

1 **Title:**

2 When increasing population density can promote the evolution of metabolic cooperation

3 **Running title:** High population density can promote cooperation

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16 **Conflict of interest:** The authors declare no conflict of interest

17 **Funding:** Richard J. Lindsay and Ivana Gudelj: European Research Council no. 647292

18 MathModExp. Bogna J. Pawlowska: Engineering and Physical Sciences Research Council Doctoral
19 training grant studentship.

20 **Subject category:** Microbial population and community ecology

21 **Abstract**

22 Microbial cooperation drives ecological and epidemiological processes and is affected by the ecology
23 and demography of populations. Population density influences the selection for cooperation, with
24 spatial structure and the type of social dilemma, namely public-goods production or self-restraint,
25 shaping the outcome. While existing theories predict that in spatially structured environments
26 increasing population density can select either for or against cooperation, experimental studies with
27 both public-goods production and self-restraint systems have only ever shown that increasing
28 population density favours cheats. We suggest that the disparity between theory and empirical studies
29 results from experimental procedures not capturing environmental conditions predicted by existing
30 theories to influence the outcome. Our study resolves this issue and provides the first experimental
31 evidence that high population density can favour cooperation in spatially structured environments for
32 both self-restraint and public-goods production systems. Moreover, using a multi-trait mathematical
33 model supported by laboratory experiments we extend this result to systems where the self-restraint
34 and public-goods social dilemmas interact. We thus provide a systematic understanding of how the
35 strength of interaction between the two social dilemmas and the degree of spatial structure within an
36 environment affect selection for cooperation. These findings help to close the current gap between
37 theory and experiments.

38 **Introduction**

39 Microorganisms engage in an impressive array of cooperative behaviours (Crespi 2001) that drive
40 ecosystem and epidemiological processes including nutrient recycling (Kaiser et al 2015), antibiotic
41 resistance (Lee et al 2010) and disease virulence (Crespi et al 2014, Griffin et al 2004, Lindsay et al
42 2016). These social interactions are shaped by the ecology and demography of populations with
43 population density known to affect selection for cooperation (Chen et al 2014, Datta et al 2013,
44 Dobay et al 2014, Greig and Travisano 2004, MacLean and Gudelj 2006, Ross-Gillespie et al 2009).

45 Whether high population density favours cooperation can depend on the spatial structure of the
46 environment (Dobay et al 2014, MacLean and Gudelj 2006) and the type of social dilemma faced

47 (Dobay et al 2014, Greig and Travisano 2004, MacLean and Gudelj 2006, Ross-Gillespie et al 2009).
48 In particular, two types of social dilemmas have been considered: public-good production and self-
49 restraint. Public-goods are extracellular factors used to perform a range of functions including nutrient
50 acquisition, biofilm formation and quorum sensing (West et al 2007). They are costly to produce and
51 benefit individuals in the locality. Therefore, public-goods are prone to exploitation by cheats who do
52 not contribute to their production, but can still reap the rewards. Self-restraint cooperation arises from
53 a metabolic trade-off between growth rate and efficiency, whereby fast growth is consequently less
54 efficient than slow growth (Pfeiffer et al 2001). Efficient use of common resources conforms to the
55 classical definition of a cooperative trait; it is beneficial to the group because more biomass is
56 produced per unit of resource, but costly to individuals because they reproduce at a slower rate. This
57 is prone to exploitation by cheats that use resources quickly but inefficiently for their own rapid
58 multiplication, at the expense of the total population yield (MacLean and Gudelj 2006).

59 The relationship between population density and cooperation is not yet fully understood with theory
60 and empirical studies in disagreement. Theory suggests that high population density could favour
61 either cooperators or cheats. For self-restraint cooperation it is argued that selection depends on the
62 biological details of the system because density alters numerous factors including the resource
63 availability per individual, the variation in resource concentrations as they are consumed, and how
64 beneficial cooperation is (Ross-Gillespie et al 2009). For public-goods systems, it is postulated that
65 high population density could favour cheats because in dense populations cheats are 'physically
66 closer' to cooperators and can thus exploit them more effectively (Ross-Gillespie et al 2009).
67 However it is also suggested that high cell density could favour cooperators if cells diffuse slower
68 than the public-goods they produce (Dobay et al 2014), or the environment is sufficiently spatially
69 structured (Lion and Gandon 2009, Lion 2010).

70 Until now empirical studies have only been able to demonstrate that in structured environments high
71 population density favours cheats both in self-restraint (MacLean and Gudelj 2006) and public-good
72 (Celiker and Gore 2012, Chen et al 2014, Datta et al 2013, Greig and Travisano 2004, Kümmerli et al
73 2009, Ross-Gillespie et al 2009) cooperative systems. Moreover, contrary to the predictions in (Dobay

74 et al 2014) experiments with motile (Ross-Gillespie et al 2009) and non-motile (Greig and Travisano
75 2004) cell populations producing public-goods both report the same outcome that high density favours
76 cheats, even when cells diffuse slower than the public-goods.

77 Could the discrepancies between theoretical and empirical studies result from experimental
78 procedures not capturing the extent of environmental variation that is predicted to affect the outcome
79 (Dobay et al 2014, Ross-Gillespie et al 2009)? To address this, we conducted a series of microbial
80 laboratory experiments using a model cooperative system with *Saccharomyces cerevisiae* which
81 exhibits both public-good (Gore et al 2009) and self-restraint cooperation (MacLean and Gudelj
82 2006). We manipulated factors suggested by theoretical models to underpin the relationship between
83 population density and cooperation, these are: the relative cost and benefits of cooperation (Dobay et
84 al 2014, Ross-Gillespie et al 2009), the physical proximity of cells to each other (Ross-Gillespie et al
85 2009) and the diffusion of public-goods and cells (Dobay et al 2014). For the first time we provided
86 experimental evidence supporting theoretical predictions that high population densities could favour
87 cooperation in spatially structured environments in both self-restraint (Ross-Gillespie et al 2009) and
88 public-goods cooperative systems (Dobay et al 2014).

89 As cooperative traits frequently interact (Brown and Taylor 2009, Harrison and Buckling 2009,
90 Lindsay et al 2016, MacLean et al 2010, Ross-Gillespie et al 2015) we next asked whether high
91 population density can favour cooperation in systems where the self-restraint and public-goods social
92 dilemmas interact. To this end we developed a multi-trait mathematical model, manipulated the
93 strength of interactions between the two social traits and showed that high population density can still
94 favour cooperation. Moreover, our model predicted that decreasing the influence of self-restraint over
95 public-goods production will increase the range of environmental structures where high population
96 density favours cooperation. We provided a mechanistic explanation for this outcome, suggesting that
97 the cost of inefficient metabolism arising in the presence of the self-restraint dilemma outweighs the
98 benefit of public-good cooperation in environments with low spatial structure where public-goods are
99 more accessible to cheats. However, reducing the strength of the self-restraint dilemma will reverse
100 the cost/benefit relationship in favour of cooperation. This was verified experimentally.

101 Our work provides the first empirical evidence that high population density can favour cooperation in
102 spatially structured environments in both single and multi-trait cooperative systems and we provide an
103 explanation as to why this result has so far been elusive.

104 **Materials and methods**

105 **The experimental system**

106 To secure nutrients microbes can cooperatively secrete enzymes, termed public goods, that break
107 down complex sugars into simple sugars that are easier to digest (Schweizer and Dickinson 2004,
108 Talbot 2010). Public-good cooperation in *S.cerevisiae* arises from the production of invertase to
109 externally hydrolyse sucrose into glucose and fructose, the preferential carbon sources (Schweizer and
110 Dickinson 2004).

111 Once simple sugars are available in the environment, microbes are constrained by a metabolic trade-
112 off between growth rate and efficiency (Pfeiffer et al 2001), which is at the core of the self-restraint
113 social dilemma. In *S.cerevisiae*, the rate at which resources are taken up alters whether they are
114 catabolised by rapid, low yielding fermentation or slower, more efficient respiration (Otterstedt et al
115 2004, Postma et al 1989).

116 There is an inevitable interaction between public-good and self-restraint cooperation during invertase-
117 mediated metabolism of sucrose by *S. cerevisiae* (MacLean et al 2010). This occurs because when
118 invertase secreting cells externally hydrolyse sucrose, they form local spikes in monosaccharide
119 concentrations. Cells exposed to high resources concentrations metabolise them relatively less
120 efficiently than when exposed to lower concentrations (Postma et al 1989, Weusthuis et al 1994), such
121 as in the regions of cells that do not produce invertase. This forms the basis of self-restraint
122 cooperation. Therefore, varying resource supply can control the strength of interaction between self-
123 restraint and public-goods dilemmas. When resources are scarce, the rate-efficiency trade-off is weak
124 (Weusthuis et al 1994, MacLean et al 2010) and public-good production is expected to be the

125 dominant constraint on growth. However, when resources are abundant, inefficient metabolism from
126 high uptake rates will constrain growth (MacLean et al 2010).

127 **Mathematical model**

128 A mathematical model was developed to examine the relationship between population density and the
129 interaction between self-restraint and public goods cooperation. The model was based on the
130 established mathematical framework developed previously (MacLean et al 2010) with spatial
131 interactions represented by a system of reaction-diffusion equations (For more details see
132 Supplementary Text).

133 **Experimentally manipulating spatial structure of the environment**

134 We established the lowest level of structure in shaken liquid cultures (Figure 1a). Note that while for
135 self-restraint systems shaken liquid cultures represent spatially unstructured environments (MacLean
136 and Gudelj 2006), for public-goods systems spatial structure is not completely absent. This is because
137 invertase producers get preferential access to the public-good (Gore et al 2009) and form small
138 clumps when dividing (Koschwanez et al 2011). An intermediate level of structure was established
139 with initially mixed subpopulations on agar plates, and a high level was established with segregated
140 subpopulations on agar plates (Figure 1a).

141 **Strains**

142 Strains of *S. cerevisiae* were those used previously to test public-good cooperation (Celiker and Gore
143 2012, Gore et al 2009) (from J. Gore, MIT). The invertase producing strain (cooperator, BY4741,
144 *SUC2*) constitutively expresses yEYFP by the *TEF1* promoter. The non-invertase producing strain
145 (JG210C, cheat, $\Delta suc2::kanMX4$) expresses tdTomato by the *PGK1* promoter.

146 Strains to test self-restraint cooperation were those used previously (MacLean and Gudelj 2006) (from
147 P. Dahl, University of Gothenburg). CEN.PK2-1C has wild-type hexose transport capabilities and is a

148 self-restraint cheat. Whereas TM6*, a mutant that has a single synthetic hexose transporter, is a self-
149 restraint cooperator. The strains are distinguishable by fluorescence with CEN.PK2-1C constitutively
150 expressing GFP and TM6* expressing mCherry, both by the *TEF1* promoter, inserted into the *URA3*
151 locus. TM6* was also used as a public-good cooperator with reduced hexose uptake ability (Figure
152 3d), but with a GFP marker.

153 To test the influence of interacting cooperative traits, in addition to competing BY4741 and JG210C
154 (Figure 4b-c), a non-invertase producing mutant of TM6* (Δ *suc2::kanMX4*) (Supplementary Figure
155 S2) was generated in the mCherry-expressing background to compete with TM6* (GFP) (Figure 4d-
156 f).

157 Details of strains are summarised in Table 1. Strains used in each competition are also indicated on
158 Figures.

159 **Competition experiments**

160 To experimentally test the influence of population density on the selection of cooperation in different
161 environmental conditions, competition experiments were conducted in each environment with three
162 different initial population densities. The nature of the relationship between cooperator fitness and
163 density was assessed for each environment. The initial frequency of cooperators (f , given in figure
164 legends) was equivalent for different spatial structures and resources concentrations tested, except
165 when specifically testing the effect of changes in frequency on the relationship between density and
166 cooperation (Supplementary Figure S5).

167 Competition experiments were conducted in defined media (DM: 6.9 g/l Yeast Nitrogen Base w/o
168 amino acids, 790 mg/l complete supplement mixture (Formedium, UK) with varying specified
169 concentrations of glucose (for self-restraint cooperation) or sucrose (for public-good and interacting
170 cooperative traits) and 16 g/l agar where applicable.

171 Strains were initially grown in 5 ml YPD (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose) shaken
172 overnight at 30°C. Cells were washed twice and resuspended in 5 ml DM lacking sugar. Spatially
173 structured competitions (intermediate and high) were performed on 9 cm diameter Petri dishes
174 containing 25 ml DM. Cells were inoculated onto agar plates in patches of 20 µl in a 4x5 array (Figure
175 1a) as described previously (MacLean et al 2010). See Supplementary Figure S1 for details. Plates
176 were sealed with parafilm to minimise evaporation and incubated at 30°C for 7 d to allow resources to
177 diffuse and be consumed. Cells were collected by flooding the plates with 5 ml PBS and colonies
178 were gently scraped from the agar into suspension and appropriately diluted for flow cytometry to
179 distinguish strain densities as detailed below. Each plate was considered a single replicate.

180 Low structure competitions were performed in DM, omitting agar, within a 48-well suspension
181 culture microplate (Bio-One Greiner) (640 µl per well). Cultures were incubated at 30 °C in a
182 FLUOstar Omega microplate reader (BMG Labtech) with shaking at 700 r.p.m. for periods to allow
183 population growth to reach approximately stationary phase based on OD_{620nm} readings. This was for
184 48 h for all competitions, except for those with low density and resources (96 h) and using TM6* (72
185 h) where growth rate was low owing to the Allee effect (Dai et al 2012) and metabolic constraints
186 (Otterstedt et al 2004).

187 Rate-efficiency trade-off tests were performed the same way for 72 h, with an initial density of 2×10^5
188 cells.well⁻¹. Population density was calibrated to OD for this system according to (Dai et al 2012). For
189 our spectrophotometer, $OD_b = 0.064$, $OD_{max} = 3.3$. For liquid cultures, each well was considered a
190 replicate. Flow cytometry was used to establish the initial and final densities of the strains. This was
191 performed with a Guava easyCyte HT System using Guava InCyte software (Merck Millipore).
192 Populations were diluted in PBS (pH 7.4) to a density of $10^4 - 5 \times 10^5$ cell.ml⁻¹. Density was
193 established by measuring events gated on FSC and SSC. Cooperator and cheat cells were gated based
194 on fluorescence (Supplementary Figure S3). Relative fitness was calculated based on the ratio of
195 Malthusian growth parameters (Lenski et al 1991), as done previously when assessing the density-
196 dependent nature of cooperator fitness (Greig and Travisano, 2004), with relative fitness = 1 denoting
197 equal fitness (see Supplementary text for details).

198 **Data analysis**

199 Statistical tests were performed using R version 3.1.1. We assess the nature and strength of the
200 relationship between density (x) and cooperator fitness (y) using linear models.. Linear models were
201 calculated and plotted using the “MASS” package (version 7.3-35) and plotted (black line) \pm s.e.
202 (shaded regions). Monotonic models were used to capture changes in selection across the range of
203 initial densities tested. Monotonic models were fitted in the form $y = x$ or $y = e^x$ with the better
204 model fit shown based on AIC values with coefficient estimates (β) reported. Though not optimal fits,
205 these were used for simplicity to readily distinguish switches in the selection for cooperation. The
206 initial density was \log_{10} transformed for the assumptions of the parametric statistical test.
207 Comparisons of the strength of association between density and fitness between levels of structuring
208 and resource concentrations (Figure 1) were performed with a GLM with density and structure or
209 resource concentration as explanatory variables with an interaction term between the two. Non-
210 monotonic relationships were examined with quadratic terms, and assessed for best fit based on AIC
211 values, with linear models using density as factor to assess relative fitness differences.

212 **Results**

213 **Self-restraint cooperation**

214 Previous experimental studies found that density-dependent selection for self-restraint cooperation is
215 positive in spatially unstructured yet negative in structured environments (MacLean and Gudelj 2006).
216 While our experiments agree for shaken liquid environments (Figure 1b), in structured environments
217 we found the opposite (Figure 1c-d), namely that high population density favours cooperation. Why is
218 our result different to that in (MacLean and Gudelj 2006)? The difference comes from the way spatial
219 structure is represented experimentally. In (MacLean and Gudelj 2006), unshaken liquid cultures were
220 used to represent a spatially structured environment while our experiments were conducted on agar
221 plates (Figure 1a).

222 We found that the extent to which increasing density selects for cooperation reduces as spatial
223 structuring increases (Figure 1c-d). This result arises, as suggested (Ross-Gillespie et al 2009),
224 because spatial structuring alters the relative magnitude of benefits gained through cooperation. In our
225 system, resources become heterogeneously distributed when the population is spatially structured
226 because self-restraint cooperators and cheats have disparate resource consumption rates (Otterstedt et
227 al 2004). These resource gradients alter the pay-offs of self-restraint cooperation through the rate-
228 efficiency trade-off and/or the antagonistic metabolic by-products generated by self-restraint cheats
229 (MacLean and Gudelj 2006, Weusthuis et al 1994). To test this, we repeated competitions when
230 spatial structuring was high, but we altered the cost-to-benefit ratio of self-restraint cooperation by
231 reducing the resource concentration (Supplementary Figure S4). When glucose concentration
232 lowered, from 111.01 to 13.88 mM, positive density-dependence of self-restraint cooperator fitness
233 remained, however the strength of this relationship was lessened (Figure 1e). When reduced further
234 (to 2.78 mM), self-restraint cooperator fitness was negatively density-dependent (Figure 1f).

235 Therefore, as suggested by theory (Ross-Gillespie et al 2009), we found that the relationship between
236 self-restraint cooperator fitness and population density depended on biological details of the system,
237 such as the relative pay-offs of self-restraint cooperation. This can be altered through spatial
238 structuring, which alters the available resource concentrations and hence the incentives to cheat. To
239 demonstrate this, we conducted competitions with low and intermediate levels of spatial structure and
240 low resources (2.78 mM). Unlike with high resource levels (111 mM), we found regions of both
241 positive and negative density-dependence of self-restraint cooperator fitness (Figure 1g-h). This
242 illustrates how the biological parameters dictate the way in which density influences the selection for
243 self-restraint cooperation (Ross-Gillespie et al 2009) and how resource concentration and spatial
244 structure interact to dictate cooperator success.

245 **Public-good cooperation**

246 In agreement with previous experimental studies with this *S.cerevisiae* system, we found that for
247 intermediate resource concentrations (29.2 mM sucrose) public-good cooperator fitness was

248 negatively density-dependent when the level of spatial structuring was low (Figure 2a) (Chen et al
249 2014, Datta et al 2013) and intermediate (Figure 2b) (Greig and Travisano 2004), a relationship that is
250 predicted by our mathematical model (Figure 2c, Supplementary Information). These findings also
251 agree with bacterial public-goods systems (Kümmerli et al 2009, Ross-Gillespie et al 2009).

252 However, theory suggests that the relationship between density and public-good cooperator fitness
253 depends on the degree of diffusion of public-goods and cells (Dobay et al 2014). To examine this, we
254 introduced a higher degree of spatial structuring (Figure 1a, high structure), and hence altered the
255 distances between strains over which public-goods diffuse. In that case, we found public-good
256 cooperator fitness was positively density-dependent (Figure 2d), in agreement with previous theory
257 (Dobay et al 2014) and predictions made by our mathematical model (Figure 2e).

258 Why can high population densities favour public-good cooperation? We reason that the higher the
259 density of public-good cooperators, the quicker they will consume the resources made available from
260 public-good production. In our highly-structured environment the available hexose is sufficiently
261 scarce and heterogeneously distributed. Therefore, at higher densities cooperators will consume a
262 larger proportion of the resources before they diffuse away to become accessible to cheats, as
263 suggested previously (Dobay et al 2014, Koschwanez et al 2011). Our model illustrated this effect
264 where in a highly-structured environment with sufficiently low resources, the overall amount of
265 hexose captured by public-good cheats was higher at low density (Figure 3a) than at high density
266 (Figure 3b).

267 To experimentally test this prediction we repeated competitions between public-good cooperators and
268 cheats in the same highly spatially structured environment. However, this time we replaced the
269 public-good cooperator (Wt *S. cerevisiae* possesses at least 20 hexose transporter genes (Wieczorke et
270 al 1999)) with a public-good cooperator, TM6*, which has just a single hexose transporter and
271 therefore its maximal hexose uptake rate is only about 10 % of the Wt (Otterstedt et al 2004). This
272 meant that TM6* was less able to take advantage of the high-density conditions to capture the
273 liberated hexose from sucrose before it diffuses away. Competitions with the TM6* public-good

274 cooperator and Wt public-good cheat (JG210C) were performed on 1.46 mM sucrose to reduce the
275 influence of the rate-efficiency trade-off (Otterstedt et al 2004) because at sufficiently low sucrose
276 concentrations the rate-efficiency trade-off is diminished (Weusthuis et al 1994) (Supplementary
277 Figure S6a-b). Unlike with the wild-type hexose transporter strain (Figure 3c), the fitness of the TM6*
278 public-good cooperator was negatively density-dependent (Figure 3d). This outcome was also
279 captured by our model when we reduced the maximal hexose uptake rate of the public-good
280 cooperator to 10 % of the wildtype (Figure 3e-f). This switch from positive to negative density-
281 dependence experimentally verifies that in structured environments as population density increases,
282 public-goods cooperators have an increased capacity to capture hexose liberated from sucrose.

283 **Interacting effects between density, spatial structure, and the rate-efficiency trade-off** 284 **on the evolution of public-good cooperation**

285 How is the relationship between population density and public-good cooperation influenced by a
286 second social trait, namely self-restraint, given that these traits can interact (MacLean et al 2010)? To
287 explore this we conducted numerical simulations of our multi-trait model (Supplementary
288 Information) in environments with varying degree of spatial structure and resource concentration
289 (Figure 4a). The latter alters the strength of self-restraint dilemma (MacLean et al 2010) whereby low
290 resource environments reduce the strength of the rate-efficiency trade-off, which underpins self-
291 restraint cooperation.

292 Our model predicted that whether density favours public-good cooperation depends both on the
293 spatial structure and resource concentration of that environment (Figure 4a). We predicted that
294 reducing the resource concentration could increase the range of spatial structures where increasing
295 population density favours public-good cooperation (Figure 4a columns C-G versus H-K). At higher
296 resource concentrations, public-good cooperators incur a cost of inefficient metabolism because of
297 local spikes in hexose concentration that are formed as they hydrolyse sucrose (MacLean et al 2010).
298 This cost outweighs the relative benefit of public-good cooperation when spatial structure is lower
299 (Figure 4a, H1:K6), as in that scenario public-goods are more accessible to cheats. However, reducing

300 the strength of the self-restraint dilemma by reducing resources, will diminish the cost of inefficient
301 metabolism incurred by public-good cooperators because the hexose concentration spikes formed will
302 be less pronounced (MacLean et al 2010). Therefore, in these environments increasing density will
303 favour public-good cooperators even when spatial structure is low (Figure 4a, C1:G6) because of an
304 enhanced ability to capture resources before they diffuse away (Figure 3a-b).

305 We tested this prediction by competing public-good cooperators and cheats with wild-type hexose
306 uptake in environments with intermediate and low spatial structuring, where increasing density had
307 earlier been found to favour public-good cheats (Figure 2a-b), however this time with reduced
308 resources. As predicted by our model (Figure 4a columns C-G), reducing resources resulted in the
309 fitness of public-good cooperators becoming positively density-dependent in both intermediate and
310 low levels of spatial structure (Figure 4b-c).

311 To verify that this direction of selection switch is caused by interactions between public-goods and
312 self-restraint, instead of reducing the resource concentration we performed competitions between
313 purely respiring, and hence metabolically efficient (Supplementary Figure S6a-b), invertase producers
314 (TM6*) and non-producers (TM6* Δ suc2). Again in sufficiently structured environments, public-
315 good cooperator fitness was positively density-dependent (Figure 4d). However unlike with the wild-
316 type respiro-fermenting strains (Figure 2b), with intermediate levels of structure public-good
317 cooperator fitness was positively density-dependent (Figure 4d), as predicted by our model
318 (Supplementary Figure S7). With low structure, a non-monotonic relationship between density and
319 public-good cooperator fitness was found (Figure 4f). This result was predicted by our model when
320 the rate-efficiency trade-off is absent at low resource concentrations where no general statement could
321 be made regarding density and selection for cooperation (Figure 4a).

322

323 **Discussion**

324 In this article we provided the first experimental evidence that high population density can favour
325 microbial cooperation in spatially structured environments in both public good production and self-
326 restraint cooperative systems as well as when the two social dilemmas interact.

327 Past theories have predicted that in spatially structured environments increasing population density
328 can either select for or against self-restraint cooperation depending on the details of the system, such
329 as how beneficial cooperation is (Ross-Gillespie et al 2009). Yet empirical studies show that high
330 population density favours self-restraint cheats (MacLean and Gudelj 2006). Similarly, for public-
331 goods systems theory predicts that high population density favours cheats when they are ‘physically
332 closer’ to cooperators (Ross-Gillespie et al 2009) and favours cooperators if microbial cells diffuse
333 slower than the public-goods (Dobay et al 2014) or the environment is sufficiently spatially structured
334 (Lion and Gandon 2009, Lion 2010). However, empirical studies with public-good cooperative
335 systems consistently show that high population density favours cheats (Chen et al 2014, Datta et al
336 2013, Greig and Travisano 2004, Kümmerli et al 2009, Ross-Gillespie et al 2009, Sanchez and Gore
337 2013).

338 We postulated that the discrepancy between theory and experiments could be due to experimental
339 procedures not capturing the extent of environmental variation predicted to affect the outcome (Dobay
340 et al 2014, Ross-Gillespie et al 2009). Indeed, our theoretical model also showed that whether high
341 population density promotes cooperation depends intricately on the degree of spatial structuring as
342 well as resource concentration in the environment (Figure 4a). However, while some empirical studies
343 systematically vary the degree of spatial structure (Boots and Meador 2007, Kümmerli et al 2009),
344 more frequently spatial structure is manipulated through an initial distribution of organisms within an
345 environment. In such cases many ‘spatially heterogeneous’ initial distributions all fall into the same
346 category of ‘structured’ environments. For instance, microbial communities can be fragmented into
347 subpopulations that are linked only through migration. Such metapopulation structure can be imposed
348 experimentally by embedding populations into microtitre plates and the initial spatial distribution and

349 migration is controlled (Dai et al 2013, Datta et al 2013). Another way of manipulating spatial
350 structure is to inoculate agar media with numerous droplets containing microbes, forming patches of
351 interacting subpopulations (Lindsay et al 2016, MacLean et al 2010, Ross-Gillespie et al 2009). In this
352 scenario, the initial spatial distribution is controlled, but the subsequent interactions between
353 subpopulations are not. Finally, homogeneous cultures can be inoculated: (a) onto agar media (Greig
354 and Travisano 2004, Griffin et al 2004, Le Gac and Doebeli 2010, Ross-Gillespie et al 2009, van
355 Gestel et al 2014); (b) into unshaken liquid cultures (Koschwanez et al 2011, MacLean and Gudelj
356 2006, Rainey and Travisano 1998) or (c) into shaken liquid cultures (Celiker and Gore 2012, Chen et
357 al 2014), for certain public-goods systems where producers get preferential access to the public-good
358 (Gore et al 2009). In all three cases (a-c) spatial structure self-emerges and both the initial spatial cell
359 distribution and the subsequent interactions between the emergent subpopulations are not controlled.
360 However, high population densities can impede the self-aggregation of cells (van Gestel et al 2014).

361 We conducted laboratory experiments using a model cooperative system with *S. cerevisiae* which
362 exhibits both public-good (Gore et al 2009) and self-restraint cooperation (MacLean and Gudelj
363 2006). We considered three levels of spatial structure and demonstrated that the relationship between
364 population density and cooperation depends on the degree of spatial structure as well resource
365 concentration within an environment (Figures 1,2,4). Therefore, we argue that previous experimental
366 studies with self-restraint (MacLean and Gudelj 2006) and public-goods (Greig and Travisano 2004,
367 Ross-Gillespie et al 2009) cooperative systems found that high population density favours cheats
368 because they were conducted in environments with either relatively low spatial structure or high
369 resource concentration.

370 Our data supports the mechanistic explanations put forward by theoretical models as to why high
371 population densities can favour cooperation. For self-restraint cooperation, the magnitude of benefits
372 gained through cooperation influences the extent to which increasing density selects for cooperation
373 (Ross-Gillespie et al 2009). In our system, resources become heterogeneously distributed when the
374 population is spatially structured because self-restraint cooperators and cheats have disparate resource
375 consumption rates (Otterstedt et al 2004). These resource gradients mean that the pay-offs of self-

376 restraint cooperation are altered through the rate-efficiency trade-off and/or the antagonistic metabolic
377 by-products generated by self-restraint cheats (MacLean and Gudelj 2006, Weusthuis et al 1994).

378 Similarly, we demonstrated that with increasing population density public-good cooperation can
379 either be selected for or against depending on whether the environment is sufficiently structured and
380 how this structure influences the amount of public-good benefit gained. Intuitively, increasing
381 population density increases the incentives to cheat because it increases the proximity to, and absolute
382 number of, public-good cooperators that can be exploited (Ross-Gillespie et al 2009). On the other
383 hand, increasing density also increases the amount of the public-goods that are generated, which can
384 be captured by cooperators before they diffuse to become available to cheats (Dobay et al 2014). The
385 scale of population structuring will alter the relative extent of these opposing forces, which in turn
386 will shape whether density selects for or against public-good cooperation. We experimentally verified
387 this mechanism by limiting the ability of public-good producers to obtain the generated benefits of
388 public-good cooperation. This predictably switched the direction of selection from increasing density
389 favouring public-good cooperators (Figure 3c) to favouring cheats (Figure 3d).

390 We also showed that high population density can favour cooperation even when the self-restraint and
391 public-goods social dilemmas interact (Figure 2d). This is important as cooperative traits frequently
392 interact (Brown and Taylor 2009, Harrison and Buckling 2009, Lindsay et al 2016, MacLean et al
393 2010, Ross-Gillespie et al 2015). We developed a multi-trait mathematical model and predicted that
394 decreasing the influence of self-restraint over public-goods production will increase the range of
395 environmental structures where high population density favours cooperation (Figure 4a columns C-G
396 versus H-K). This was verified experimentally. For example, when resources were sufficiently low,
397 high population density favoured cooperators in environments with low (Figure 4c), intermediate
398 (Figure 4b) and high (Figure 3c) spatial structure. However, for sufficiently high resource
399 concentrations, high population density favoured cooperators only when spatial structure was high
400 (Figure 2d).

401 We reason that at higher resource concentrations, public-goods cooperators incur a cost of inefficient
402 metabolism. Therefore in environments with sufficiently low spatial structure where public-goods are
403 more accessible to cheats, the cost of inefficient metabolism outweighs any personal benefit of public-
404 goods production. Reducing the resource concentration and therefore reducing the strength of the self-
405 restraint dilemma diminishes the cost of inefficient metabolism incurred by public-goods cooperators,
406 tipping the balance in favour of cooperators even in environments with low spatial structure.

407 For certain environmental conditions our model predicts that increasing population density may
408 favour public-good cooperators or cheats depending on the details of the system (Figure 4a, yellow
409 panels). This is in line with empirical observations of a non-monotone relationship between
410 population density and cooperator relative fitness (Figure 4f).

411 Our results are also of relevance to the study of cooperation in populations undergoing an increase in
412 the geographical area they occupy, known as range expansion. Recent studies have found that the
413 advantage public-good cooperators can gain at low density may enrich cooperation at the front of
414 expanding populations (Datta et al 2013). Our results suggest that this may not be the case if the
415 environment is sufficiently structured or has low resources. Microbes inherently exist in structured
416 communities, whether as clonal patches such as colonies or hyphal networks (Koschwanez et al 2011,
417 Nadell et al 2010), or they inhabit spatially structured substrates (Boddy 2000, Ettema and Wardle
418 2002), which may frequently be nutrient depleted (Greig and Leu 2009). In these environments, rather
419 than being promoted at the low-density expanding front of a population, cooperation may be more
420 resistant against an invasion of cheats in the established bulk population where density is high as the
421 community proliferates. Conversely, this means that if public-good cheats arise through mutation they
422 may be more likely to gain a competitive advantage in the low-density advancing front of a
423 population during range expansion. This “allele surfing” phenomenon may facilitate the maintenance
424 of metabolic diversity within a population (Excoffier and Ray 2008), even for potentially deleterious
425 mutations (Travis et al 2007), until environmental conditions become more preferential for public-
426 good cheats. This diversity could enable adaptability to environmental change, improve the outcome

427 of ecosystem processes (Kaiser et al 2015), and maximise population fitness (Lindsay et al 2016,
428 MacLean et al 2010).

429

430 **Acknowledgments**

431 We would like to thank Robert Beardmore for comments and helpful discussions.

432 **Conflict of interest**

433 The authors declare no conflict of interest

434 **Supplementary information is available at The ISME Journal website**

435

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570

571 **Figure Legends:**

572 **Figure 1. The relationship between self-restraint cooperator fitness and population**
573 **density depends on the degree of spatial structuring in the environment and the extent**
574 **of the rate-efficiency trade-off.**

575 **a** Schematic of degrees of spatial structure used for experiments. Low is shaken liquid, intermediate is
576 patches of mixed strains, and high is made up of patches of either cooperators or cheats (for details
577 see Supplementary Figure S1). In a low level of spatial structuring we found a positive relationship
578 between density and cooperator fitness ($p < 1.06 \times 10^{-12}$, $F_{(1,16)} = 395.1$, $\text{Adj } R^2 = 0.9586$, $\beta = 0.08962$
579 ± 0.00451 (s.e.), $n = 6$, $f = 0.3$) **(b)**. When competed on agar plates, the relationship was also positive
580 for both intermediate **(c)** ($p < 2.66 \times 10^{-7}$, $F_{(1,7)} = 365.7$, $\text{Adj } R^2 = 0.9785$, $\beta = 1.07 \pm 0.0560 \times 10^{-5}$
581 (s.e.), $n = 3$, $f = 0.3$) and high structure **(d)** ($p < 8.56 \times 10^{-8}$, $F_{(1,7)} = 508.2$, $\text{Adj } R^2 = 0.9845$, $\beta = 3.84 \pm$
582 0.170×10^{-5} (s.e.), $n = 3$, $f = 0.3$), but the strength of this association reduced (from intermediate to
583 high, interaction term: $p < 0.0002$, $F_{(1,14)} = 25.11$). With high structure, the relationship between
584 density and cooperator fitness depended on the resource concentration. It was positive when glucose
585 concentration was high (111.01 mM), and remained positive with intermediate glucose (13.88 mM)
586 **(e)** ($p < 0.0004$, $F_{(1,7)} = 40.02$, $\text{Adj } R^2 = 0.8299$, $\beta = 0.00803 \pm 0.00127$ (s.e.), $n = 3$, $f = 0.3$), but the
587 strength of this association was reduced (interaction term : $p < 0.00276$, $F_{(1,14)} = 13.141$). However,
588 this relationship became negative when glucose concentrations were further reduced (2.78 mM) **(f)** (p
589 $< 3.34 \times 10^{-5}$, $F_{(1,7)} = 87.34$, $\text{Adj } R^2 = 0.9152$, $\beta = -0.0130 \pm 0.00139$ (s.e.), $n = 3$, $f = 0.3$). When
590 resources were reduced (2.78 mM) with an intermediate level of structure, we found regions of
591 positive and negative density-dependence **(g)** (quadratic fit: $p < 5.83 \times 10^{-3}$, $F_{(2,6)} = 13.68$, $\text{Adj } R^2 =$
592 0.7601 , $n = 3$, $f = 0.2$) with cooperator relative fitness lower at intermediate cell densities (approx. 10^6
593 cells) compared to low (approx. 10^4 , mean difference \pm s.e. = 0.0871 ± 0.0173 , $p < 2.37 \times 10^{-3}$) and
594 high (approx. 10^8 , mean difference \pm s.e. = 0.0645 ± 0.0173 , $p < 9.70 \times 10^{-3}$). With low structure,
595 density-dependent fitness of cooperator fitness remained positive in 13.88 mM glucose **(h)** ($p < 4.33 \times$
596 10^{-8} , $F_{(1,13)} = 127.5$, $\text{Adj } R^2 = 0.9004$, $\beta = 6.70 \pm 0.593 \times 10^{-4}$ (s.e.), $n = 5$, $f = 0.3$) and regions of
597 positive and negative density-dependence were found in 2.78 mM **(i)** (quadratic fit: $p < 1.61 \times 10^{-10}$,
598 $F_{(2,12)} = 251.3$, $\text{Adj } R^2 = 0.9728$, $n = 5$, $f = 0.3$), cooperator relative fitness is lower at intermediate cell

599 densities (approx. 3×10^4 cells) compared to low (approx. 10^3 , mean difference \pm s.e. = $0.0196 \pm$
600 0.00709 , $p < 0.0173$) and high (approx. 2×10^6 , mean difference \pm s.e. = 0.146 ± 0.00709 , $p < 9.62 \times$
601 10^{-11}).

602 **Figure 2. The influence of population density and spatial structuring on the selection for**
603 **public-good cooperation.**

604 The selection for public-good cooperation (BY4741 v JG210C) is negatively-density-dependent when
605 the spatial structuring of the environment is low **(a)** ($p < 0.00482$, $F_{(1,10)} = 12.99$, $\beta (\pm \text{s.e.}) = -0.00793$
606 ± 0.00220 , $\text{Adj. } R^2 = 0.5214$, $n = 4$, $f = 0.2$) and intermediate **(b)** ($p < 6.98 \times 10^{-9}$, $F_{(1,7)} = 1047$, $\beta (\pm$
607 $\text{s.e.}) = -5.88 \pm 0.182 \times 10^{-5}$, $\text{Adj. } R^2 = 0.992$, $n = 3$, $f = 0.2$), as predicted by model simulations (Degree
608 of Spatial Structuring (DSS) = 0, see Supplementary Information for definition) **(c)**, and positively-
609 density-dependent when structure is high **(d)** ($p < 7.93 \times 10^{-7}$, $F_{(1,7)} = 266.1$, $\beta (\pm \text{s.e.}) = 1.60 \pm 0.0981$
610 $\times 10^{-4}$, $\text{Adj. } R^2 = 0.971$, $n = 3$, $f = 0.2$) as predicted by model simulations ($DSS = 0.8$) **(e)**. Labels
611 indicate level of structuring and [sucrose]. Experimental repeats generated equivalent qualitative
612 trends, which were also found to be robust to differing initial frequencies of public-good cooperators
613 (Supplementary Figure S5). For simulations, initial density is $g \text{ biomass.l}^{-1}$, $S_0 = 50$, $f = 0.4$.

614 **Figure 3. Positive density-dependence of public-good cooperator fitness in structured**
615 **environments results from a higher capacity to capture public-goods.**

616 Average hexose uptake rates (over all spatial locations) of public-good (PG) cooperators and cheats in
617 a spatially structured environment at low population density ($N_0 = 6 \times 10^{-6} \text{ g biomass.l}^{-1}$) **(a)** and high
618 population density ($N_0 = 6 \times 10^{-2} \text{ g biomass.l}^{-1}$) **(b)**, as predicted by model simulations ($DSS = 0.7$, $n =$
619 5 , $p = 1$, $S_0 = 5.85$). The amount of hexose captured by PG cheats is higher in low-density than high-
620 density populations. The selection for cooperation in 1.46 mM sucrose is positively-density-dependent
621 in highly structured environments with wild-type hexose capture ability (BY4741) ($p < 1.12 \times 10^{-5}$,
622 $F_{(1,7)} = 121.6$, $\beta (\pm \text{s.e.}) = 7.57 \pm 0.686 \times 10^{-4}$, $\text{Adj. } R^2 = 0.9378$, $n = 3$, $f = 0.3$) **(c)**, but becomes
623 negatively-density-dependent when the capacity for public-good producers to capture the generated
624 hexoses is reduced (TM6*) ($p < 1.29 \times 10^{-5}$, $F_{(1,7)} = 116.6$, $\beta (\pm \text{s.e.}) = -6.40 \pm 0.593 \times 10^{-5}$, $\text{Adj. } R^2 =$

625 0.9353, $n = 3$, $f = 0.3$) **(d)**. Labels indicate level of structuring and [sucrose]. Experimental repeat can
 626 be seen in Supplementary Figure S6. Based on growth rate data from this study, with calculations and
 627 invertase activity data from (Gore et al., 2009), we estimate that the glucose capture efficiency of
 628 TM6* is $69.6 \pm 0.939\%$ of wild-type capabilities in 1.46 mM sucrose (TM6* < wild-type: $p < 0.001$, t
 629 = 32.3, $n = 3$, Welch's two-tailed t-test). Our model predicts the same outcome for PG cooperator
 630 relative fitness in structured environments ($S_0 = 1.46$, $DSS = 0.7$, $f = 0.2$, initial density is g biomass.l⁻¹
 631 ¹), with positive-density-dependence with wild-type hexose uptake **(e)** and negative density-
 632 dependence when hexose uptake is reduced ($Vh/10$) **(f)**.

633 **Figure 4. The effect of population density on public-good cooperation as a function of**
 634 **spatial structure and strength of rate-efficiency trade-off: simulations and empirical**
 635 **results.**

636 **a** Competitions were simulated between public-good cooperators and cheats in 88 environmental
 637 conditions (8 degrees of spatial structure in 11 resource concentrations) at three initial population
 638 densities. The outcomes of the competitions in relation to increasing population density are illustrated
 639 in the matrix (yellow panels: increasing density may favour co-operators or cheats depending on the
 640 details of the system). See Supplementary Information for details on how the outcome of numerical
 641 simulations were assessed. Labels within panels indicate the figure in this article that represents
 642 experimental support that replicates predictions from the mathematical model. Predictions of the
 643 simulation were tested experimentally (b-f; labels within plots indicate hexose uptake ability of both
 644 competitors, degree of structure and [sucrose]). **b** When the impact of the rate-efficiency trade-off is
 645 reduced by lowering resources (from 29.2 to 1.46 mM sucrose) we find public-good cooperator
 646 fitness becomes positively density-dependent with intermediate spatial structure ($p < 0.0173$, $F_{(1,7)} =$
 647 9.613 , $\beta (\pm \text{s.e.}) = 0.0215 \pm 0.00694$, $\text{Adj } R^2 = 0.5185$, $n = 3$, $f = 0.2$). Test for non-monotonicity is
 648 not-significant (NS, $p > 0.434$) (c.f. Figure 2b). **c** With low structure at 1.46 mM, public-good
 649 cooperator fitness becomes positively density-dependent ($p < 2.42 \times 10^{-16}$, $F_{(1,16)} = 1154$, $\beta (\pm \text{s.e.}) =$
 650 $5.38 \pm 0.158 \times 10^{-4}$, $\text{Adj } R^2 = 0.9855$, $n = 6$, $f = 0.2$) (c.f. Figure 2a). **d** In competitions between
 651 exclusively respiring public-good cooperators (TM6*) and cheats (TM6* $\Delta\text{suc}2$) with high structure,

652 public-good cooperator fitness was positively density-dependent ($p < 1.50 \times 10^{-6}$, $F_{(1,7)} = 221$, $\text{Adj-R}^2 =$
653 0.9649 , $\beta = 0.0321 \pm 0.00216$, $n = 3$, $f = 0.3$) like wild-type hexose transporting strains (BY4741 v
654 JG210C) (Figure 2d). **e** However, unlike wild-type hexose transporting strains with intermediate
655 structure (Figure 2b) this relationship remained positive ($p < 4.25 \times 10^{-6}$, $F_{(1,7)} = 162.4$, $\text{Adj-R}^2 =$
656 0.9528 , $\beta = 6.07 \pm 0.476 \times 10^{-5}$, $n = 0.3$, $f = 0.3$). These results were predicted by our model when
657 hexose uptake rate is diminished (Supplementary Figure S7). **f** In low structure, we found that density
658 has a non-monotonic relationship with public-good cooperator fitness (quadratic: $p < 5.00 \times 10^{-14}$, $F_{(2,$
659 $15)} = 437.7$, $\text{Adj-R}^2 = 0.9809$, $n = 6$, $f = 0.3$). Public-good cooperator relative fitness was lower at
660 intermediate densities (approx. 5×10^4) compared with low (approx. 10^3 cells, mean difference \pm s.e.
661 $= 0.107 \pm 0.00399$, $p < 4.64 \times 10^{-14}$) and high density (approx. 2.5×10^6 , mean difference \pm s.e.
662 $= 0.00923 \pm 0.00399$, $p < 0.0356$, linear model with density as factor).

Degree of Spatial Structure

Figure 1

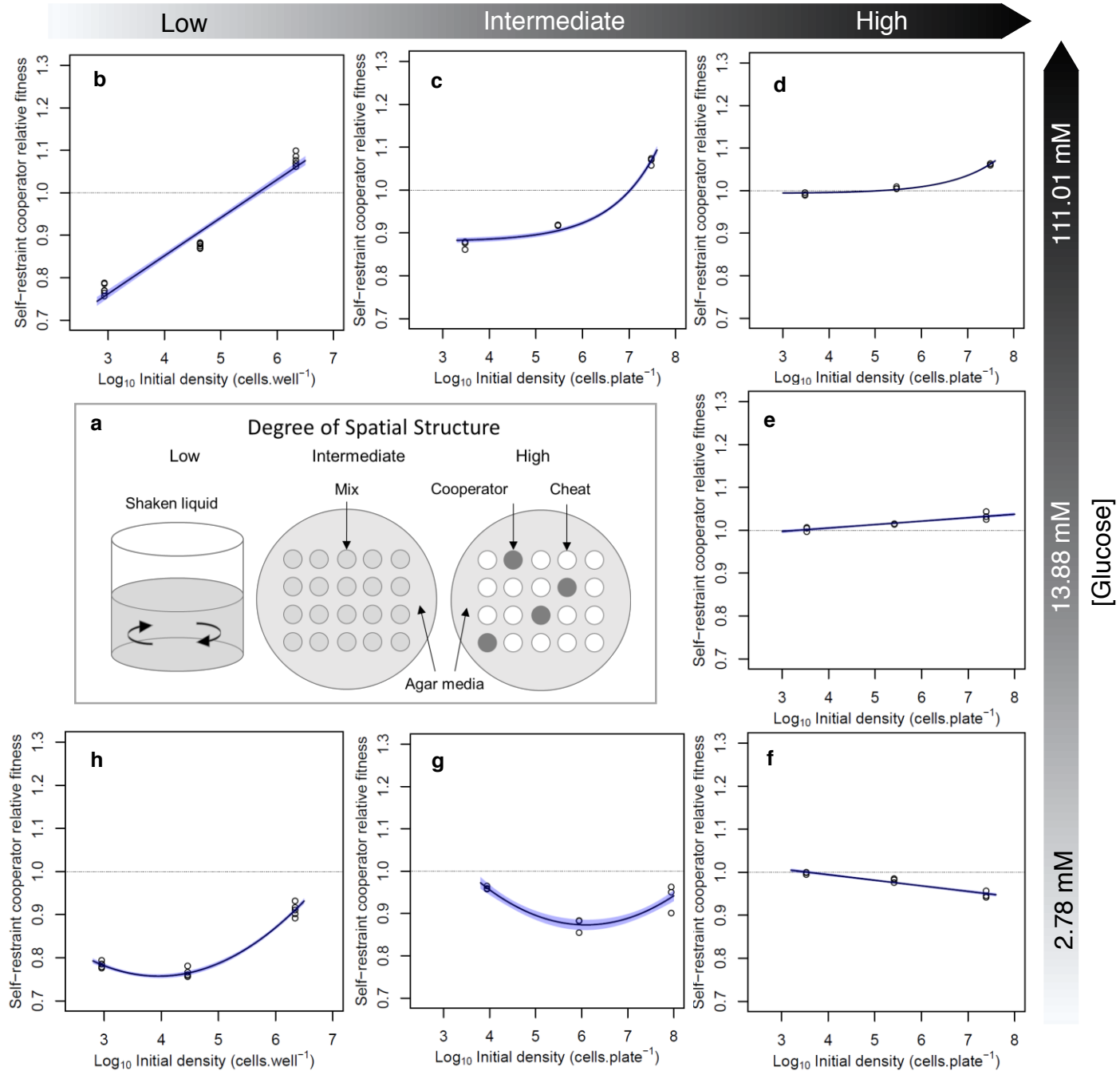


Figure 2

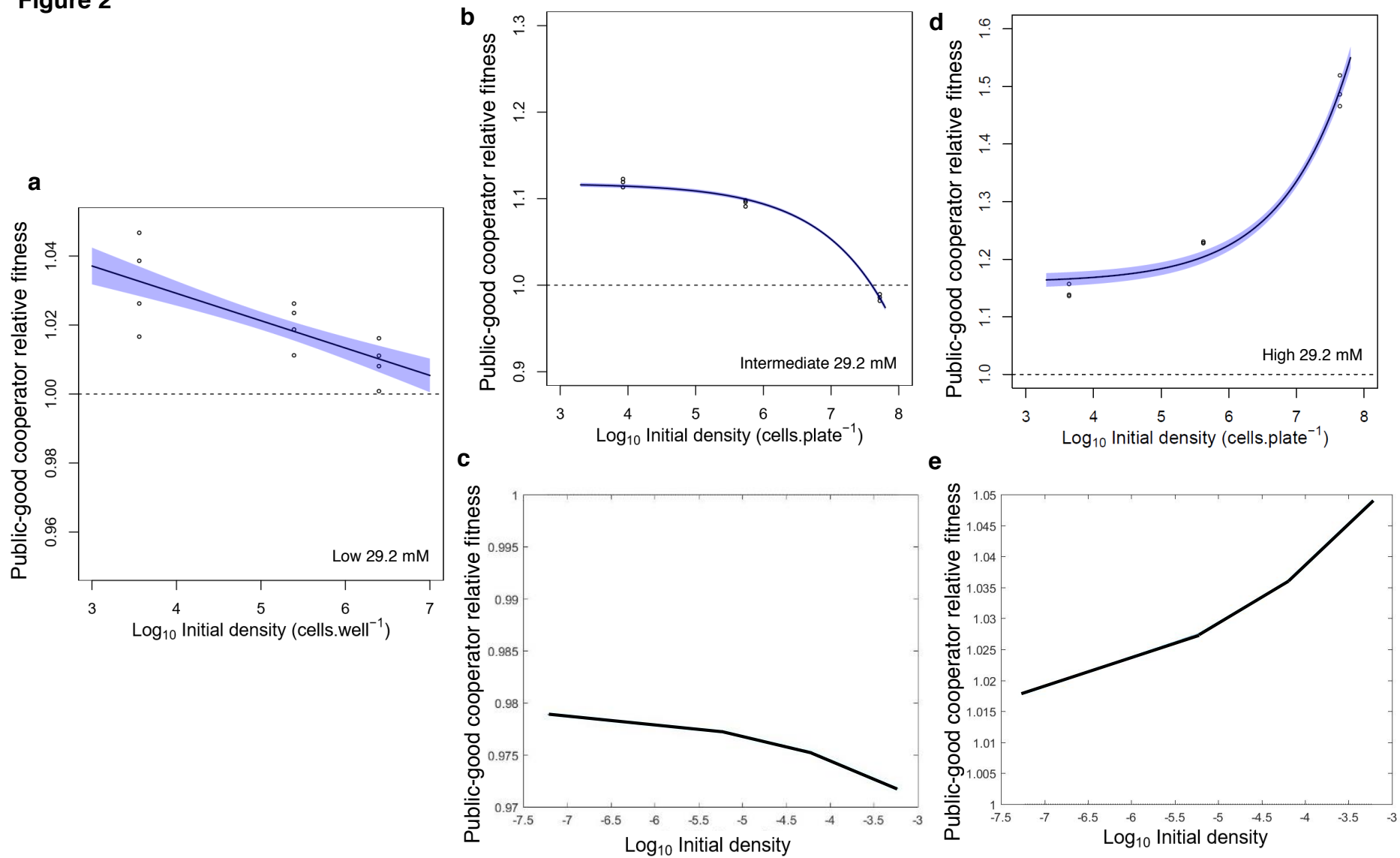


Figure 3

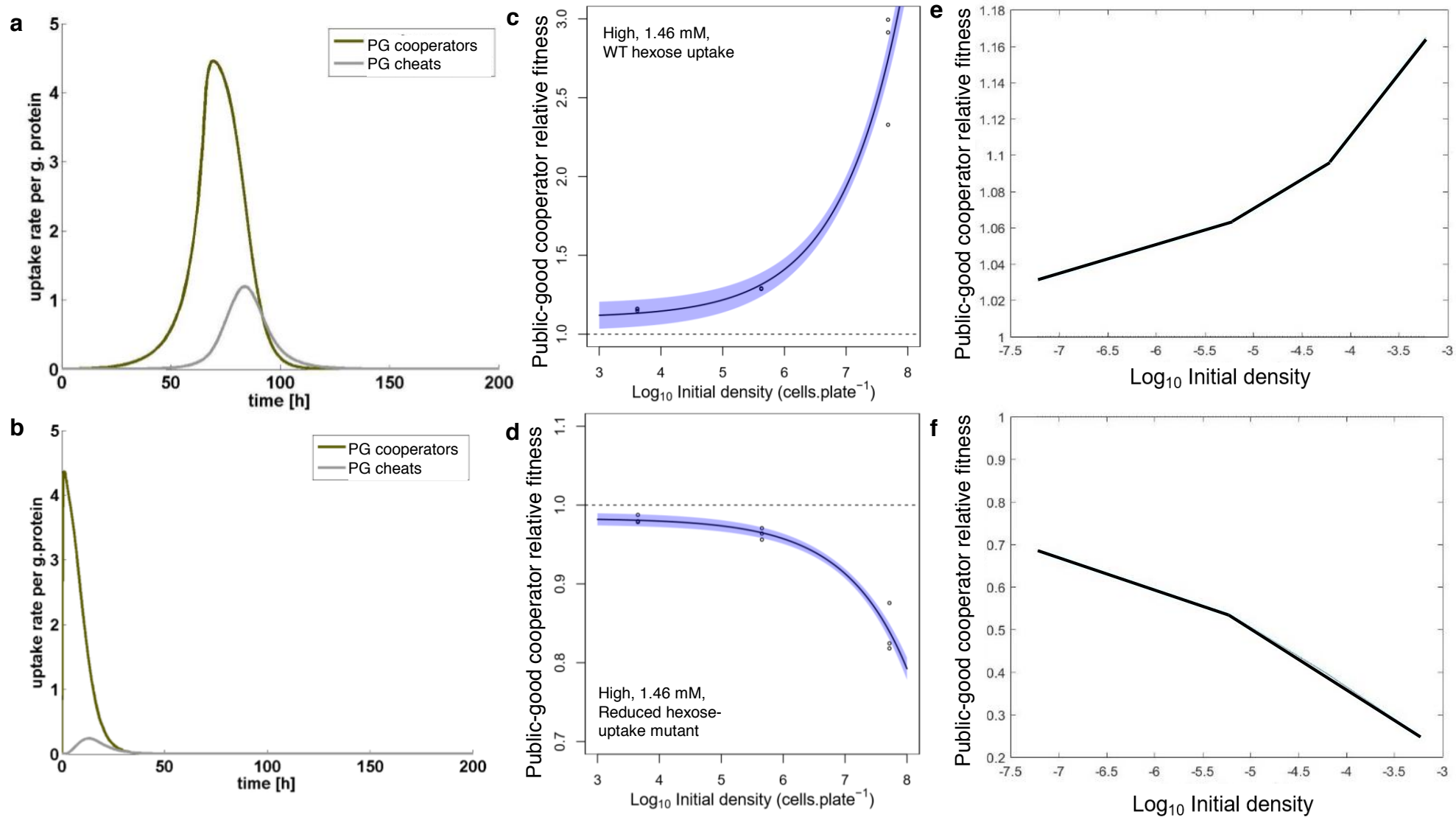


Figure 4

