Mixing selects for predation resistance in lab-evolved communities of bacterial prey and social predator *Myxococcus xanthus*

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Abstract

Population mixing, and transmission modes strongly influence social interactions. However, the impact of repeated mixing on the evolutionary dynamics of microbial predator-prey interactions remains underexplored[1,2]. Hence, we conducted a laboratory evolution experiment with bacterial predator-prey communities under two transfer regimens: repeated mixing (horizontal transfer) versus no mixing (vertical transfer). For this, *Myxococcus xanthus* served as the generalist predator[3,4] and *Escherichia* coli as prey. We show that prey populations from vertical regimen were less resistant to predation than the ones from horizontal regimen. This was because prey isolates in the vertical treatment showed varying resistance levels relative to ancestors, while those in the horizontal treatment demonstrated less variation. Moreover, analysis of prey populations over evolutionary time revealed that the populations from horizontal treatment showed increasing levels of resistance to predation over time, whereas the ones from vertical treatment did not show similar trend. The differences in the outcomes of the two treatments was because the variants better at intraspecies competition, can only be maintained in the vertical treatment, whereas in horizontal treatment the benefits of superior intraspecies competitive fitness are nullified because of population mixing, as predicted by mathematical modeling approach. These predictions were empirically confirmed. Moreover, contrary to our expectations, predatory efficiency of evolved *M. xanthus* isolates was either the same or lesser than the ancestor. Together, we demonstrate that mixing affects the evolution of prey bacteria but has little effect on the hunting ability of the social predator *M. xanthus*.

Introduction:

The influence of mixing on the evolution of microbial interactions and their effects on community assembly has garnered significant interest in recent times [1,2]. This interest seems justified, given that the mixing has been shown to strongly affect the evolution of antagonistic as well as synergistic interactions within populations (i.e., groups of individuals of single species) [1,2,5-9]. Microbial communities in nature are frequently mixed because of human activities, abiotic, and biotic factors [10-12]. However, despite the prevalence of mixing events, we have limited understanding of the effects of repeated mixing on the evolution of microbial interactions both in the context of simple communities such as predator-prey interaction, and complex multispecies communities [1,2,13].

Evolutionary trajectories of antagonistically interacting species, like host-parasite and predator-prey systems follow the Red Queen (RQ) dynamics in which interacting partners coevolve in an evolutionary arms-race [14-16]. The RQ hypothesis has gained significant prominence over the years and has been proposed to explain the evolution of sex, antagonist-driven emergence of diversity, symbiosis, and antimicrobial resistance [8,17,18]. Originally proposed by Van Valen (1973) [19], the Red Queen hypothesis is intentionally focused only on the influence of the biotic factor. However, in nature, a combination of both biotic and abiotic factors will likely influence the outcome of antagonistic evolution. Therefore, extensions of this theory that incorporate both the effects of biotic and abiotic factors, such as diversity, migration, and changes in environmental conditions have attracted considerable attention[7]. For example, studies using host-parasite interactions suggest a significant effect of population mixing on the Red Queen dynamics between co-evolving antagonists [18,20]. While predator-prey coevolutionary dynamics has been the focus of multiple studies in the past [21-24], surprisingly the influence of repeated mixing, across habitats or even ecosystem, on the predator-prey coevolutionary dynamics is relatively less explored among microbes [1].

Here we asked whether repeated mixing of distinct microbial predator-prey consortia will affect their coevolutionary dynamics. For within-species interactions, theory and empirical evidence suggest that the coevolutionary dynamics without mixing events are likely to promote synergistic interactions [25]. In contrast, repeated mixing of microbial consortia, that are growing independent of each other are likely to promote antagonism [25]. Hence, we predicted that for interspecies interactions (such as predator-prey) too, repeated mixing will result in the evolution of predators that show better predatory performance relative to the ones that evolved in communities that did not experience repeated mixing events. Similarly, prey populations that experience repeated mixing events will evolve to be more resistant to predation than when predator-prey communities are propagated independent of mixing events. This is because of differences in the partner-fidelity between the two treatments. We also hypothesized that the populations from mixed (horizontal) and unmixed (vertical)

treatments would follow distinct evolutionary strategies. This is because the mixing events will increase the supply of mutation which might affect the fixation probabilities (by selecting larger effect mutations) because of changes in the clonal interference. To test these hypotheses, bacterial predator-prey communities of *M. xanthus* and *E. coli* were propagated in a lab evolution experiment.

M. xanthus is a generalist bacterial predator that kills prey bacteria by both contact-dependent and contact-independent mechanisms [4,26-30]. This includes the production of enzymes [27,31], antibiotics [3], and secretion systems [28,30]. In the presence of *M. xanthus,* coevolving *E. coli* populations have been shown to diversify and maintain predation-resistant mucoid variants along with the predation-sensitive types over the evolutionary timescale [24,32]. Further, as expected in a winnerless Red Queen dynamic, *M. xanthus* evolves higher predatory performance[24].

Here, we demonstrate that coevolution with a social microbial-predator results in an asymmetric arms-race where only the prey continues to evolve better survival strategies over evolutionary time in response to predation. Further, we show that the mixing of predator-prey populations during each transfer results in the evolution of resistance to predation. Whereas prey populations that do not experience mixing events maintained greater diversity in prey phenotype based on their varying degree of resistance to predation, indicating maintenance of diverse strategies. Most interestingly, evolved populations of predators from both regimens show the emergence of within-population diversity which harbors genotypes with similar predatory performance as their ancestors and the genotypes with reduced predatory performance relative to their ancestors. The mathematical modelling approach revealed that the differences in the outcomes between the two treatments are because of changes in the clonal interference caused by mixing events. These predictions were further confirmed by performing a laboratory evolution experiment.

Results:

Repeated mixing results in increased resistance to predation in the evolved prey population

The evolution experiment designed to test the effect of mixing on the evolution of prey and predator populations involved repeated mixing of predator-prey consortia every fourth day (Supplementary figure S1) for eighty days. Whereas control populations were propagated without mixing. To perform these experiments glucose was used as a carbon source. Since *E. coli* can use glucose as a carbon source but *M. xanthus* cannot, predation on *E. coli* population was the only source of nutrients for *M. xanthus*. The evolved communities after sixty-four days of serial transfers were analysed for the changes in the resistance to predation in the evolved *E. coli* and changes in the predatory performance of evolved *M. xanthus*.

To test the effect of repeated mixing, we measured the growth of evolved (after sixteen rounds of propagation in the lab evolution experiment) and ancestral *E. coli* populations when cocultured with ancestral *M. xanthus* in conditions identical to the ones during the evolution experiment. This measure accounts for the growth and survival of prey in the presence of *M. xanthus*. In line with our expectations, *E. coli* populations from the horizontal regimen on average exhibit better growth in the presence of predators than their ancestors (1.09 log-fold relative to ancestor, one sample t-test, $p^{[FDR\ corrected]} < 0.001$). Whereas growth of populations from vertical treatment did not show a significant difference relative to the ancestor (1.03 log-fold relative to ancestor, one sample t-test, $p^{[FDR\ corrected]} > 0.05$). Thus, *E. coli* population from vertical treatment were in general less resistant to predation than the ones from horizontal treatment (Figure 1A, independent sample t-test, $p = 0.057$). However, one of the four vertically coevolved populations (population 3) did evolve higher levels of resistance to predation, which was comparable to horizontally transmitted lines (Figure 1A, Supplementary figure S2). Together, vertically propagated prey populations seem to evolve different mechanisms of survival in the presence of predators, which in most cases increases the sensitivity of the prey populations to predators.

Differences between the vertically coevolved and horizontally coevolved prey populations can emerge in response to the presence of predators or as an outcome of adaptation to abiotic factors. However, since mono-evolved *E. coli* populations (propagated in the absence of *M. xanthus,* Supplementary figure S1C and S1D) from the vertical as well as horizontal regimen showed no difference in the overall levels of resistance to predation (Figure 1B and Supplementary figure S2, one sample Wilcoxon test, $p > 0.05$), we concluded that the differences between vertical and horizontal *E. coli* populations propagated along with *M. xanthus* were not the result of adaptation to abiotic factors. Together, these results demonstrate a strong effect of the transfer regimen on the evolution of the prey population.

Evolution of reduced predatory performance in co-evolved *M. xanthus* populations

It is difficult to reliably measure the growth of *M. xanthus* in our experimental setup (See methods). Hence, to assess changes in the evolved predator populations, the growth of ancestral prey was used as a measure of the predatory performance of *M. xanthus*. This measure of predation is a combination of growth and death of *E. coli* in the presence of the predator. Contrary to our expectations and previously reported findings [24], we observed that the ancestral *E. coli* was able to grow to a similar density in the presence of evolved as well as ancestral *M. xanthus* populations (Figure 1C, and Supplementary figure S3, one sample t-test $p > 0.05$). Further, no significant differences between coevolved *M. xanthus* populations (after sixteen transfers) from horizontal and vertical regimens

148 (Figure 1C, independent sample t-test for differences between prey growth in the presence of evolved
149 predators, $p > 0.3$) were observed. Together, the results suggest that the predator populations were
150 probably under relatively weaker selection to evolve enhanced hunting ability. Thus, adaptive
151 response other than the increased predatory performance as measured in our experiments might
152 have been selected in our experiments.

153 It is expected that the evolved populations are internally diverse and hence population-level data
154 might not be reflective of the intra-population diversity. Hence, we measured the predatory
155 performance of distinct clones isolated from evolved *M. xanthus* populations. To do so, four *M.*
156 *xanthus* clones were randomly isolated from each of the replicate lines from the vertical regimen and
157 sixteen clones were isolated from the horizontal regimen. Surprisingly, a minority of the clones from
158 both the transfer regimens showed reduced hunting performance relative to the ancestors
159 (Supplementary figure S3, ANOVA and SNK *post hoc* test). Whereas the majority showed no
160 difference.

161 However, genomic analysis of the evolved clones revealed mutations specific to the co-evolved
162 clones that were absent in the ancestors. Most of these mutations were either located in the genes
163 that putatively code for non-ribosomal peptide synthetases (NRPS) or transporter proteins
164 (Supplementary table T4). Previous reports have demonstrated that NRPS is involved in the synthesis
165 of antimicrobial peptides and hence is a major component of predatory behavior of *M. xanthus* [33,34].
166 Thus, mutation in this locus is in line with the selection pressure during the evolution experiment.
167 While in the bad predators (clones with lower predatory efficiency) most consistent mutations were
168 in the genes that putatively code for chemotaxis proteins (Supplementary table T4)**.** Mutations in the
169 chemotactic pathways like in the CheA gene, which is a part of the Che3 cluster of chemotaxis proteins
170 in *M. xanthus*, also explain reduced swarming efficiencies of the *M. xanthus* clones that have evolved
171 reduced predatory efficiency (Supplementary table T4). The evolution experiments were conducted
172 in a spatially structured environment, thus *M. xanthus* cells might need the ability to find the next
173 patch of prey populations. Thus, chemotactic mutants that cannot swarm towards the prey show
174 lower prey-killing efficiencies. Interestingly, despite the similarities in the predatory efficiencies of the
175 predators from both regimens, the overall mutations were more similar in the horizontally coevolved
176 *M. xanthus* isolates, compared to the vertically coevolved ones (Supplementary table T4). These
177 results therefore strongly suggest that the predators' populations have also evolved differentially.
178 However, the differential evolution has not affected the prey-killing efficiency of the predator
179 population**.**
180

181 **Coevolved *E. coli* clones from vertical regimen exhibit large within-population differences in their**
182 **sensitivity to *M. xanthus* predation than the ones from horizontal treatment**

183 Since evolved prey populations from the two treatments were significantly different from each
184 other, we further tested whether the population level differences were representative of the
185 phenotypes of distinct clones within each population. To do so, four *E. coli* clones were isolated from
186 each of the vertically propagated lines and sixteen clones were isolated from the horizontally evolved
187 population. We observed significant variation among the isolates derived from vertically coevolved
188 populations than between clones isolated from horizontal transfer regimen (Supplementary figure S4,
189 average variance among vertical clones = 0.414, average variance across horizontal clones = 0.130,
190 independent sample t-test for differences between variance $p < 0.02$). Importantly, out of sixteen
191 clones from the vertical regimen, five clones seem to have higher resistance and four clones seem to
192 show lower resistance to predation relative to ancestors (Figure 2 and Supplementary figure S4).
193 Whereas all clones from the horizontal regimen were more resistant to predation than the ancestors.

194 The evolution of *E. coli* strains sensitive to *M. xanthus* predation in vertically evolved populations
195 suggested that the evolution of resistance to predation might not be the only adaptive strategy. This
196 was indeed the case, as pairwise competition experiments between evolved *E. coli* clones and their
197 respective Kanamycin-marked fitness-neutral ancestors (Supplementary figure S8) revealed that
198 irrespective of their sensitivity to *M. xanthus* predation, most coevolved clones showed higher
199 competitive fitness than ancestors in the presence of *M. xanthus* (Figure 3, one sample Wilcoxon test
200 $p^{[FDR\ corrected]} < 0.0005$). Moreover, the fitness of co-evolved clones was in general significantly higher in
201 the presence of *M. xanthus* than in the absence of it (Figure 3, Kruskal Wallis and Dunn's *post hoc*
202 test).

203 Similar experiments with strains from mono-evolved lines of *E. coli* showed that all the clones had
204 lower resistance against *M. xanthus* than their ancestors (Figure 2, an average of prey survival of
205 mono-evolved clones was 1.03-fold lower than ancestors, one sample Wilcoxon test, $p < 0.0003$).
206 Further, we did not detect differences in the relative fitness of mono-evolved *E. coli* in the presence
207 of *M. xanthus* than in its absence (Figure 3, Kruskal Wallis, Dunn's *post hoc*).

208 Further analysis of the data from predation experiments (Figure 2) and competition experiments
209 (Figure 3) revealed a positive correlation between resistance to predation and relative fitness for
210 vertically coevolved clones of *E. coli* (Supplementary figure S5, Pearson's correlation coefficient (r) =
211 0.6, $p < 0.019$), but not for *E. coli* coevolved in the horizontal regimen. Thus, though the evolution of
212 high levels of resistance to predation is not essential for competitive advantage against ancestors,
213 strains with higher resistance will generally have higher fitness than the ones with lower levels of
214 resistance to predation.

215 Genomic analysis of the evolved and ancestral prey clones from both coevolved and mono-evolved
216 treatments revealed several mutations that are specific to coevolved prey clones (Supplementary
217 table T3). Amongst these, insertions at different locations in and after fliPQR operon in RcsB binding
218 site were most common [35]. This binding site is important for the regulation of RcsA gene that is
219 involved in Rcs-phosphorelay[35]. Previous studies have demonstrated a diverse set of functions for Rcs
220 signal transduction system that include regulation of motility, capsule formation and virulence [35-37]. A
221 detailed understanding of how these mutations affect the sensitivity of prey bacteria in our
222 experiments will require exhaustive analysis that is not part of the current manuscript. Further, a
223 higher genetic diversity was observed in the vertically coevolved prey, that is consistent with their
224 phenotypic diversity. Whereas the horizontally coevolved prey was more genetically similar with
225 comparable mutations in the majority of the clones.
226
227 **Mathematical modelling reveals prey evolution in horizontal treatment is mainly driven by**
228 **adaptation to the presence of predator rather than intraspecific competition between prey**
229 Together, our experiments demonstrate that horizontally propagated populations, which
230 were mixed, only evolved predator avoidance, while vertically propagated ones, with no mixing,
231 evolved both predator avoidance and prey competitive ability (Figure 2 and Figure 3). To explain why
232 mixing suppressed prey competitive ability and amplified predator avoidance, we formulated a simple
233 mathematical model of our communities. Briefly, our model partitioned each growth cycle into two
234 phases: an initial period of prey growth, and a later period of predator growth and prey death (Figure
235 4A), consistent with experimental data of the dynamics within a growth cycle (Supplementary figure
236 S6).
237 The relative fitness of prey in each of the two phases operated along two distinct measures of
238 fitness in our model. During prey growth, prey fitness was determined primarily by prey competition,
239 i.e., the growth rate of each prey subpopulation relative to the others (Figure 4B). During predator
240 growth, prey fitness was set primarily by predator avoidance, i.e., the death rate of each prey
241 subpopulation (Fig. 4B and Methods for details of the model). Thus, the fitness for each prey
242 subpopulation was determined by two parameters. Our model simulated population dynamics over
243 these growth cycles for a population comprising several prey subpopulations whose prey competition
244 (growth rate) and predator avoidance (death rate) were randomly picked from an appropriate
245 distribution (details in Methods). To simulate mixing (horizontal) and no mixing (vertical) regimens,
246 we mimicked our experimental protocol (Methods).
247 Simulations of our model reproduced our experimental observations: mixed populations
248 showed greater predator avoidance and lower relative prey competitive ability (Figure 4C and 4D).

249 Further, the model explained why mixing suppressed prey competition. The key idea is that mixing
250 increases the number of competing prey subpopulations, which increases clonal interference (Figure
251 4D). This slows down the takeover of prey with a growth advantage and favors prey with improved
252 predator avoidance. Thus, our model suggests that mixing increases clonal interference among
253 competing prey, potentially explaining the different experimental outcomes in vertical and horizontal
254 regimens.
255
256 **Prey populations from vertical and horizontal treatment show distinct evolutionary dynamics**
257 Together, empirical observations and theoretical predations suggest that the prey populations in
258 the vertical transfer regimen are under fluctuating selection. This was because during each growth
259 cycle interference competition against conspecifics was under selection during the first growth phase
260 and resistance to predation was under selection in the second growth phase (Figure 4A). This resulted
261 in the emergence of distinct genotypes with different degrees of resistance to *M. xanthus* predation
262 in vertical treatment. Since the benefits of better intra-prey population competitive ability during the
263 growth phase were limited in the horizontal treatment, prey populations from the horizontal regimen
264 were probably under directional selection, which selects for lesser phenotypic diversity. If true,
265 horizontally evolved prey populations should over evolutionary time keep on either increasing or
266 maintaining high levels of resistance against *M. xanthus*. While vertically co-evolved prey populations
267 are likely to not show a trend of increasing resistance to predation over evolutionary time.
268 To test this hypothesis, we isolated *M. xanthus* populations after twenty rounds of coevolution.
269 These *M. xanthus* populations were then used to test the resistance of evolved *E. coli* populations
270 from three distinct evolutionary time points (i.e., after ten, sixteen and twentieth transfer). In line
271 with our hypothesis, we observed that after sixteen transfers, populations of horizontally coevolved
272 prey were on average 1.06 log-fold more resistant to predators than the ones from the tenth transfer.
273 Further, though not statistically significant, differences were observed between prey at the sixteenth
274 and twentieth transfer, prey at the sixteenth transfer on average seemed more sensitive to predation
275 than the ones after twenty transfers (Figure 5A, ANOVA and Tukey's post-hoc). Whereas the vertically
276 coevolved prey did not show differences in the levels of resistance to predation from transfer-ten to
277 transfer-twenty (Figure 5A, ANOVA and Tukey's post-hoc). These data, coupled with our observations
278 that within-population variation among clones from the horizontally coevolved prey populations is
279 less (Figure 2), and that vertically evolved prey populations are internally heterogeneous (i.e., show
280 diversity in predation resistance phenotypes) (Figure 2), supports our hypothesis that the horizontally
281 co-evolved prey populations tend to either continuously evolve higher level of resistance of maintain

282 the levels of resistance over evolutionary time. Whereas vertically co-evolved prey populations evolve
283 two strategies, i.e. evolution of superior intraspecies competitive fitness and resistance to predation.
284 However, when *M. xanthus* population isolated from different evolutionary time points (i.e., tenth,
285 sixteenth and twentieth) were cultured with *E. coli* population isolated after the twentieth transfer,
286 no significant differences were observed in the predatory ability of the predators over evolutionary
287 time from both the treatments. Here, the survival of terminally coevolved *E. coli* (CFU/mL) in the
288 presence of predators from distinct evolutionary timepoints within respective treatments, was taken
289 as a read-out of the predatory abilities of respective predator population (Figure 5B, ANOVA and
290 Tukey's post-hoc)
291
292 **Switching the mixing regimen between the terminally evolved horizontal and vertical populations**
293 Predictions from our model suggest that the distinct outcomes in the two selection regimens
294 depend on the continuation of the selection regime. Thus, if mixing indeed resulted in directional
295 selection in prey survival strategies, repeating the same evolution experiment again with the
296 diversified populations from the vertical regimen should result in one or both of the following
297 possibilities. First, reduced variation among clones after being propagated in the mixing treatment.
298 And second, increased resistance to predation both at the population and isolate levels. To test these
299 hypotheses, we propagated a terminally coevolved population (i.e., after twenty transfers) by
300 Switching the transfer regimen - i.e., populations from the horizontal regimen were propagated
301 vertically, and the vertically evolved populations were propagated in the horizontal regimen -
302 (Supplementary figure S7). Similar to the experiments above, prey populations were isolated from the
303 evolved prey-predator consortia (See methods) after they were propagated for sixteen transfers. On
304 testing the ability to survive predation in the evolved preys against mixing, not only evolved a
305 significantly higher ability to survive predation than their ancestors (Figure 6A, ANOVA and Tukey's
306 post-hoc) but also exhibited a lower within-population diversity than their ancestors (Figure 6B,
307 ANOVA and Tukey's post-hoc for difference between groups and also for differences within each of
308 the groups). Moreover, consistent with our previous results, while the overall resistance to predation
309 did not change much at a population level (Figure 6A, ANOVA and Tukey's Post-hoc), in the prey
310 population that was propagated under vertical regimen exhibited a greater degree of within-
311 population variation than their ancestors (Figure 6B, ANOVA and Tukey's post-hoc for difference
312 between groups and also for differences within each of the groups). Together, these experiments
313 reveal that irrespective of preexisting standing diversity, prey populations that experience repeated
314 mixing will evolve to have reduced within-population diversity and higher resistance than the ones
315 that evolved in the absence of mixing events.

316 **Discussion**

317 Events that affect the mixing of communities are common in nature [1,9,13,38-40]. However, their
318 influence on the evolution of bacterial predator-prey interaction remains less explored [1]. Here we
319 used model predator-prey communities of bacteria to show that repeated mixing of predator-prey
320 communities can result in the evolution of enhanced prey survival ability in the presence of the
321 predator, relative to when communities are allowed to evolve in the absence of repeated mixing
322 events. Surprisingly though, we could not detect increased predatory performance in the evolved
323 populations of predators. Moreover, contrary to expectations we observed the evolution of reduced
324 predatory performances in certain predator isolates. Hence the observations reported in our study do
325 not follow the coevolutionary pattern of the evolutionary arms-race as expected from host-parasite,
326 and host-pathogen interactions [16,20,41]. Several factors such as social predation by *M. xanthus*, inter-
327 vs intra-species competition, differences in the effective mutation supply in the two selection
328 regimens, and asymmetry in the rate of evolution of prey and predator can explain the observed
329 outcomes.

330 Two aspects of *M. xanthus* biology can introduce intra-species competition among predators. First,
331 *M. xanthus* is a social predator that uses diffusible compounds to kill prey [3,4,29,42]. Moreover, digestion
332 of the prey is done extracellularly [27,43]. Hence, both the killing factors, as well as the digested prey, are
333 available as public goods to the individuals in the neighborhood. In our experiments, we find that most
334 of the individual isolates from both treatments show similar predatory performance as their
335 ancestors, except for a few clones, that perform worse than their ancestors. Hence, it seems possible
336 that the apparent decrease in population productivity is mediated by the exploitation of public goods
337 by the non-producers. If this is true, the use of non-social predators in a similar experimental setup
338 should result in the evolution of increased predatory performance. Indeed, this is the case, predator-
339 prey coevolution experiments using non-social *M. xanthus* isolates have resulted in enhanced
340 predatory performance relative to the ancestral variants [24]. Though not the focus of the current
341 manuscript, it is an interesting opportunity to test the interaction dynamics between the coevolved
342 efficient predator and coevolved less-efficient predator. Such population studies coupled with
343 mechanistic understanding might reveal ecological and evolutionary mechanisms that lead to the
344 evolution of scavenging strategies. That is the evolution of reduced prey-killing ability coupled with a
345 strong dependence on the presence of efficient predators for better survival.

346 Microbial antagonism is pervasive in complex microbial communities [44,45]. Hence, studies of such
347 interactions have attracted tremendous interest in recent years to combat microbial infections and
348 contaminations [46-48], and to better the understanding of microbial ecology [24,32,49,50]. To our surprise,
349 studies have used the terms host-parasite and predator-prey interactions interchangeably for

350 identical interactions (for example bacteria and phage) [38,51,52]. However, predator-prey interactions
351 are distinct from host-parasite interactions in many ways [51,54-58] (for example: relationship intimacy,
352 relationship duration, generation time, population size, and trophic level). Among them we feel two
353 are relatively more significant than others. First, the population size of the exploiter and exploited is
354 inverse for predator-prey interactions relative to that of the host-parasite interactions. Second, the
355 generation time of the predator and prey is similar, whereas the generation time of parasites is
356 significantly smaller than their hosts. Thus, the exploited partner has a faster rate of evolution in
357 predator-prey interactions, which is the opposite for host-parasite interactions where the exploiter
358 has a faster rate of evolution relative to the exploited partner. Here, we did not observe the evolution
359 of increased predation efficiency among *M. xanthus* populations, whereas bacteria-phage (host-
360 parasite) coevolution experiments have repeatedly shown the evolution of increased virulence [1,20, 53].
361 The differences we find here relative to the ones reported in the previous studies [1,19,52] in which the
362 exploiter (parasite) evolves faster might be attributed to the differences in the relative rate of
363 evolution of exploiter to the exploited partner.

364 The effect of population mixing has been studied in multiple instances [1,59]. Whereas the effect of
365 mixing of predator-prey communities has not been studied in as much detail. Interestingly, we
366 observed that the evolution of prey bacteria followed a similar fate to previous reports in which the
367 effect of population mixing was studied on the evolution of bacterial host-parasite interaction [1]. Albeit
368 with one key difference, i.e., unlike in the host-parasite evolution experiment in which vertical
369 propagation either selected for resistance to phage in some replicate lineages or intraspecies
370 competitive fitness in others, we observed that both of these strategies simultaneously evolved within
371 each of the replicate population. Further, we observed that repeated mixing resulted in directional
372 selection on the evolution of resistance against the exploiting partner. And unmixed communities, like
373 in previous report [24,32], selected for the coexistence of distinct survival strategies that include the
374 evolution of resistance and sensitivity to the exploiter, which is suggestive of fluctuating selection. If
375 these trends observed in our experiments are indeed solely a result of the repeated mixing of
376 communities, it is expected that when the diversified vertically coevolved communities are
377 propagated under horizontal treatment regimen, homogenization of the survival strategies should be
378 observed conferring overall increased resistance to predation. When the transfer regimen was flipped
379 from horizontal to vertical, diverse survival strategies should emerge from a more homogeneously
380 distributed population. Consistent with our expectation we observe an increased resistance to
381 predation in our flipped horizontal treatment and diversification in our vertical treatments. Thus,
382 supporting our previous observation and further establishing that mixing predominantly will result in

383 an increased resistance to predation in prey irrespective of whether the ancestral population is
384 isogenic or has a standing diversity.

385 Though the experimental approaches to studying the effect of population mixing are numerous,
386 subtle differences between them introduce differential importance to the spatial structure. For
387 example, population mixing within a single spatially structured arena will allow the mixing of distinct
388 genotypes but will not affect the overall availability of diffused shared resources such as public-good
389 [60-62]. Whereas mixing of populations as done in this study will result in the mixing of distinct lineages
390 repeatedly relative to the vertically propagated communities in which identical genotypes interact
391 with each other over evolutionary time. Separation of populations during the growth phase and
392 mixing at each transfer will result in increased mutational supply even after segregation of the
393 populations. Thus, the design of the evolution experiment allows the horizontal population to behave
394 as a single large metapopulation. Thus, we used a mathematical modelling approach to demonstrate
395 that the mixing events, as expected, will effectively allow the horizontal populations (using the growth
396 phase) to sample a larger number of mutations. The model further shows that this results in changes
397 in the dynamics of interference competition. These changes in the dynamics of interference
398 competition resulted in the selection for resistance to predation. This was because of the diminishing
399 adaptive benefits of resource competition. The model further predicted that the outcomes of the
400 evolution experiment depend on the continuation of regimens. That is, if the populations evolved in
401 mixed treatment are propagated as unmixed, then it is expected that the populations will evolve
402 diverse adaptive strategies like in the vertical treatment. We demonstrate that this outcome is indeed
403 true by performing additional evolution experiments in which the selection regimens were switched.

404 The logistics of our evolution experiment involved aspects of evolution in the spatially structured
405 as well as unstructured environment. For example: limited diffusion of resources is an important
406 distinguisher between structured and unstructured environments, which has been shown to strongly
407 affect the evolution of synergistic [25,63] as well as antagonistic interactions [64]. The transfer protocol used
408 in this study involved population mixing even in the vertically transmitted regimen, thus limiting the
409 evolution of interaction networks. However, the probability of the evolution of synergistic interactions
410 was most likely higher in the vertically propagated regimen than in the horizontal regimen. This is
411 because of the mixing of multiple lineages before each transfer in the latter.

412 Interestingly, one of the four vertically propagated coevolving prey populations also demonstrated
413 high levels of resistance to predation, which was comparable to horizontally propagated populations.
414 Thus, vertically propagated prey populations might explore distinct evolutionary fates that range from
415 the evolution of higher levels of resistance to *M. xanthus*, to the evolution of within-population
416 diversity with both resistant and sensitive individuals. This finding is not surprising as similar effects

417 of differential effects of bacterial predators, protists, and phages on bacteria have been demonstrated
418 previously [32,65-67]. Nonetheless, unlike prey populations that evolved in horizontal regimen, the vertical
419 population with high levels of resistance to predation harboured intraspecies diversity (prey with low
420 and high resistance to predation) which was similar to the other vertically propagated coevolving *E.*
421 *coli* populations. Together, it seems that the evolution of the homogenous predation-resistant-prey
422 population in horizontally propagated lines indicates that the predation-resistant individuals will
423 outcompete predation-sensitive ones when communities are repeatedly mixed.
424 In recent years the call for harnessing the potential of microbial predators to control microbial
425 contaminations and infections has generated tremendous interest. Thus, it is important to understand
426 the effect of microbial predation on the evolution of prey species. In this context, it is unavoidable
427 that distinct predator-prey populations might experience mixing events which might change the
428 effective supply of mutations. Here we demonstrate that because of mixing events microbial predator-
429 prey interactions might have a strong influence on the evolutionary outcomes for prey populations
430 and that mixing will most likely have a stronger influence on the evolution of prey bacteria but not the
431 predator.
432

Acknowledgements

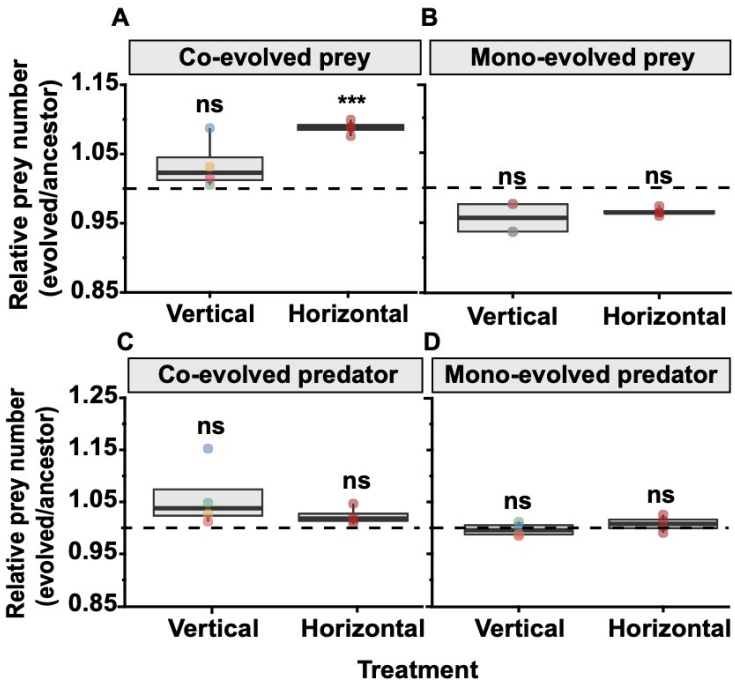
The authors thank Deepa Agashe and Gregory Velicer for providing *E. coli* (MG1655) and *M. xanthus* (GJV1) strains, members of the Bacterial Ecology and Evolution lab at the Indian Institute of Science for helpful discussion. We also thank DST-FIST for support to the Department of Microbiology and Cell Biology, UGC Centre for advanced study, and the DBT-IISc partnership. AG acknowledges support from the Ashok and Gita Vaish Junior Researcher Award, as well as the Government of India's DBT Ramalingaswami Fellowship. This study was mainly supported by the funds from India Alliance Intermediate fellowship to SP (IA/I/20/1/504921) and HGK-IYBA grant (BT/13/IYBA/2020/15) to SP.

Author contribution

SP conceived the project. SS, BB, and SP designed experiments. SS, BB, and JL performed experiments. SS, BB and JL analyzed the data. AG performed the mathematical modelling. SS wrote the first draft of the manuscript. SP edited the manuscript.

448 **Figures and figure legends**

449



450

451

452 **Figure 1: Effect of selection regimen on the evolution of resistance to predation in prey and predatory**
453 **performance of *M. xanthus* populations.** (A and B) Growth of evolved *E. coli* relative to the growth of
454 ancestral *E. coli* in the presence of ancestral predator is shown. (A) Prey populations coevolved in the
455 vertical transfer regimen were more susceptible to predation than the populations that evolved in the
456 horizontal transfer regimen (paired t-test for differences between vertically evolved *E. coli* relative to
457 horizontally co-evolved populations, p*** < 0.001). (B) Whereas mono-evolved populations showed no
458 significant differences both between treatments as well as relative to the ancestors (Wilcoxon-test for
459 differences between vertically and horizontally mono-evolved populations, $p > 0.05$, n = 4). Color of dots
460 in the vertical regimen represents distinct lineages. (C) Predator populations coevolved in vertical and
461 horizontal treatment exhibited similar predatory performance, which was not significantly different from
462 their respective ancestors. Relative numbers (evolved/ancestor) are log(CFU/ml) of ancestral *E. coli* when
463 cocultured with evolved predator population relative to their respective ancestor (one sample T-test for
464 differences between coevolved predator population and their ancestors, $p > 0.05$, n=4). (D) Mono-evolved
465 predator populations showed no significant differences both between treatment as well as from ancestors
466 $p > 0.05$, n=4). Relative numbers (evolved/ancestor) in log (CFU/ml) of ancestral *E. coli* when cocultured
467 with evolved predator population relative to their respective ancestor. Different color of dots in the vertical
468 regimen represents distinct lineages.

469

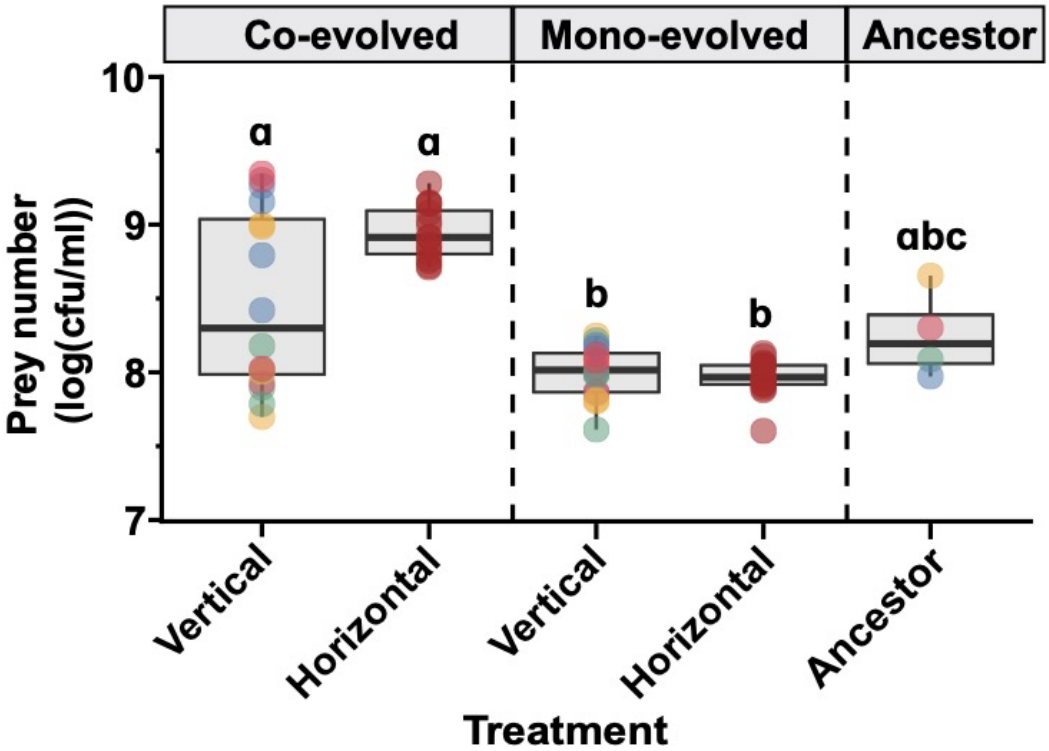
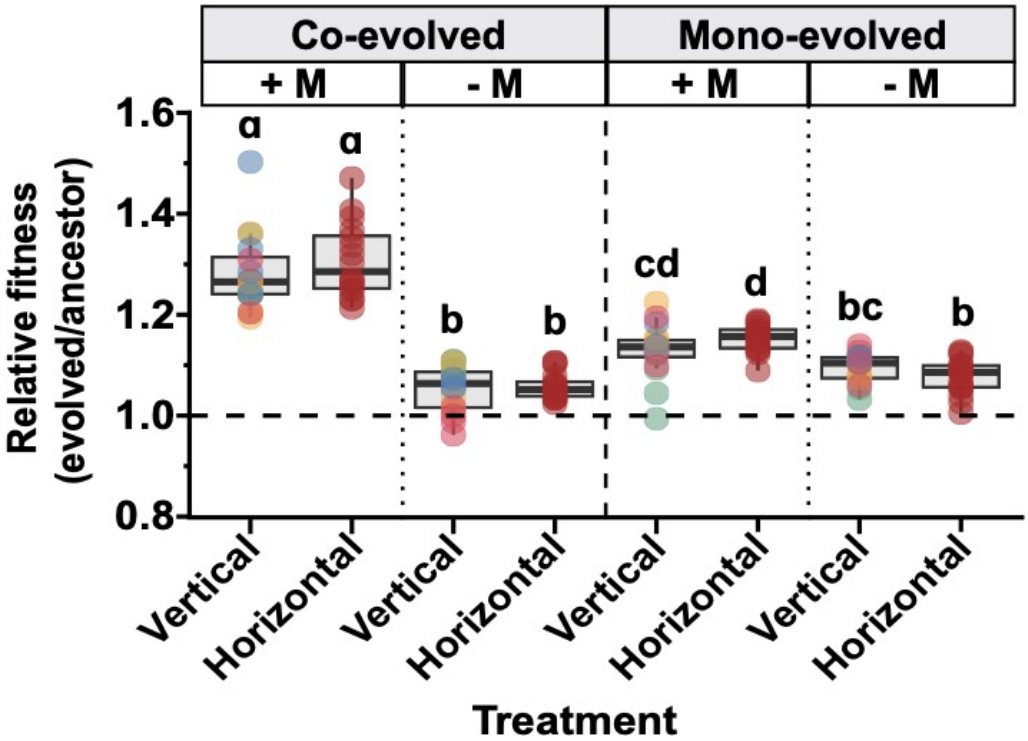


Figure 2: Effect of the selection regimens on the evolution of resistance to predation in prey clones. Survival of evolved and ancestor *E. coli* clones is shown as log(CFU/ml) of prey numbers when cocultured with the ancestral *M. xanthus.* Letters indicate significant difference between groups (Kruskal-Wallis, Dunn's post hoc test, n =3 for each clone studied). Different color of dots in the vertical and ancestral regimen represents a distinct lineage that started from the ancestor shown in the same color.

481



482
483

484 **Figure 3: Effect of the selection regimens on the competitive fitness of prey clones relative to their**
485 **ancestors**. Relative fitness of evolved *E. coli* clones (dots) is shown. All groups are significantly different
486 from 1.0 (one sample Wilcoxon test for differences between average relative-fitness of all clones
487 relative to 1.0, $p^{[FDR\ corrected]} < 0.01$, n = 3), and letters indicate significant differences between groups
488 (Kruskal-Wallis, Dunn's post hoc test for differences among four treatments). Different color of dots
489 in the vertical treatment represents distinct lineages.

490

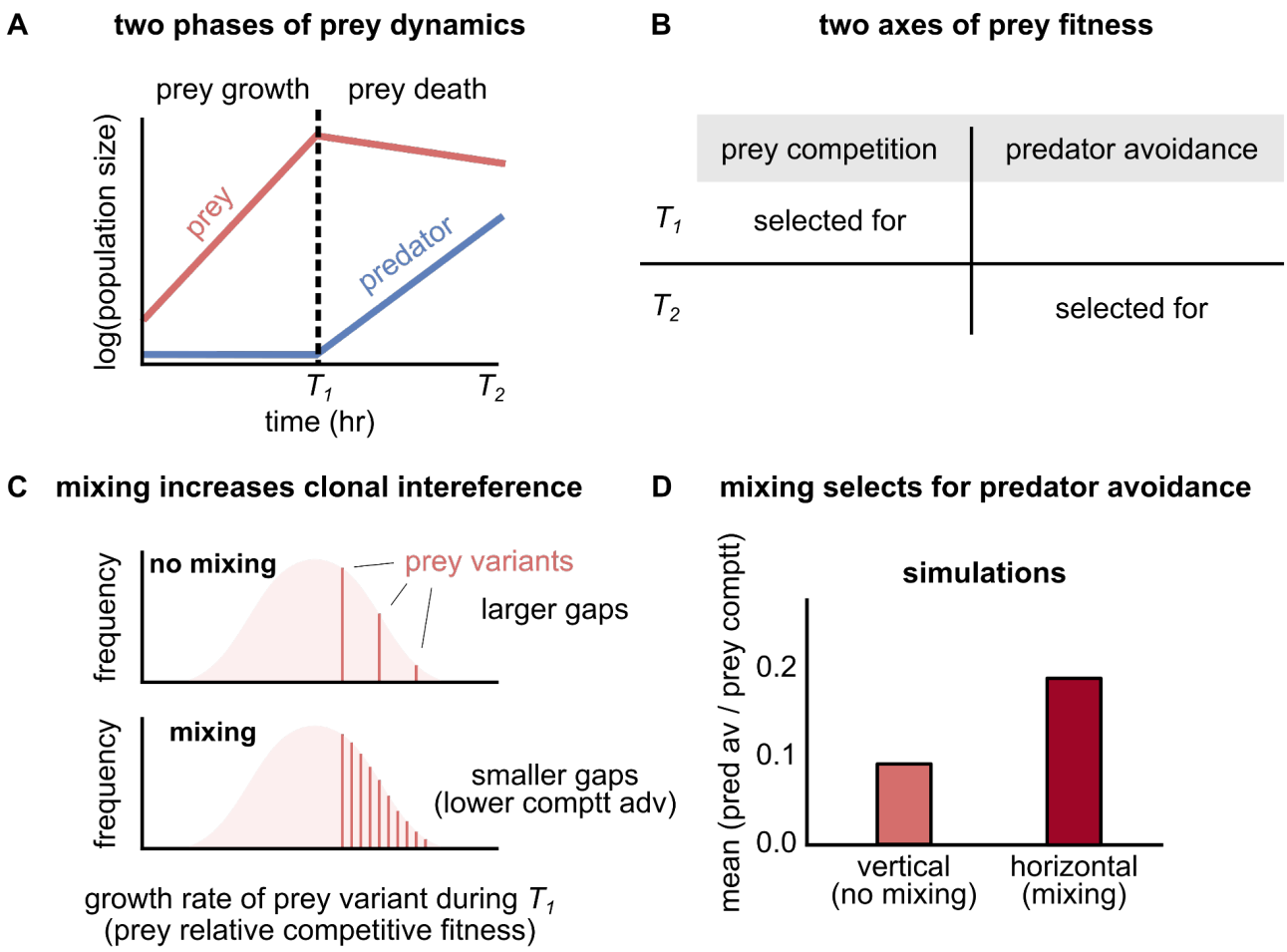


Figure 4: Mathematical model of population dynamics suggests that mixing increases clonal interference and preferentially selects for predator avoidance. A) Schematic illustrating the two dynamic phases of the prey and predator in each growth cycle. The first phase, lasting time T_1, exclusively involves exponential prey growth (linear on a log axis), while the second phase, lasting time T_2, involves predator growth and consequent prey death (Methods). B) In each dynamic phase, different aspects of the prey affect its fitness. In the prey growth phase, prey competition dominates, while during prey death, predator avoidance dominates. C) Schematic illustrating how mixing increases clonal interference and slows the evolution of prey competition. Shown are hypothetical growth rate distributions for prey subpopulations. Vertical lines show hypothetical prey variants. Mixing increases the diversity of competing prey variants, lowering the gaps between their growth rates and lowering the relative competitive advantage of prey variants with large growth rates. Thus, prey variants with decreased death rates have a larger selective advantage. D) Results from simulations of our model (for details see Methods) suggest that mixing selects for variants with increased predator avoidance over prey competition, providing an explanation for our observations.

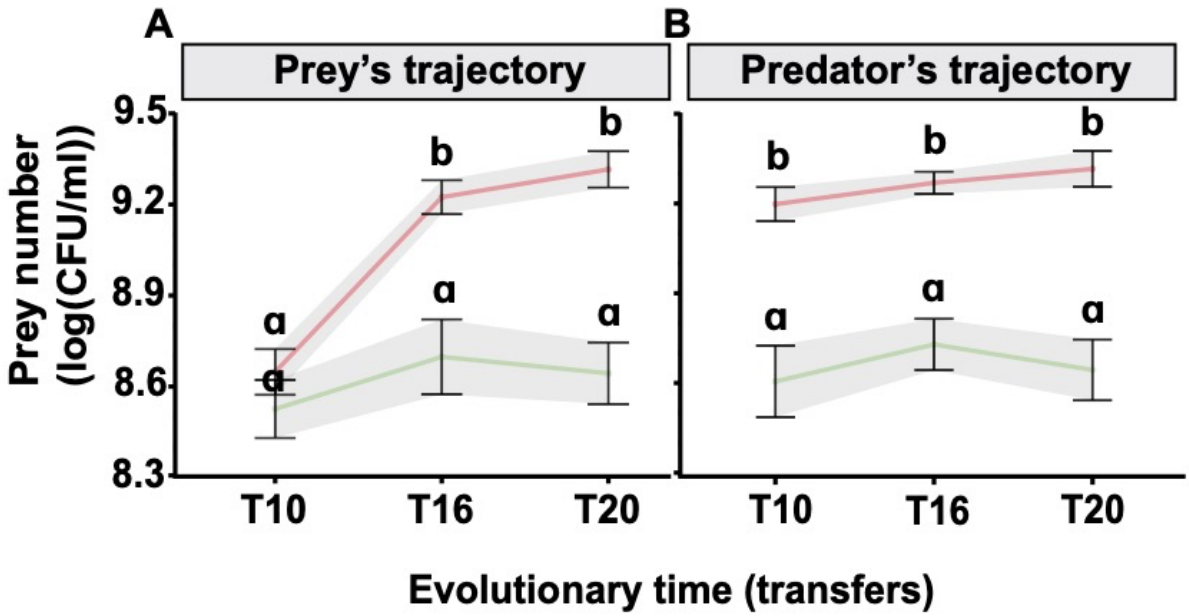


Figure 5: Prey from the horizontal selection regimen become increasingly more resistant over evolutionary time whereas the ones from the vertical regimen do not. A) Coevolved *E. coli* populations after ten (T10), sixteen (T16), and twenty (T20) transfers from vertical (green line) and horizontal (red line) regimens were analysed for changes in the resistance to predation against terminally evolved (after twenty cycles) respective *M. xanthus* populations. B) Coevolved *M. xanthus* populations after ten (T10), sixteen (T16) and twenty (T20) transfers from vertical (green line) and horizontal (red line) regimens were analysed for the change in the predatory performance against terminally evolved (after twenty cycles) respective *E. coli* populations from either vertical or horizontal regimen. Data shown is the average log(CFU/ml) for prey survival against respective *M. xanthus* from both regimens. Error bars are 95% confidence interval, letters indicate significant differences within the regimen (ANOVA, Tukey's post hoc test, n = 3).

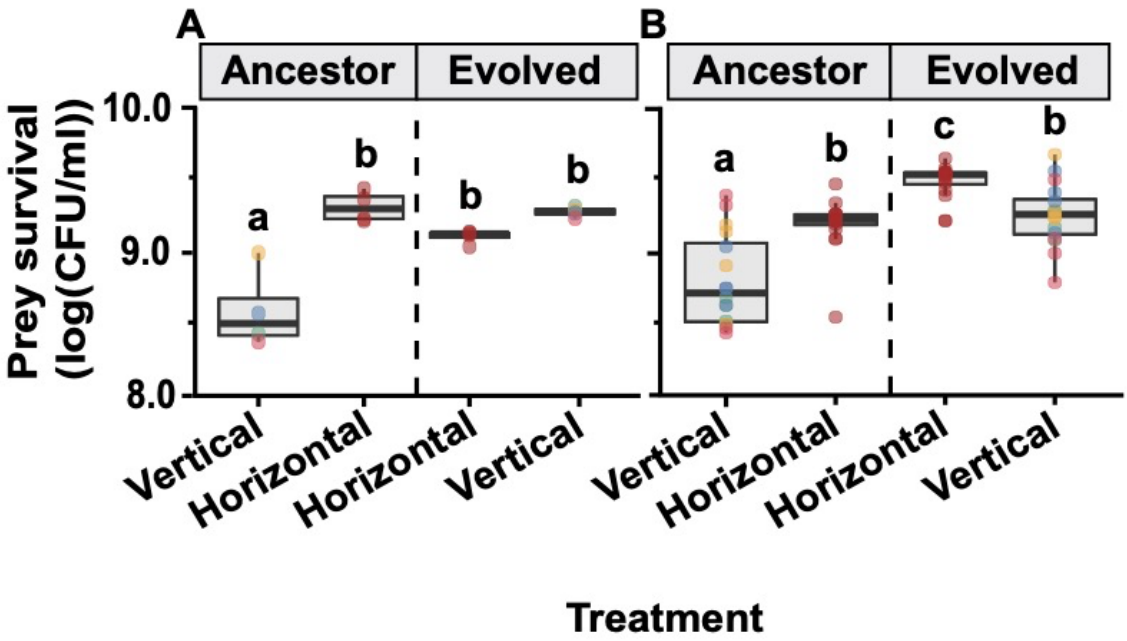


Figure 6: Mixing communities always results in evolution of higher resistance to predation while isolated communities evolve diversified survival strategies in prey population. A) Growth of ancestral vertically coevolved and horizontally coevolved *E. coli* population (T20 ancestors) along with the *E. coli* population after sixteen transfers in flipped regimen (Flipped: where vertically coevolved communities were further propagated under horizontal regimen and horizontally coevolved clones were further propagated under vertical treatment regimen), in presence of ancestral *M. xanthus* (ANOVA, Tukey's post hoc test, n = 4). B) Growth of *E. coli* clones isolated from ancestral vertically coevolved and horizontally coevolved *E. coli* population (T20 ancestors) along with the *E. coli* clones isolated after sixteen transfers in flipped regimen (Flipped) in presence of ancestral *M. xanthus* (ANOVA, Tukey's post hoc test, n = 4). Different color of dots in the vertical treatment represents distinct lineages.

539 **Methods**

540 **Semantics**

541 We define predation-prey interaction as an antagonist interaction in which the exploiting partner
542 kills the exploited partner to acquire nutrients. Predator-prey interactions are distinct from host-
543 parasite interactions in many ways [51,54-58] (for example: relationship intimacy, relationship duration,
544 generation time, population size, and trophic level). Importantly, we argue that two factors most likely
545 to differentiate the evolutionary dynamics of host-parasite interactions relative to that of predator-
546 prey interactions are.

547 1) The overall population size of the predator is similar or lower relative to the prey. Thus, prey
548 is generally present in higher numbers than predators.

549 2) Unlike host-parasite interactions in which parasites have smaller generation time, predator
550 and prey organisms generally have similar generation times.

551 Thus, unlike host-parasite or host-pathogen interactions, the exploited organisms evolve faster
552 than the exploiters in predator-prey communities.

553

554 **Strains used**

555 *M. xanthus* (GJV1) and *E. coli* (MG1655) were used as predator and prey respectively. *M. xanthus*
556 strain used in this study was provided by Dr. Gregory Velicer's laboratory at ETH Zurich, Zurich,
557 Switzerland. Whereas *E. coli* (MG1655) strain was provided by Dr. Deepa Agashe's laboratory at the
558 National Centre for Biological Sciences, Bangalore, India.

559

560 **Culture condition**

561 All plates were incubated at 32 °C, all liquid cultures were incubated at 32 °C, 200 RPM. *M. xanthus*
562 strains were stored as frozen glycerol (20%) stocks in CTT [68] medium, and *E. coli* strains were stored in
563 frozen glycerol (20%) stocks in LB medium (HIMedia Laboratories Pvt. Ltd, India).

564 To initiate the evolution experiment *M. xanthus* glycerol stock was inoculated on CTT medium with
565 1.5 % agar, incubated for 3-5 days, inoculated from colony edge into 8 mL liquid CTT medium, and
566 incubated for 24 h at 200 RPM. Grown liquid culture of *M. xanthus* was dilution plated on CTT 0.5 %
567 agar plates and colonies (subclones) were isolated, stored as freezer stock and distinct subclones were
568 used as biological replicates to initiate the evolution experiment.

569 Similarly, *E. coli* strains were inoculated on LB medium with 1.5 % agar, incubated for 1-day,
570 individual colonies were inoculated into 8 mL liquid LB medium, and incubated for 24 h at 200 RPM.
571 Each colony (subclone) of *E. coli* served as an ancestor for an independent replicate line.

572 For experiments performed to analyse evolved population/clones of *M. xanthus*, glycerol stock of
573 *M. xanthus* was spotted on CTT medium with 1.5% agar plates and incubated at 32°C for 3-4 days.
574 Once the swarm appeared, cells from the outer edge (actively growing and swarming) were inoculated
575 in CTT liquid and incubated under shaking conditions at 32 °C for 20 h. For *E. coli*, cultures were grown
576 in M9 (MP Biomedicals, United States) + 0.2% glucose (ThermoFisher Scientific, India) liquid, prior to
577 setting all experiments.
578
579
580 **Evolution experiment 1:**
581 Protocol for the evolution experiment is demonstrated in Figure S1. Evolution experiment was
582 performed in 50 mL flasks containing 10 mL M9 medium + 1.5 % agar medium (M9 salts (MP
583 Biomedicals) + 0.2 % glucose + 0.01mM $CaCl_2$ (ThermoFisher Scientific, India) + 0.2mM $MgSO_4$ + 1.5%
584 bacto agar (DIFCO, BD Biosciences, India)). Glucose in the M9 medium supports the growth of *E. coli*
585 whereas *M. xanthus* cannot utilise glucose as a carbon source, and hence depend on their predatory
586 ability to feed on the growing population of *E. coli.*
587 To initiate the evolution experiment, the densities of *E. coli* and *M. xanthus* were adjusted to ~10^6
588 cells/mL and ~$2*10^{10}$ cells/mL respectively by resuspending in M9 buffer (M9 salts (MP Biomedicals)
589 + 0.01mM $CaCl_2$ + 0.2mM $MgSO_4$). 50 µL culture of prey and predator was spread across the agar bed
590 with 9-12 sterile glass beads. For the *E. coli* only control 50 µL of *E. coli* culture was spread on the agar
591 beds along with 50 µL of M9 buffer (M9 salts (MP Biomedicals, United States) + 0.01mM $CaCl_2$ + 0.2mM
592 $MgSO_4$). For predator-only control, agar beds were supplemented with 5% dead *E. coli.* Dead *E. coli*
593 were obtained by autoclaving 10 mL of 1.0 OD culture of *E. coli* for 20 minutes. All agar beds were
594 prepared 1 day prior to the transfers. After 96 h of incubation at 32°C the predator-prey communities
595 were harvested by adding 4 mL of M9 buffer followed by shaking for ~30 mins at 200 RPM to
596 resuspend the growing culture. For the vertical transfer regimen, 200 µL (5 %) of the resuspended
597 culture from each flask was transferred to a fresh flask and spread using glass beads. In horizontal
598 transfer regimen, all four replicate cultures of the respective treatment were pooled in a single 15 mL
599 conical centrifuge tube, mixed, 200 µL of these pooled cultures were redistributed in each of the four
600 replicate flasks with fresh medium and spread with the help of 9-12 glass-beads. The evolution
601 experiment was carried out for 20 such transfer cycles with populations being stored at regular
602 intervals in 20 % glycerol stocks at -80 °C.
603
604
605

606 **Isolation of clones from evolved populations of *M. xanthus* and *E. coli***

607 To isolate the evolved population of *E. coli*, a fraction of frozen glycerol stock was inoculated in 5
608 mL LB liquid medium (with 1 % NaCl instead of 0.5 %), incubated at 32 °C at 200 RPM for 7-8 h, and
609 frozen as 20 % glycerol stock. *M. xanthus* cells are sensitive to the presence of salt and hence do not
610 grow in these conditions, whereas cells of *E. coli* do.

611 To isolate *M. xanthus* population, frozen glycerol stock was inoculated in 8 mL CTT liquid with
612 Gentamicin (Sisco Research Laboratories Pvt. Ltd, India) (10 µg/mL), incubated at 32 °C at 200 RPM for
613 18-20 h, and frozen as 20 % glycerol stock. Being naturally resistant to Gentamicin, *M. xanthus* can
614 grow in the presence of Gentamicin.

615 Next, to isolate *E. coli* clones glycerol stocks of frozen *E. coli* populations were dilution plated on
616 LB + 0.5 % agar (Agar agar, ThermoFisher Scientific, India), incubated at 32 °C for 18 h. Distinct colonies
617 were isolated as clones, inoculated in liquid LB medium, incubated at 32 °C at 200 RPM for 7-8 h, and
618 stored as glycerol (20 %) stocks at -80 °C.

619 To isolate *M. xanthus* clones glycerol stocks of frozen *M. xanthus* populations were pour-platted in
620 CTT + 0.5 % agar, and incubated at 32 °C for 96 h. Distinct colonies were isolated as subclones,
621 inoculated in liquid CTT medium, incubated at 32 °C at 200 RPM for 20 h, and stored at glycerol (20 %)
622 stocks at -80 °C.

623

624 **Predation assay**

625 Predatory performance can be measured as the growth of M. *xanthus* when prey is the only source
626 of nutrient, or growth of *E. coli* in the presence of *M. xanthus* relative to its absence. In our study, we
627 used growth of *E. coli* as a measure of predation by *M. xanthus*. This is because *M. xanthus* is a sticky
628 biofilm forming bacterium. Therefore, estimates of Colony Forming Units (CFU) are generally
629 unreliable, and a single colony might emerge from a group of cells resulting in significant
630 underestimation of *M. xanthus* cell numbers.

631 All predation assays were performed on 10 mL M9 + 1.5 % agar beds (M9 salts (MP Biomedicals) +
632 0.2 % glucose + 0.01 mM $CaCl_2$ + 0.2 mM $MgSO_4$ +1.5 % Bacto agar) in 50 mL conical flasks with 9-12
633 sterile glass beads. Media was poured into the flask one day prior to the experiment. All *E. coli* cultures
634 were grown to log phase in liquid M9 media (M9 salts (MP Biomedicals) + 0.2 % glucose + 0.01 mM
635 $CaCl_2$ + 0.2 mM $MgSO_4$) prior to setting up the experiments. The *M. xanthus* cultures were grown to
636 log phase in liquid CTT media. The prey cell density was adjusted to 0.1 OD (~10^6 cells/mL) and
637 predator cell density was adjusted to $5*10^9$ cells/mL in TPM buffer (10 mM Tris pH 8.0, 8 mM $MgSO_4$,
638 1 mM K_2HPO_4 – KH_2PO_4), 50µL of these cultures were then inoculated on the M9+1.5 % agar beds and
639 spread with sterile glass beads. For monoculture of *E. coli,* 50 µL of cell density adjusted culture was

640 inoculated along with 50 µL TPM buffer and spread on the M9+ 1.5 % agar medium. The cultures were
641 incubated for 96 h at 32 °C. Following the incubation period, cells were harvested by adding 4 mL of
642 TPM buffer with 9-12 sterile glass beads followed by ~30 min shaking at 200 RPM. Viable number of
643 *E. coli* was determined by dilution plating the resuspended cell suspension in LB + 0.5 % agar.
644
645 **Competition assay**
646 **Competition between ancestors with and without Kanamycin marker**
647 *E. coli* ancestral strains with and without Kanamycin marker were grown to log phase in LB liquid
648 medium and 1:1 ratio of 0.1 OD adjusted culture of each strain was co-inoculated to initiate the
649 experiment. The competition experiment was performed on 10 mL M9 + 1.5 % agar beds (M9 salts
650 (MP Biomedicals) + 0.2 % glucose + 0.01 mM $CaCl_2$ + 0.2 mM $MgSO_4$ + 1.5 % Bacto agar) in 50 mL
651 conical flasks with 9-12 sterile glass beads. The culture was spread evenly on the agar-beds using glass
652 beads and incubated for 24 h at 32 °C. Post incubation, the cells were harvested by adding 4 mL TPM
653 buffer with ~30 min shaking at 200 RPM. Viable counts of Kanamycin sensitive and resistant *E. coli*
654 were determined by dilution plating on LB + 0.5 % agar and LB + 0.5 % agar with Kanamycin (40 µg /
655 mL) (Merck Germany).
656
657 **Competition between evolved clones and Kanamycin marked ancestors**
658 Four *E. coli* clones were isolated from each of the vertically evolved population, sixteen from each
659 from horizontally co-evolved and mono-evolved populations. Each of these clones were competed
660 against their respective ancestors, in the presence or absence of ancestral *M. xanthus.* For these
661 experiments we used Kanamycin-resistant ancestor *E. coli*, which allowed us to distinguish them from
662 the evolved clones. Competition experiments were performed on 10 mL M9 + 1.5 % agar beds (M9
663 salts (MP Biomedicals) + 0.2 % glucose + 0.01 mM $CaCl_2$ + 0.2 mM $MgSO_4$ + 1.5 % Bacto agar) in 50 mL
664 conical flasks with 9-12 sterile glass beads. All *E. coli* cultures were grown to log phase in M9 liquid
665 media, 25 µL of 0.1 OD (~10^6 cells/mL) adjusted culture of each evolved and ancestor *E. coli* were co-
666 inoculated, either with (50 µL of $5*10^9$ cells/mL) or without (50 µL TPM buffer) ancestral *M. xanthus*,
667 spread on the agar medium with sterile glass beads. These were incubated for 96 h at 32 °C and
668 harvested by adding in 4 mL TPM buffer with ~30 min shaking at 200 RPM. Viable counts of ancestor
669 and evolved *E. coli* were determined by dilution plating on LB + 0.5 % agar and LB+0.5% agar with
670 Kanamycin (40 µg/mL) (Merck Germany).
671
672
673

674 **Determination of prey-predator growth dynamics in co-cultures experiments**

675 To demonstrate the relative growth dynamics of the prey and predator when co-cultured together in
676 media containing only glucose as a carbon source, the prey numbers and predator densities were
677 tracked for 4 days. Since only *E. coli* can utilise glucose and *M. xanthus* cannot. The only available
678 source of nutrition for *M. xanthus* was the prey population. Additionally, given the differences in
679 generation times of *E. coli* and *M. xanthus*, their growth in the coculture is expected to be temporally
680 segregated. Hence, it is expected that *E. coli* will have distinct growth and death phases. Thus, to
681 determine the changes in the population size of prey bacteria during the four-day transfer cycle, prey
682 counts were obtained every 24 h from 0 to 96 h. To do so, 50 µL of 0.1 OD (~10^6 cells/mL) adjusted
683 culture of ancestor *E. coli* was co-inoculated, either with (50 µL of $5*10^9$ cells/mL) or without (50 µL
684 TPM buffer) ancestral *M. xanthus*, and spread on 10 mL of M9 + 1.5 % agar beds (M9 salts (MP
685 Biomedicals) + 0.2 % glucose + 0.01 mM $CaCl_2$ + 0.2 mM $MgSO_4$ + 1.5 % Bacto agar). The cultures were
686 spread on the agar medium with the help of sterile glass beads, incubated at 32 °C, and harvested by
687 adding in 4 mL TPM buffer with ~30 min shaking at 200 RPM. Viable counts of *E. coli* were determined
688 by dilution plating on LB + 0.5 % agar. Because of the destructive sampling approach, distinct prey-
689 predator microcosms were set up for each time point.

690 Since exopolysaccharides secreted by *M. xanthus* result in the formation of biofilms that are difficult
691 to disrupt, it reduces the reliability of the obtained *M. xanthus* viable counts, using similar methods
692 as for *E. coli*. Therefore, we used the swarm sizes as a proxy to determine the relative densities of *M.*
693 *xanthus* in the cocultures. Swarming of *M. xanthus* on hard agar surfaces is known to be a positive
694 density-dependent trait. Hence, it is expected that as the relative numbers increase, the swarm sizes
695 will also increase. To estimate the swarm sizes, post harvesting of the cocultures in TPM buffer (as
696 described above), 10 µL of the culture was spotted on CTT hard agar (1.5 Bacto agar) surface in 90 mm
697 plates. The initial swarm was marked after 24 h and the final swarms were marked after 120 h. The
698 average distance between the initial and final swarms was recorded and presented as the swarm sizes.
699

700 **Mathematical model of evolution in predator-prey communities**

701 To explain our surprising experimental observation that variants with superior competitive fitness that
702 did not show any increase in their ability to resist predation evolved only in the vertical treatment and
703 not in the horizontal treatment, we developed a mathematical model of population dynamics. Our
704 model followed the dynamics of prey subpopulations explicitly and the predator populations
705 implicitly. Specifically, we assumed each growth cycle (which lasted time T) had two distinct periods:
706 the first where prey subpopulations grew (lasting time T_1) and the second where they died due to
707 predation (lasting time T_2). In each period, each subpopulation (labeled α) had an exponential growth

708 rate g_α and death rate d_α respectively, both drawn randomly and independently from a normal
709 distribution with means 0.7 hr^{-1} and 0.2 hr^{-1} respectively, and standard deviations 0.3 hr^{-1} and 0.2 hr^{-1}
710 respectively (disallowing negative rates). With these the growth of each subpopulation was described
711 by the following equation:

712 $$N\alpha(T) = N_\alpha(0) \exp(g_\alpha T_1 - d_\alpha T_2)$$

713 Inspired by evolutionary ecology, our model implemented evolution by introducing distinct
714 "mutants" as prey subpopulations at the beginning of population dynamics and following their fate
715 over time. We modeled 4 distinct communities, each seeded with 5 subpopulations of prey (chosen
716 arbitrarily). At the beginning of each simulation, each subpopulation started with equal abundance
717 0.1 (arbitrary units) and grew according to the dynamics described above. After each growth cycle
718 (time *T*), all subpopulations in each population were diluted by a factor *D = 20* (5% bottleneck) and
719 grown again in a new cycle. In the vertical regimen, different communities were not mixed, while in
720 the horizontal one, they were mixed and redistributed equally, mimicking our experiment. We
721 continued these dynamics across multiple growth-dilution cycles till communities reached a steady
722 state, after which we compared the mean ratio of the relative growth rates (quantifying prey
723 competition) and death rates (quantifying predator avoidance) of each surviving subpopulation. We
724 repeated and averaged our results over such 100 simulations with randomly chosen parameters and
725 plotted the results (Fig. 4D).
726
727 **Time-course assay**
728 Predation assays (were performed with populations of coevolved prey and predator from both the
729 transfer regimes (vertical and horizontal), respectively isolated from different evolutionary time. The
730 populations of both prey and predator were isolated from the glycerol stocks stored at different
731 transfers, namely, after transfer 10 (T10), transfer 16 (T16) and transfer 20 (T20). The prey, and the
732 predator populations were isolated using a similar protocol as described earlier (See isolation of
733 population and clones). To determine the change in prey survival over evolutionary time against the
734 terminally coevolved predator, *E. coli* populations from different evolutionary times (T10, T16 and
735 T20) were inoculated with the terminally evolved *M. xanthus* (after 20 transfers). The predation assay
736 for each combination of prey and predator population was set up as discussed earlier (See predation
737 assay). The change in prey survival over time was recorded for prey population by dilution plating on
738 LB + 0.5 % agar. Similarly, the *M. xanthus* populations from all time points (T10, T16 and T20) were
739 pitched against the terminally co-evolved *E. coli* population, to determine the changes in the
740 predatory ability over evolutionary time. The survival of the terminally coevolved prey against *M.*
741 *xanthus* from different evolutionary time-point was used as a measure of change in the resistance to

742 predation over evolutionary time. Here, a lower prey survival implies better predatory ability, and a
743 higher prey survival indicates worse predatory ability.
744
745 **Flipped-regime evolution experiment (Evolution experiment 2)**
746 Experimental protocol for the second evolution experiment is demonstrated in Supplementary
747 figure S7. Evolution experiment was performed in 50 mL flasks containing 10 mL M9 + 1.5 % agar
748 medium (M9 salts (MP Biomedicals) + 0.2 % glucose+ 0.01mM CaCl2 + 0.2mM MgSO4 + 1.5 % bacto
749 agar). To initiate the second evolution experiment, glycerol stocks of the T20 population from
750 evolution experiment 1 were thawed on ice and 5% of the culture was washed with M9 buffer two
751 times and then resuspended in 200 µL M9 buffer. These cultures were then spread on fresh M9 + 1.5
752 % agar beds with the help of 9-12 sterile glass beads. Similar treatment was done for all populations
753 from both mixed communities of prey and predator and monoculture prey (control) treatments. The
754 mixed communities of prey and predator (coevolved) /prey only (mono-evolved) population were
755 incubated in fresh media at 32 ^{O}C for 96 h. This initial 96 h incubation was done to allow the
756 stabilization of the communities after revival from freezer stocks. Following this both the mixed
757 communities of prey-predator and prey only treatments were harvested in 4 mL of M9 buffer and
758 shaking at ~200 RPM for 30 mins to resuspend the growing cultures. 5 % of the resuspended culture
759 was transferred to fresh M9 medium + 1.5 % agar medium under two different transfer regimens:
760 Vertical transfer regimen and Horizontal transfer regimen (similar to the first experiment). However,
761 in this experiment the transfer regimens of the evolved population from evolution experiment-1 were
762 interchanged. The vertically coevolved/mono-evolved population were propagated under the
763 horizontal transfer regimen, i.e., the population from replicate lines were pooled in a 15 mL centrifuge
764 tube and then 5% of the resuspended culture was transferred to each of the 4 flasks with fresh media,
765 at every transfer (ensuring a periodic mixing of population). Whereas the horizontally
766 coevolved/mono-evolved population were propagated under the vertical transfer regimen, where
767 suspended cultures from the 4 replicate lines were serially transferred to respective fresh M9 + 1.5 %
768 agar medium. All the cultures were spread on the agar beds using the glass beads. The experiment
769 was carried out for 20 such cycles with population being stored at regular intervals in the form of 20
770 % glycerol stocks in -80 °C. Evolved population of prey and predator was isolated after 16 transfer
771 cycles using similar protocols as used for the isolation of population in the previous experiments.
772
773 **Genomic analysis**
774 For the isolation of genomic DNA, *E. coli* and *M. xanthus* clones were grown in M9 + 0.2 % glucose
775 and CTT liquid medium respectively at 32 ^{o}C, 200 RPM till they achieved optical density between 0.2

776 to 0.8 at 600 nm. These cultures were used for the extraction of genomic DNA using the QIAGEN
777 genomic DNA isolation kit using 20/G column. Manufacturer's instructions were followed for the
778 isolation of genomic DNA. Next steps for sequencing were performed at Macrogen Inc (Korea). Before
779 TrueSeq library preparation the quality control of the samples was done using Agilent TapeStation[R]
780 DNA Screen Tape (Agilent). The DNA samples were sequenced on HiSeq platform, for 150 bp paired-
781 end sequencing. Ambiguous low- quality sequences were trimmed using Trimmomatic v0.39. The
782 Genomic libraries were analysed using breseq[69] against reference genome of *E. coli* MG1655 (GenBank
783 Accession No. GCA_000005845.2) or *M. xanthus* DK1622 (GenBank Accession No.GCA_000012685.1)
784 from NCBI genomic data bank. The breseq pipeline was used with all default parameters.
785
786 **Statistical analysis**
787 All statistical analysis were performed in R (version 4.0.4). Levene's test was performed with each
788 set of data to check for the homogeneity of variance. For a $p > 0.05$ for Levene's test, either an
789 independent sample t-test or one sample t-test was performed wherever applicable, and the
790 Benjamini-Hochberg method was used for correcting for multiple testing. Significant ANOVA in all
791 analysis was followed by an SNK *post hoc* test. However, for a $p < 0.05$ for Levene's test nonparametric
792 tests were used for data analysis. The significant Kruskal-Wallis test was followed by Dunn's *post hoc*
793 test. The statistical test for the respective data set is mentioned in the figure legends.
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