Title: Symbiont virulence is a poor predictor of impacts on host population dynamics

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MKD, KMM, MHC, and MAD designed the research, MKD, KMM, ESD, FEC, TN, PM, RTM, EB, NFS, MR, ED, KJM, CH, LS, and KD conducted the experiments, MKD conducted data analysis, MKD wrote the manuscript with contributions from MHC and MAD, all authors proofread the manuscript.

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Abstract

Symbionts are classified as parasites, commensals, or mutualists based on their individual-level effects on host traits such as mortality or reproduction rates. However, the classification of symbiosis might change when the focal interaction is investigated at the host population scale, for multiple generations, or under different environmental contexts. We investigated the individual- and population-level effects of a microsporidian, *Ordospora pajunii,* and a yeast, *Metschnikowia bicuspidata,* on the key freshwater grazer *Daphnia dentifera*. Specifically, we tested whether the microsporidian, which has low individual-level virulence, can switch to mutualism and protect its host when the highly virulent yeast is in the environment. We tracked population dynamics of *Daphnia* and infection prevalence in populations exposed to each symbiont separately, both symbionts together, or unexposed controls. We found that *O. pajunii* infection reduced *M. bicuspidata* outbreaks, but we also found that the seemingly mildly virulent *O. pajunii* strongly reduced host population size, while the highly virulent *M. bicuspidata* had no detectable negative effects on host population size. The harmfulness of *O. pajunii* was most likely due to transgenerational virulence – reduced survival of unexposed offspring of exposed mothers. The lack of adverse effects, and short-term positive effects, of *M. bicuspidata* on *Daphnia* populations were consistent with a hydra effect. Our study shows that investigating symbioses at the population-level, with consideration of transgenerational effects and ecological context, can change the qualitative conclusions regarding the impacts of symbionts, and that studies that fail to do so risk focusing mitigation and control measures on the wrong symbionts.

Significance

Endosymbionts are often classified as parasites (those that cause harm) or mutualists (those that provide benefits) based on their effects on the individual host they reside within. Understanding how the individual-level effects of the symbionts scale up to affect the entire host population is vital in the fields of epidemiology, conservation, and agriculture. Our study shows that relying on individual-level, single generation studies can make extremely harmful pathogens seem benign and pathogens with low impacts on host populations seem like a great threat. Accurate estimation of the consequences of disease outbreaks and symbiosis outcome on host populations requires multigenerational, population-level studies and awareness of the role of ecological context.

Introduction

Scientists have long recognized that symbionts can have major effects on host fitness (1), classifying symbionts with net positive impacts as mutualists and those with net negative impacts as parasites (2). This classification is typically based on individual-level measurements of changes in fitness-related traits of the host, like symbiont-induced changes in host survival (3–6) or reproductive success (e.g., (5, 7–9)). A common measure of the impact of symbionts on hosts is virulence, a term that has a variety of definitions, but in a broad sense is defined as damage inflicted on the host by the symbiont (e.g., (5)), and in a narrow sense as symbiont-induced increases in mortality (3–6). Throughout this manuscript we use the broad sense definition, but emphasize that both definitions of virulence refer to an individual-level measure of symbiosis outcome. Measuring virulent effects of parasites on life-history traits helps assess the impact of a given symbiont on its host, and is commonly used in human and veterinary medicine, conservation, and organismal biology (4, 10–12). However, besides the individual-level impacts, it is also important to identify how symbionts affect host population trajectories. Accurate prediction of the large-scale, long-term consequences of symbiosis outcomes is crucial to identify risks and benefits of symbiont

97 spread through host populations, and can be used in improving management practices in
98 agriculture and conservation biology (13, 14). In addition, incorrectly labeling a symbiont as
99 beneficial or harmful can lead us astray when we attempt to assess the food web- and ecosystem-
100 level impacts of symbionts, or when we try to decide which symbionts should be combated or
101 propagated. There is growing evidence that effects at the individual-level can poorly predict effects
102 at the population-level (14), and that symbionts that seem benign at the individual-level can have
103 harmful population-level effects and vice versa (e.g., (15, 16)). To improve our understanding of
104 the role symbionts play in host populations, food webs and ecosystems, we need to recognize that
105 the outcomes of symbiosis can vary depending on the time scale, level of biological organization,
106 and ecological context in which the outcome is measured (e.g., (14–16)).
107
108 Studying symbiosis at a larger time scale (i.e., for multiple generations) and higher level of
109 biological organization (i.e., host populations instead of individuals) can change its outcome due
110 to discrepancies between individual- and population-level effects of symbiosis. Individual-level
111 studies fail to grasp patterns related to transmission, host and parasite density-dependent effects,
112 or transgenerational effects of the symbionts; these effects can strongly influence the net impact
113 of symbionts on host populations. For example, higher host density can reduce the short-term risk
114 of infection but increase it in the long term, due to density-dependent transmission (17), and host
115 density-dependent intraspecific competition can change the impact of a symbiont from benign to
116 highly virulent (15). In addition, symbionts can induce a multitude of transgenerational effects,
117 which alter host offspring and can translate into population-level effects (18). At the population-
118 level, some parasites can induce hydra effects – where increased host reproduction
119 overcompensates for infection-related mortality – which results in increased host population size

120 despite the symbiont-driven high mortality (19, 20). Hence, estimating symbiosis outcomes on a
121 population-level and over multiple generations – instead of relying solely on individual-level
122 effects – might dramatically change our perspective on the nature of the impact of a symbiont on
123 its host.
124
125 Ecological context can also alter the net outcome of host-symbiont interactions. It is now well-
126 established that symbioses can shift along the parasitism-mutualism continuum in response to
127 changing environmental factors (16, 21–25). For example, one of the most well-studied species of
128 bacteria, *Escherichia coli*, is a natural and beneficial member of the gut microbiome, but can
129 rapidly turn parasitic when present in other tissues (26). In contrast, viruses are considered
130 parasites, given their need to exploit host cells to proliferate, but some viruses become extremely
131 beneficial, e.g., when other pathogens are infecting their host (27). There are many examples of
132 symbionts whose effects on hosts change with ecological context (e.g., (3, 16, 21)), highlighting
133 the need for considering environmental context when predicting outcomes of symbiosis.
134 Additionally, the context-dependent changes in symbiosis outcome can differ when investigated
135 at the individual- vs. population-level (e.g., (14)). Hence, to fully understand the nature and
136 outcome of a symbiotic relationship, one must consider population-level effects, multigenerational
137 effects and ecological context.
138
139 In this study, we explored how the net impact of symbiosis may change depending on the time
140 scale, level of biological organization, and ecological context, using the freshwater zooplankton
141 *Daphnia dentifera* as a model organism. *Daphnia*, like all organisms, are subject to and dependent
142 on symbiotic interactions; some of these interactions are vital for their survival (e.g., the gut

143 microbiome is necessary for survival, (28)), while others are detrimental (e.g., highly virulent
144 parasites, (29)). A well-studied symbiont of *Daphnia* spp. is *Metschnikowia bicuspidata*, a yeast
145 pathogen considered highly virulent mostly due to its large reduction in host lifespan (30–33). A
146 recently discovered and seemingly much less virulent microsporidian symbiont of *Daphnia*
147 species, *Ordospora pajunii* (34, 35), shows potential to become a mutualist when *M. bicuspidata*
148 is present in the environment (36). *Ordospora pajunii* infection reduces the penetrability of
149 *Daphnia* guts to attacking *M. bicuspidata* spores (36, 37). Additionally, an experimental
150 investigation of potential drivers of context-dependent mutualism revealed that *O. pajunii* causes
151 *Daphnia* to become a dead-end host for *M. bicuspidata* upon sequential co-infection due to
152 increased host mortality (37). However, *O. pajunii* has also been found to greatly reduce offspring
153 quality through transgenerational effects, which should decrease host population size over longer
154 time scales (38). Therefore, our expectation was that the net impact of *O. pajunii* might change
155 depending on whether the impacts were measured over short timescales and on individuals vs.
156 over longer time scales and at the population-level, as well as depending on whether the ecological
157 context included the highly virulent *M. bicuspidata*.
158
159 First, we investigated the short-term, individual-scale effects of each symbiont on the host with a
160 life-table experiment, then we tested for potential shifts in outcomes based on ecological context
161 and longer timescales (spanning generations) using a population-level experiment. In the life table
162 experiment, we exposed *D. dentifera* to *O. pajunii*, *M. bicuspidata*, or a sham (control) and
163 quantified offspring production and lifespan. Based on the life-table experiment results and
164 previous studies of these symbionts, we predicted that the highly virulent yeast *M. bicuspidata*
165 would strongly reduce the population density of *Daphnia*. The individual-level experiment also

166 suggested that *O. pajunii* should have negligible effects on *Daphnia* populations. However, we
167 know that *O. pajunii* induces negative transgenerational effects (i.e., transgenerational virulence,
168 (38)), and therefore, we expected that the microsporidian could negatively impact *Daphnia*
169 population density over multiple generations. We also hypothesized that *O. pajunii* would prevent,
170 or at least reduce, *M. bicuspidata* outbreaks, benefiting the host. Hence, we exposed *Daphnia*
171 populations to *O. pajunii*, *M. bicuspidata*, both, or neither as a control. Because *O. pajunii*
172 outbreaks in natural lakes occur before those of *M. bicuspidata* (37), and the hypothesized
173 protection mechanism against *M. bicuspidata* requires established infection of *O. pajunii* (36, 37),
174 we added *O. pajunii* spores at the start of the experiment and waited until day 31 for its epidemics
175 to be established at ~15% prevalence to add *M. bicuspidata*. We analyzed the growth rates and
176 densities of host populations, as well as prevalences of each parasite. We also investigated
177 differences in host demographics, expecting that transgenerational virulence of *O. pajunii* would
178 reduce the proportion of juveniles in the populations. Additionally, we quantified the standing
179 stock of algae in the microcosms to determine how the symbionts altered host-resource
180 interactions.
181
182 **Results**
183 At the individual-level, infections affected host reproduction (parasite:clutch interaction F=43.52,
184 p<0.001) and lifespan (parasite effect F=29.35, p<0.001), but the effects of each symbiont strongly
185 differed. Specifically, the yeast *M. bicuspidata* was highly virulent to the host, reducing its
186 reproduction by 28-46% in clutches 2-4 (t-ratio>5.4 and p<0.001 for each comparison, Fig. 1A)
187 and lifespan by 63.5% (t-ratio=6.22, p<0.001, Fig. 1B), the latter resulting in the host producing
188 no more than four clutches. *Ordospora pajunii* infection, on the other hand, was less harmful to

189 host individuals. The infections of *O. pajunii* had no effect on the number of offspring in the first
190 three clutches, but this microsporidian had a negative effect on later reproduction, with the size of
191 clutches 4-8 reduced by 8-29% (t-ratio>3.2 and p<0.003 for each comparison, Fig. 1A) and
192 clutches 9-14 reduced by 31-54% (t-ratio>11.2 and p<0.001 for each comparison). *O. pajunii* did
193 not significantly reduce host lifespan (t-ratio=1.74, p<0.27, Fig. 1B).
194
195 At the population-level, the effects of single- and co-exposure to the symbionts were assessed
196 during two time intervals: the initial growth phase (until day 23) and the post-peak phase (days 30-
197 93). The separation was determined by both population dynamics and the experimental design: *M.*
198 *bicuspidata* spores were added on day 31, when *O. pajunii* epidemics were established (~15%
199 prevalence) and the host populations had peaked in abundance. Thus, we used the time before the
200 *M. bicuspidata* exposure to quantify how *O. pajunii* affects initial host population growth rate, and
201 we combined the two groups exposed to *O. pajunii* into one group (OP and OPMB, labeled 'OP'
202 in Fig. 2A), and the two unexposed groups into a separate group (C and MB, labeled 'C' in Fig.
203 2A). The post-peak phase was used to analyze the impact of both symbionts on host population
204 trajectories, with the four treatments analyzed separately.
205
206 Contrary to what the individual-level measurements of virulence would predict, we found strong
207 negative effects of *O. pajunii* on the host populations and no negative effects of *M. bicuspidata*.
208 In the initial growth phase of the experiment, *O. pajunii* reduced the population growth rate by
209 13% (χ^2 = 17.37, p<0.001, Fig. 2A). This negative impact of *O. pajunii* on hosts continued after
210 the peak in densities. In populations with only *O. pajunii*, host population size on days 30-64 was
211 reduced by, on average, 47% (t-ratio>2.7 and p<0.034 for each comparison between OP and C on

212 each day); in the co-exposure treatment, host population size was reduced by, on average, 42% (*t*-
213 ratio>2.8 and $p<0.026$ for each comparison; Fig. 2B). Conversely, *M. bicuspidata* had no
214 observable negative effects on the host populations; instead, later in the experiment (days 71-83),
215 the *Daphnia* population size was larger in comparison to the control populations by, on average,
216 41% (*t*-ratio<-2.84 and $p<0.028$ for comparisons on each day; Fig. 2B). Additionally, we found
217 that the impact of parasite outbreaks on the population size of *Daphnia* were non-linear and
218 changed over time (parasite:day interaction $F=2.76$, $p=0.042$; significant smoothers for C and MB,
219 which indicate significant change in densities of these two populations over time).
220
221 *Ordospora pajunii* reduced the prevalence of *M. bicuspidata*, as expected (Fig. 3A). Despite this,
222 co-exposed populations had similar host population dynamics as the populations exposed just to
223 *O. pajunii* (Fig. 2B), even though infection prevalence of each pathogen was lower in the co-
224 exposed populations than the single-exposed populations. Specifically, the prevalence of *M.*
225 *bicuspidata* was lower in co-exposure from day 56 of the experiment onward, and reached a 66%
226 reduction by the end of the experiment (treatment:day interaction *z*-value=-2.33, $p=0.020$; Fig.
227 3A). *Ordospora pajunii* was also less prevalent in the co-exposure treatment from day 44 onward,
228 reaching a 57% reduction at the end of the experiment (treatment:day interaction $F=15.39$,
229 $p<0.001$; Fig. 3B).
230
231 We hypothesized that the discrepancy between the modest individual-level virulence of the
232 microsporidian (*O. pajunii*) and its strong impacts on host population size might result from a
233 recently described phenomenon called transgenerational virulence: offspring gestated by females
234 during exposure to the microsporidian have much shorter life expectancy (high mortality before

235 and shortly after maturation), despite their lack of direct contact with the microsporidian (38).
236 Hence, we predicted that the microsporidian-induced transgenerational virulence would reduce
237 survival of juvenile *Daphnia*, causing an altered proportion of juveniles in populations exposed to
238 *O. pajunii*. The proportion of juveniles decreased throughout the experiment in all treatments (day
239 effect F=116.83, p<0.001). Notably, and consistent with predicted impacts of transgenerational
240 virulence, *Daphnia* populations undergoing *O. pajunii* epidemics had lower proportions of
241 juveniles than those that were not exposed to this symbiont (treatment effect F=9.66, p<0.001; Fig.
242 4).
243
244 We also hypothesized that some of the discrepancy between the individual-level effects on host
245 fitness and population-level patterns could be explained by host-resource interactions.
246 Specifically, strong negative effects of *O. pajunii* could be the result of an infection-driven increase
247 in resource demand that led to starvation and smaller population sizes. Contrary to this, *O. pajunii*
248 exposed populations (single and co-exposure) had leftover algae on days 30-51 (treatment effect
249 χ^2>19.48 and p<0.001 for each comparison; Fig. 5), when the host populations were undergoing
250 the strongest reductions in density. This indicates that the decrease in population size of *Daphnia*
251 exposed to the microsporidian was not driven by resource depletion and starvation. In contrast,
252 control and *M. bicuspidata*-exposed *Daphnia* populations consumed all the available food (Fig.
253 5). Previous work has suggested that infection-driven decreases in foraging rates in *M.*
254 *bicuspidata*-infected hosts can allow for increased resource uptake by other hosts, driving a
255 phenomenon known as a disease-induced hydra effect (20). The lack of a standing stock of algae
256 in the *M. bicuspidata*-exposed treatment suggests that the very high densities of hosts consumed
257 all the available food; if infected hosts consumed less food, that would leave additional food for

258 the uninfected hosts, fueling a hydra effect, which is consistent with the periodically higher
259 population size of the yeast-exposed population in comparison to the control (Fig. 2B).
260
261 **Discussion**
262 In this study, we investigated whether the microsporidian *O. pajunii* is a context-dependent
263 mutualist that protects its host from a more virulent pathogen *M. bicuspidata*. At the same time,
264 we tested how the individual-level impacts of symbionts on their hosts scale up to the population-
265 level. Virulence measurements of each symbiont at the individual-level showed that the yeast *M.*
266 *bicuspidata* is substantially more harmful to the infected host than the microsporidian *O. pajunii*.
267 However, looking at the population-level effects of *M. bicuspidata* and *O. pajunii* in this study,
268 we found that *M. bicuspidata* had no negative effects on host population size, while *O. pajunii*
269 strongly reduced host population size. Hence, the population-level experiment yielded two patterns
270 at odds with the results obtained in the individual-level experiment: i) *O. pajunii* is much more
271 harmful to its host population than indicated by the traditional individual-level measures of
272 virulence, and ii) *M. bicuspidata* inflicts no harmful effects on the host population, despite its high
273 virulence for infected individuals.
274
275 Given the contrasting effects of *O. pajunii* across scales, we considered two potential explanations
276 for the population-level harm. One possibility is that the microsporidian infections elevate host
277 resource demands. That could increase foraging and cause faster resource depletion when
278 individuals in the population compete for limited resources; such a pattern would not have been
279 detected in the life-table experiment where *Daphnia* were fed *ad libitum*. In fact, some models
280 indicate that intraspecific host competition for resources can amplify the virulence of seemingly

281 benign parasites (15). However, our measurements of standing stock algae indicate that
282 populations undergoing *O. pajunii* epidemics had excess algae when the reduction in host
283 population size was the strongest (days 30-51), suggesting those *Daphnia* were not resource-
284 limited. Conversely, it is possible that the exposed *Daphnia* were undergoing illness-mediated
285 anorexia (39, 40), which would lower the overall food consumption. In a different study, we found
286 that *O. pajunii* can depress host feeding rates (41). Hence, the unexpected harmfulness of *O.*
287 *pajunii* is not likely to be a result of resource shortage. We acknowledge that *O. pajunii* infection
288 might hamper host nutrition through processes independent of food density in the environment,
289 although assimilation efficiency of hosts seems to be unaffected by the microsporidian (41).
290
291 Instead, the most likely explanation for the decimation of *Daphnia* populations by *O. pajunii* is
292 transgenerational virulence (i.e., negative effects of maternal exposure on offspring fitness, even
293 when those offspring are not themselves exposed (38)). A mathematical model estimating the
294 population-level impacts of transgenerational virulence predicted an approximately 30% reduction
295 in population size (38), which is similar to the effect we observed in the population experiment.
296 Additionally, we found a lower proportion of juveniles in the *O. pajunii*-exposed populations,
297 which is consistent with high juvenile mortality caused by transgenerational virulence. Adverse
298 transgenerational effects of maternal exposure to stressors have been observed before, as a result
299 of processes such as altered embryo nutrition or epigenetic inheritance (42–44) or, for parasites,
300 vertical transmission (45, 46). We do not know yet the process is responsible for the
301 transgenerational virulence of *O. pajunii*, but we do know that the microsporidian is not vertically
302 transmitted and that the embryos produced by exposed mothers are more fragile (38).
303

Similar to *O. pajunii*, *M. bicuspidata* had differing individual- and population-level effects. However, in the case of *M. bicuspidata*, the host individuals suffered from high individual-level virulence that did not negatively affect host populations. Instead, population size was, if anything, higher when *M. bicuspidata* was present. We suspect that this is related to how this symbiont affects the host's interactions with its resources. *M. bicuspidata* infection can induce foraging reduction in *Daphnia*, resulting in more food available to the population undergoing epidemics (20, 39, 47). By inducing strong foraging depression, *M. bicuspidata* epidemics can free up resources for uninfected hosts, resulting in a hydra effect (20). In our experiment, we did not observe algae accumulation in the *M. bicuspidata*-exposed treatment, which might suggest that there was no foraging depression. However, because *M. bicuspidata* exposure occurred at high *Daphnia* densities, when resource competition between *Daphnia* was strong (a necessary condition for a hydra effect, (19)), any algae not consumed by infected individuals with reduced foraging would have been rapidly consumed by the many other individuals in the population. Additionally, during *M. bicuspidata* epidemics, the older and larger *Daphnia* experience the greatest infection-driven mortality. Old and large adults are effective grazers (48), but contribute very little to the population growth rate (49). Therefore, their increased mortality would release a fraction of resources from their control, and nourish the juveniles, increasing their survival, and stabilizing the whole population. Hence, we suspect that a joint effect of increased resource availability due to foraging depression, and age-dependent mortality eliminating large grazers, could be responsible for the lack of negative effects of *M. bicuspidata* at the population-level despite its high individual-level virulence.

326 This study was motivated by the hypothesis that *O. pajunii* is a context-dependent mutualist whose
327 impact switches from mildly negative to positive in the presence of the more harmful yeast
328 symbiont. Consistent with prior work on *O. pajunii* (36, 37) and on its close relative (50), *M.*
329 *bicuspidata* prevalence was reduced in our experiment in the presence of *O. pajunii*, and *O. pajunii*
330 prevalence was lower during *M. bicuspidata* outbreaks. However, our assumption that *O. pajunii*
331 could be beneficial for *Daphnia* is not supported because *O. pajunii* had a strong negative
332 population-level effect whereas *M. bicuspidata* did not. Citing Francis Bacon "*the remedy is worse*
333 *than the disease*".
334
335 Awareness of how ecological context affects the outcome of symbiosis is crucial for epidemiology,
336 environmental management and conservation, medicine, and agriculture (16). While there is
337 growing consideration of context-dependent changes in symbiosis outcomes, fewer studies
338 consider the role that time span and level of biological organization play in the functioning of
339 symbiosis. Switching from individual-level analysis to population-level studies done over multiple
340 generations can dramatically change the perspective on the outcome or even nature of the focal
341 symbiosis. For example, anthelmintic treatment that was predicted to help the buffalo population
342 fight off tuberculosis infection actually increased the spread of bovine tuberculosis (14).
343 Furthermore, *Wolbachia* is known to have complex transgenerational effects (51); for example, in
344 *Drosophila melanogaster,* grandmother age determines the strength of the cytoplasmic
345 incompatibility impact on fitness of the offspring of *Wolbachia*-infected males (52). These
346 examples, and many other studies (e.g., (31, 33, 53–56)), show that individual-level, single
347 generation studies are burdened with a risk of overlooking important patterns and interactions,
348 such as transgenerational inheritance, selection of host and symbiont genotypes, or shifts in

349 symbiosis outcomes stemming from population/community level interactions. These effects have
350 the potential to change our classification of a focal symbiosis from mutualistic to parasitic or vice
351 versa. Here, we present how transgenerational virulence inflicted by a microsporidian that does
352 minimal harm to the infected host can decimate the host population, and how a highly virulent
353 yeast parasite that was expected to be extremely harmful to the host population turned out to be
354 benign. We would not have been able to predict such strong population-level consequences if we
355 had relied solely on measurements of virulence in a single generation.
356
357 Understanding the large-scale and long-term consequences of symbiosis is crucial for ecology,
358 evolutionary biology, and epidemiology. The strong population-level effects of *O. pajunii* detected
359 in this study indicate that long-lasting epidemics of this widespread microsporidian (as have been
360 seen in nature; (36, 57, 58)) have the potential to decrease the abundance of *Daphnia*; given their
361 central role in lake food webs, this likely has consequences for primary producers and ecosystem
362 functioning. Indeed, our finding that there was excess algae in populations exposed to *O. pajunii*
363 but not in control populations or those exposed to only *M. bicuspidata* suggest cascading effects
364 on the food web. More generally, we found that switching between individual- and population-
365 level studies revealed patterns that change our understanding of how a focal symbiosis functions
366 and how it should be classified. Our study emphasizes that individual-based methods of virulence
367 estimation are not enough to make accurate predictions about the impact of a symbiont on a host
368 population, and hence parametrizing models using only within-generation, individual-level
369 measurements may not be sufficient to predict the population- and community-level effects of
370 epidemics. To combat the spread and harmful effects of infectious diseases in humans, wildlife
371 and agriculture (e.g., (59–61)), and to use symbiosis in disease biocontrol (e.g., (62–65)), we

372 require a deep understanding of the outcomes of symbioses at levels beyond individuals. Such
373 understanding comes from determining the impacts of symbionts at the population scale over
374 longer time scales, as well as the role of ecological context in shaping those interactions.
375 Incorporating these factors into our investigations of infectious diseases and symbioses will be
376 crucial not only for furthering our knowledge of the ecology and evolution of symbionts but also
377 for decision making in agriculture, conservation, and management.
378

379 **Methods**

380 In this study, we focused on the tripartite interaction between the zooplankton host *Daphnia*
381 *dentifera* and two endosymbiotic microorganisms: *Metschnikowia bicuspidata* and *Ordospora*
382 *pajunii*. Freshwater crustaceans of the genus *Daphnia* are common model organisms for studying
383 evolutionary ecology and disease ecology, having great utility in experimental work due to their
384 parthenogenetic reproduction (66). Here, we briefly describe the experimental methods and
385 analyses. More detailed information about the study system, experiments, and analyses can be
386 found in the Supplement.

387 We carried out two experiments to investigate the interaction between hosts and two
388 symbionts at the individual- and population-level. We studied the individual-level effects using a
389 life table experiment with three treatment groups: C - control *Daphnia* not exposed to either one
390 of the symbionts, MB - *Daphnia* exposed to *M. bicuspidata*, and OP - *Daphnia* exposed to *O.*
391 *pajunii*. The exposure lasted two days for all treatments, after which we changed water and beakers
392 for each *Daphnia*, and continued the experiment until all *Daphnia* had died. We monitored the
393 *Daphnia* daily for survival and offspring production, and we counted and removed the offspring
394 each day. We checked each parasite-exposed *Daphnia* for infection at death or on day 20,

395 whichever came first (20 days is enough for individuals exposed to either pathogen to show visible
396 symptoms of infection). For both symbionts, 100% of the exposed animals who lived long enough
397 for infections to develop were infected.

398 We analyzed the number of offspring produced by *Daphnia* per clutch (reproductive event)
399 using a generalized additive model (GAM), with number of offspring as the response variable,
400 parasite treatment, clutch number, and their interaction as explanatory variables, and clutch
401 number as a common smoother for all the treatments. Based on a comparison of Akaike
402 Information Criterion (AIC), we determined that there was no justification to use separate
403 smoothers for each treatment. Post-hoc pairwise comparisons were conducted with estimated
404 marginal means and Bonferroni correction using the R package *emmeans* (67). Mean lifespan of
405 *Daphnia* was analyzed using a generalized least squares model with lifespan (measured in days)
406 as the response variable, parasite treatment as the explanatory variable and as a variance covariate
407 due to heterogeneity of variances between the treatments. We conducted pairwise comparisons
408 with *emmeans* and using Bonferroni correction.

409 In the population-level experiment, we tested the effects of *M. bicuspidata* and *O. pajunii*
410 on *Daphnia* populations, as well as the protective effect of *O. pajunii* against *M. bicuspidata*
411 outbreaks. We used the same genotypes of host and symbionts for this experiment as for the life
412 table experiment. This experiment had four treatments (10 replicates each): C - control treatment
413 with only *Daphnia* and no parasites; OP - *Daphnia* exposed to *O. pajunii* spores on day 1; MB -
414 *Daphnia* exposed to *M. bicuspidata* spores on day 31; OPMB - *Daphnia* exposed to *O. pajunii*
415 spores on day 1 and then to *M. bicuspidata* spores on day 31. These were started in beakers and
416 then moved to pitchers filled with 5L of filtered lake water after 2 days, in which *Daphnia*
417 populations were kept for the rest of the experiment. Beginning on day 9, we measured *Daphnia*

density, infection prevalence, demographic structure (females, males, juveniles), and algae concentration once per week.

Using the initial growth phase of the experiment, we calculated population growth rates with the following equation: $r = \ln(\text{density}_{t23} - \text{density}_{t0})/n$, where density_{t23} is *Daphnia* density at day 23, density_{t0} is *Daphnia* density at the beginning of the experiment and n is number of days. Due to influential outliers and violation of normal distribution of residuals, we analyzed the data with a non-parametric Kruskall-Wallis test. We also analyzed the change in population size over time in the second phase of the experiment (after day 30), using a GAM with *Daphnia* density as a response variable, treatment, day of experiment, and their interaction as explanatory variables, and day as a smoothing factor fitted separately for each treatment. Due to significant interactions between the terms, we conducted a pairwise comparison with Bonferroni correction to determine at which days population densities are significantly different, using the package *emmeans*. We compared *M. bicuspidata* infection prevalence between the MB and OPMB treatments using a beta regression model. We analyzed *O. pajunii* infection prevalence in the OP and OPMB treatments using a GAM model with common smoothers. We compared the proportion of juveniles (juvenile density/total density) and algae concentration between all four treatments using a linear model for the former, and a Kruskal-Wallis test separate for each sampling day for the latter. Additional information on these analyses can be found in the Supplement. All analyses and figures were conducted in RStudio using R version 4.4.1, and the figures were plotted using the package *ggplot2* (68).

Acknowledgements

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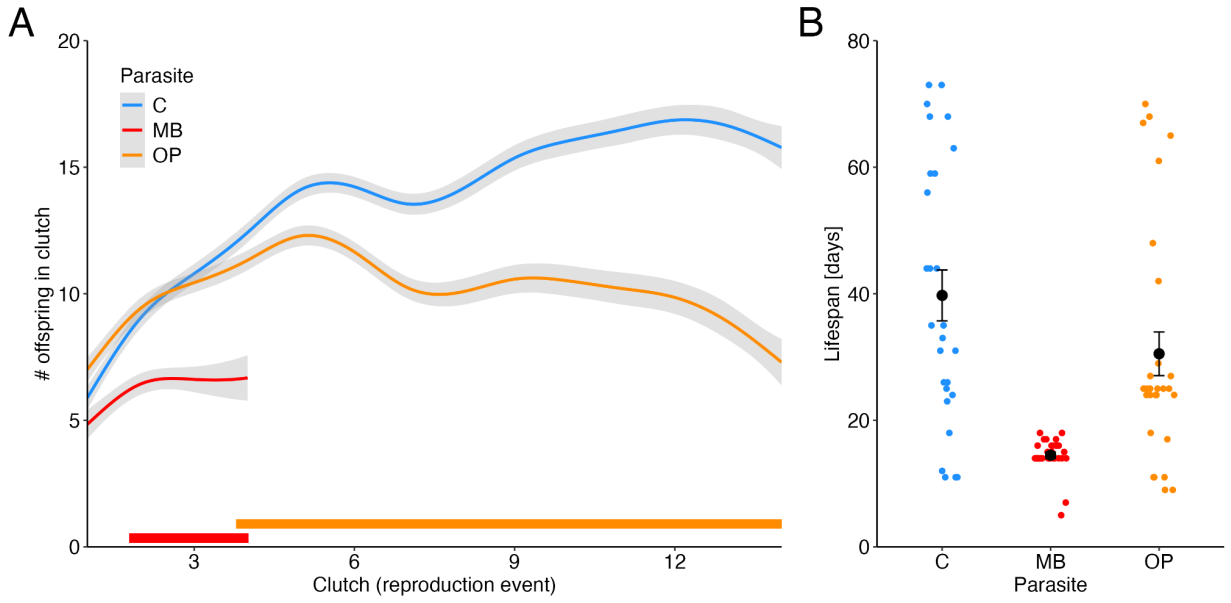
598 **Figures**



599

600 Figure 1. The number of offspring per clutch (A) and the lifespan (B) of *Daphnia* were strongly
601 reduced by *M. bicuspidata* (red), while *O. pajunii* (orange) reduced clutch size only in late life
602 stages (after clutch 3) and had no effect on host lifespan. Panel A shows fitted values±SE of clutch
603 size (number of offspring per reproduction event) in consecutive clutches. Thick lines on the
604 bottom of the figure indicate clutches at which the difference in clutch size between control and
605 the treatment of corresponding color is significant. Panel B shows mean±SE lifespan (black points
606 and error bars) of *Daphnia* in each treatment, with individual data points color-coded by treatment.
607

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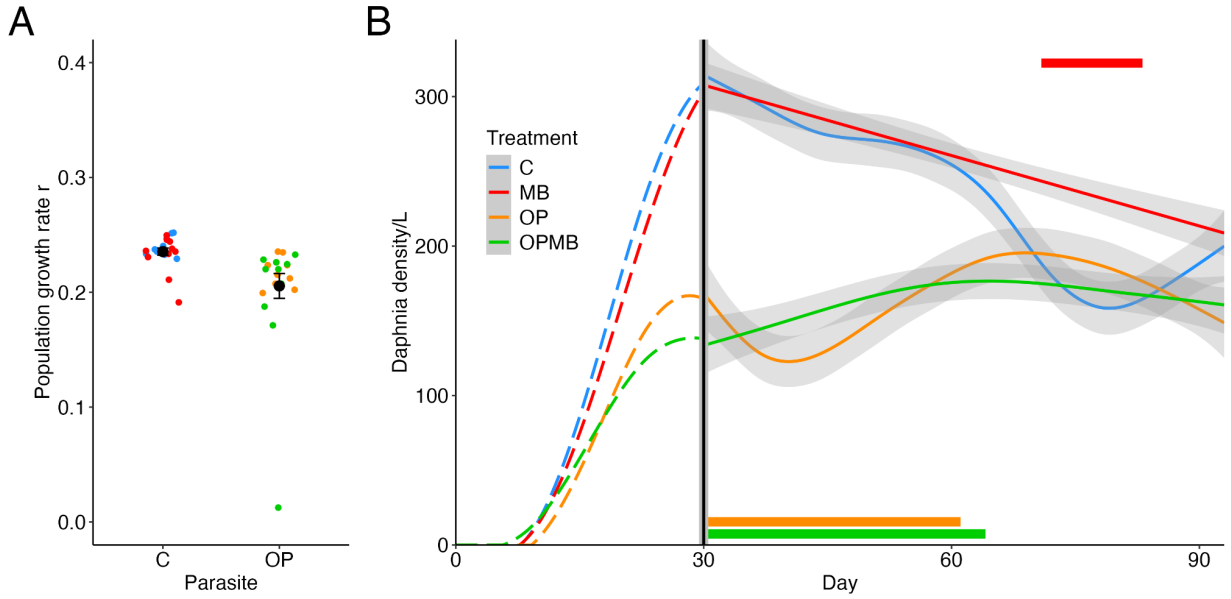

Figure 2. *Daphnia* initial population growth rate (panel A) and population size (panel B) were reduced by *O. pajunii* whereas *M. bicuspidata* increased population size. Panel A shows population growth rate during the initial growth phase (through day 23) in groups unexposed (C and MB) and exposed to *O. pajunii* (OP and OPBM), grouped into parasite treatment C or OP, respectively, because the population growth rates were measured before *M. bicuspidata* dosing (dashed lines on panel B). The black points and error bars indicate mean±SE, the data points are plotted underneath with colors corresponding to treatments. Panel B shows GAM modeled population densities in each treatment, the vertical gray line with black stripe on day 30 separates the time before the populations peaked (dashed lines) and after they peaked (solid lines); *M. bicuspidata* was dosed to the system on day 31, around when densities peaked. Only the modeled population sizes after day 30 were analyzed with the GAM. The population size in the single infection (OP) treatment was reduced on days 30-61, while, in the co-infection (OPMB) treatment, it was reduced on days 30-64. *M. bicuspidata* (MB) had a positive effect on the host population on days 71-83. The solid lines and gray ribbons indicate fitted values±SE, thick lines on top and bottom of the figure indicate the time span at which the treatment of the corresponding color had significantly larger or smaller host population size, respectively, in comparison to the control.

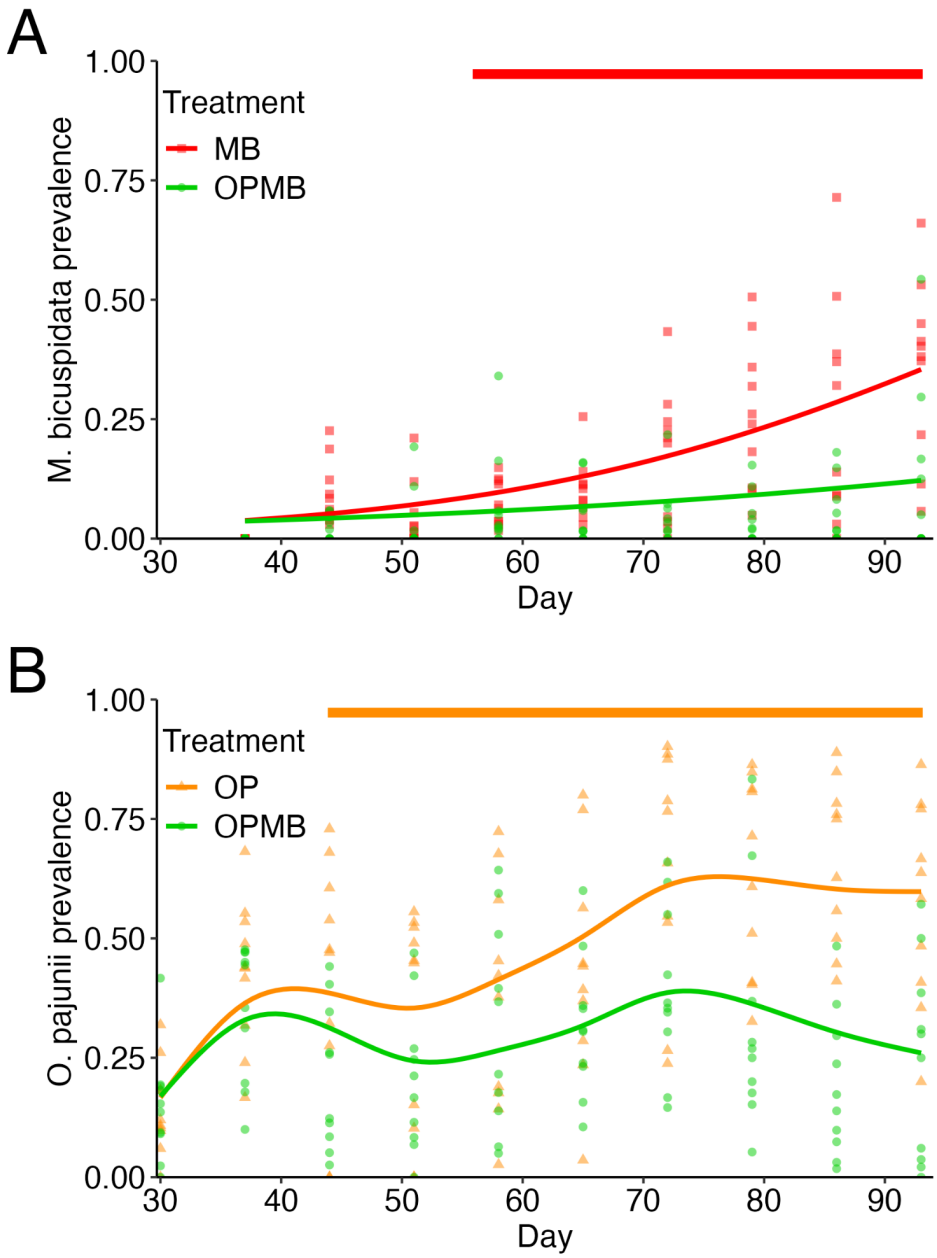

Figure 3. Prevalence of *M. bicuspidata* (panel A) and *O. pajunii* (panel B) were lower in the co-infection treatment in comparison to the single infection treatments. The plots show fitted curves of beta regression for *M. bicuspidata* (panel A) and GAM for *O. pajunii*, with data points plotted underneath; the thick lines above the figures show the time span when the prevalence of the respective parasite was higher in the single infection treatment than in the co-infection treatment.

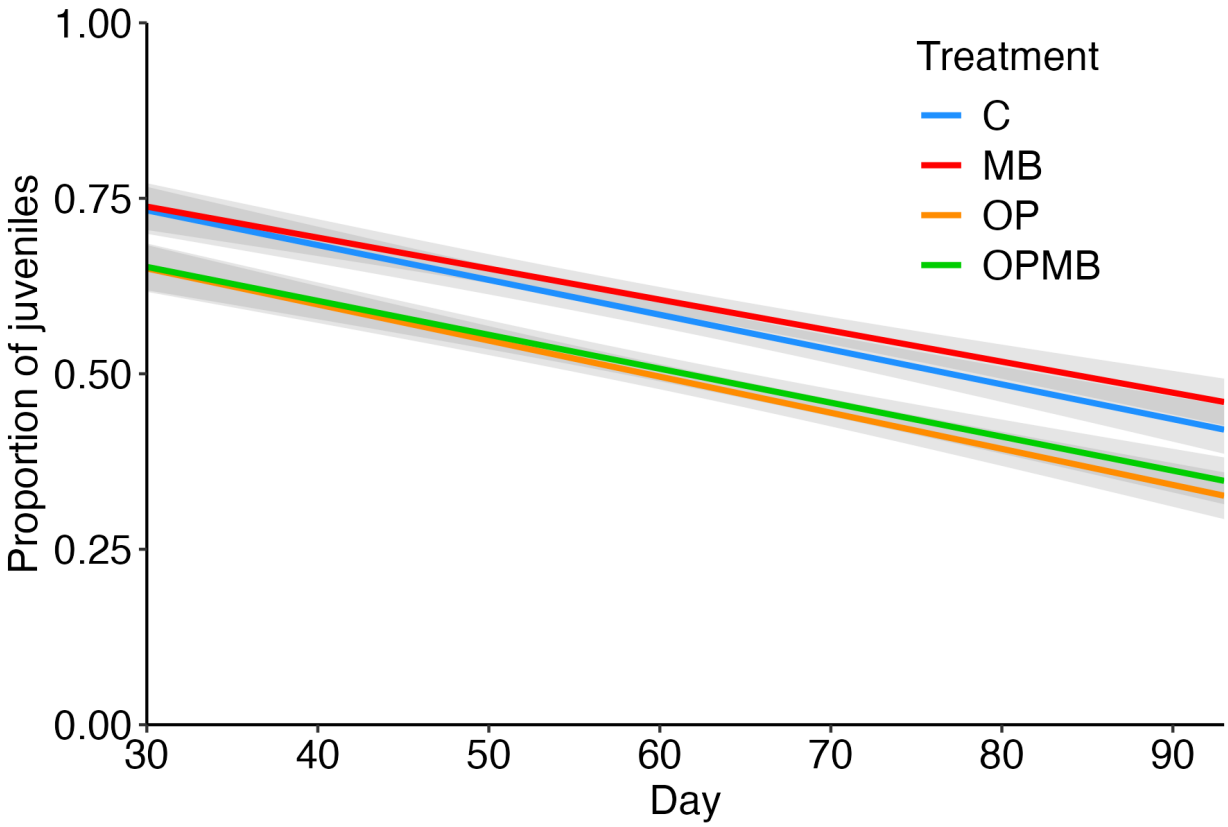

Figure 4. Proportion of juveniles decreased over time in all treatments, but populations with *O. pajunii* outbreaks consistently had a lower proportion of juveniles than either control populations or *M. bicuspidata* exposed populations. Fitted values of linear regression±SE are presented.

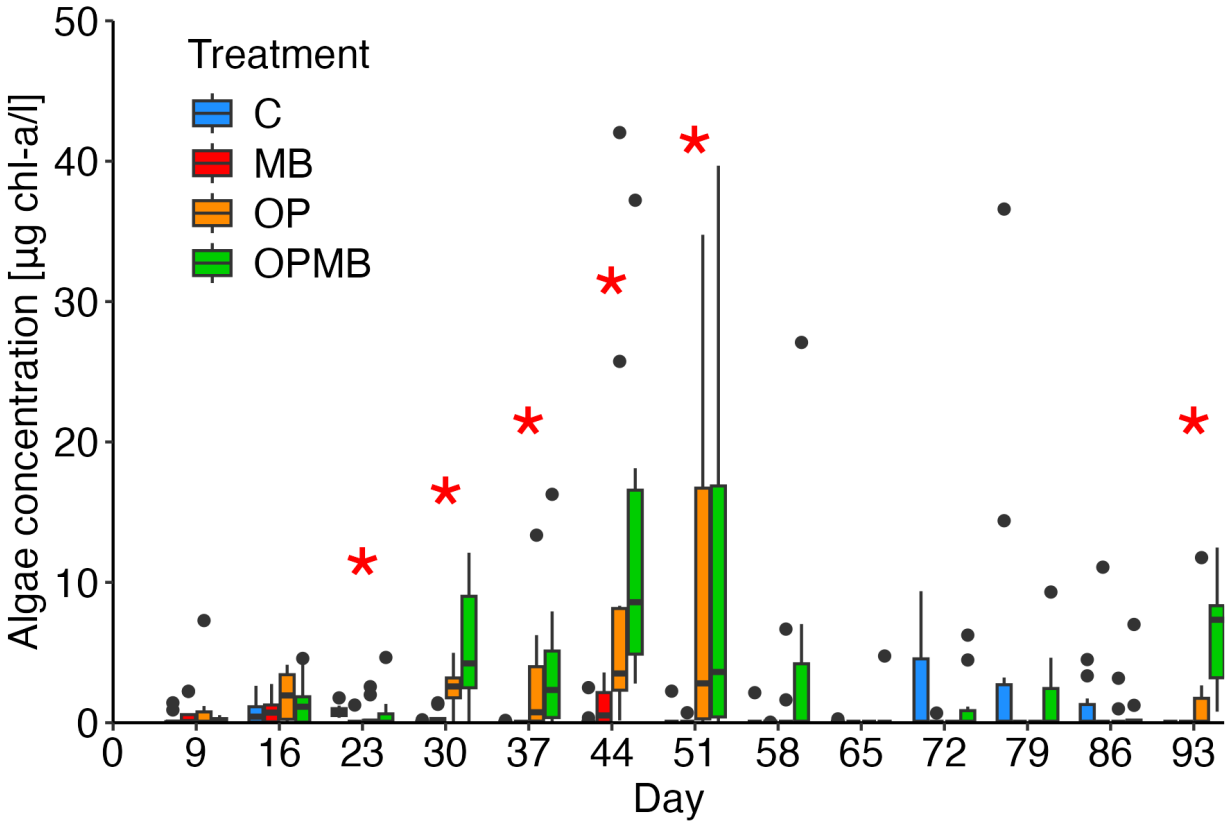

Figure 5. Algae were mostly depleted in control and *M. bicuspidata*-exposed treatments, but treatments with *O. pajunii* outbreaks had leftover algae present in the pitchers on days 30-51. The OPMB treatment had excess algae on day 93 as well, while the MB treatment had significantly less algae than C on day 23. The boxplot shows median (middle line), 25-75 percentile (box), largest values (no larger than 1.5x interquartile range, whiskers) and outliers (black points). Red stars indicate a significant treatment effect based on Kruskall-Wallis test.

Supplemental Methods

Study system

In this study we exposed *Daphnia dentifera* to two symbionts: *Metschnikowia bicuspidata* and *Ordospora pajunii*. The yeast *M. bicuspidata* is an obligate killer that is ingested by the host during feeding, then penetrates the host's gut to get inside the body cavity, where it proliferates (1). Infected host lifespan is reduced (c.a. 12-17 days at 20°C, Fig. 1), and parasite spores are only

670 released after host death (2). The spores of the microsporidian *O. pajunii* are also consumed with
671 food, but once they are present in the host's gut lumen, they invade the gut epithelial cells where
672 they reproduce, eventually causing the cell burst and releasing spores back into both the gut lumen
673 and the environment (3). As this process is not lethal, *O. pajunii* can reinfect the same host multiple
674 times, which leads to perpetual production and shedding of parasite spores. *O. pajunii* is considered
675 a symbiont of very low virulence, as it has little to no effect on host lifespan or reproduction: Fig.
676 1 indicates reduction in offspring production only in late life stages – around and past the 5th clutch
677 – and no significant impact on lifespan, while previous studies indicate that it can be completely
678 benign or even beneficial (3–5). However, we also observed substantial early mortality of offspring
679 of the *Daphnia* exposed to *O. pajunii*, suggesting that its virulence is mostly transgenerational
680 rather than intragenerational (6).
681
682 We carried out two experiments, a life table (or individual-level) experiment and a population
683 experiment. In both experiments, we used the *D. dentifera* clone "S" isolated from a lake in Barry
684 County (Michigan, USA) and kept in the lab for over two decades, the *M. bicuspidata* strain
685 "Standard" isolated from Baker Lake (Barry County, Michigan, USA) in 2003, and the *O. pajunii*
686 isolate "BDWalsh" collected from Walsh Lake (Washtenaw County, Michigan, USA) in 2021.
687 The S clone was chosen for this study due to its susceptibility to both symbionts and the substantial
688 prior work on it.
689
690 *Life table experiment: experimental methods*
691 To estimate the virulence of both symbionts, we designed a life table experiment with three
692 treatment groups: C - control *Daphnia* not exposed to either one of the symbionts, MB - *Daphnia*

693 exposed to *M. bicuspidata*, and OP - *Daphnia* exposed to *O. pajunii*. We note that these data
694 overlap with some of the data presented in (5); specifically, the *M. bicuspidata* treatment (MB)
695 individuals in the current experiment are the same as the 'MB high' treatment animals in that
696 experiment (presented in Figure 2 in (5)), and the control group (C) in this experiment was also
697 used in supplementary Figure S3 of that article. Prior to the experiment, maternal lines of *Daphnia*
698 were kept singly for three generations in 100 ml of water from North Lake (Washtenaw County,
699 Michigan, USA) filtered with Pall AE glass microfiber filters, fed three times a week with 20,000
700 cells/ml of green algae *Ankistrodesmus falcatus* (a saturating level of food), and stored in an
701 incubator set at 20°C and 16:8 light:dark conditions.
702
703 The experiment started when we collected 0-24h old *Daphnia* of the third clutch of the (uninfected
704 and unexposed) experimental mothers. We pooled the animals in one dish and distributed
705 haphazardly among 90 beakers filled with 100 ml of filtered lake water, having a single *Daphnia*
706 per beaker. Each beaker was randomly assigned to one of the three treatments (C, MB or OP),
707 resulting in 30 replicates per treatment. *Daphnia* were placed in the incubator (same conditions as
708 described above for the stock cultures) and fed 20,000 cells/ml of the green algae daily.
709
710 On the third day, the water in all experimental beakers was replaced, with the volume reduced to
711 50 mL, and *Daphnia* in each group were exposed to their respective treatment: MB received 250
712 spores/mL of *M. bicuspidata*, OP received a dose of spores equivalent to a single heavily infected
713 donor (which is c.a. 15,000 spores, see (5)) and C received a placebo dose composed of ground-
714 up unexposed *Daphnia*, with each animal receiving the equivalent of 1 unexposed animal. To dose
715 *M. bicuspidata* spores for the MB treatment, we harvested *Daphnia* infected with *M. bicuspidata*

716 from our stock cultures, ground them with a motorized pestle, counted the mature spore
717 concentration under a compound scope at x400 magnification using a hemocytometer, diluted the
718 spores with filtered lake water and dosed each beaker with 12,500 spores. To expose the OP
719 treatment, we screened *Daphnia* from lab *O. pajunii* stock cultures under a dissecting microscope
720 and harvested heavily infected individuals, placed them in a 1.5 ml centrifuge tube filled with 100
721 µl of filtered lake water, then ground them with a pestle into a slurry. The slurry was resuspended
722 in filtered lake water and dosed into the beakers in a ratio of 1 donor:1 recipient, so each beaker
723 received a volume of slurry equivalent to a single ground-up donor *Daphnia*. We did not count the
724 *O. pajunii* spores due to their small size (~2µm length), which makes it impossible to determine
725 whether a given spore is mature/infective or not. To ensure that each beaker received a dose of
726 similar composition, we dosed all the beakers from the same spore slurry, as we have done in other
727 experiments (e.g., (5)). We prepared the placebo solution by collecting uninfected *Daphnia* from
728 cultures that had never been exposed to *O. pajunii* in a 1.5 ml centrifuge tube, grinding them with
729 a motorized pestle and resuspending the slurry in filtered lake water; we dosed the C treatment
730 beakers with the placebo slurry in a ratio of one ground-up *Daphnia* per beaker. The exposure
731 lasted two days for all treatments, after which we changed water and beakers for each *Daphnia*,
732 and carried out the experiment until all *Daphnia* had died. We monitored the *Daphnia* daily for
733 survival and offspring production, and we counted and removed the offspring each day. We
734 checked each parasite-exposed *Daphnia* for infection at death or on day 20, whichever came first
735 (20 days is enough for individuals exposed to either pathogen to show visible symptoms of
736 infection). For both symbionts, 100% of the exposed animals who lived long enough for infections
737 to develop were infected.
738

Life table experiment: statistical analysis

First, we note that some information from the main text is repeated here so that all of the information is in one place. To compare the virulence of both symbionts, we analyzed the number of offspring produced by *Daphnia* per clutch (reproductive event) and the host's lifespan in each treatment. For the reproduction analysis, we used a generalized additive model (GAM) from the package *mgcv* (7) because, as *Daphnia* grow, their clutch size increases, but this increase eventually stops and *Daphnia* starts producing fewer offspring due to senescence; hence the change of clutch size over time is non-linear. We also trimmed out the data past clutch fourteen because the estimates would have been unreliable due to high mortality and low number of replicates later in the experiment. We used number of offspring as the response variable, parasite treatment, clutch number, and their interaction as explanatory variables, and clutch number as a common smoother for all the treatments. Based on a comparison of Akaike Information Criterion (AIC), we determined that there was no justification to use separate smoothers for each treatment. The model was investigated for normality of residuals distribution, homogeneity of variances, and influential outliers using diagnostic plots. Post-hoc pairwise comparisons were conducted with estimated marginal means and Bonferroni correction using the R package *emmeans* (8). The GAM model diagnostics showed a slight increase in the response variable plotted against the fitted values because the first few *Daphnia* clutches naturally have fewer offspring than later clutches (due to small size of the mothers). To be sure that this small inconsistency in variance does not make our results qualitatively erroneous, we conducted an additional set of non-parametric Kruskal-Wallis tests of the relationship between the number of offspring per clutch and parasite treatment at each clutch, followed by a Dunn test from package *dunn.test* (9) for pairwise comparisons. The results of this test were consistent with the GAM results, although a bit more conservative; we do not

762 show the non-parametric test results in the manuscript, but they are available in the R code, which
763 is publicly accessible on GitHub (https://github.com/marcinkdziuba/Pitcher-
764 experiment/releases/tag/v1.1) and through Zenodo (https://doi.org/10.5281/zenodo.15186214).
765 Mean lifespan of *Daphnia* was compared between the treatments using a generalized least
766 squares model with lifespan (measured in days) as the response variable, parasite treatment as the
767 explanatory variable and as a variance covariate due to heterogeneity of variances between the
768 treatments. We checked model assumptions with diagnostic plots and conducted pairwise
769 comparisons with *emmeans* and using Bonferroni correction.
770
771 *Population experiment: experimental methods*
772 We designed this experiment to test the effects of *M. bicuspidata* and *O. pajunii* on *Daphnia*
773 populations, as well as the protective effect of *O. pajunii* against *M. bicuspidata* outbreaks. We
774 used the same genotypes of host and symbionts for this experiment as for the life table experiment.
775 The maternal lines of *Daphnia* were prepared following the same protocol as the life table
776 experiment. Our population-level experiment started when the (uninfected and unexposed)
777 mothers produced their second clutch of offspring, which we collected 0-24h after birth. We
778 pooled the offspring in one dish and distributed them haphazardly among 40 beakers filled with
779 100 ml of filtered lake water, adding one individual per beaker, then a second to each beaker and
780 so on until each beaker had 10 juvenile *Daphnia*. We assigned each beaker to one of four
781 treatments (10 replicates each): C - control treatment with only *Daphnia* and no parasites; MB -
782 *Daphnia* exposed to *M. bicuspidata* spores on day 31; OP - *Daphnia* exposed to *O. pajunii* spores
783 on day 1; OPMB - *Daphnia* exposed to *O. pajunii* spores on day 1 and then to *M. bicuspidata*
784 spores on day 31. After assigning the beakers to treatments, we exposed OP and OPMB treatments

785 to spores of *O. pajunii*. The spore slurries were dosed the same way as in the life table experiment,
786 except this time the dosing ratio was 1 donor : 2 recipients, so each beaker containing 10 juvenile
787 *Daphnia* received a volume of slurry equivalent to 5 ground-up donor *Daphnia*. After dosing, all
788 treatments were fed 20,000 cells/ml of algae and placed in an experimental room maintained at
789 20°C and with 16:8 light:dark conditions. After two days, we poured the content of each beaker
790 into a separate 5L pitcher filled with 5L of filtered lake water, in which *Daphnia* populations were
791 kept for the rest of the experiment. Each 5L pitcher was dosed with 10,000 cells/ml of algae on
792 this initial transfer day and then three times a week throughout the experiment.

793 For each pitcher, we took weekly measurements of *Daphnia* density, infection prevalence,
794 demographic structure (females, males, juveniles), and algae concentration, beginning on day 9 of
795 the experiment. The screening protocol was as follows: the content of each pitcher was transferred
796 into a clean 5L pitcher (to prevent filamentous algae buildup), the water was refilled when needed
797 to account for evaporative losses, the contents of the pitcher were gently stirred using a 150 ml
798 ladle to homogenize *Daphnia* distribution across the water column, and then subsampled with the
799 ladle. The subsamples were at least 300 ml (with an exception of day 30 when we subsampled 150
800 ml as *Daphnia* densities were extremely high) and had at least 20 *Daphnia*. We did not subsample
801 more than 1000 ml. In the case of densities below detection, we quantified the number of *Daphnia*
802 in the whole pitcher. A subsample was poured into a 2L watch glass, and each *Daphnia* from the
803 subsample was then transferred with a glass pipette onto a microscope slide and checked under a
804 dissecting microscope for infection and demographic identity. Processed *Daphnia* were returned
805 to their pitchers. After checking all *Daphnia*, we measured the concentration of green algae in 50
806 ml of water using a FluoroProbe (BBE Moldanke). The sample to determine algal standing stock
807 was always collected on the third day after the previous feeding and right before the next feeding.

808 The rest of the water from the subsample was poured from the watch glass back into the respective
809 pitcher. Each treatment had a separate ladle, and the glassware was changed between each
810 treatment to avoid spore transfer between treatments.

811 Screening on day 30 revealed that *O. pajunii* epidemics were established, with ~15%
812 parasite prevalence in the exposed populations. Therefore, on day 31, treatments MB and OPMB
813 were exposed to *M. bicuspidata* spores. The *M. bicuspidata* dosing was staggered because we were
814 testing the hypothesis that *M. bicuspidata* is less successful when the host is already infected with
815 *O. pajunii* (5). Additionally, our field data indicated that *O. pajunii* epidemics occur before *M.*
816 *bicuspidata* outbreaks (5). Thus, we needed the *O. pajunii* epidemics to be established before
817 introducing *M. bicuspidata*. We prepared the *M. bicuspidata* slurries following the same protocol
818 as the life table experiment. However, we dosed the pitchers with 12.5 spores/ml of water (62,500
819 spores per pitcher), which is lower than the dose used in the individual-level experiment (i.e., 250
820 spores/ml), because previous studies showed that higher experimental volumes required lower
821 spore concentrations to yield high infection levels (10). After dosing the pitchers with *M.*
822 *bicuspidata*, we continued screening the 40 populations for prevalence of both parasites and the
823 other parameters until day 93 of the experiment. We terminated the experiment at day 93 because
824 some of the beakers started growing filamentous algae in which the *Daphnia* were getting tangled
825 and stuck, leading to their starvation and death. We note that, in the OPMB treatment, very
826 advanced and severe *M. bicuspidata* infections could have obscured the diagnosis of *O. pajunii*
827 infections later in the experiment. Spores of *M. bicuspidata* in heavily infected hosts overgrow the
828 hemocoel around the gut, blocking our view of the gut. Therefore, there is a risk that some animals
829 that were actually co-infected in the OPMB treatment late in the experiment were scored as singly

infected with only *M. bicuspidata*, which could have resulted in underestimation of *O. pajunii* prevalence in the OPMB treatment.

Population experiment: statistical analysis

The density of *Daphnia* was analyzed as the total number of all *Daphnia* (infected and uninfected, adults and juveniles, females and males) per liter of water. Based on the density data at the beginning of the experiment and on day 23 – the last screening day of the initial growth phase – we calculated population growth rates using the following equation: $r = \ln(\text{density}_{t23} - \text{density}_{t0})/n$, where density_{t23} is *Daphnia* density at day 23, density_{t0} is *Daphnia* density at the beginning of the experiment and n is number of days. Because we analyzed the time period prior to *M. bicuspidata* spore dosing, we effectively had only two groups of populations: exposed to *O. pajunii* OP (containing treatments OP and OPMB) and unexposed C (containing treatments C and MB), and we analyzed the data as such. Due to influential outliers and violation of normal distribution of residuals, we analyzed the data with a non-parametric Kruskall-Wallis test.

To investigate the change in population size over time (after day 30), we used a GAM with *Daphnia* density as a response variable, treatment, day of experiment, and their interaction as explanatory variables, and day as a smoothing factor fitted separately for each treatment. After fitting the model, we inspected diagnostic plots to ensure the assumptions of normal distribution of residuals and homogeneity of variance were met. Due to significant interactions between the terms, we conducted a pairwise comparison with Bonferroni correction to determine at which days population densities are significantly different, using the package *emmeans*.

To analyze *M. bicuspidata* prevalence in treatments MB and OPMB, we used a beta regression model from the package *betareg* (11) with the day of experiment, treatment, and their interaction as explanatory variables. We used beta regression because *M. bicuspidata* prevalence data were strongly right-skewed (many measurements close to zero), and beta regression handles the skewed distribution of proportion data much better than a GAM. To analyze the prevalence of *O. pajunii* in groups OP and OPMB, we used a GAM with day of experiment, treatment, and their interaction as explanatory variables, and day as a smoothing factor common for both groups (based on AIC score for common vs. separate smoothers). For both prevalence analyses, we conducted model diagnostics and used the *emmeans* package and Bonferroni correction to determine at which days the prevalence differed between the compared treatments.

The proportion of juveniles (juvenile density divided by total density) was analyzed using a linear model with day of experiment, treatment, and their interaction as explanatory variables, checking model diagnostics and conducting pairwise comparisons using *emmeans* and Bonferroni correction.

The data on algae concentration in the pitchers had a large proportion of zeros (indicating that *Daphnia* in those populations consumed all the algae provided). Thus, we used a non-parametric Kruskal-Wallis test for comparison within each screening day, using treatment as an explanatory variable. When there was a significant effect of treatment, we conducted a Dunn test with Bonferroni correction for pairwise comparisons.

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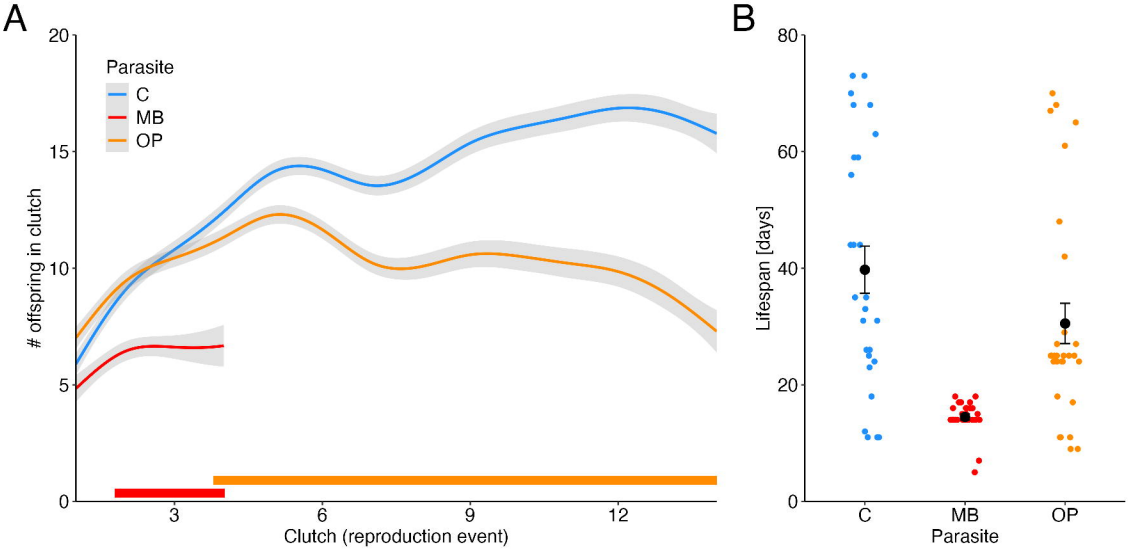
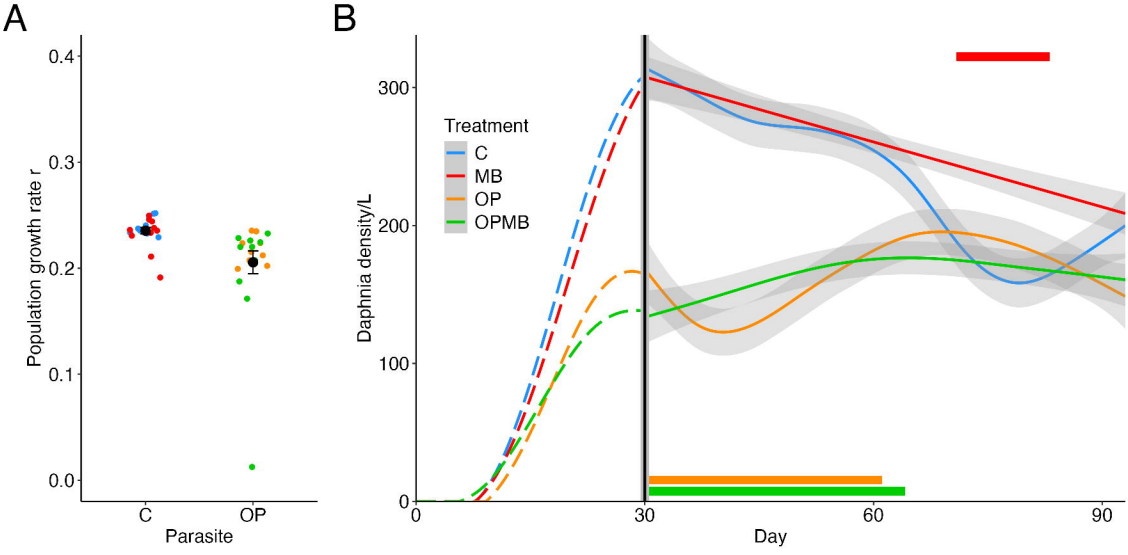
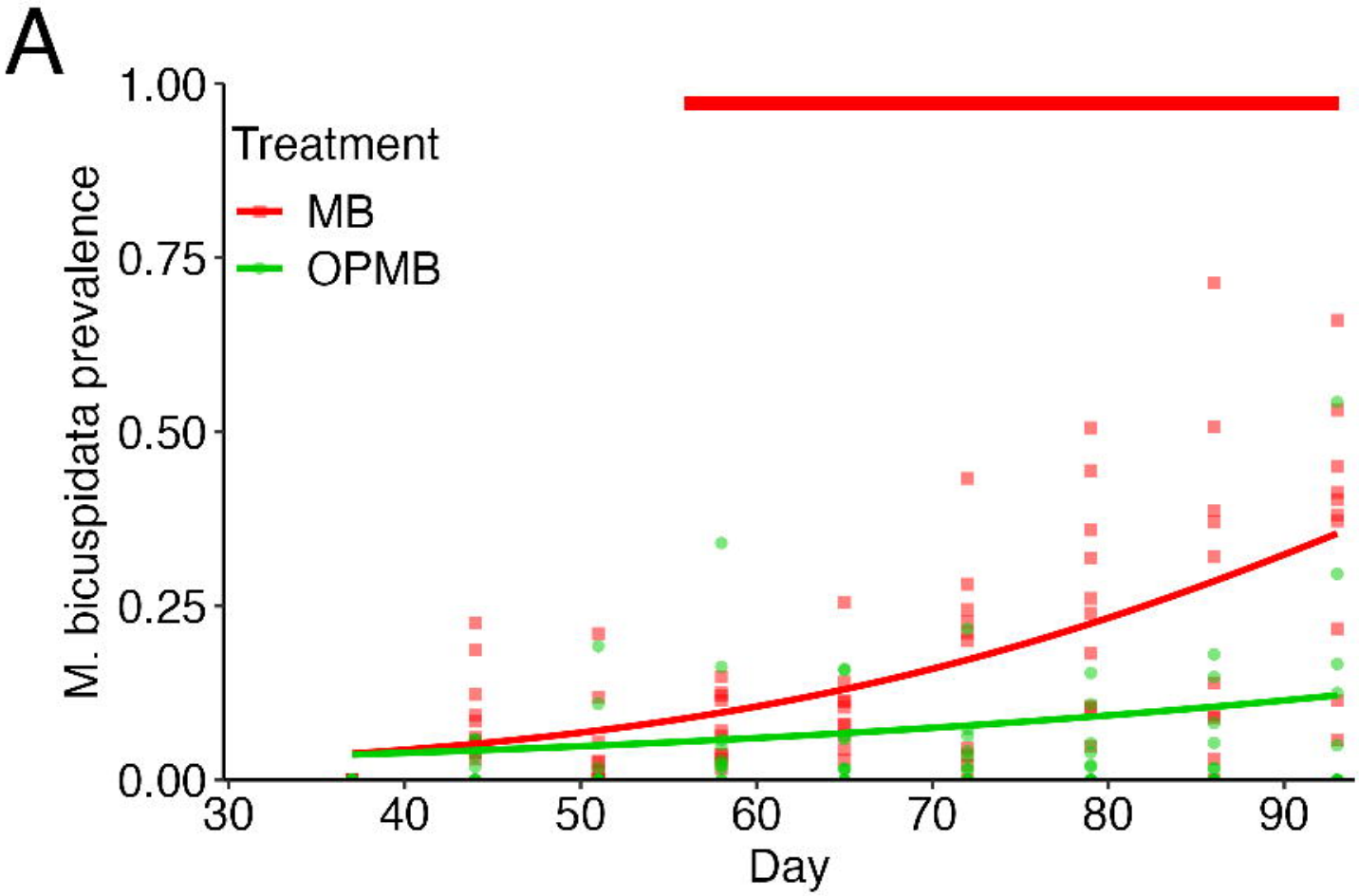
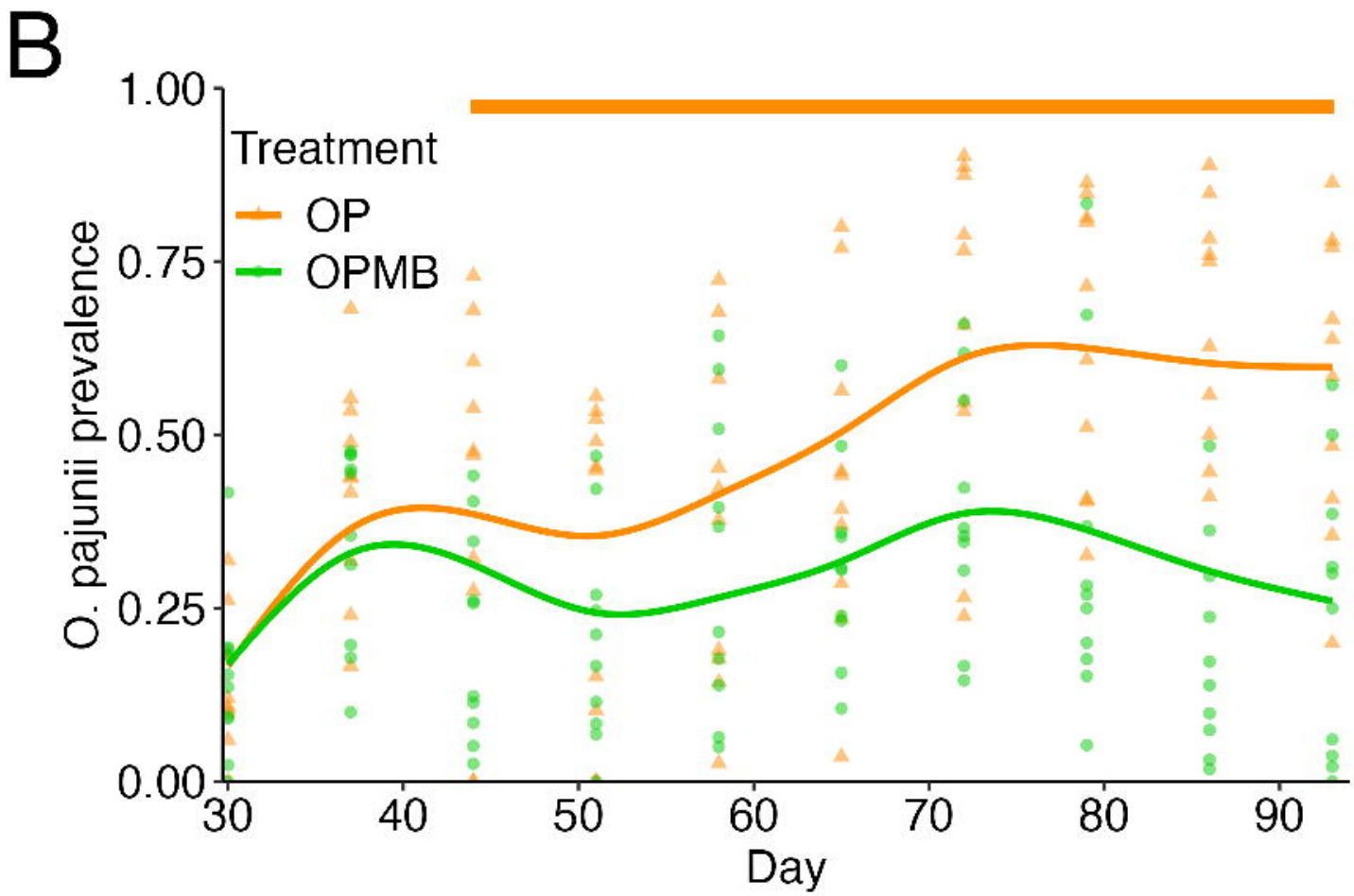
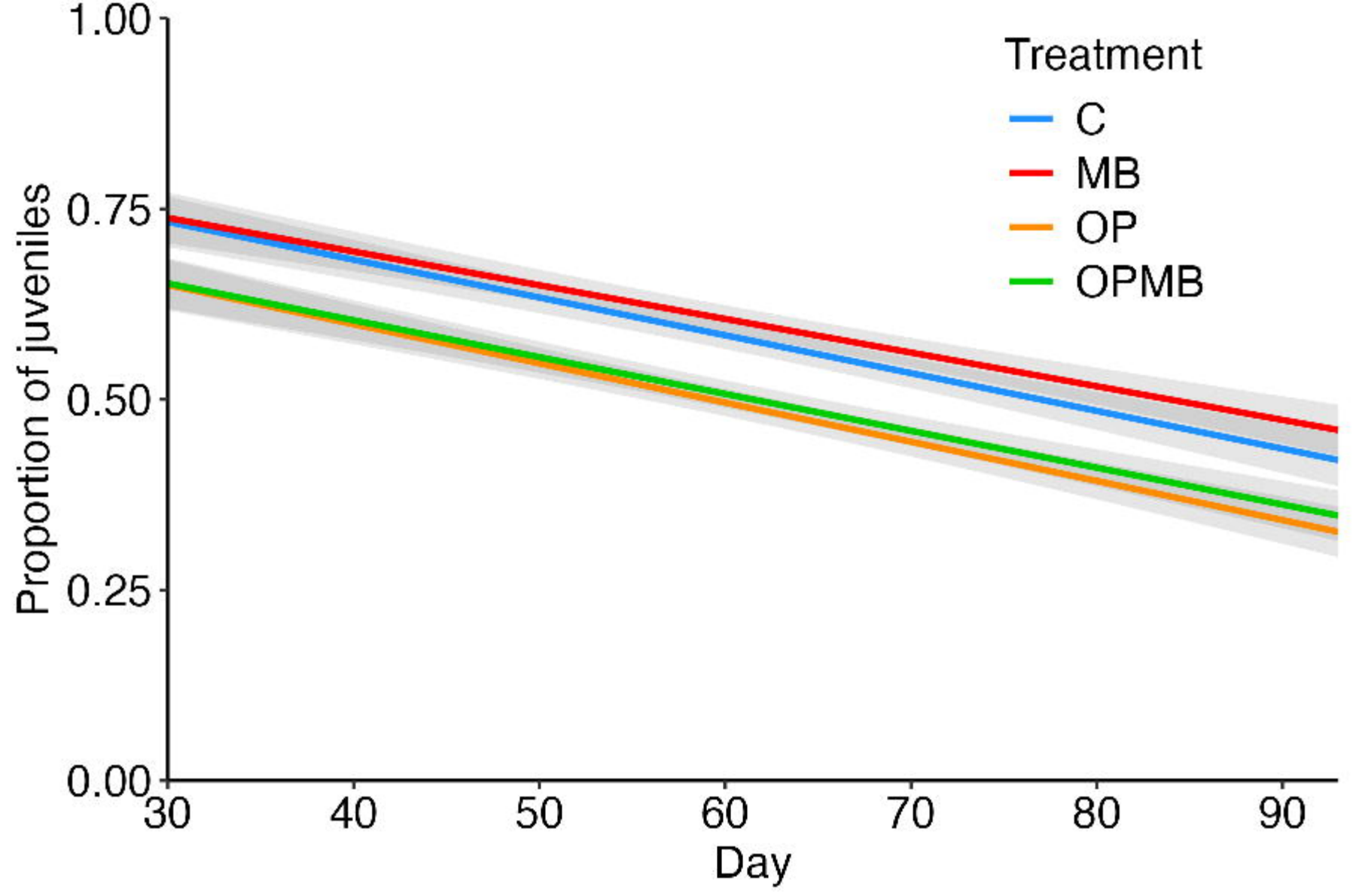
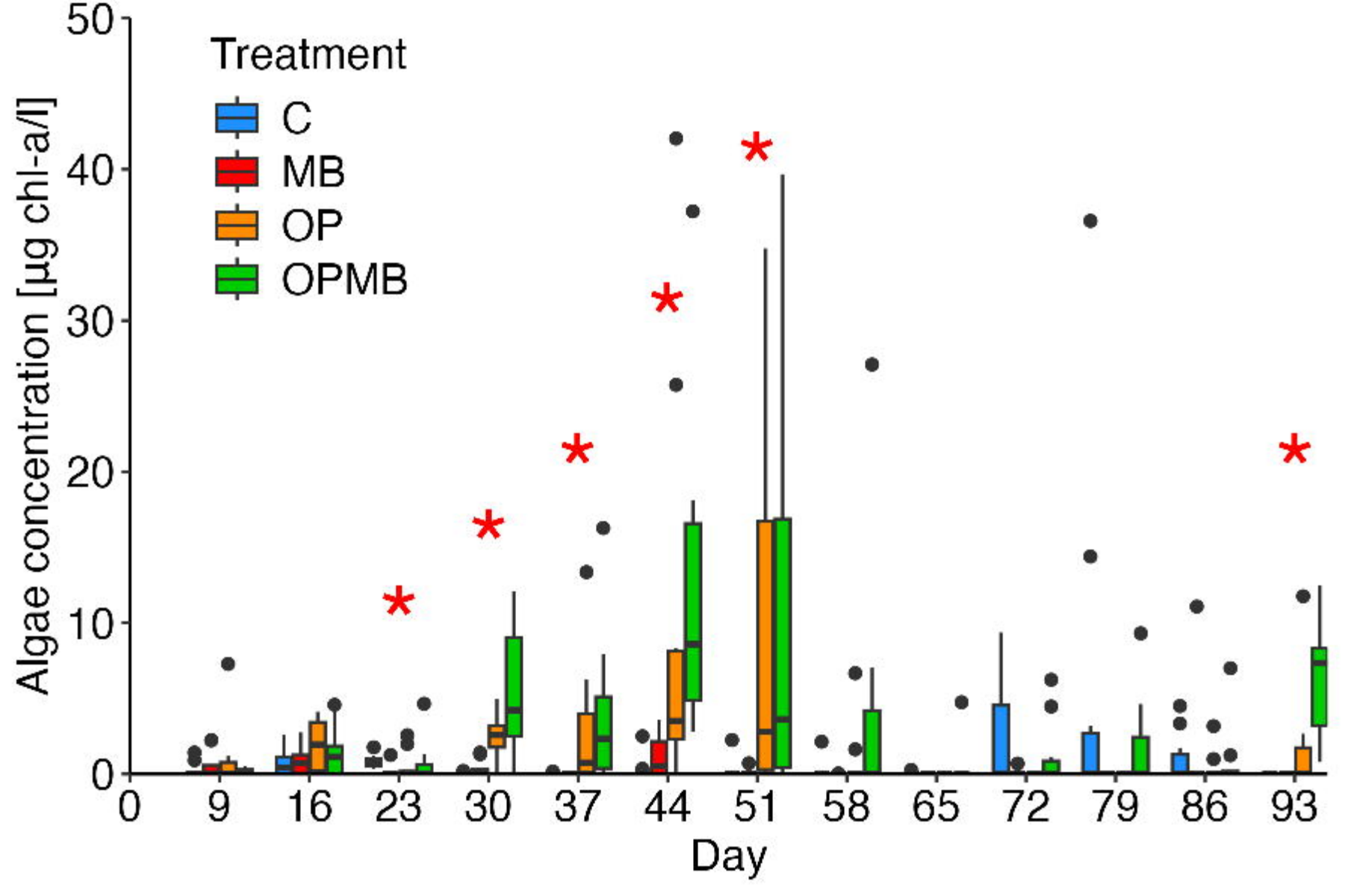

A
Parasite
C
MB
OP
offspring in clutch
Clutch (reproduction event)
0
5
10
15
20
3
6
9
12
B
Lifespan [days]
Parasite
C
MB
OP
0
20
40
60
80


A
Population growth rate r
0.4
0.3
0.2
0.1
0.0
C
OP
Parasite
B
Daphnia density/L
300
200
100
0
Treatment
C
MB
OP
OPMB
0
30
60
90
Day


A
1.00
0.75
0.50
0.25
0.00
M. bicuspidata prevalence
Treatment
MB
OPMB
30
40
50
60
70
80
90
Day


B
1.00
0.75
0.50
0.25
0.00
O. pajunii prevalence
Treatment
OP
OPMB
30
40
50
60
70
80
90
Day


Treatment
C
MB
OP
OPMB
Proportion of juveniles
1.00
0.75
0.50
0.25
0.00
30
40
50
60
70
80
90
Day


Treatment
C
MB
OP
OPMB
Algae concentration [µg chl-a/l]
50
40
30
20
10
0
0
9
16
23
30
37
44
51
58
65
72
79
86
93
Day