Meta-population structure and the evolutionary transition to multicellularity

Caroline J. Rose[1,2]*, Katrin Hammerschmidt[1,3]* & Paul B. Rainey[1,4,5]

[1]New Zealand Institute for Advanced Study, Massey University, Auckland, New Zealand
[2]Present address: Centre d'Écologie Fonctionnelle et Évolutive (CEFE), CNRS, Montpellier, France
[3]Present address: Institute of General Microbiology, Kiel University, Kiel, Germany
[4]Present address: Max Planck Institute for Evolutionary Biology, Department of Microbial Population Biology, Plön 24306, Germany and [5]École Supérieure de Physique et de Chimie Industrielles de la Ville de Paris (ESPCI Paris-Tech), CNRS UMR 8231, PSL Research University, Paris, France.

*These authors contributed equally to this work.

Author ORCIDs
Caroline J Rose https://orcid.org/0000-0001-7133-035
Katrin Hammerschmidt https://orcid.org/0000-0003-0172-8995
Paul B Rainey https://orcid.org/0000-0003-0879-5795

Correspondence and requests for materials should be addressed to PBR rainey@evolbio.mpg.de

Classification: BIOLOGICAL SCIENCES

Keywords: Multicellularity, Meta-population, Group Selection

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

Life is hierarchically structured. Multicellular organisms are comprised of cells, cells contain organelles; the nucleus contains chromosomes comprised of genes (1, 2). The origin of this structure reflects successive evolutionary transitions in individuality (ETIs) with lower level particles being subsumed within higher-level self-replicating entities (2). Central to each transition was the emergence of Darwinian properties at the new (group) level. With the emergence of such properties came new kinds of biological individuals that participated directly in the process of evolution by natural selection (2-5).

Explanations for the evolution of multicellular life centre on the origins of group reproduction (6-9). Modes that involved early discovery of life cycles replete with reproductive specialisation are of particular significance (1, 5, 8, 10). Such cycles establish a second (longer) timescale over which selection operates (5, 11-13). This permits the possibility of a birth death process at the group level. Additionally, life cycles require a developmental process – an altogether new trait that is a property of the group – that necessarily becomes a focus of selection.

While a developmental life cycle is important (9), it is not sufficient. The mere existence of the fundamental requirements for the evolution of multicellularity (e.g., a bottleneck and reproductive specialisation) does not guarantee selection at the higher level. Selection simultaneously continues to operate at the lower level, favouring the reproductive success of cells at the expense of the group to which they belong. This poses a general problem, which lies at the heart of any ETI – how can the higher level successfully constrain the lower level? This poses a very particular challenge during early phases of an ETI. Theoretical studies on the origins of cells provide clues (14, 15). Theoretical models show that selection at the higher level can be achieved through specific population structures: when replicators are individually isolated, selection remains focussed on the lower level – that of the individual replicators (14). However, when replicators are localized in reproducing compartments within a meta-population of compartments, the focus of selection is the higher level (15, 16). We therefore hypothesize that a specific population structure that embraces innovation in primordial life cycles while escaping the

burden of lower level selection is a crucial requirement for the transition to multicellularity. Competition is key, however the level at which rudimentary groups compete depends primarily upon meta-population structure and the partitioning of variation that this population structure imposes.

Our experiments using a model bacterial system (*Pseudomonas fluorescens*) have shown how life cycles can emerge from negative frequency dependent interactions between two ecologically distinct phenotypes that each give rise to the other (9). One type is defined by sticky cells that produce a soma-like mat at the air-liquid interface allowing access to oxygen, while the other is marked by non-sticky dispersing cells that function as proxy for a germ line. The mat forming type performs an ecological role, but also gives rise to propagules that are seeds of the next generation of mats. Selection operating at the level of the nascent multicellular organisms via a group-level death-birth process led to enhanced fitness of groups (relative ability of groups to give rise to offspring groups), while fitness of the individual cells was reduced relative to the ancestral types.

These findings gave substance to the suggestion that life cycles, in conjunction with a death-birth process at the level of collectives (lineage selection), provide opportunities for selection to transition to higher levels of organisation and that a two-phase life cycle (underpinned by a developmental programme) is of particular importance, but much remains unexplored. Of particular relevance is the effect of the population structure: the nature of the environment affects how variation is partitioned within and between groups. A highly structured environment as in the experiments of Hammerschmidt *et. al.* (9), which is analogous to models of compartment structure of early replicators (15), eliminates the possibility of mixing between evolving lineages thus ensuring strong competition between groups. Less structure would likely intensify competition within groups and possibly decrease opportunity for group-level evolution.

Here we empirically consider the impact of population structure on the emergence of individuality – that is, we examine the relative emphasis of interactions within and between groups – on the fitness spectrum of the two levels. We contrast the life cycle

from our previously published results (9) (Non-Mixed Ecology treatment; Figure 1a) – with an identical two-phase life cycle that incorporates competition (mixing) during the propagule phase of emerging multicellular groups. This environmental manipulation, which we term the Mixed Ecology treatment (Figure 1b), was performed simultaneously with the earlier study. We show that competition effected during the propagule phase of a two-stage life cycle leads selection to favour traits that promote cell growth at the expense of traits underlying group fitness. This conflict between the two levels of selection is due to a trade-off between traits underlying the fitness of groups and their cells. While the existence of a germ line can bring about the decoupling of fitness required to achieve a higher level of individuality, intense competition between cells nevertheless skews selection towards traits that enhance the competitive ability of cells, rather than towards traits that enhance the functioning of the life cycle as a whole.

Results

The evolution of simple groups has been observed in experiments using populations of *P. fluorescens* propagated in spatially structured microcosms (17-20). Such groups constitute a plausible starting position from which to explore transitions in Darwinian individuality from cells to multicellular groups (7, 8, 18). In these experiments, cooperative groups arise from single mutant "wrinkly spreader" (WS) cells that overproduce a cell-cell glue; the failure of cells to separate at cell division leads to the formation of mats that colonize the air-broth interface (19, 21-23). Glue production is costly to individual cells, but the trait spreads because cells within the mat reap a reward (access to oxygen) that is denied to free-living cells (24). Cooperating groups are therefore inevitably short-lived (18, 25-29). Selection continues to act at the level of individual cell by favouring mutant "smooth" (SM) types that cheat by no longer producing glue, but nonetheless gain the advantages of being part of the group (18).

The negative frequency-dependent selection described above creates a cycling between cooperative WS cells and non-cooperative SM cells (10). When viewed from the long-term perspective of the group, this oscillation drives a rudimentary life cycle that confers

on the group the potential to reproduce by means of dispersing 'germ line' cells (8). The group may thus evolve by natural selection (9). Each generation begins with WS cells forming a mat at the air-liquid interface (Figure 1). For a mat to reproduce it must be both viable and fecund, i.e., produce SM germ line cells (Phase I in Figure 1).

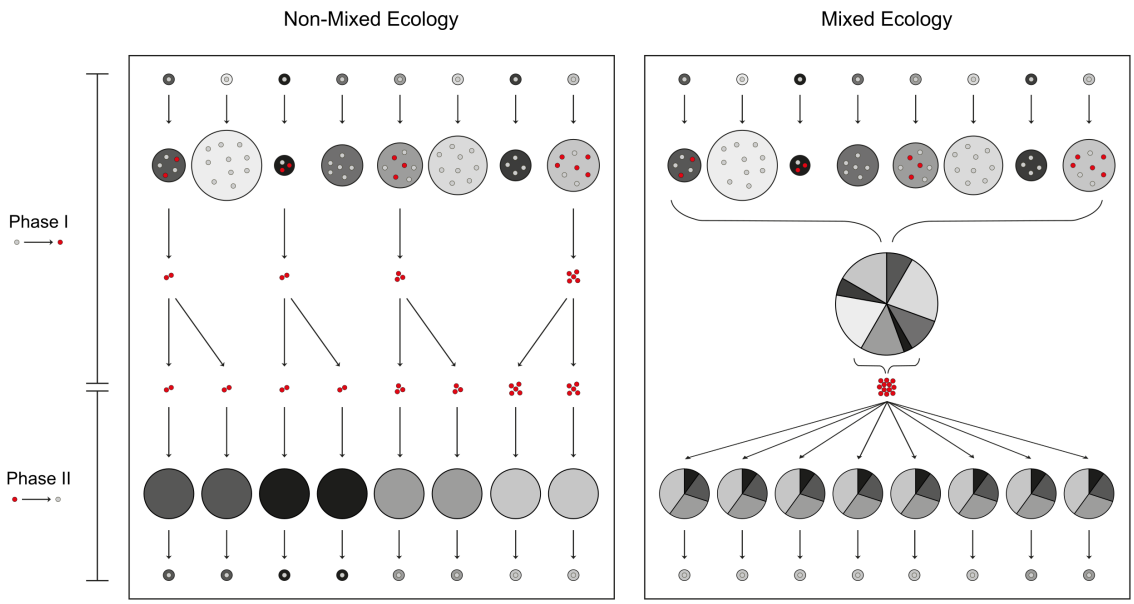


Figure 1. Schematic depiction of a population of eight genetically distinct groups (indicated by different shades of grey) proceeding through one life cycle within their respective ecologies. Grey circles = WS "soma" cells, red circles = SM "germ" cells. Variation is maintained between groups in the Non-Mixed Ecology, while a single-cell bottleneck curtails within-group variation. Propagule mixing eliminates between-group variation during Phase II of the Mixed Ecology.

We have shown previously that this life cycle results in the 'decoupling' of fitness[1] between groups and their cells (9), however this alone does not guarantee the emergence of individuality. In the present study we examine this life cycle in two ecological scenarios in order to understand how the partitioning of variation (and therefore competition and selection) can help or hinder the emergence of individuality. In the Non-

[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.

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).

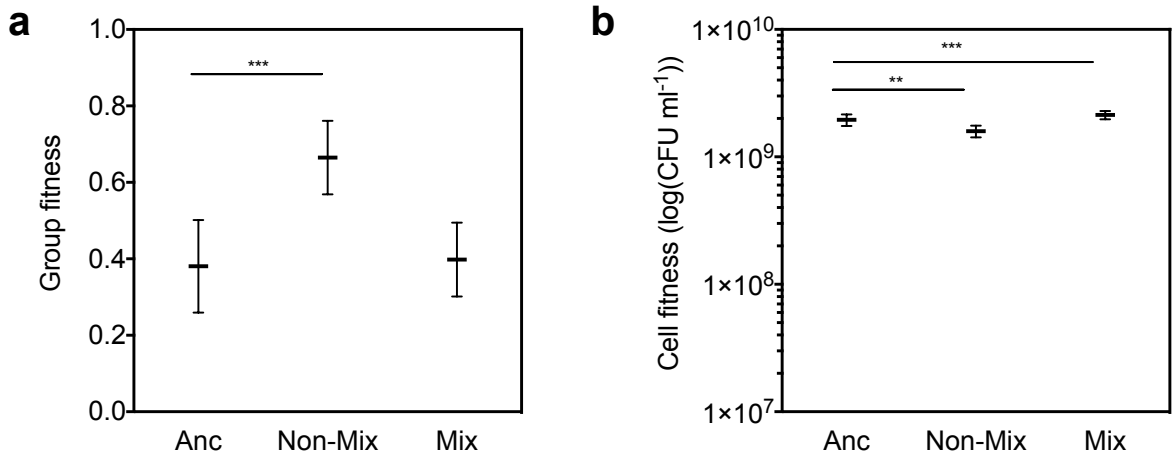


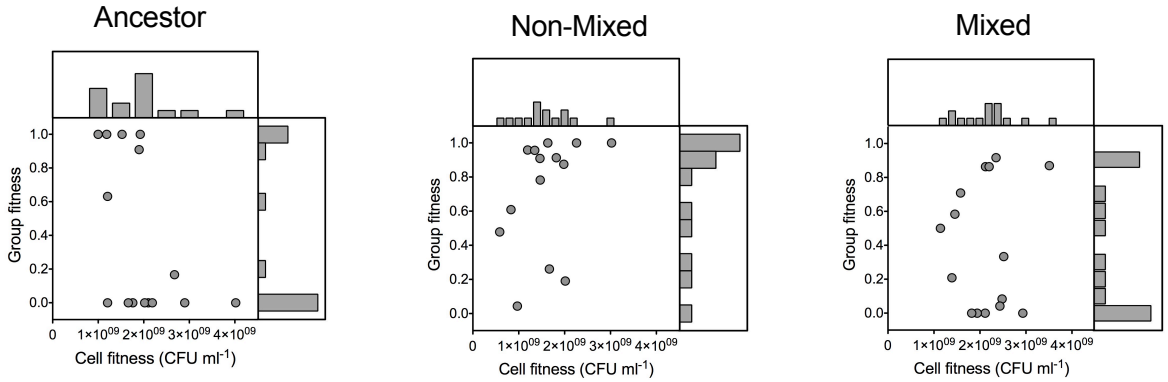
Figure 2. Changes in group (a) and cell (b) fitness in the Non-Mixed Ecology (Non-Mix) and Mixed Ecology (Mix) regimes compared to ancestral populations (Anc). Group fitness is the proportion of derived offspring mats relative to a genetically marked reference genotype. Error bars are s.e.m., based on 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.

Trade-off between group and cell fitness

Figure 3 (left panel) illustrates a negative relationship between cell and group fitness in the ancestral lines (χ^2=4.246, d.f.=1, P=0.0393). Ancestral clones also displayed a bimodal distribution of group fitness, suggesting that a trade-off exists between traits underpinning cell and group fitness. Ten generations of selection in the Non-Mixed Ecology shifted the distribution towards the 'high group fitness/low cell fitness' corner of the graph (Figure 3, middle panel), indicating that group-level selection was more potent than cell-level selection under the Non-Mixed population ecology. In the Mixed Ecology (Figure 3, right panel), the absence of change in group fitness and increase in cell fitness, in addition to the trade-off between cell and group fitness inherent in the life cycle, suggests that tension exists between the two levels of selection when groups are propagated under this regime.

The contrasting response to selection of groups subjected to the Non-Mixed and Mixed ecologies likely results from the two ways in which groups interact, which has major consequences for the resulting distribution of variation. Only cells within the Mixed groups interact physically, however groups in the Non-Mixed Ecology nevertheless

208 participate in fitness-affecting interactions; the death of one group directly affects the
209 fitness of the other groups in the population.
210



211
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

Finally, the rate of transition between WS and SM cells dramatically increased under the Non-Mixed Ecology (χ^2=114.198, d.f.=1, P<0.0001, and also to a lesser extent under the Mixed Ecology (χ^2=12.459, d.f.=1, P=0.0004; Figure 4e). This is consistent with the requirement - in both ecologies - for groups to cycle through the life cycle by transitioning between the two cell states.

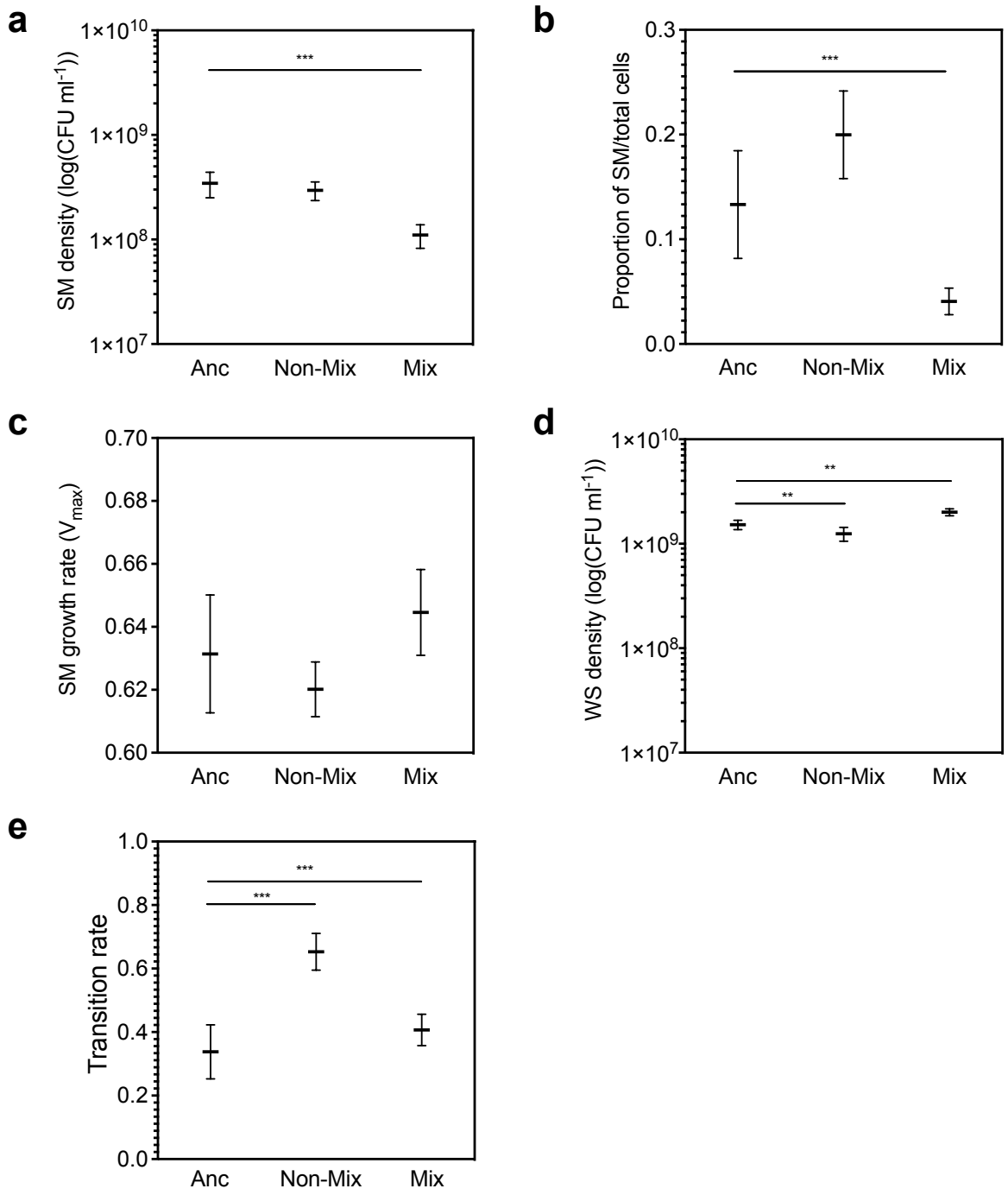


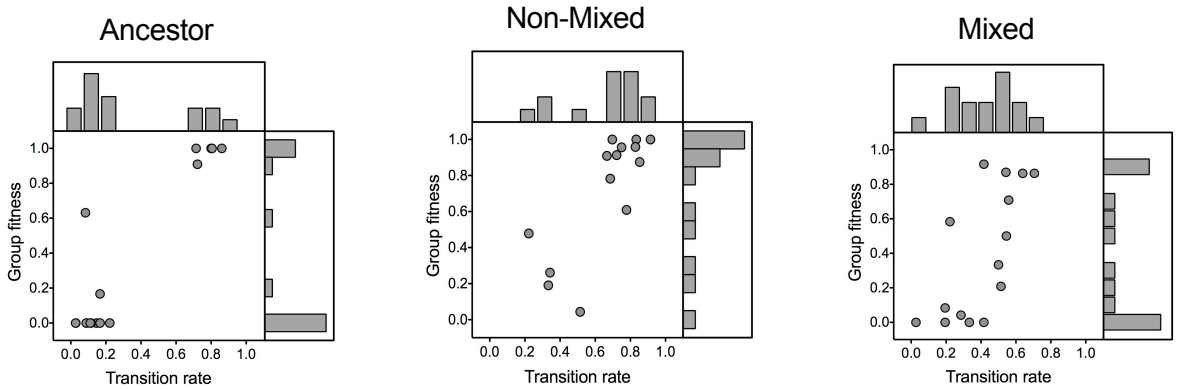
Figure 4. Changes in life cycle traits in the Non-Mixed (Non-Mix) and Mixed (Mix) Ecologies compared to the ancestral populations (Anc): (a) SM density, (b) Proportion of SM, (c) SM growth rate, (d) WS density, (e) Transition rate. Error bars are s.e.m., based on n = 14 (Non-Mix) and n = 15 (Anc, Mix).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

Identification of parameters linked to group and cell fitness

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

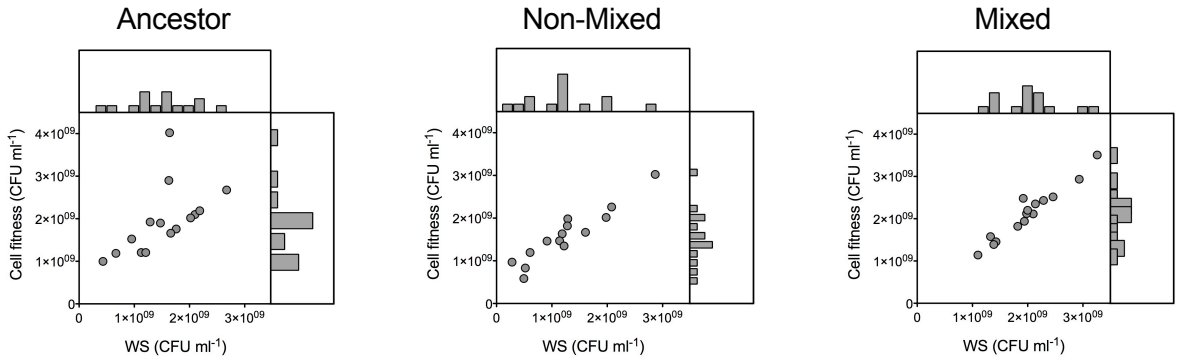
Cell Fitness in the ancestral lines is strongly associated with the Density of WS cells (F_1 = 6.673, P = 0.023; Figure 5b, left panel). The distribution of both parameters increased during the Mixed Ecology (F_1 = 200.931, P < 0.0001; Figure 5b, right panel) and decreased during the Non-Mixed Ecology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

288 **a**



289

290 **b**



291

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

301

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

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

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

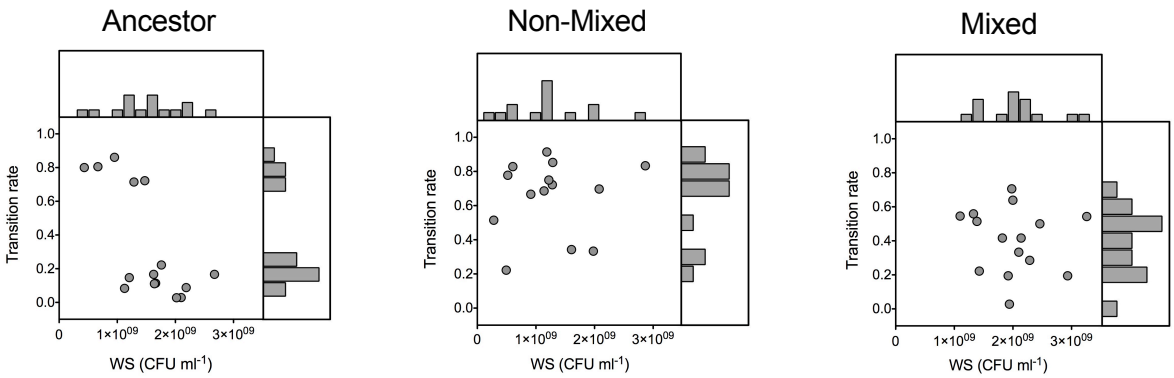


Figure 6. Relationship between WS density and transition rate in the ancestral populations, and in the Non-Mixed and Mixed Ecologies. Dots represent the mean of eight lines per replicate population, which were assessed three independent times.

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

Table 1. Effects of the meta-population structure on the level of selection. The red box highlights selection during different phases of the Mixed Ecology for two incompatible traits (parameters that are 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

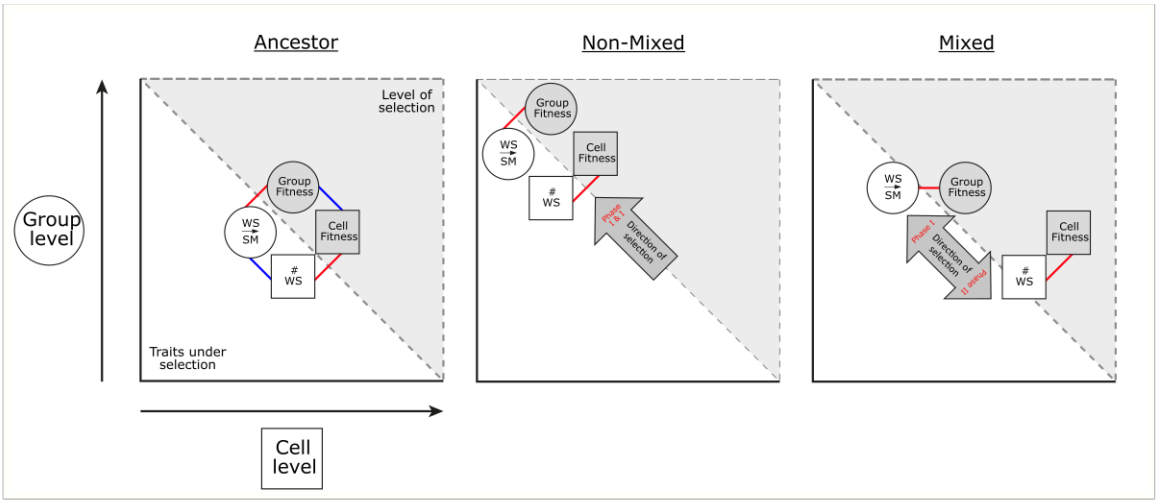


Figure 7. Relationships between the traits underpinning cell and group fitness. Cell and group fitness are depicted in grey, while the traits underpinning them are white. Red lines indicate a positive correlation; blue lines indicate a negative association. After ten generations of selection in the Non-Mixed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

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

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.
404

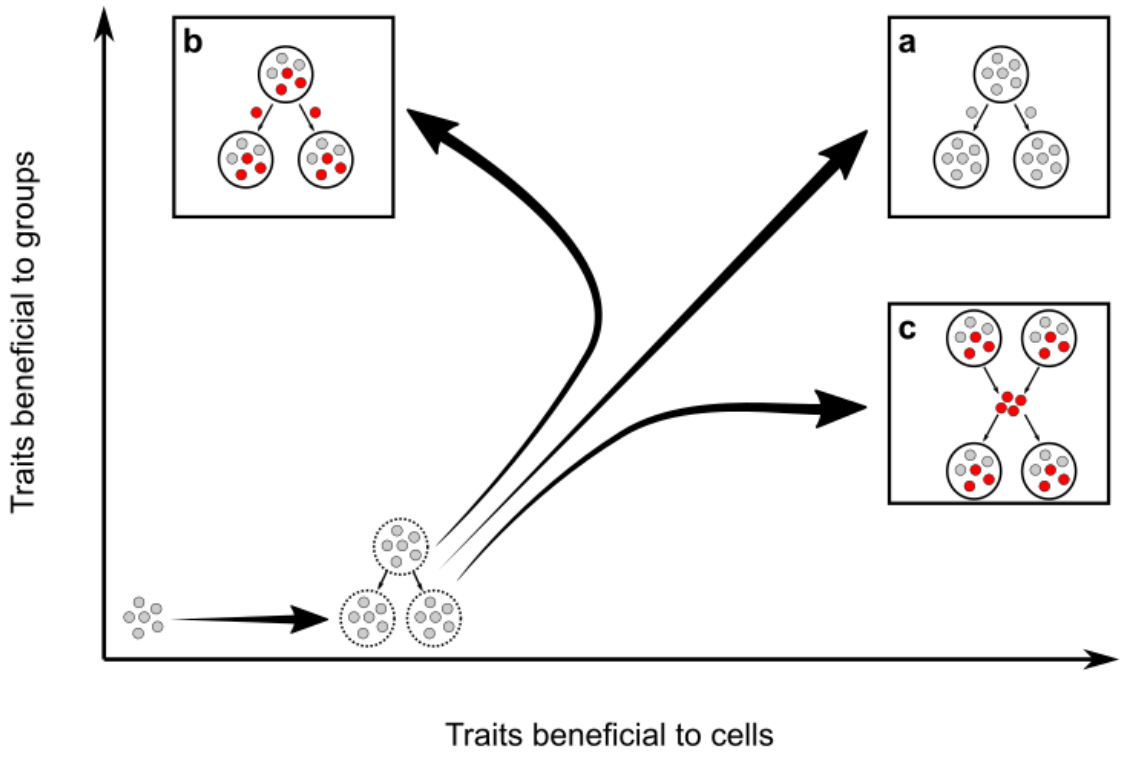


Figure 8. The origins of life cycles and the notion of fitness decoupling. Mode of group reproduction via a) fragmentation, b) a germ line (red) in a highly structured population and c) a germ line with propagule mixing, affects the emergence of individuality. Mode of group reproduction impacts the relationship between two levels of selection: the cell level (relative to the free-living state), and that of the emerging group. a) illustrates an example of a group that reproduces by fragmentation where fitness is 'coupled': group fitness is a by-product of the fitness of the constituent cells. Larger groups contain more cells and produce more offspring. This holds even when the reproductive life cycle involves a single-celled bottleneck (9) – a feature that is expected to reduce within-group competition. b) and c) show examples of groups that reproduce via a life cycle involving two cell types – one soma-like and the other germ-like. Such two-phase life cycles allow possibility for traits determining a necessary developmental programme to evolve independent of the growth rate of cells that comprise the nascent organism. This paves the way for the emergence of new kinds of biological individual where group fitness 'decouples' from cell fitness. Another possibility explored in the present study considers the impact of population structure on the fitness spectrum of the two levels. Contrast the life cycle illustrated b) (Non-Mixed Ecology) with an identical two-phase life cycle that incorporates competition during the propagule phase of emerging multicellular groups (c), Mixed Ecology). While the existence of a germ line can effect the decoupling of fitness required to achieve a higher level of individuality, competition between cells skews selection towards traits that enhance the competitive ability of cells, rather than towards traits that enhance functioning of the life cycle as a whole.

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

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

Integration is an important component, alongside a bottleneck and a germline, for paradigmatic group reproduction (5). Innovations that contribute to the integration of groups encompass features such as non-reproductive division of labour, the mutual dependence (loss of autonomy) of parts and a boundary surrounding the group (5). After a period of selection for traits that favour the persistence of the group in a *K*-selected 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

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

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

516 # Methods

517 **Experimental regime**

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

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

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

544

545 **Fitness assay and life cycle parameters**

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

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

567

568 **Statistical analysis**

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

Ecology, and representative clone (nested within Ecology). Posthoc tests revealed differences between the evolved and ancestral lines. Relationships between the traits and cell and group-level fitness were tested using the mean per representative type accounting for regime. Pearson correlations and regressions were performed. The sample size was chosen to maximise statistical power and ensure sufficient replication. Assumptions of the tests, i.e., normality and equal distribution of variances, were visually evaluated. All tests were two-tailed. Effects were considered significant at the level of $P = 0.05$. All statistical analyses were performed with JMP 9. Figures were produced with GraphPad Prism 5.0, Adobe Illustrator CC 17.0.0 and Inkscape 0.92.3.

585 **References**

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

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

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

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

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

595 6. Griesemer J (2001) The Units of Evolutionary Transition. *Selection* 1(1-
596 3):67–80.

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

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

601 9. Hammerschmidt K, Rose CJ, Kerr B, Rainey PB (2014) Life cycles, fitness
602 decoupling and the evolution of multicellularity. *Nature* 515(7525):75–
603 79.

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

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

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

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

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

615 15. Szathmáry E, Demeter L (1987) Group selection of early replicators and
616 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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

72(1):143–146.

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

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

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

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

Acknowledgements

We thank B. Kerr, E. Libby and Y. Pichugin for discussion, and Pierrick Bourrat for comments on drafts of the manuscript. The work was directly supported by the Marsden Fund Council from government funding administered by the Royal Society of New Zealand, and in part by grant RFP-12-20.

Author contributions

All authors contributed to the conception and design of the study. K.H. and C.J.R. performed research, undertook data analysis and prepared figures. All authors wrote the paper.

Additional information

Competing financial interests: The authors declare no competing financial interests.