1 # Meta-population structure and the evolutionary transition to

2 # multicellularity

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Abstract

The evolutionary transition to multicellularity has occurred on numerous occasions, but transitions to complex life forms are rare. While the reasons are unclear, relevant factors include the intensity of within versus between group selection that are likely to shape the course of life cycle evolution. A highly structured environment eliminates the possibility of mixing between evolving lineages thus ensuring strong competition between groups. Less structure intensifies competition within groups decreasing opportunity for group-level evolution. Here we present experiments that contrast two ecological frameworks that differ in the way in which nascent multicellular groups, and their constituent cells, compete. Groups of the bacterium *Pseudomonas fluorescens* were propagated under a regime requiring reproduction via a life cycle with developmental and dispersal phases. By controlling the extent of mixing during the dispersal phase it was possible to alter the relative emphasis on the two phases. While all groups possessed 'paradigmatic' features of multicellular individuals (e.g., bottleneck and germ line), the mode of group interaction substantially affected the strength and direction of selection operating at both group and cell levels. Constraints on meta-population structure may therefore explain the observation that multicellular aggregates rarely complete the transition to individuality.

Introduction

49

50 Life is hierarchically structured. Multicellular organisms are comprised of cells, cells

51 contain organelles; the nucleus contains chromosomes comprised of genes (1, 2). The

52 origin of this structure reflects successive evolutionary transitions in individuality (ETIs)

53 with lower level particles being subsumed within higher-level self-replicating entities

54 (2). Central to each transition was the emergence of Darwinian properties at the new

55 (group) level. With the emergence of such properties came new kinds of biological

56 individuals that participated directly in the process of evolution by natural selection (2-5).

57

58 Explanations for the evolution of multicellular life centre on the origins of group

59 reproduction (6-9). Modes that involved early discovery of life cycles replete with

60 reproductive specialisation are of particular significance (1, 5, 8, 10). Such cycles

61 establish a second (longer) timescale over which selection operates (5, 11-13). This

62 permits the possibility of a birth death process at the group level. Additionally, life cycles

63 require a developmental process – an altogether new trait that is a property of the group –

64 that necessarily becomes a focus of selection.

65

66 While a developmental life cycle is important (9), it is not sufficient. The mere existence

67 of the fundamental requirements for the evolution of multicellularity (e.g., a bottleneck

68 and reproductive specialisation) does not guarantee selection at the higher level. Selection

69 simultaneously continues to operate at the lower level, favouring the reproductive success

70 of cells at the expense of the group to which they belong. This poses a general problem,

71 which lies at the heart of any ETI – how can the higher level successfully constrain the

72 lower level? This poses a very particular challenge during early phases of an ETI.

73 Theoretical studies on the origins of cells provide clues (14, 15). Theoretical models

74 show that selection at the higher level can be achieved through specific population

75 structures: when replicators are individually isolated, selection remains focussed on the

76 lower level – that of the individual replicators (14). However, when replicators are

77 localized in reproducing compartments within a meta-population of compartments, the

78 focus of selection is the higher level (15, 16). We therefore hypothesize that a specific

79 population structure that embraces innovation in primordial life cycles while escaping the

80   burden of lower level selection is a crucial requirement for the transition to

81   multicellularity. Competition is key, however the level at which rudimentary groups

82   compete depends primarily upon meta-population structure and the partitioning of

83   variation that this population structure imposes.

84

85   Our experiments using a model bacterial system (*Pseudomonas fluorescens*) have shown

86   how life cycles can emerge from negative frequency dependent interactions between two

87   ecologically distinct phenotypes that each give rise to the other (9). One type is defined

88   by sticky cells that produce a soma-like mat at the air-liquid interface allowing access to

89   oxygen, while the other is marked by non-sticky dispersing cells that function as proxy

90   for a germ line. The mat forming type performs an ecological role, but also gives rise to

91   propagules that are seeds of the next generation of mats. Selection operating at the level

92   of the nascent multicellular organisms via a group-level death-birth process led to

93   enhanced fitness of groups (relative ability of groups to give rise to offspring groups),

94   while fitness of the individual cells was reduced relative to the ancestral types.

95

96   These findings gave substance to the suggestion that life cycles, in conjunction with a

97   death-birth process at the level of collectives (lineage selection), provide opportunities for

98   selection to transition to higher levels of organisation and that a two-phase life cycle

99   (underpinned by a developmental programme) is of particular importance, but much

100   remains unexplored. Of particular relevance is the effect of the population structure: the

101   nature of the environment affects how variation is partitioned within and between

102   groups. A highly structured environment as in the experiments of Hammerschmidt *et. al.*

103   (9), which is analogous to models of compartment structure of early replicators (15),

104   eliminates the possibility of mixing between evolving lineages thus ensuring strong

105   competition between groups. Less structure would likely intensify competition within

106   groups and possibly decrease opportunity for group-level evolution.

107

108   Here we empirically consider the impact of population structure on the emergence of

109   individuality – that is, we examine the relative emphasis of interactions within and

110   between groups – on the fitness spectrum of the two levels. We contrast the life cycle

4

111   from our previously published results (9) (Non-Mixed Ecology treatment; Figure 1a) –

112   with an identical two-phase life cycle that incorporates competition (mixing) during the

113   propagule phase of emerging multicellular groups. This environmental manipulation,

114   which we term the Mixed Ecology treatment (Figure 1b), was performed simultaneously

115   with the earlier study. We show that competition effected during the propagule phase of a

116   two-stage life cycle leads selection to favour traits that promote cell growth at the

117   expense of traits underlying group fitness. This conflict between the two levels of

118   selection is due to a trade-off between traits underlying the fitness of groups and their

119   cells. While the existence of a germ line can bring about the decoupling of fitness

120   required to achieve a higher level of individuality, intense competition between cells

121   nevertheless skews selection towards traits that enhance the competitive ability of cells,

122   rather than towards traits that enhance the functioning of the life cycle as a whole.

123

124   **Results**

125   The evolution of simple groups has been observed in experiments using populations of *P.*

126   *fluorescens* propagated in spatially structured microcosms (17-20). Such groups

127   constitute a plausible starting position from which to explore transitions in Darwinian

128   individuality from cells to multicellular groups (7, 8, 18). In these experiments,

129   cooperative groups arise from single mutant "wrinkly spreader" (WS) cells that

130   overproduce a cell-cell glue; the failure of cells to separate at cell division leads to the

131   formation of mats that colonize the air-broth interface (19, 21-23). Glue production is

132   costly to individual cells, but the trait spreads because cells within the mat reap a reward

133   (access to oxygen) that is denied to free-living cells (24). Cooperating groups are

134   therefore inevitably short-lived (18, 25-29). Selection continues to act at the level of

135   individual cell by favouring mutant "smooth" (SM) types that cheat by no longer

136   producing glue, but nonetheless gain the advantages of being part of the group (18).

137

138   The negative frequency-dependent selection described above creates a cycling between

139   cooperative WS cells and non-cooperative SM cells (10). When viewed from the long-

140   term perspective of the group, this oscillation drives a rudimentary life cycle that confers

141   on the group the potential to reproduce by means of dispersing 'germ line' cells (8). The

142   group may thus evolve by natural selection (9). Each generation begins with WS cells

143   forming a mat at the air-liquid interface (Figure 1). For a mat to reproduce it must be both

144   viable and fecund, i.e., produce SM germ line cells (Phase I in Figure 1).

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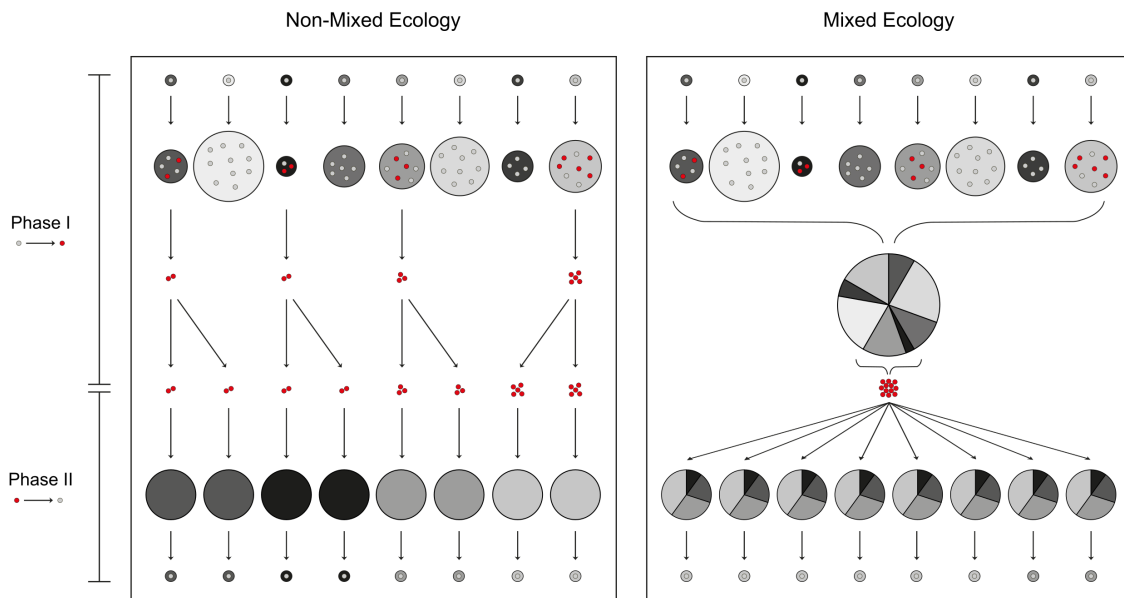
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147   **Figure 1. Schematic depiction of a population of eight genetically distinct groups (indicated by different**

148   **shades of grey) proceeding through one life cycle within their respective ecologies**. Grey circles = WS

149   "soma" cells, red circles = SM "germ" cells. Variation is maintained between groups in the Non-Mixed

150   Ecology, while a single-cell bottleneck curtails within-group variation. Propagule mixing eliminates

151   between-group variation during Phase II of the Mixed Ecology.

152

153   We have shown previously that this life cycle results in the 'decoupling' of fitness[1]

154   between groups and their cells (9), however this alone does not guarantee the emergence

155   of individuality. In the present study we examine this life cycle in two ecological

156   scenarios in order to understand how the partitioning of variation (and therefore

157   competition and selection) can help or hinder the emergence of individuality. In the Non-

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[1]'Fitness decoupling' (30) is the notion that during evolutionary transitions to multicellularity the long-term success of emerging groups is no longer a direct consequence of the growth rate (short-term fitness) of the cells of which they are composed.

6

158 Mixed Ecology treatment, competition between groups resulted from a death-birth
159 process: following an extinction event (usually due to the lack of SM production (9)), a
160 group was randomly replaced by a surviving competitor group. SM cells were harvested
161 separately from each surviving group at the end of the Maturation Phase (Phase I).
162 Extinction/replacement of groups occurred with high frequency (9) and therefore imposed
163 potent between-group selection. By contrast, competition between surviving groups in the
164 Mixed Ecology resulted from a mixing step: following Phase I extinction events all
165 groups were mixed and SM cells harvested. The mixture of SM cells seeded all eight
166 groups in the Dispersal Phase (Phase II). In both ecologies, SM germ line cells competed
167 to produce WS types, and ultimately for mat formation. To avoid chimeric mat organisms,
168 a population bottleneck was achieved by transferring one colony of the most dominant
169 WS type to a fresh microcosm (Phase II in Figure 1) (9).

170

171 **Changes in group and cell fitness**

172 Differences in the partitioning (and amount) of variation within and between groups
173 (Figure 1) is predicted to generate differences in competition, and therefore the outcome
174 of natural selection, within (cell level) and between (group level) groups. After ten group
175 generations, changes in both cell and group level fitness were estimated by competition
176 with neutrally marked ancestral lines.

177

178 Derived lines in the Non-Mixed Ecology significantly increased group fitness (ability to
179 leave group offspring) relative to the ancestral types (χ^2=32.660, d.f.=1, P<0.0001;
180 Figure 2a), whereas cell fitness (number of cells present immediately prior to dispersal)
181 decreased (F_1 = 10.612, P = 0.002; Figure 2b). In contrast, under the Mixed Ecology,
182 group fitness did not change (χ^2=3.137, d.f.=1, P=0.077; Figure 2a), whereas cell fitness
183 increased (F_1 = 56.214, P < 0.0001; Figure 2b).

a

b

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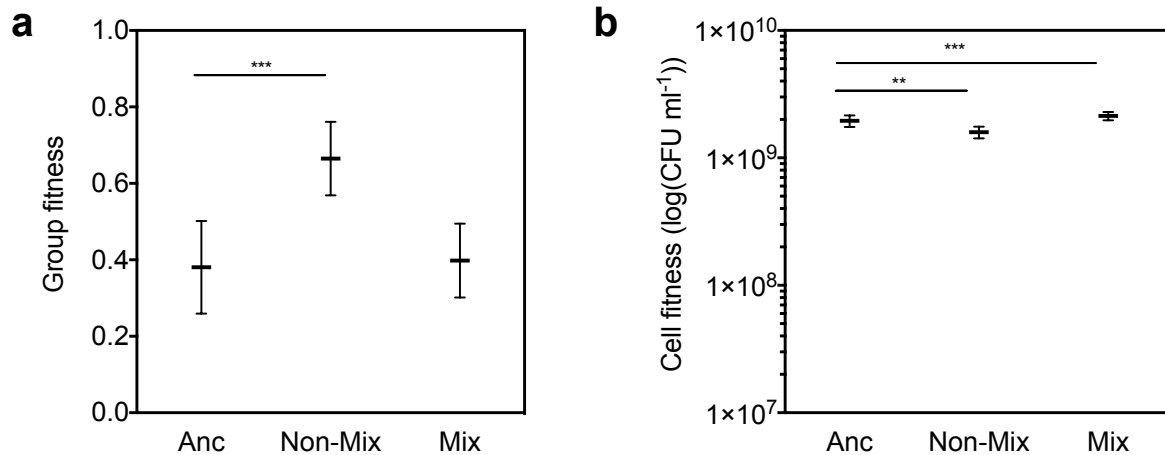
Figure 2. Changes in group (a) and cell (b) fitness in the Non-Mixed Ecology (Non-Mix) and Mixed Ecology (Mix) regimes compared to ancestral populations (Anc). Group fitness is the proportion of derived offspring mats relative to a genetically marked reference genotype. Error bars are s.e.m., based on n = 14 (Non-Mix) and n = 15 (Anc, Mix). ** denotes significance at the level of P = 0.001 - 0.01, and *** at the level of P <0.001.

190

191 **Trade-off between group and cell fitness**

192 Figure 3 (left panel) illustrates a negative relationship between cell and group fitness in

193 the ancestral lines (χ^2=4.246, d.f.=1, P=0.0393). Ancestral clones also displayed a

194 bimodal distribution of group fitness, suggesting that a trade-off exists between traits

195 underpinning cell and group fitness. Ten generations of selection in the Non-Mixed

196 Ecology shifted the distribution towards the 'high group fitness/low cell fitness' corner of

197 the graph (Figure 3, middle panel), indicating that group-level selection was more potent

198 than cell-level selection under the Non-Mixed population ecology. In the Mixed Ecology

199 (Figure 3, right panel), the absence of change in group fitness and increase in cell fitness,

200 in addition to the trade-off between cell and group fitness inherent in the life cycle,

201 suggests that tension exists between the two levels of selection when groups are

202 propagated under this regime.

203

204 The contrasting response to selection of groups subjected to the Non-Mixed and Mixed

205 ecologies likely results from the two ways in which groups interact, which has major

206 consequences for the resulting distribution of variation. Only cells within the Mixed

207 groups interact physically, however groups in the Non-Mixed Ecology nevertheless

8

208 participate in fitness-affecting interactions; the death of one group directly affects the

209 fitness of the other groups in the population.

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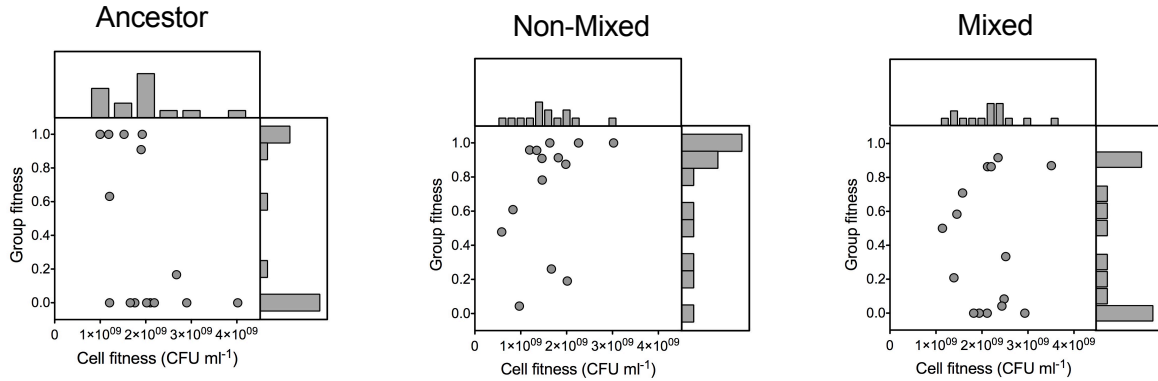
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212 **Figure 3. Relationship between cell and group fitness in the Non-Mixed and Mixed Ecologies compared**
213 **to ancestral populations**.   Group fitness is the proportion of derived offspring mats relative to a
214 genetically marked reference genotype.   Dots represent the mean of eight lines per replicate population,
215 which were assessed in three independent competition assays.

216

217 In both ecologies, the life cycle began in Phase I with a single-celled bottleneck, and

218 therefore variation existed only between groups (regardless of population ecology) until

219 within-group variation emerged during maturation (Figure 1). Phase II Non-Mixed

220 groups are also seeded with dispersal cells originating from single parent groups,

221 resulting again in high levels of between-group variation and relatively low levels of

222 within-group variation. This between-group variation led to competition and therefore

223 selection between groups, resulting in increased group fitness. A consequence of strong

224 group-level selection was a reduction in cell fitness due to the negative relationship

225 between cell and group fitness (Figure 3).

226

227 The direction of selection along this negative fitness spectrum was more complicated in

228 the Mixed Ecology. Phase II began with eight identical groups so variation, competition

229 and therefore selection occurred only within groups (Figure 1), leading to increased cell

230 fitness. The mixing step has consequences not only for the partitioning of variation but

231 also has downstream effects on the overall amount of variation. Competition within

232 groups (between cells) during Phase II likely resulted in the same single WS cell type

233   ultimately seeding all eight groups in Phase I of the next generation. Consequently, the

234   limited between-group competition in Phase I did not overcome the considerable within-

235   group selection towards the 'high cell fitness/low group fitness' corner of the fitness

236   spectrum during Phase II, because between-group variation is essentially removed during

237   Phase II of the previous generation.

238

239   **Changes in life cycle parameters**

240   To explore specific adaptations contributing to differences in fitness between the two

241   ecologies, we assayed parameters expected to be key to thriving in the multicellular life

242   cycle. Despite the importance that the life cycle places on the appearance of SM cells

243   during mat maturation, the Density, Proportion, and Growth Rate of SM cells did not

244   change significantly during evolution under the Non-Mixed Ecology (density: $F_1 = 1.278$,

245    $P = 0.2663$; proportion: $F_1 = 2.702$, $P = 0.1095$; growth rate: $F_1 = 2.116$, $P = 0.1522$), and

246   in fact SM Density and Proportion of SM cells decreased during the Mixed Ecology

247   (density: $F_1 = 56.214$, $P < 0.0001$; proportion: $F_1 = 102.217$, $P < 0.0001$; growth rate: F_1

248    $= 2.664$, $P = 0.1103$; Figure 4a,b,c).

249

250   Interestingly, the Density of WS cells decreased under the Non-Mixed Ecology ($F_1 =$

251   8.036, $P = 0.0065$), and increased under the Mixed Ecology ($F_1 = 9.904$, $P = 0.0027$;

252   Figure 4d. The two population ecologies reflect the differences in the emphasis of

253   selection (or lack thereof) on the number of WS cells arising during Phase II. Phase II

254   culminates in selection of the single most dominant WS type to enter the bottleneck and

255   seed Phase I of a next generation. Therefore, competition between WS types is intensified

256   when groups are mixed prior to entering Phase II; WS cells arising from one parent group

257   must outcompete alternative WS cell types arising from *all* other groups in order to

258   survive the Mixed Ecology. In contrast, the WS cells arising in Phase II of the Non-

259   Mixed Ecology need only outcompete any alternative WS cell types that may (or may

260   not) arise within the same group.

261

262   Finally, the rate of transition between WS and SM cells dramatically increased under the

263   Non-Mixed Ecology (χ^2=114.198, d.f.=1, P<0.0001, and also to a lesser extent under the

264   Mixed Ecology (χ^2=12.459, d.f.=1, P=0.0004; Figure 4e). This is consistent with the

265   requirement - in both ecologies - for groups to cycle through the life cycle by

266   transitioning between the two cell states.

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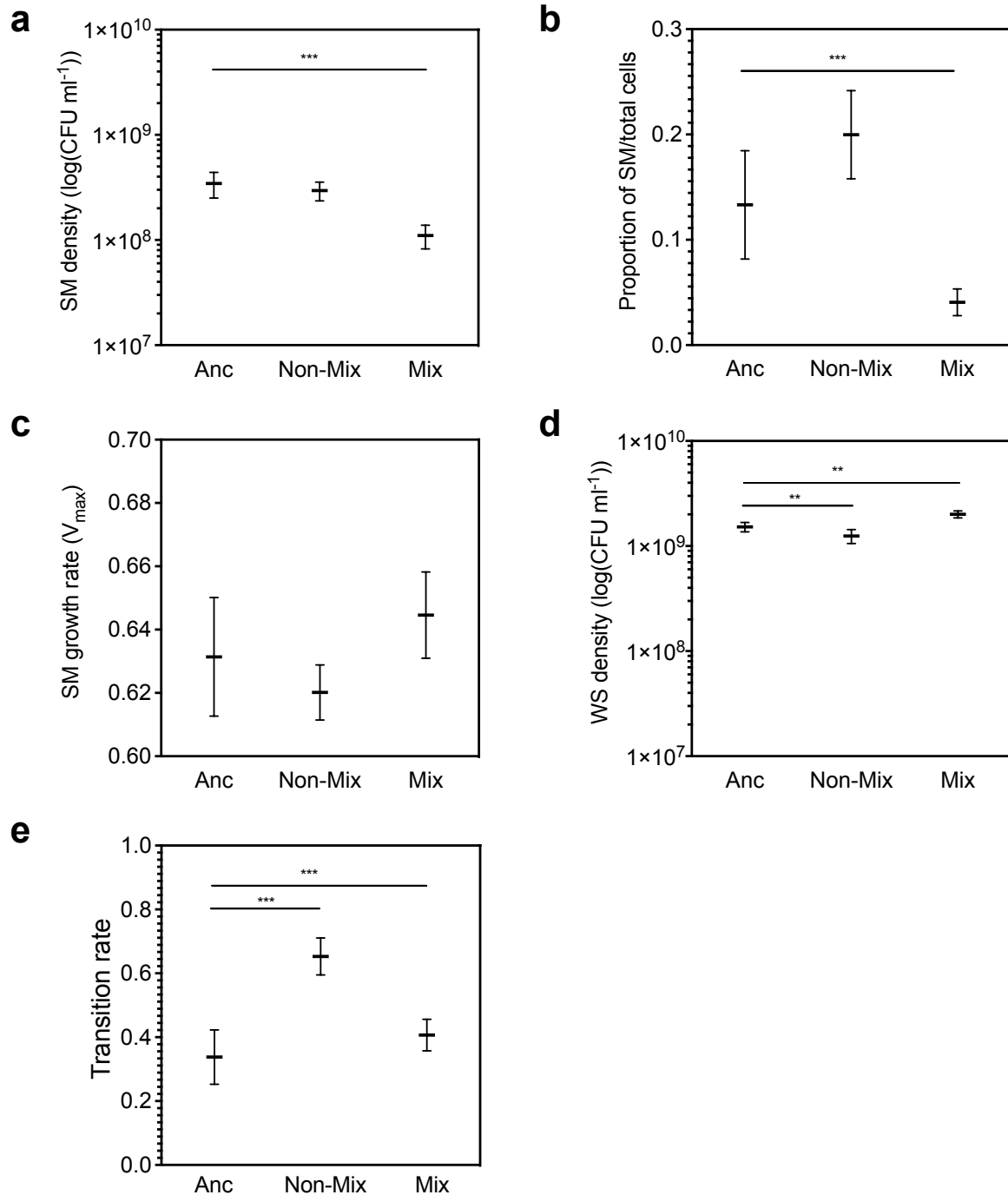
268 **Figure 4. Changes in life cycle traits in the Non-Mixed (Non-Mix) and Mixed (Mix) Ecologies compared to**
269 **the ancestral populations (Anc)**: (a) SM density, (b) Proportion of SM, (c) SM growth rate, (d) WS density,
270 (e) Transition rate. Error bars are s.e.m., based on n = 14 (Non-Mix) and n = 15 (Anc, Mix). ** denotes
271 significance at the level of *P* = 0.001 - 0.01, and *** at the level of *P* <0.001.
272

273 **Identification of parameters linked to group and cell fitness**

274 The fact that the WS-SM cell transition rate is the only measured parameter to increase in
275 the Non-Mixed Ecology led us to speculate that the WS-SM transition rate is associated
276 with group fitness. Indeed, these two factors are positively correlated in the ancestral
277 lines (χ^2=28.029, d.f.=1, *P*<0.0001; Figure 5a, left panel). During evolution in the Non-
278 Mixed Ecology, the distribution has shifted towards the 'High Group Fitness/High
279 Transition Rate' corner of the spectrum with the two parameters still associated
280 (χ^2=13.657, d.f.=1, *P*=0.002; Figure 5a, middle panel).

281

282 Cell Fitness in the ancestral lines is strongly associated with the Density of WS cells (F_1
283 = 6.673, *P* = 0.023; Figure 5b, left panel). The distribution of both parameters increased
284 during the Mixed Ecology (F_1 = 200.931, *P* < 0.0001; Figure 5b, right panel) and
285 decreased during the Non-Mixed Ecology (F_1= 97.359, *P* < 0.0001; Figure 5b, middle
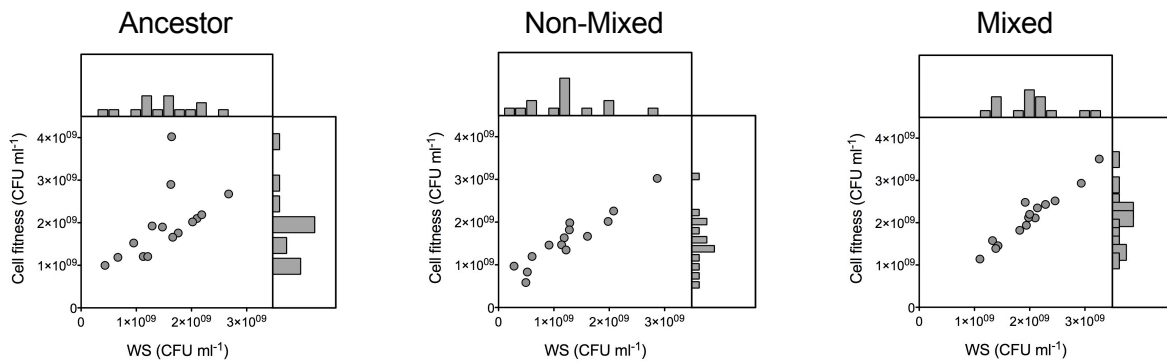286 panel).

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288   **a**

289

290   **b**

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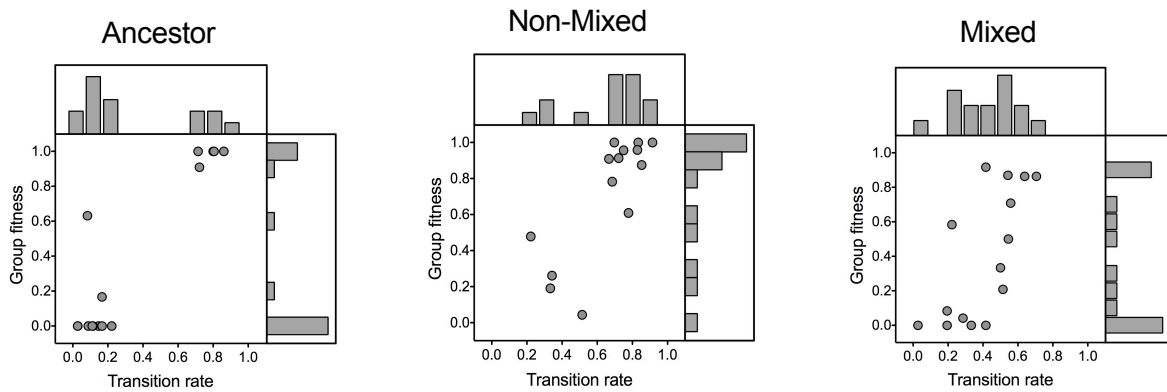
Figure 5. Relationship between life cycle traits and group and cell fitness. (a) Association of transition rate and group fitness in the ancestral populations, and in the Non-Mixed and Mixed Ecologies. (b) WS density is positively associated with cell fitness in the ancestral populations, and in the Non-Mixed and Mixed Ecologies. Group fitness is the proportion of derived offspring mats relative to a genetically marked reference genotype. Dots represent the mean of eight lines per replicate population, which were assessed in three independent competition assays. Cell Fitness in the ancestral lines is strongly associated with the Density of WS cells (F_1 = 6.673, P = 0.023; 55b, left panel). The distribution of both parameters increased during the Mixed Ecology (F_1 = 200.931, P < 0.0001; Figure 5b, right panel) and decreased during the Non-Mixed Ecology (F_1 = 97.359, P < 0.0001; Figure 5b, middle panel).

Trade-off between WS-SM cell transition rate and WS density

A negative relationship (trade-off) exists between WS Density (which is linked to cell fitness) and WS-SM transition rate (which is linked to group fitness) in the ancestral population (r=-0.705, P=0.003, N=15; Figure 6, left panel). The nature of the association between these two traits explains both the negative relationship between the two levels of fitness observed above (Figure 3), and the opposing direction of selection in the two

13

308 ecologies. While cells were required to survive an identical two-phase life cycle
309 regardless of meta-population structure, these two traits were driven in opposite
310 directions under the two ecologies because of differences in the emphasis of cell and
311 group level selection.

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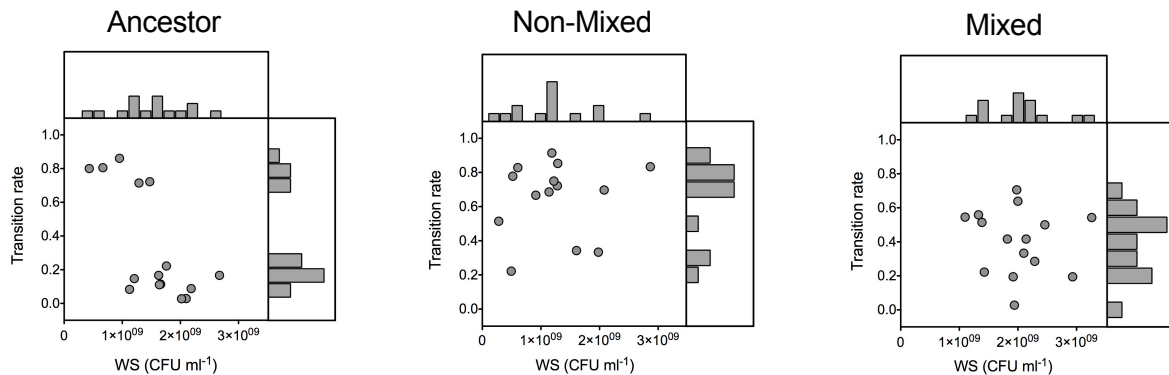
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314 **Figure 6. Relationship between WS density and transition rate in the ancestral populations, and in the**
315 **Non-Mixed and Mixed Ecologies.** Dots represent the mean of eight lines per replicate population, which
316 were assessed three independent times.

317

318 Table 1 summarises the differences between ecologies in the partitioning of variation
319 across the meta-populations, and the downstream consequences on the traits selected
320 during Phase I and Phase II. Selection during both phases of the Non-Mixed Ecology
321 favours a higher WS-SM transition rate. However, the trade-off apparent in Figure 6
322 suggests that there is conflict between the two phases of the Mixed Ecology in terms of
323 the direction of selection within the scope of these two traits. Adaptations may arise that
324 allow groups to survive Phase I of the Mixed Ecology (*i.e.*, high WS-SM transition rate),
325 only to be extinguished during Phase II due to a low competitive ability resulting from
326 reduced WS density. The red box highlights this conflict between selection of two
327 incompatible traits. The relationships between these traits and their effect on the two
328 levels of fitness are illustrated in Figure 7.

329

330

331 **Table 1. Effects of the meta-population structure on the level of selection**. The red box highlights

332 selection during different phases of the Mixed Ecology for two incompatible traits (parameters that are

333 negatively correlated), leading to a conflict between levels of selection.

Ecology	Life cycle phase	Distribution of Variation	Level of Selection	Life-history requirement(s)	Trait selected
Non-Mixed	**PHASE I**	Between groups	Between groups	Produce SM cells	WS-SM transition rate
	PHASE II	Between groups	Between groups	Produce WS cells	WS-SM transition rate
Mixed	**PHASE I**	Between groups (low)	Between groups (weak)	Produce SM cells	WS-SM transition rate
	PHASE II	Within groups	Between cells	Produce WS cells AND Outcompete WS produced by other groups	WS density

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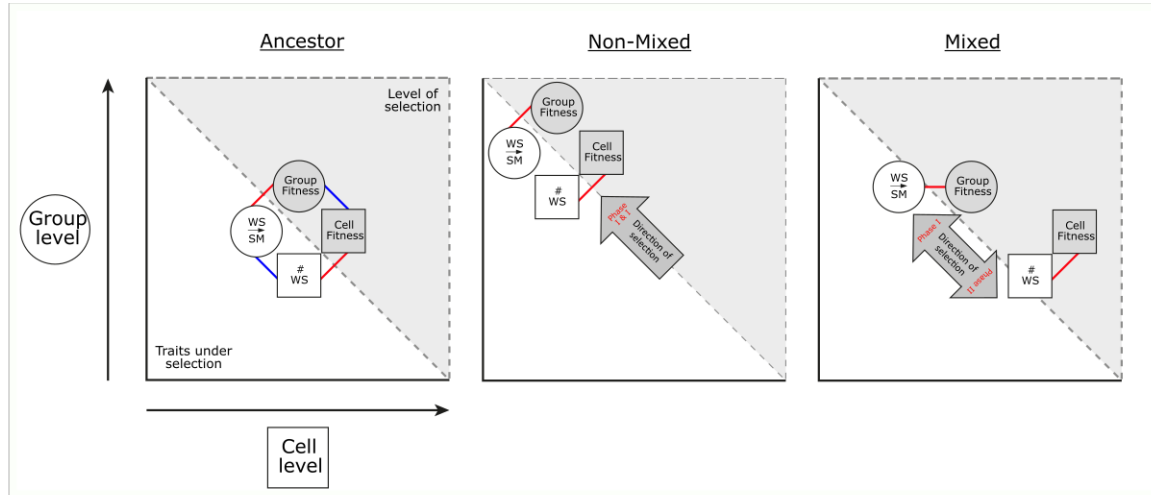
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335 **Figure 7. Relationships between the traits underpinning cell and group fitness**. Cell and group fitness are
336 depicted in grey, while the traits underpinning them are white. Red lines indicate a positive correlation;
337 blue lines indicate a negative association. After ten generations of selection in the Non-Mixed Ecology
338 (middle panel), group fitness and its associated trait (WS-SM transition rate) increased, while cell fitness
339 and its associated trait (WS Density) were reduced, most likely due to the trade-offs that exist between
340 these traits in the ancestral population (left panel, blue lines). In contrast, after ten generations of
341 selection in the Mixed Ecology (right panel), cell fitness and its associated trait (WS Density) increased due
342 to within group selection during Phase II, while group fitness did not change (although its associated trait,
343 WS-SM transition rate, increased slightly).

344
345
346

16

Discussion

Life cycles underpin evolutionary transitions in individuality (1, 7-9). The particular mode by which the earliest multicellular groups acquired the capacity to reproduce has implications for their ability to transition to groups that come to participate in the process of evolution by natural selection in their own right (Figure 8) (1, 5, 8, 10, 31). The emergence of developmental life cycles involving reproductive specialisation removed the constraints on the evolution of complexity in plants, animals and fungi, making the germ line one of the key innovations enabling the evolution of integrated multicellular organisms (32). A germ line enables selection to act on the developmental life cycle as a unit, rather than on a particular cell state – leading to the accumulation of traits that enhance the functioning of the group over the longer timescale (9).

Nascent multicellular organisms lack functional integration during early stages of the transition. An ETI requires a population structure that supports the cohesiveness of early groups and simultaneously constrains the rampant growth of constituent cells. In the present study we experimentally addressed the relevance of population structure for an ETI by imposing group competition on two ecological frameworks that differ in the relative extent of fitness-affecting interaction within and between groups. All groups reproduced via identical life cycles incorporating the hallmarks of a new level of individuality (a bottleneck and germline) but in one experimental treatment (Mixed Ecology) groups were pooled prior to dispersal. Both treatments had a multilevel selection aspect, and by pooling groups the availability of niche space to offspring groups was manipulated, thus altering the relative emphasis of selection on the two phases of this life cycle.

Groups in the Non-Mixed Ecology could multiply to occupy new niches only upon death of a competing group. Selection favoured persistence of the entire life cycle because the availability of a group's own niche was always guaranteed. In both phases of the life cycle, the appearance of the alternative cell type (SM during maturation, WS during the dispersal phase) was sufficient to ensure that a group could persist and enter the next generation. Selection favoured an increased rate of transition between the two cell types

17

379   (Figure 4e), which facilitated the almost guaranteed detection of a new cell type. The

380   emphasis on persistence over cell number in this structured environment is akin to a *K*-

381   selected ecological framework (33). In contrast, mixing populations prior to dispersal

382   created an environment resembling an *r*-selected ecology in which cells from all groups

383   within each meta-population competed for all niches in the next generation (33). Groups

384   in the two ecologies experienced identical environments during maturation, however in

385   the dispersal phase of the Mixed Ecology the most numerous WS cell type from all eight

386   groups in the population was selected to seed the next generation. The resulting selection

387   for increased WS cell density was therefore crucial for groups to prevail under such

388   ecological pressure. Whereas the *r*-selected ecology experienced by populations in the

389   Mixed Ecology placed the emphasis of selection on cell numbers, the *K*-selected Non-

390   Mixed groups were released from the pressure for fast growth, providing an opportunity

391   for innovation.

392

393   The two ecologies in this study are distinguished by the partitioning of variation across

394   meta-populations. Competition drives natural selection, and variation is crucial for

395   competition. It follows then that the distribution of variation within and between groups

396   determines the relative intensity of natural selection within and between groups. The

397   importance of a bottleneck in multicellular life cycles has been attributed to its effect on

398   the distribution of variation. A bottleneck redistributes variation (innovation) from within

399   the parent to between its offspring (30), allowing selection to operate on different

400   innovations between higher-level individuals. While both ecologies here experience a

401   single-celled bottleneck during the life cycle, the mixing stage negated this effect because

402   it prevents the redistribution of variation among offspring; on the contrary, all post-

403   mixing offspring are identical.

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Traits beneficial to cells

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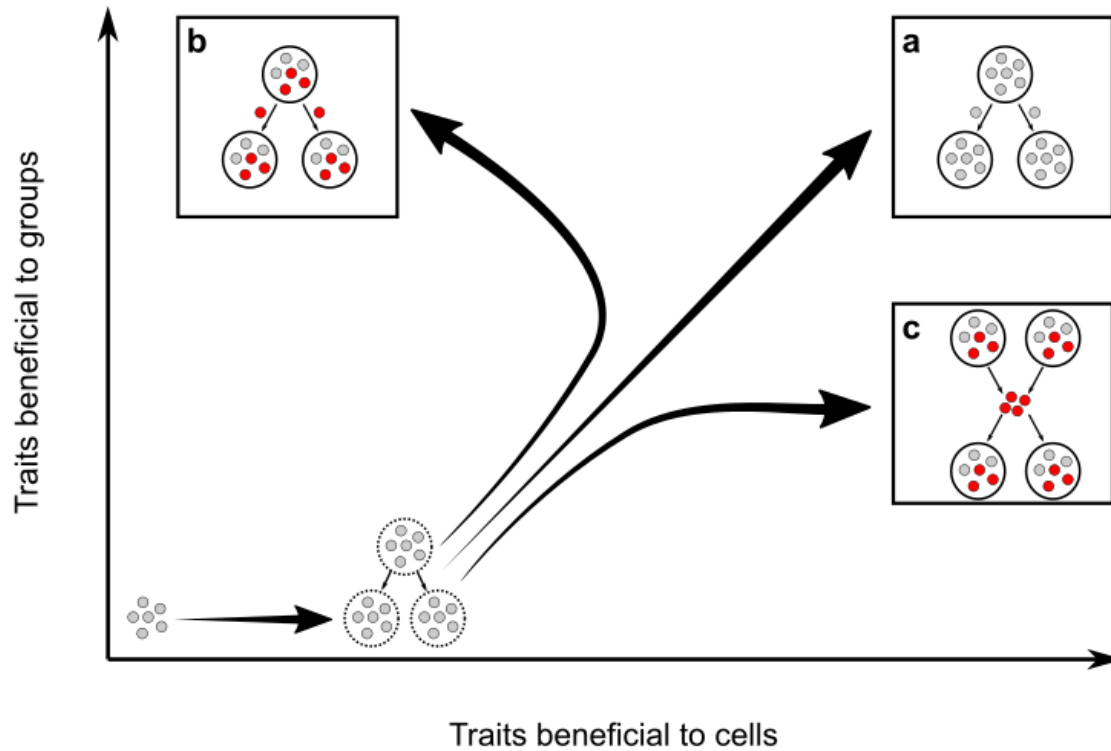
406 **Figure 8. The origins of life cycles and the notion of fitness decoupling**. Mode of group reproduction via

407 a) fragmentation, b) a germ line (red) in a highly structured population and c) a germ line with propagule

408 mixing, affects the emergence of individuality. Mode of group reproduction impacts the relationship

409 between two levels of selection: the cell level (relative to the free-living state), and that of the emerging

410 group. a) illustrates an example of a group that reproduces by fragmentation where fitness is 'coupled':

411 group fitness is a by-product of the fitness of the constituent cells. Larger groups contain more cells and

412 produce more offspring. This holds even when the reproductive life cycle involves a single-celled

413 bottleneck (9) – a feature that is expected to reduce within-group competition. b) and c) show examples

414 of groups that reproduce via a life cycle involving two cell types – one soma-like and the other germ-like.

415 Such two-phase life cycles allow possibility for traits determining a necessary developmental programme

416 to evolve independent of the growth rate of cells that comprise the nascent organism. This paves the way

417 for the emergence of new kinds of biological individual where group fitness 'decouples' from cell fitness.

418 Another possibility explored in the present study considers the impact of population structure on the

419 fitness spectrum of the two levels. Contrast the life cycle illustrated b) (Non-Mixed Ecology) with an

420 identical two-phase life cycle that incorporates competition during the propagule phase of emerging

421 multicellular groups (c), Mixed Ecology). While the existence of a germ line can effect the decoupling of

422 fitness required to achieve a higher level of individuality, competition between cells skews selection

423 towards traits that enhance the competitive ability of cells, rather than towards traits that enhance

424 functioning of the life cycle as a whole.

19

425 While the number of WS cells (and total number of cells) was expected to increase over
426 the course of the Mixed Ecology, it is curious that this ecology also resulted in a
427 reduction in the number of SM cells (Figure 4a). One might have predicted that the
428 number of SM cells would also increase in both ecologies because the life cycle
429 necessitates switching between the two cell states. Furthermore, the Mixed Ecology
430 places additional pressure on competition between SM cells during the propagule mixing
431 step, yet the number of SM cells was not maximised. This surprising outcome becomes
432 understandable when the trade-off brought about by the two-phase life cycle is
433 considered. A negative correlation between the traits linked to group fitness (WS-SM
434 transition rate) and cell fitness (WS density) (Figure 6) reveals that groups in the Mixed
435 Ecology could not increase the total number of both WS and SM cells simultaneously.
436 Intense competition between WS cells in the Mixed Ecology inadvertently reduced the
437 number of SM cells, to the detriment of the functioning of the life cycle as a whole.

438

439 Conflict between the two levels of selection is an inevitable consequence of the trade-off
440 underpinning the 'decoupled' fitness that is essential for an ETI. The importance of
441 ecology during an ETI becomes more apparent when we consider that a brand new
442 maladapted level of selection emerges from well-adapted lower-level individuals. The
443 new level has not yet accumulated adaptations to ensure their own survival when
444 confronted with opposing pressure from the lower level. Nevertheless, new fragile
445 higher-level individuals may withstand this 'threat from below' if they have the
446 opportunity to evolve in a physically separated K-selected environment. Such conditions
447 favour persistence of the group and promote the accumulation of adaptations that
448 contribute to the stability and integration of the group as a whole.

449

450 Integration is an important component, alongside a bottleneck and a germline, for
451 paradigmatic group reproduction (5). Innovations that contribute to the integration of
452 groups encompass features such as non-reproductive division of labour, the mutual
453 dependence (loss of autonomy) of parts and a boundary surrounding the group (5). After
454 a period of selection for traits that favour the persistence of the group in a K-selected
455 environment, more integrated and well-adapted higher-level individuals may be equipped

456   to withstand a less structured ecology. In other words, a structured environment can

457   provide the ecological scaffolding to support persistence during an initial period of

458   evolution in which complex adaptations can arise and prevail over selection solely for

459   growth rate. Upon removal of the scaffold, such features, such as boundaries that

460   demarcate groups, allow groups to continue to function as evolutionary individuals in a

461   less structured environment.

462

463   Upon the emergence of a degree of integration, groups are likely to withstand a higher

464   level of within-group conflict. Extant multicellular organisms tolerate varying degrees of

465   cell-level selection, as evidenced by the diverse modes of multicellular reproduction that

466   incorporate intense competition at the gamete level. Many plants, for example, engage in

467   synchronous seed dispersal – a life cycle not unlike that depicted in Figure 8c. Cancer is a

468   classic example of lower-level selection subsisting in multicellular organisms that is

469   largely contained by selection at the higher level (cancers generally arise later in life,

470   after reproduction (34)). In polyandrous animals, sexual selection also occurs at two

471   levels: a higher level with competition between individuals for mating, and a lower level

472   with competition between sperm for fertilization of eggs within female genital tracts. This

473   lower level has often been shown to account for a large fraction of total variance in male

474   fitness (and hence of the opportunity for selection); for example, 46% in red jungle fowl

475   (35), or 40% in snails (36). Competition between units of the lower level (i.e., germ cells)

476   is extreme in many aquatic invertebrates during broadcast spawning. Here, the animals

477   (higher level) never meet as sperm and eggs (lower level) are released into the water

478   column, where competition for fertilization takes place.

479

480   Contrast the innovation made possible by evolution in a *K*-selected environment with

481   extant multicellular assemblages that have evolved in less physically structured

482   environments. True slime molds (Myxomycetes), and social Myxobacteria, for example,

483   exhibit rather sophisticated features such as 'wolf-pack feeding' that allow cells to benefit

484   from group-living (37). Cellular slime molds such as the Dictyostelids can form

485   multicellular fruiting bodies when their food supply is exhausted (38). All of these groups

486   exhibit rudimentary multicellular life cycles with cellular differentiation, and yet they

487   have remained relatively simple for millions of years and appear not to have become
488   paradigmatic units of selection at the group level. This may be due, at least in part, to
489   ecological factors that maintain a high degree of competition between cells from different
490   groups during certain phases of their respective life cycles. It is also likely that the
491   aggregative mode of group formation ('coming together') inhibits the process of selection
492   at the aggregate level, compared to groups that form by growth from propagules ('staying
493   together') (39). It is interesting to note that in the experiments presented here, the benefits
494   (to group fitness) of 'staying together' were negated in the Mixed Ecology, which had
495   more resemblance to the 'coming together' mode of group organisation during Phase II of
496   the life cycle.

497

498   The notion of natural selection occurring between groups in a meta-population has
499   historically been shrouded by controversy. This is partly a consequence of the term
500   'group selection' being used to describe both selection on groups (where groups are units
501   of selection in their own right (34, 40, 41), and selection on individuals within group-
502   structured populations (where traits costly to individuals are the focus of attention (5, 42-
503   44). Here, as previously (9), we have experimentally demonstrated that group selection in
504   which groups compete with one another via a death-birth process can transition
505   individuality, whereas meta-populations that experience a 'trait-group' ecology do not,
506   despite the evolving collectives experiencing life cycles involving both bottleneck and a
507   germ line phases. Nowadays, the theoretical possibility of paradigmatic group selection is
508   generally recognised, but often dismissed as a rare occurrence that is unlikely to be of
509   importance in nature (44). However, the very existence of a biological hierarchy in which
510   higher-level individuals are composed of groups of lower-level individuals demonstrates
511   that during the course of life's evolution, groups have acquired Darwinian properties and
512   become units of selection in their own right. The likelihood of this kind of group
513   selection is of little consequence to its implications – complex multicellular organisms
514   are known to have evolved just three times in 3.5 billion years.

515

Methods

Experimental regime

We have previously published the Non-Mixed Ecology treatment in a study that compared its effect relative to a life cycle without reproductive specialisation (9). Here we compare the effect of meta-population structure on the potential for an ETI. Groups of cells ('microcosms') in both the Non-Mixed and Mixed meta-population ecologies of the present study experience identical two-phase life cycles driven by frequency-dependent selection. More specifically, each of the Non-Mixed and Mixed meta-population ecologies comprised of 15 replicates of eight competing groups that were founded with *P. fluorescens* strain SBW25 (45), and propagated through ten generations of evolution (one generation equated to one WS-SM-WS life cycle (9).

Phase I (Maturation Phase**):** Each group was founded by a single WS colony. Microcosms were incubated under static conditions for six days, after which they were checked for the presence of an intact mat at the air-liquid interface. If the mat was not intact, that line was deemed extinct. All microcosms with viable mats were mixed by vortexing and then, either individually diluted and plated on solid media (Non-Mixed Ecology), or pooled prior to plating (Mixed Ecology). Agar plates were subsequently screened for SM colonies. Lines without SM colonies were deemed extinct, while those with SM propagules proceeded to Phase II.

Phase II (Dispersal Phase)**:** All SM colonies were individually transferred to 200 ml liquid medium and incubated for 24 h under static conditions. Thereafter they were pooled and used to inoculate Phase II microcosms. After three days of incubation under static conditions (during which new WS mats emerged), all microcosms in both treatments were individually plated on solid agar. The most dominant WS morphotype on each agar plate was selected to inoculate the next generation of the life cycle. If there were no WS colonies on the plate, the microcosm was deemed extinct. Figure 1 contrasts the death-birth process of group competition in the Non-Mixed Ecology, with the physical mixing mode of competition in the Mixed Ecology.

23

Fitness assay and life cycle parameters

Cell-level and group-level fitness were assayed after ten life cycle generations: 15 representative clones (one per replicate population) were generated from each of the evolved treatments, in addition to 15 ancestral WS lines (each independently isolated from the earliest mats to emerge from the ancestral SM strain SBW25) (as described in detail in (9). Three replicate competition assays were performed for one group generation against a neutrally marked ancestral competitor (46).

Our proxy for group-level fitness is the proportion of evolved 'offspring' mats relative to the marked reference strain, and cell-level fitness the total number of cells in the mat after Phase I. Density of WS and SM cells, and Proportion of SM cells were also assayed after Phase I. The growth rate of SM cells was determined from three biological replicate SM colonies per line (for details on how the SM were obtained, see (9)) in 96-well microtitre plates shaken at 28°C, and absorbance (OD600) measured in a microplate reader (BioTek) for 24h. The experiment was repeated three times and the maximum growth rate (Vmax) was calculated from the maximum slope of absorbance over time. The transition rate between WS and SM cells, i.e., the level of SM occurrence in Phase I, and WS occurrence in Phase II, was determined in a separate experiment, where static microcosms were individually inoculated with single colonies of the representative WS types (Phase I). Phase I was extended from 6 to 12 days, and Phase II from 3 to 6 days. At day six, SM cells were collected for Phase II, and microcosms inoculated. Each day, three replicate microcosms per line were destructively harvested and the occurrence, i.e. the microcosms with SM, and number of SM and WS colony forming units recorded.

Statistical analysis

For detecting differences in group-level fitness and transition rate between cells of the evolved and ancestral lines, generalized linear models (error structure: binomial; link function: logit) with the explanatory variables Ecology, and representative clone (nested within Ecology) were calculated. Analyses of variance (ANOVA) were used to test for differences in cell-level fitness, density of WS cells, and density, proportion, and growth rate of SM cells between the evolved and ancestral lines. Explanatory variables were

24

575 Ecology, and representative clone (nested within Ecology). Posthoc tests revealed

576 differences between the evolved and ancestral lines. Relationships between the traits and

577 cell and group-level fitness were tested using the mean per representative type accounting

578 for regime. Pearson correlations and regressions were performed. The sample size was

579 chosen to maximise statistical power and ensure sufficient replication. Assumptions of

580 the tests, i.e., normality and equal distribution of variances, were visually evaluated. All

581 tests were two-tailed. Effects were considered significant at the level of $P = 0.05$. All

582 statistical analyses were performed with JMP 9. Figures were produced with GraphPad

583 Prism 5.0, Adobe Illustrator CC 17.0.0 and Inkscape 0.92.3.

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References

1. Buss LW (1987) *The Evolution of Individuality* (Princeton University Press).

2. Smith JM, Szathmáry E (1995) *The Major Transitions in Evolution* (Oxford University Press).

3. Michod RE (1999) *Darwinian Dynamics* (Princeton University Press).

4. Okasha S (2006) *Evolution and the Levels of Selection* (Oxford University Press).

5. Godfrey-Smith P (2009) *Darwinian Populations and Natural Selection* (OUP Oxford).

6. Griesemer J (2001) The Units of Evolutionary Transition. *Selection* 1(1-3):67–80.

7. Rainey PB (2007) Unity from conflict. *Nature* 446(7136):616–616.

8. Rainey PB, Kerr B (2010) Cheats as first propagules: A new hypothesis for the evolution of individuality during the transition from single cells to multicellularity. *BioEssays* 32(10):872–880.

9. Hammerschmidt K, Rose CJ, Kerr B, Rainey PB (2014) Life cycles, fitness decoupling and the evolution of multicellularity. *Nature* 515(7525):75–79.

10. Libby E, B Rainey P (2013) A conceptual framework for the evolutionary origins of multicellularity. *Physical Biology* 10(3):035001.

11. Bourrat P (2015) Levels of selection are artefacts of different temporal fitness measures. *Ratio*, 2*8*(1): 40-50.

12. Rainey PB, Remigi P, Farr AD, Lind PA (2017) Darwin was right: where now for experimental evolution? *Current Opinion in Genetics & Development* 47:102–109.

13. P B (2015) Levels of selection are artefacts of different temporal fitness measures. *Ratio* 28:40–50.

14. Michod RE (1983) Population Biology of the First Replicators: On the Origin of the Genotype, Phenotype and Organism. *Am Zool* 23(1):5–14.

15. Szathmáry E, Demeter L (1987) Group selection of early replicators and the origin of life. *Journal of Theoretical Biology* 128(4):463–486.

617   16.   Szathmáry E (2015) Toward major evolutionary transitions theory 2.0.
618   *Proc Natl Acad Sci USA* 112(33):10104–10111.

619   17.   Rainey PB, Travisano M (1998) Adaptive radiation in a heterogeneous
620   environment. *Nature* 394(6688):69–72.

621   18.   Rainey PB, Rainey K (2003) Evolution of cooperation and conflict in
622   experimental bacterial populations. *Nature* 425(6953):72–74.

623   19.   McDonald MJ, Gehrig SM, Meintjes PL, Zhang XX, Rainey PB (2009)
624   Adaptive divergence in experimental populations of *Pseudomonas*
625   *fluorescens*. IV. Genetic constraints guide evolutionary trajectories in a
626   parallel adaptive radiation. *Genetics* 183(3):1041–1053.

627   20.   Lind PA, Farr AD, Rainey PB (2015) Experimental evolution reveals
628   hidden diversity in evolutionary pathways. *eLife* 4:441.

629   21.   Spiers AJ, Kahn SG, Bohannon J, Travisano M, Rainey PB (2002) Adaptive
630   divergence in experimental populations of *Pseudomonas fluorescens*. I.
631   Genetic and phenotypic bases of wrinkly spreader fitness. *Genetics*
632   161(1):33–46.

633   22.   Goymer P, et al. (2006) Adaptive divergence in experimental
634   populations of *Pseudomonas fluorescens*. II. Role of the GGDEF regulator
635   WspR in evolution and development of the wrinkly spreader phenotype.
636   *Genetics* 173(2):515–526.

637   23.   Bantinaki E, et al. (2007) Adaptive divergence in experimental
638   populations of *Pseudomonas fluorescens*. III. Mutational origins of
639   wrinkly spreader diversity. *Genetics* 176(1):441–453.

640   24.   Trivers RL (1971) The evolution of reciprocal altruism. *The Quarterly*
641   *Review of Biology* 46(1):35–57.

642   25.   Maynard Smith J. *Evolutionary Progress and Levels of Selection*, ed Nitecki
643   MH, pp 219–230.

644   26.   Sober E, Wilson DS *Unto Others: The Evolution and Psychology of*
645   *Unselfish Behavior* (Harvard University Press).

646   27.   Strassmann JE, Zhu Y, Queller DC (2000) Altruism and social cheating in
647   the social amoeba *Dictyostelium discoideum*. *Nature* 408(6815):965–967.

648   28.   Velicer GJ, Kroos L, Lenski RE (2000) Developmental cheating in the
649   social bacterium *Myxococcus xanthus*. *Nature* 404(6778):598–601.

650   29.   Pfeiffer T, Schuster S, Bonhoeffer S (2001) Cooperation and competition

651   in the evolution of ATP-producing pathways. *Science* 292(5516):504–
652   507.

653   30.   Michod RE, Roze D (1999) Cooperation and conflict in the evolution of
654   individuality. III. Transitions in the unit of fitness. *Mathematical and*
655   *Computational Biology Computational Morphogenesis, Hierarchical*
656   *Complexity, and Digital Evolution*, ed Nehaniv CL (eebweb.arizona.edu),
657   pp 47–92.

658   31.   van Gestel J, Tarnita CE (2017) On the origin of biological construction,
659   with a focus on multicellularity. *Proc Natl Acad Sci USA* 114(42):11018–
660   11026.

661   32.   Grosberg RK, Strathmann RR (2007) The Evolution of Multicellularity: A
662   Minor Major Transition? *Annu Rev Ecol Evol Syst* 38(1):621–654.

663   33.   MacArthur RH, Wilson EO (1967) *Monographs in Population Biology*
664   (Princeton University Press).

665   34.   Nunney L (1999) Lineage selection and the evolution of multistage
666   carcinogenesis. *Proc Biol Sci* 266(1418):493–498.

667   35.   Collet J, Richardson DS, Worley K, Pizzari T (2012) Sexual selection and
668   the differential effect of polyandry. *Proc Natl Acad Sci USA*
669   109(22):8641–8645.

670   36.   Pélissié B, Jarne P, Sarda V, David P (2014) Disentangling precopulatory
671   and postcopulatory sexual selection in polyandrous species. *Evolution*
672   68(5):1320–1331.

673   37.   Bonner JT (1998) The origins of multicellularity. *Integrative Biology:*
674   *Issues, News, and Reviews* 1(1):27–36.

675   38.   Strmecki L, Greene DM, Pears CJ (2005) Developmental decisions in
676   *Dictyostelium discoideum*. *Developmental Biology* 284(1):25–36.

677   39.   Tarnita CE, Taubes CH, Nowak MA (2013) Evolutionary construction by
678   staying together and coming together. *Journal of Theoretical Biology*
679   320:10–22.

680   40.   Wynne-Edwards VC (1962) *Animal Dispersion, in Relation to Social*
681   *Behavior*.

682   41.   Doebeli M, Ispolatov Y, Simon B (2017) Towards a mechanistic
683   foundation of evolutionary theory. *eLife* 6:347.

684   42.   Wilson DS (1975) A theory of group selection. *Proc Natl Acad Sci USA*

685   72(1):143–146.

686 43.   Hamilton WD (1964) The genetical evolution of social behaviour. II.
687   *Journal of Theoretical Biology* 7(1):17–52.

688 44.   West SA, Griffin AS, Gardner A (2007) Evolutionary Explanations for
689   Cooperation. *Current Biology* 17(16):R661–R672.

690 45.   Silby MW, et al. (2009) Genomic and genetic analyses of diversity and
691   plant interactions of *Pseudomonas fluorescens*. *Genome Biology*
692   10(5):R51.

693 46.   Zhang X-X, Rainey PB (2007) Construction and validation of a neutrally-
694   marked strain of *Pseudomonas fluorescens* SBW25. *Journal of*
695   *Microbiological Methods* 71(1):78–81.

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Author contributions

704 All authors contributed to the conception and design of the study. K.H. and C.J.R.
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707
Additional information

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