibiotics revealed that female attractiveness is mediated by commensal microbes  
192 (83). These laboratory studies provide a critical model for how microbe-assisted modifications in  
193 a signaling pathway, ensuing behavioral changes, and mating assortment can potentiate  
194 behavioral isolation and possibly speciation. Indeed, natural populations of *D. melanogaster*  
195 express positive assortative mating and differential signal production based on food sources (84),  
196 and a bacterial role in these instances should be explored.

197         Microbe-assisted signaling also occurs in laboratory mice (*Mus musculus*), in which  
198 bacterial conversion of dietary choline into trimethylamine (TMA) leads to attraction of mice  
199 while also repelling rats (85). Antibiotic treatment decreases TMA production, and genetic  
200 knockout of the mouse receptor for TMA leads to decreased attraction in mice (85). Antibiotic  
201 treatment and subsequent depletion of TMA in mice could in turn result in a decrease in  
202 repellence of rats (85), though this possibility has not yet been tested *in vivo*. Another study  
203 found that female mice are more attracted to males not infected with *Salmonella enterica*  
204 infected compared to those that are, yet females mated multiply and equally in mating choice  
205 tests with the two types of males (86).

206         Mate preference based on infection status fits well with the Hamilton-Zuk hypothesis of  
207 parasite-mediated sexual selection, which posits that traits related to infection status can  
208 influence mating success (87). One seminal study showed that male jungle fowl infected with a

209 parasitic roundworm produce less developed ornamentation and are less attractive to females  
210 (87). In house finches, male plumage brightness indicates their quality of broodcare and is  
211 associated with resistance to the bacterial pathogen *Mycoplasma gallicepticum* (88). The  
212 Hamilton-zuk hypothesis has been reviewed in detail (89).

213

## 214 **MICROBE-SPECIFIC SIGNALS**

215 Microbe-specific signals frequently involve the release of volatile microbial metabolites,  
216 often through excretions from specialized glands on the host's body (Fig. 1B). Microbial  
217 volatiles can transmit information utilized for social signaling (13, 90) and intra- or interspecies  
218 mate recognition (85, 91). For example, beetles (91), termites (51), nematodes (92), hyenas (64),  
219 meerkats (27), and badgers (93) produce and recognize bacterial metabolites in communication  
220 that can modulate their behavior. In termites, fecal metabolites produced by intestinal bacteria  
221 (51) coat the termite body and hive walls to signal colony membership. Termite holobionts  
222 lacking colony-specific metabolite profiles are attacked and killed by the hive (51). In contrast,  
223 some beetles and mammal species excrete bacterial metabolites from colleterial and anal scent  
224 glands, respectively (27, 64, 91). For example, female grass grub beetles house bacteria within  
225 their colleterial glands peripheral to the vagina that are used to attract males to mate (91).

226 An exciting area of research regarding microbe-specific bacterial signaling involves  
227 mammalian fermentation. The mammalian fermentation hypothesis (27, 64) states that  
228 fermentative bacteria within mammalian scent glands produce odorous metabolites involved in  
229 recognition. For example, hyena subcaudal scent pouches store bacteria that are mostly  
230 fermentative (64). When marking territory, hyenas deposit species-specific, bacterial-derived  
231 volatile fatty acids from this gland onto grass stalks (64). Bacterial metabolite secretions are

232 more variable in the social hyena species, presumably because the complexity of signals from  
233 social species improves intraspecies identification (64). Alternatively, social hyenas may  
234 permissively transmit more diverse bacteria leading to diverse metabolite profiles. Hyena  
235 microbiomes also covary with group membership, sex, and reproductive state (64). Similarly,  
236 bacterial communities in meerkat anal scent secretions vary with host sex, age, and group  
237 membership (27). In both cases, the signal diversity may allow animal holobionts to recognize  
238 diverse biotic characteristics.

239 Humans also carry bacteria related to odor production. Breath (94, 95), foot (96), and  
240 underarm (97) odor covary with oral and skin microbiomes, respectively. Many diseases (e.g.,  
241 smallpox, bacterial vaginosis, syphilis, etc.) are associated with distinct odors, and have  
242 historically been used by physicians in diagnosis (98). Clothing made from different materials  
243 even carry different odor profiles based on material-specific bacterial colonization (99, 100).  
244 Male odor has been associated with women's interpretation of a male's attractiveness (101–103),  
245 possibly influencing their choice in a mate.

246 The salient theme among the aforementioned cases is that host-associated microbes  
247 frequently emit odors, and sometimes this microbe-specific chemosensory information can affect  
248 mate choice. Reciprocally, ample evidence shows that chemical signals mediate sexual isolation  
249 (104), and a full understanding of whether these signals are traceable to host-associated microbes  
250 is worthy of serious attention. Germ-free experiments and microbial inoculations should be a  
251 prerequisite for such studies; otherwise they risk missing the significance of microbes in  
252 chemosensory speciation (104). Additional behaviors involved in speciation, such as habitat  
253 choice and pollinator attraction, are also likely to be influenced by microbe-specific products.  
254 Indeed, classic model systems of speciation await further experimentation in this light. For

255 example, food-specific odors on apples and hawthorn translate directly into premating isolation  
256 of incipient host races of fruit flies of the genus *Rhagoletis* (105). Furthermore, the fruit fly  
257 *Drosophila sechellia* exclusively reproduces on the ripe fruit of *Morinda citrifolia*, which is toxic  
258 to other phylogenetically-related *Drosophila* species, including *D. melanogaster* and *D.*  
259 *simulans*. Some of the volatile compounds involved in these interactions, such as isoamyl  
260 acetate, have been associated with fermentative bacteria like *Lactobacillus plantarum* (106),  
261 suggesting that food-based premating isolation may be related to bacterial associations with the  
262 food source, though this requires further study. In summary, new challenges necessitate the  
263 concerted effort of scientists of diverse backgrounds to explore questions at the boundaries of  
264 many biological disciplines and to develop the tools to untangle and interpret this intricate web  
265 of interactions. Critical topics to be explored in the future include determining the microbial role  
266 in animal mate choice, quantifying the extent to which microbe-induced mating assortment  
267 impacts the origin of species, and identifying the mechanisms involved in these interactions.

268

## 269 **ENDOSYMBIONTS AND MATE CHOICE**

270 *Wolbachia*, *Spiroplasma*, *Rickettsia*, *Cardinium*, and several other endosymbiotic  
271 bacteria can change animal sex ratios and sex determination mechanisms to increase their  
272 maternal transmission and thus frequency in the host population from one generation to the next.  
273 Notably, these reproductive alterations affect mate choice (107), and here we highlight a few  
274 prominent examples and discuss how endosymbiotic bacteria can influence behavioral isolation  
275 and the origin of species.

276 Cytoplasmic Incompatibility: *Wolbachia* are the most well-studied reproductive distorters  
277 (108, 109) and are estimated to infect approximately 40% of all arthropod species (110). Across

278 the major insect orders, *Wolbachia* cause cytoplasmic incompatibility (CI), a phenomenon in  
279 which *Wolbachia*-modified sperm from infected males leads to post-fertilization embryonic  
280 lethality in eggs from uninfected females or from females infected with a different strain of  
281 *Wolbachia*, but not in eggs from infected females (111).

282 In this context, *Wolbachia*-induced CI can promote the evolution of mate discrimination  
283 between populations or species because females can be selected to avoid males that they are not  
284 compatible with (Fig. 2C). Among closely related species of mushroom-feeding flies,  
285 *Wolbachia*-infected *Drosophila recens* and uninfected *D. subquinaria* contact each other and  
286 interspecifically mate in their sympatric range in Eastern Canada. However, gene flow between  
287 them in either cross direction is severely reduced due to the complementary action of CI and  
288 behavioral isolation. *Wolbachia*-induced CI appears to be the agent for evolution of behavioral  
289 isolation as asymmetric mate discrimination occurs in flies from the zones of sympatry but not in  
290 flies from the allopatric ranges (112). A similar pattern of *Wolbachia*-induced mate  
291 discrimination occurs among strains of the two-spotted spider mite, *Tetranychus urticae* (113)  
292 and *D. melanogaster* cage populations (45). Moreover, discrimination between particular  
293 semispecies of *D. paulistorum* is associated with their *Wolbachia* infections (44). In cases where  
294 host populations or species harbor different *Wolbachia* infections that are bidirectionally  
295 incompatible, for example in different *Nasonia* species that exist sympatrically (114, 115),  
296 reciprocal mate discrimination has evolved (114, 116). In contrast to these examples,  
297 interspecific mate discrimination in *Nasonia giraulti* is diminished when non-native transfections  
298 of *Wolbachia* spread throughout the whole body including to the brain, suggesting that  
299 *Wolbachia* can also inhibit pre-existing mate discrimination (117).

300           These cases reveal, to varying degrees, that *Wolbachia* can be causal to the evolution of  
301 assortative mating within and between species. Indeed, population genetic theory demonstrates  
302 that mate choice alleles spread quicker in populations or species with CI than those with nuclear  
303 incompatibilities (118). This is primarily due to the dominance of these *Wolbachia*-induced  
304 incompatibilities since CI causes F1 inviability, while nuclear incompatibilities are typically  
305 expressed in the F2 hybrids due to the recessive nature of hybrid incompatibility alleles.

306           Male killing: Male killing is the most common form of endosymbiont-induced sex-ratio  
307 manipulation and can occur during embryonic (119, 120) or larval development (121, 122). The  
308 effect of male killing is to increase the number of female hosts in a population, thereby  
309 increasing endosymbiont transmission rates. To prevent complete fixation of females and  
310 population extinction (123), selection can favor hosts to (i) suppress male killing via genes that  
311 reduce *Wolbachia* densities or functions (25, 124–126) or (ii) electively choose mates whereby  
312 uninfected males preferentially mate with uninfected females (127, 128). If mate choice evolves  
313 as a behavioral adaptation to avoid male killing, it could begin to splinter infected and uninfected  
314 populations and initiate the first steps of the speciation process (Fig. 2A). One significant caveat  
315 in this conceptual model is that the infected population will go extinct without uninfected males  
316 to mate with. Thus, if mate preference based on infection status was complete, it would cause  
317 speciation between the infected and uninfected populations, resulting in the immediate extinction  
318 of the infected population that requires uninfected males to reproduce. We term this phenomena  
319 "behavioral extinction" (Figure 2).

320           *Wolbachia*-induced male killing can reach a state of equilibrium, as suggested by their  
321 long-term maintenance in natural populations of butterflies (129). Discriminatory males  
322 occasionally mate with infected females allowing for the infection to remain in the population

323 (127), and eventually an equilibrium is reached (129). However in some cases, the infection rate  
324 is high (>95%), and male preference for uninfected females has not been identified (123). It is  
325 not known what mechanisms are involved in preventing male killing from reaching fixation in  
326 these situations.

327 Feminization: Feminization, or the conversion of genetic males to morphological and  
328 functional females, has similar evolutionary consequences to male killing (Fig. 2B). This process  
329 occurs in many different arthropod species including butterflies (130, 131), leafhoppers (132),  
330 and woodlouse (133). Resistance to these effects in the pillbug *Armadillidium vulgare* has  
331 evolved in the form of feminization suppressors and male preference towards uninfected  
332 females. Males that mate with infected females produce feminized males (24, 134). Ultimately, a  
333 female-biased sex-ratio in feminized woodlouse populations results in an increase in male mate  
334 choice, male mating multiplicity, and sperm depletion. In the context of sperm depletion, initial  
335 mating encounters are normal, but upon increased mating frequency, males provide less sperm to  
336 subsequent females. Moreover, infected females are curiously less fertile at lower sperm  
337 densities possibly because they are less efficient at utilizing small quantities of sperm (128).  
338 Insufficient sperm utilization and slight differences in infected female courtship behaviors can  
339 result in male preference for uninfected females within the population (133). Just as with male  
340 killing, assortative mating within infected and uninfected populations may initiate the early  
341 stages of speciation and lead to behavioral extinction (Figure 2)

342 Parthenogenesis: Microbial-induced parthenogenesis is common among haplodiploid  
343 arthropods such as wasps, mites, and thrips (135–137), wherein unfertilized eggs become  
344 females (138, 139). As we previously discussed (140), parthenogenesis-induced speciation by  
345 endosymbiotic bacteria falls neatly with the Biological Species Concept because parthenogenesis



346 can sever gene flow and cause the evolution of reproductive isolation between sexual and  
347 asexual populations. Microbe-induced parthenogenesis does not necessarily exclude sexual  
348 capability of parthenogenetic females, but instead removes the necessity of sexual reproduction  
349 and can potentially drive divergence in sexual behaviors and mate choice (141). Speciation  
350 therefore commences between sexual and asexual populations under two models: (i) Sexual  
351 Degeneration and (ii) Relaxed Sexual Selection (140) (Fig. 2D).

352         The Sexual Degeneration model posits that the asexual population becomes incompetent  
353 to engage in sexual interactions due to mutational accumulation and thus trait degeneration while  
354 the sexual population remains otherwise the same (140). In this case, parthenogenetic lineages  
355 accumulate mutations in genes involved in sexual reproduction. Traits subject to mutational  
356 meltdown may span secondary sexual characteristics, fertilization, mating behavior, signal  
357 production, among others (142–144). For instance, long-term *Wolbachia*-induced  
358 parthenogenesis in mealybugs and some parasitoid wasps prevents females from attracting mates  
359 or properly expressing sexual behaviors (144, 145). Similarly in primarily asexual populations,  
360 male courtship behavior and sexual functionality is often impaired (142, 146, 147). The accrual  
361 of these mutations prevents sexual reproduction, thus causing the parthenogenetic population to  
362 become "locked in" to an asexual lifestyle. While this model is an attractive hypothesis for the  
363 onset of reproductive isolation between asexual and sexual populations, it is not always easily  
364 distinguishable from the alternative Relaxed Sexual Selection model (140). In this model, the  
365 sexual population diverges by evolving new or altered mating factors (e.g., courtship sequence,  
366 signals, etc.) while the asexual population does not degrade, but rather stays the same and thus  
367 can no longer mate with individuals from the diverging sexual population (140)

368

369 **CONCLUSIONS**

370 Over the past decade, biology has stood *vis-à-vis* with what Carl Woese referred to as the  
371 "sleeping giant" of biology - the microbial world (1). During this period of groundbreaking  
372 research, a new vision for the increasing importance of microbiology in many subdisciplines of  
373 the life sciences has emerged. As such, studies of animal and plant speciation that do not account  
374 for the microbial world are incomplete. We currently know that microbes are involved in a  
375 multitude of host processes spanning behavior, metabolite production, reproduction, and  
376 immunity. Each of these processes can in theory or in practice cause mating assortment and  
377 commence population divergence, the evolution of reproductive isolation, and thus speciation.  
378 Understanding the contributions of microbes to behavior and speciation will require concerted  
379 efforts and exchanges among these biological disciplines, namely ones that embrace the recent  
380 "unified microbiome" proposal to merge disciplinary boundaries (148).

381

382

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936 **FIGURE LEGENDS**

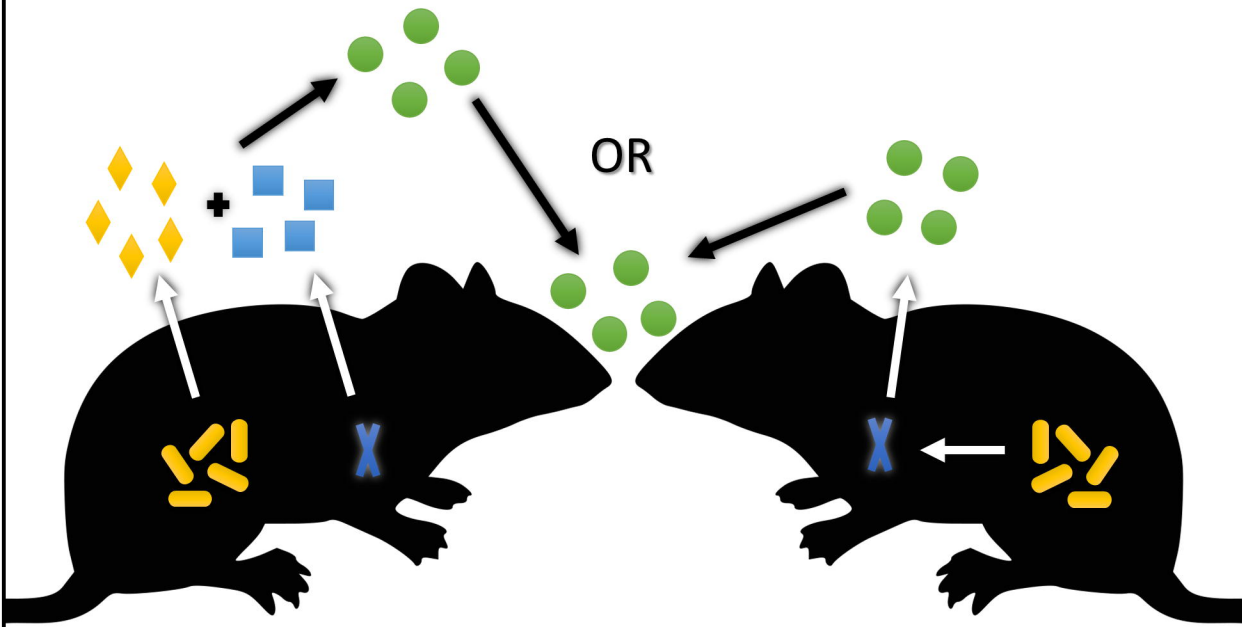
937  
938 **Figure 1. Microbe-assisted and microbe-specific signaling.** (A) Microbe-assisted processes  
939 denote the production of a host signal with input from the microbiome. It occurs in two possible  
940 scenarios. On the left, the host and microbial symbionts produce products that interact or  
941 combine to form a signaling compound; on the right, microbial symbionts modify host signal  
942 expression, but they do not make a specific product directly involved in the signal itself. (B)  
943 Microbe-specific processes denote the production of a microbial signal without input from the  
944 host. It occurs in two possible scenarios. On the left, the host and microbial symbionts produce  
945 products that are both required to elicit a response; on the right, microbial symbionts produce  
946 compounds used by the host for signaling. Mouse image source: Wikimedia Commons, Angelus.

947  
948 **Figure 2. Endosymbiont-induced behavioral isolation and extinction.** U (blue) and I (pink)  
949 represent the uninfected and infected populations, respectively. Horizontal solid arrows represent  
950 the direction of gene flow (from males to females) and vertical dashed arrows represent  
951 divergence time. Different subscript numbers for U and I represent evolutionary change in traits  
952 involved in behavioral extinction and behavioral isolation. Behavioral changes induced by (A)  
953 Male Killing (MK) and (B) Feminization (FM) evolve in response to selection on uninfected  
954 males to mate preferentially with uninfected females. If male preference is completely penetrant,  
955 then total loss of mating between the uninfected and infected population ensues, effectively  
956 leading the infected population to extinction since infected females rely on (the now  
957 discriminating) uninfected males to reproduce. We term this model "Behavioral Extinction". In  
958 contrast, behavioral changes induced by (C) Cytoplasmic Incompatibility (CI) and (D)  
959 Parthenogenesis Induction (PI) can result in reduced or no gene flow between the infected and  
960 uninfected populations. CI-assisted reproductive isolation can be enhanced by the evolution of  
961 mate discrimination and specifically uninfected female mate choice for uninfected males. While  
962 this model does not sever gene flow in reciprocal cross directions, asymmetric isolation barriers  
963 can act as an initial step in speciation. PI-assisted reproductive isolation is mediated by two  
964 possible mechanisms: (i) Sexual Degeneration which involves the degeneration of sexual traits in  
965 the infected population that ultimately lock the populations into uninfected sexual and infected  
966 parthenogenetic species, and (ii) Relaxed Sexual Selection which involves the evolution of new

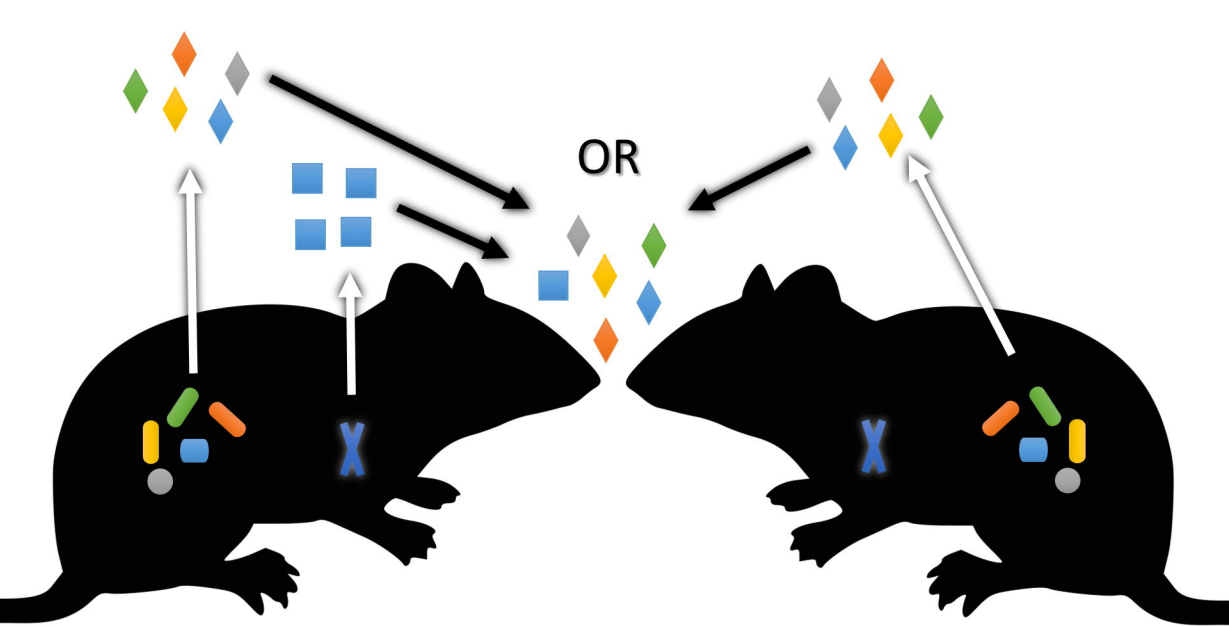
967 sexual characteristics in the uninfected sexual population that prevent mating with the infected  
968 parthenogenetic population. *Wolbachia* image source: Tamara Clark, Encyclopedia of Life,  
969 *Wolbachia* page.

<b>Table 1. Microbe-induced traits that associate with or cause changes in behavior and barriers to interbreeding</b>					
<b>Traits</b>	<b>Host Species</b>	<b>Common Name</b>	<b>Symbiont(s)</b>	<b>Behavior or reproductive outcome</b>	<b>References</b>
<b>Host signal modification</b>	<i>Drosophila bifasciata</i>	Fruit fly	Unknown	Assortative mating based on familiarity	(149)
	<i>Drosophila subobscura</i>	Fruit fly	Unknown	Assortative mating based on kinship	(149)
	<i>Drosophila melanogaster</i>	Fruit fly	<i>Lactobacilli plantarum</i>	Assortative mating based on diet	(43, 82)
	<i>Mus musculus</i>	House mouse	Unknown gut bacteria	Species recognition	(85)
<b>Bacterial metabolite production</b>	<i>D. melanogaster</i>	Fruit fly	<i>L. brevis</i> , <i>L. plantarum</i>	Assortative mating based on diet	(28)
	<i>Reticulitermes speratus</i>	Termite	Unknown gut bacteria	Exclusion of non-colony members	(51)
	<i>Costelytra zealandica</i>	Grass grub	Unknown bacteria in colleterial glands	Mate attraction	(91)
	<i>Crocota crocuta</i>	Spotted hyena	Unknown bacteria in anal scent glands	Clan, age, sex, and reproductive status recognition	(64)
	<i>Hyaena hyaena</i>	Striped hyena	Unknown bacteria in anal scent glands	Clan, age, sex, and reproductive status recognition	(64)
	<i>Meles meles</i>	European badger	Unknown bacteria in anal scent glands	Possible mate discrimination	(93)
	<i>Suricata suricatta</i>	Meerkat	Unknown bacteria in anal scent glands	Group, age, and sex recognition	(27)
	<b>Odor production</b>	<i>M. musculus</i>	House mouse	<i>Salmonella enterica</i>	Initial avoidance of infected males
<i>Homo sapiens</i>		Humans	Unknown	Attractiveness	(101–103)
<b>Cytoplasmic incompatibility</b>	<i>Drosophila paulistorum</i>	Fruit fly	<i>Wolbachia</i>	Assortment within semispecies	(44)
	<i>D. recens</i> & <i>D. subquinaria</i>	Fruit fly	<i>Wolbachia</i> in <i>D. recens</i>	Asymmetric mating isolation	(112)
	<i>D. melanogaster</i>	Fruit fly	<i>Wolbachia</i>	Increased mate discrimination	(45)
	<i>Nasonia giraulti</i>	Parasitoid wasp	<i>Wolbachia</i>	Decreased mate discrimination	(117)
	<i>Tetranychus urticae</i>	Two-spotted spider mite	<i>Wolbachia</i>	Uninfected females prefer uninfected males	(113)
<b>Male killing</b>	<i>Armadallidium vulgare</i>	Pillbug	<i>Wolbachia</i>	Reduce sperm count and female fertility	(128)
	<i>D. melanogaster</i>	Fruit fly	<i>Spiroplasma poulsonii</i>	Evolved suppressors to prevent male killing	(125)
	<i>Acraea encedon</i>	Common Acraea butterfly	<i>Wolbachia</i>	Male mate-choice	(127)
	<i>A. encedon</i>	Common Acraea butterfly	<i>Wolbachia</i>	Populations with high infection rates are not discriminatory	(123)
	<i>Hypolimnas bolina</i>	Great eggfly butterfly	<i>Wolbachia</i>	Reduced female fertility	(126, 129)
	<i>H. bolina</i>	Great eggfly butterfly	<i>Wolbachia</i>	Evolved suppressor gene to prevent male killing	(25)
<b>Feminization</b>	<i>A. vulgare</i>	Pillbug	<i>Wolbachia</i>	Males reproductively female but masculine males prefer true females	(133)
	<i>Eurema hecabe</i>	Grass yellow butterfly	<i>Wolbachia</i>	Males reproductively female	(130, 131)
	<i>Zyginidia pullula</i>	Leafhopper	<i>Wolbachia</i>	Males reproductively female	(132)
<b>Parthenogenesis</b>	<i>Apoanagyrus diversicornis</i>	Mealybug parasite	<i>Wolbachia</i>	Females less attractive to males	(145)
	<i>Asobara japonica</i>	Parasitoid wasp	<i>Wolbachia</i>	Females less attractive to males	(144)
	<i>Leptopilina clavipes</i>	Parasitoid wasp	<i>Wolbachia</i>	Reduction in male and female sexual traits and fertility	(143, 147)
	<i>Muscidifurax uniraptor</i>	Parasitoid wasp	<i>Wolbachia</i>	Reduction in sexual traits	(142)
	<i>Neochrysocharis Formosa</i>	Parasitoid wasp	<i>Wolbachia</i>	Female biased sex ratio	(139)
	<i>Galeopsomyia fausta</i>	Parasitoid wasp	Unknown	Females not receptive	(150)
	<i>Franklinothrips vespiformis</i>	Thrips	<i>Wolbachia</i>	Male sperm presumably do not fertilize female eggs	(137)

### A. Microbe-Assisted Modification of Signals



### B. Microbe-Specific Signals



■ Host-derived product

◇ Microbe-derived product

● Final signal

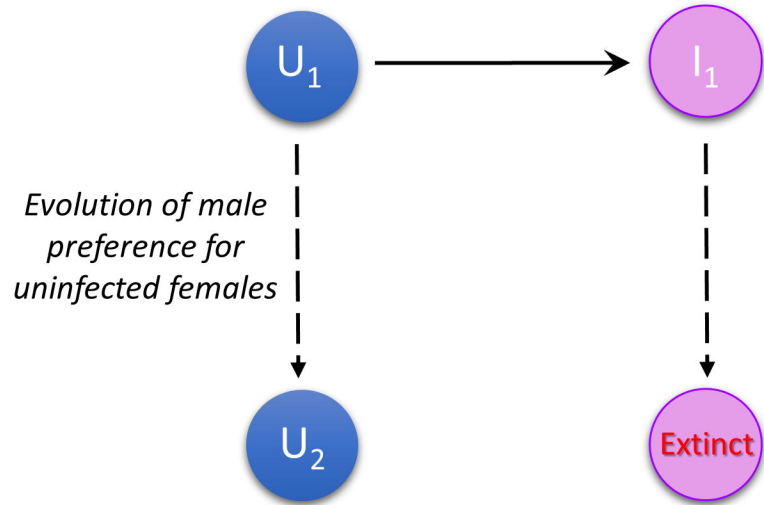
X Host genetics

○ ● ● Microbes

Behavioral Extinction

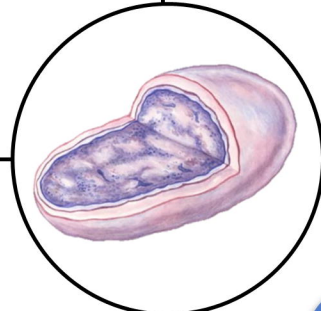
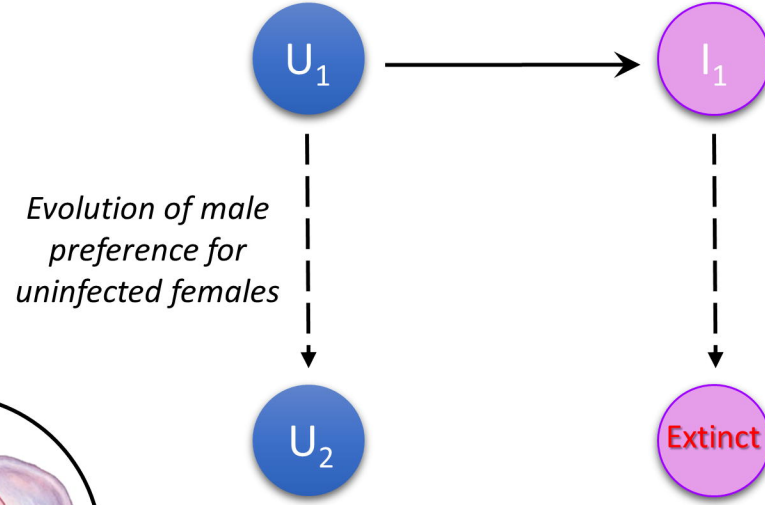
A. MK

Male Mate Choice



B. FM

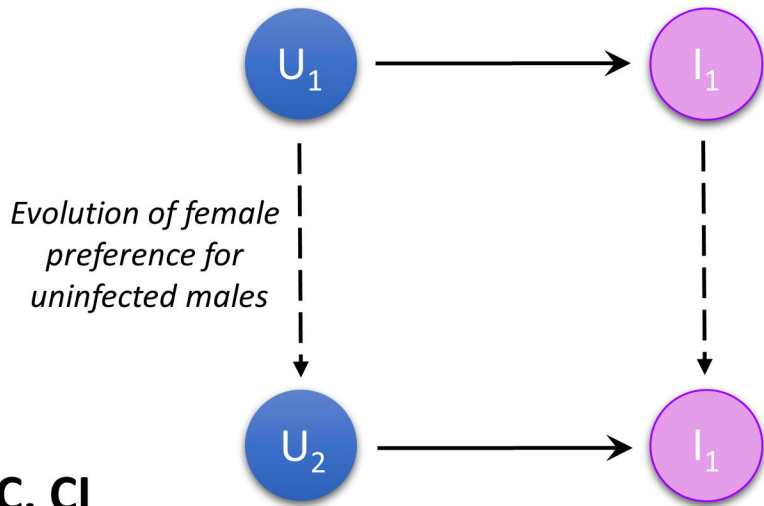
Male Mate Choice



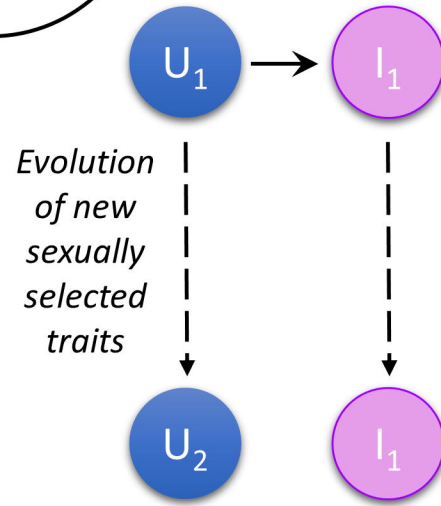
Behavioral Isolation

C. CI

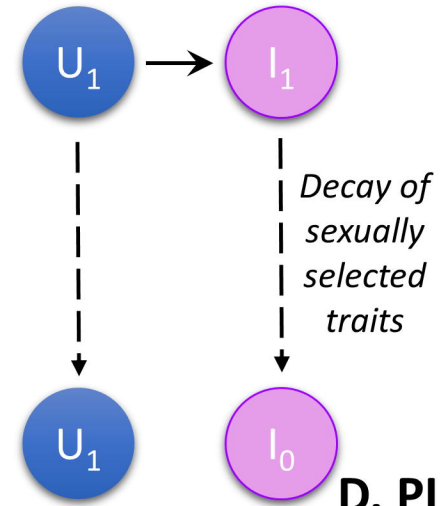
Female Mate Choice



Relaxed Sexual Selection



Sexual Degeneration



D. PI