Meta-population structure and the evolutionary transition to multicellularity

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Abstract

The evolutionary transition to multicellularity occurred on numerous occasions, but transitions to complex life forms were rare. 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 the relative emphasis on the developmental phase versus the dispersal phase was altered. While all groups possessed paradigmatic features of multicellular individuals including bottlenecks and germ lines, the mode of group interaction substantially impacted both the strength and direction of selection operating at group and cell levels. Constraints on meta-population structure may therefore explain the observation that multicellular aggregates rarely complete the transition to individuality.

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[5,11,12]. This permits the possibility of a birth death process at the group level. Additionally, life cycles require a developmental process – an altogether new phenotypic trait that is a property of the group – that necessarily becomes a focus of selection.

Over the long term, selection rewards those cells that facilitate persistence of the group. This means cells whose reproductive fate aligns with that of the most successful groups will ultimately enjoy greatest success. Relative to the ancestral free-living single-celled state, cells of the nascent multicellular organism, when viewed from the perspective of the shorter time scale of cell doubling time, are likely to appear less fit[9]. This comparison is the basis of the concept of fitness "decoupling"[13] – 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.

A recent experiment using a model bacterial system showed 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 process of lineage

selection led to enhanced fitness of groups, while fitness of the individual cells was reduced relative to the ancestral types. In a separate experiment where the same mat-forming cells transitioned through a life cycle involving fragmentation of the mat to a single cell, and with exclusion of the non-sticky propagule state, enhanced fitness of groups was also observed, but this was explained by increased cell fitness.

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 are ecological factors that shape the evolution of life cycles. Indeed, ecology is central: 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] eliminates the possibility of mixing between evolving lineages thus ensuring strong competition between groups. Less structure would intensify competition within groups and possibly decrease opportunity for group-level evolution.

Here we extend previous work by examining the impact of ecological structure on the emergence of individuality. We contrast our previously published results (Non-Mixed Ecology treatment; Hammerschmidt *et. al.*[9] (where it was referred to as the "cheat-embracing" regime)) with an environmental manipulation that was performed simultaneously with the earlier study and which we term the Mixed Ecology treatment (Figure 1). We show that competition effected during the propagule phase of a two-state life cycle leads selection to favour traits that promote cell growth at the expense of traits underlying group fitness.

Results

The evolution of simple groups has been observed in experiments using populations of *Pseudomonas fluorescens* propagated in spatially structured microcosms[14-17]. Such groups constitute a plausible starting position from which to explore transitions in Darwinian individuality from cells to multicellular groups[7,8,15]. 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[16,18-20]. 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[21]. Cooperating groups are therefore inevitably short-lived[15,22-26]. 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[15].

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 itself, 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). SM germ line cells compete to transition to WS, and ultimately for mat formation. To avoid chimeric mat organisms, a population bottleneck is achieved by transferring one colony of the most dominant WS type to a fresh microcosm (Phase II in Figure 1)[9].

Changes in group and cell fitness

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

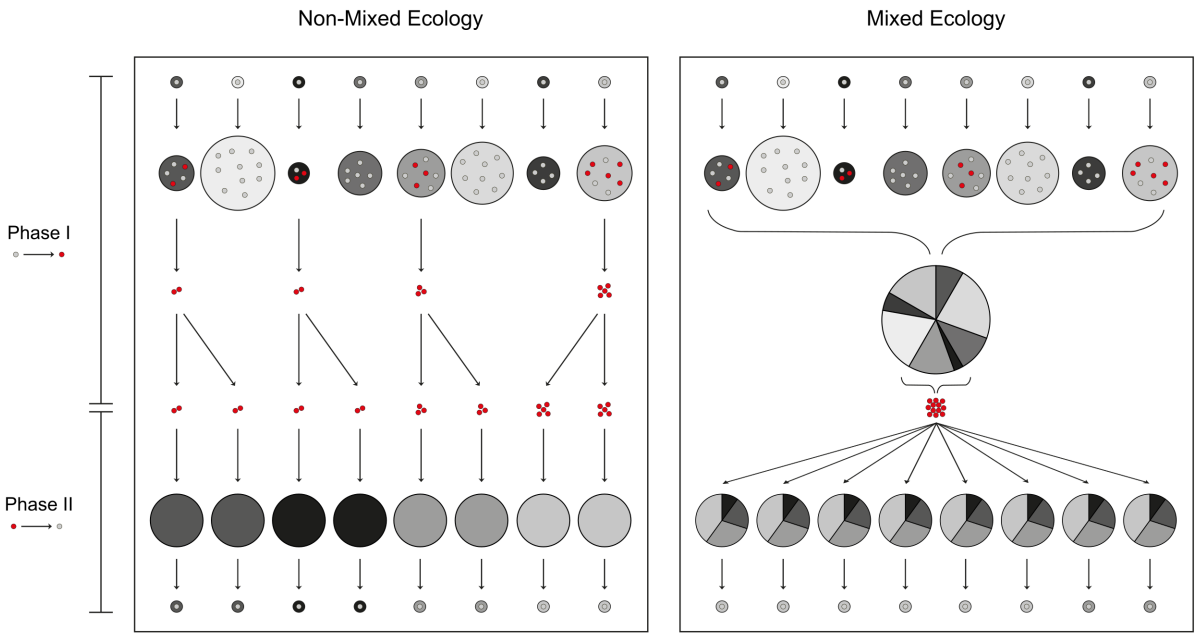

Figure 1. Schematic depiction of a population of eight genetically distinct groups (indicated by different shades of grey) proceeding through one life cycle within their respective ecologies. Phase I (Maturation Phase**):** Each group was founded by a single WS (grey) colony. Microcosms were incubated 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 (red) 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. Non-Mixed Ecology: Competition between groups resulted from a death-birth process: following an extinction event (usually due to the lack of SM production[9]), a group was randomly replaced by a surviving competitor group. SM cells were harvested separately from each surviving group at the end of Phase I. Extinction/replacement of groups occurred with high frequency[9] and therefore imposed potent between-group selection. Mixed Ecology: Following Phase I extinction events, competition between surviving groups resulted from a mixing step: All groups were mixed and SM cells harvested. The mixture of SM cells seeded all eight groups in Phase II.

Derived lines in the Non-Mixed Ecology significantly increased group fitness (ability to leave group offspring) relative to the ancestral types (χ^2=32.660, d.f.=1, P<0.0001; Figure 2a), whereas cell fitness (number of cells present immediately prior to dispersal) decreased (F_1 = 10.612, P =

0.002; Figure 2b). In contrast, under the Mixed Ecology, group fitness did not change (χ^2=3.137, d.f.=1, P=0.077; Figure 2a), whereas cell fitness increased (F_1 = 56.214, P < 0.0001; Figure 2b).

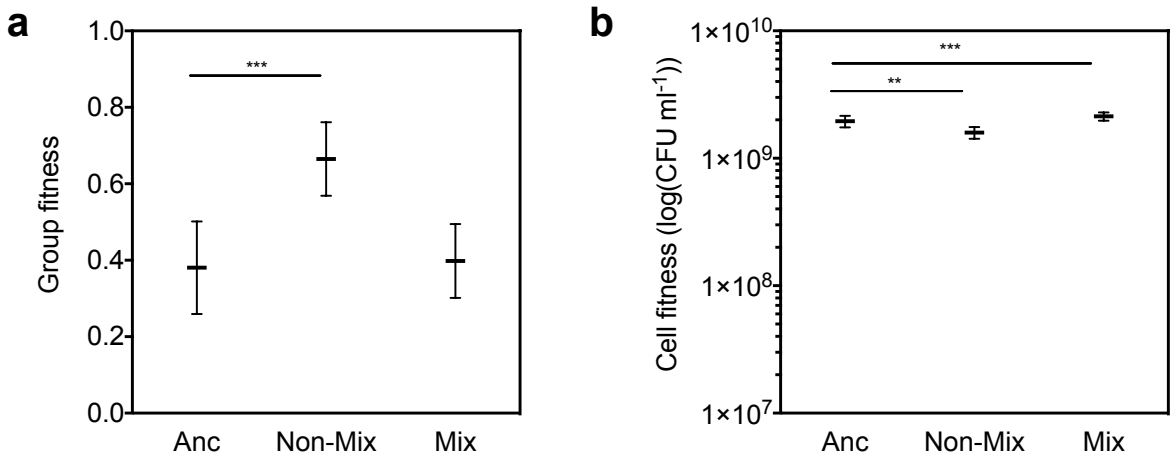

Figure 2. Changes in group (a) and cell (b) fitness in the Non-Mixed Ecology (Non-Mix) and Mixed Ecology (Mix) regimes compared to ancestral populations (Anc). Group fitness is the proportion of derived offspring mats relative to a genetically marked reference genotype. Error bars are s.e.m., based on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

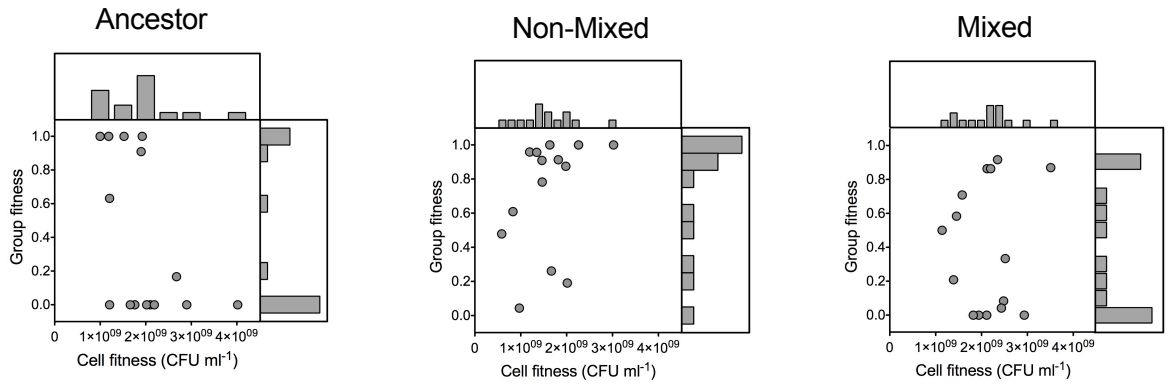

Figure 3. Relationship between cell and group fitness in the Non-Mixed and Mixed Ecologies compared to ancestral populations. Group fitness is the proportion of derived offspring mats relative to a genetically marked reference genotype. Dots represent the mean of eight lines per replicate population,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

In both ecologies, the life cycle began in Phase I with a single-celled bottleneck, and therefore variation existed only between groups (regardless of population ecology) until within-group variation emerged during maturation (Figure 1). Phase II Non-Mixed groups are also seeded with dispersal cells originating from single parent groups, resulting again in high levels of between-group variation and relatively low levels of within-group variation. This between-group variation led to competition and therefore selection between groups, resulting in increased group fitness. A consequence of strong group-level selection was a reduction in cell fitness due to the negative relationship between cell and group fitness (Figure 3).

The direction of selection along this negative fitness spectrum was more complicated in the Mixed Ecology. Phase II began with eight identical groups so variation, competition and therefore selection occurred only within groups (Figure 1), leading to increased cell fitness. The mixing step has consequences not only for the partitioning of variation but also has downstream effects on the overall amount of variation. Competition within groups (between cells) during Phase II likely resulted in the same single WS cell type ultimately seeding all eight groups in

Phase I of the next generation. Consequently, the limited between-group competition in Phase I did not overcome the considerable within-group selection towards the 'high cell fitness/low group fitness' corner of the fitness spectrum during Phase II, because between-group variation is essentially removed during Phase II of the previous generation.

Changes in life cycle parameters

To explore specific adaptations contributing to differences in fitness between the two ecologies, we assayed parameters expected to be key to thriving in the multicellular life cycle. Despite the importance that the life cycle places on the appearance of SM cells during mat maturation, the Density, Proportion, and Growth Rate of SM cells did not change significantly during evolution under the Non-Mixed Ecology (density: $F_1 = 1.278$, $P = 0.2663$; proportion: $F_1 = 2.702$, $P = 0.1095$; growth rate: $F_1 = 2.116$, $P = 0.1522$), and in fact SM Density and Proportion of SM cells decreased during the Mixed Ecology (density: $F_1 = 56.214$, $P < 0.0001$; proportion: $F_1 = 102.217$, $P < 0.0001$; growth rate: $F_1 = 2.664$, $P = 0.1103$; Figure 4a,b,c).

Interestingly, the Density of WS cells decreased under the Non-Mixed Ecology ($F_1 = 8.036$, $P = 0.0065$), and increased under the Mixed Ecology ($F_1 = 9.904$, $P = 0.0027$; Figure 4d. The two population ecologies reflect the differences in the emphasis of selection (or lack thereof) on the number of WS cells arising during Phase II. Phase II culminates in selection of the single most dominant WS type to enter the bottleneck and seed Phase I of a next generation. Therefore, competition between WS types is intensified when groups are mixed prior to entering Phase II; WS cells arising from one parent group must outcompete alternative WS cell types arising from *all* other groups in order to survive the Mixed Ecology. In contrast, the WS cells arising in Phase II of the Non-Mixed Ecology need only outcompete any alternative WS cell types that may (or may not) arise within the same group.

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.

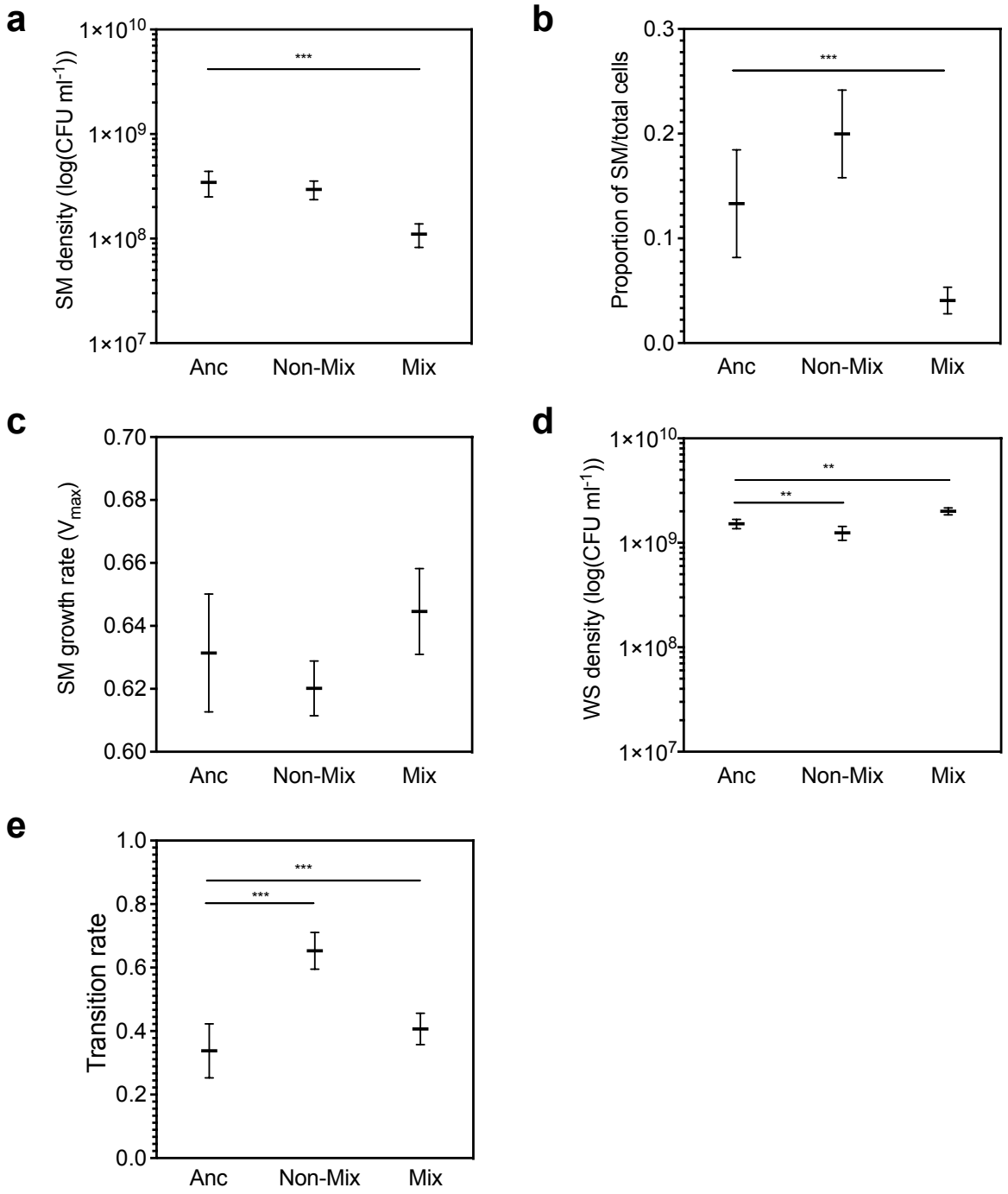

Figure 4. Changes in life cycle traits in the Non-Mixed (Non-Mix) and Mixed (Mix) Ecologies compared to the ancestral populations (Anc): (a) SM density, (b) Proportion of SM, (c) SM growth rate, (d) WS density, (e) Transition rate. Error bars are s.e.m., based on n = 14 (Non-Mix) and n = 15 (Anc, Mix).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

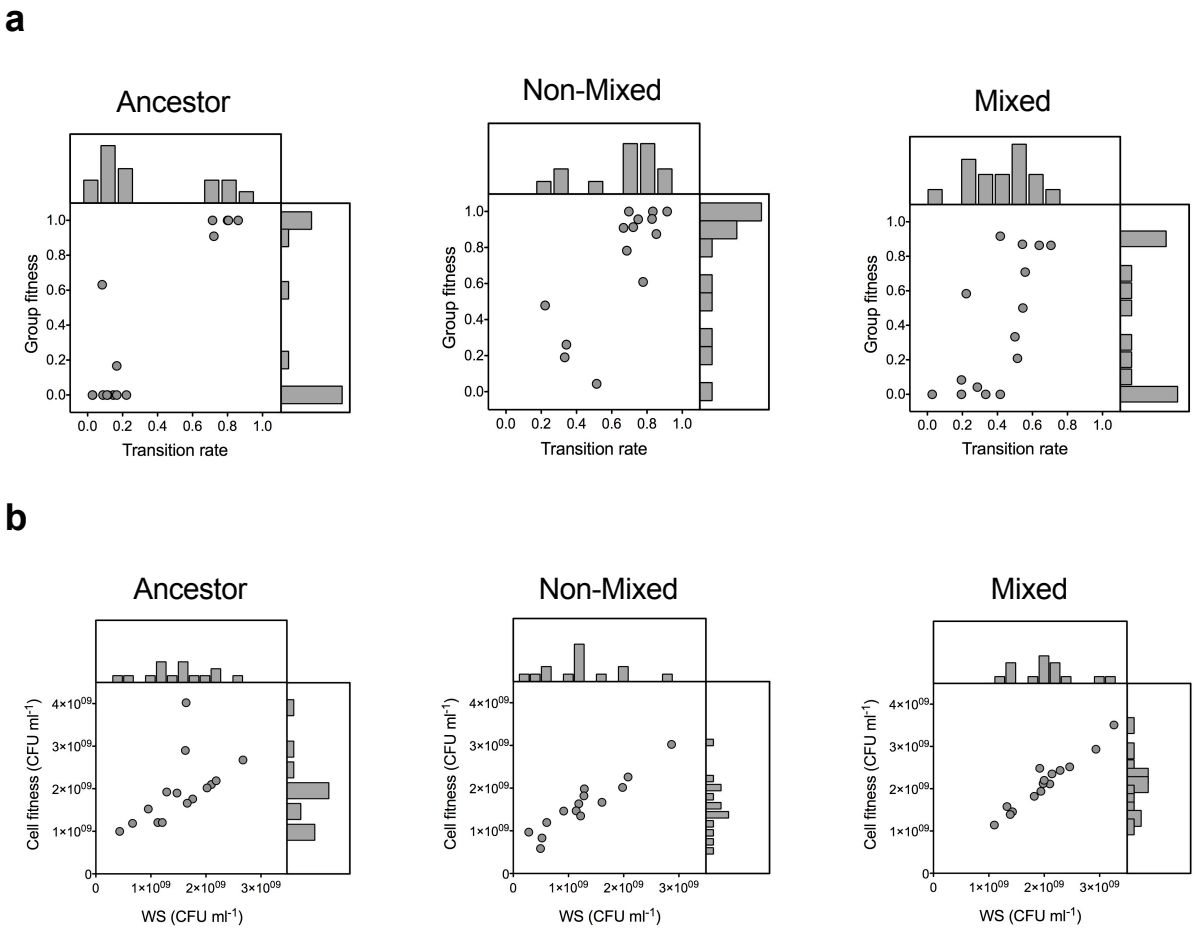

Figure 5. Relationship between life cycle traits and group and cell fitness. (a) Association of transition rate and group fitness in the ancestral populations, and in the Non-Mixed and Mixed Ecologies. (b) WS density is positively associated with cell fitness in the ancestral populations, and in the 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; 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).

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 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.

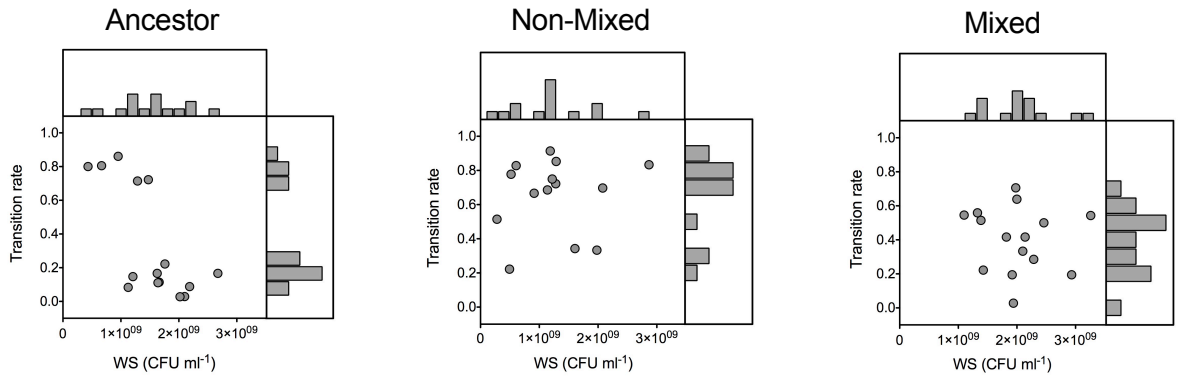

Figure 6. Relationship between WS density and transition rate in the ancestral populations, and in the Non-Mixed and Mixed Ecologies. Dots represent the mean of eight lines per replicate population, which were assessed three independent times.

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 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

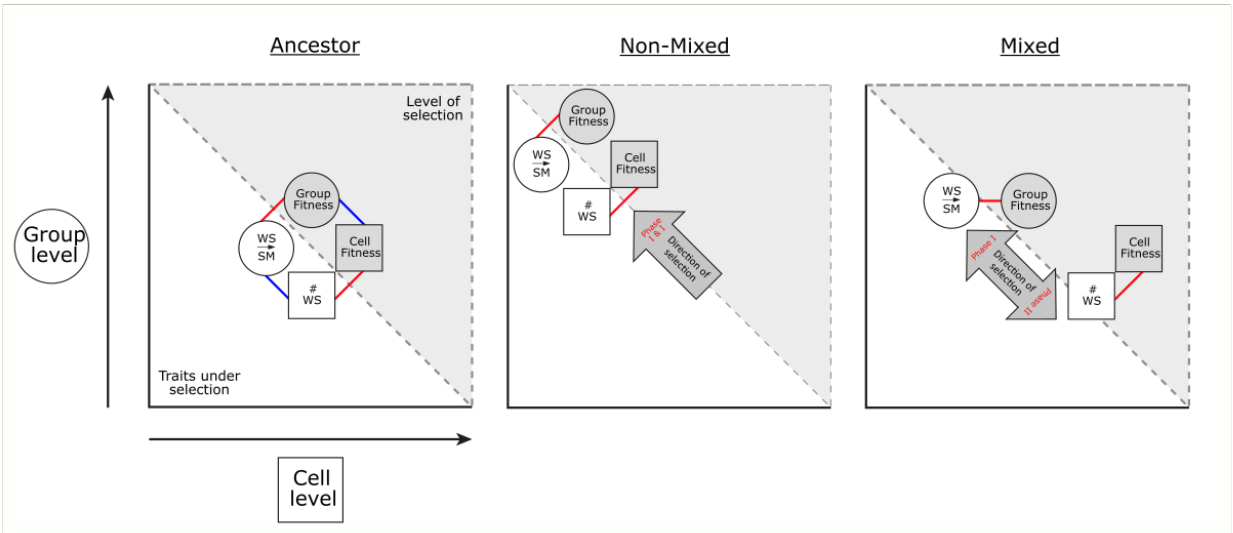

Figure 7. Relationships between the traits underpinning cell and group fitness. Cell and group fitness are depicted in grey, while the traits underpinning them are white. Red lines indicate a positive correlation; blue lines indicate a negative association. After ten generations of selection in the Non-Mixed 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 capacity to reproduce has implications for their ability to transition to groups that come to participate in natural selection in their own right (Figure 8)[1,5,8,10,27]. 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[28]. 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].

While a developmental life cycle is necessary to transition individuality, it is not sufficient. The mere existence of the fundamental requirements for the evolution of multicellularity (e.g., a bottleneck, reproductive specialisation) does not guarantee selection at the higher level. As multicellularity emerges, any trade-off that exists between traits linked to cell and group fitness allows for the possibility of selection for traits that enhance group fitness at the expense of cell fitness (Figure 8b). However, selection also continues to operate at the lower level, favouring the reproductive success of cells at the expense of the group to which they belong. Therefore, another crucial requirement for an evolutionary transition in individuality is specific environmental conditions that embrace innovation in primordial life cycles while escaping the burden of lower level selection. Competition is key, however the level at which rudimentary groups compete depends primarily upon the meta-population structure and the partitioning of variation that this population structure entails. This can lead to selection favouring either the lower or higher level when the fitnesses of the two levels are in opposition (compare Figure 8b to Figure 8c).

In the present study, we imposed group competition in 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, germline, and the resulting 'decoupled' fitness) but in one experimental treatment groups were pooled prior to dispersal. Both treatments had a multilevel selection aspect, and by

pooling groups we were able to manipulate the availability of niche space to offspring groups, thus altering the relative emphasis of selection on the two phases of this life cycle.

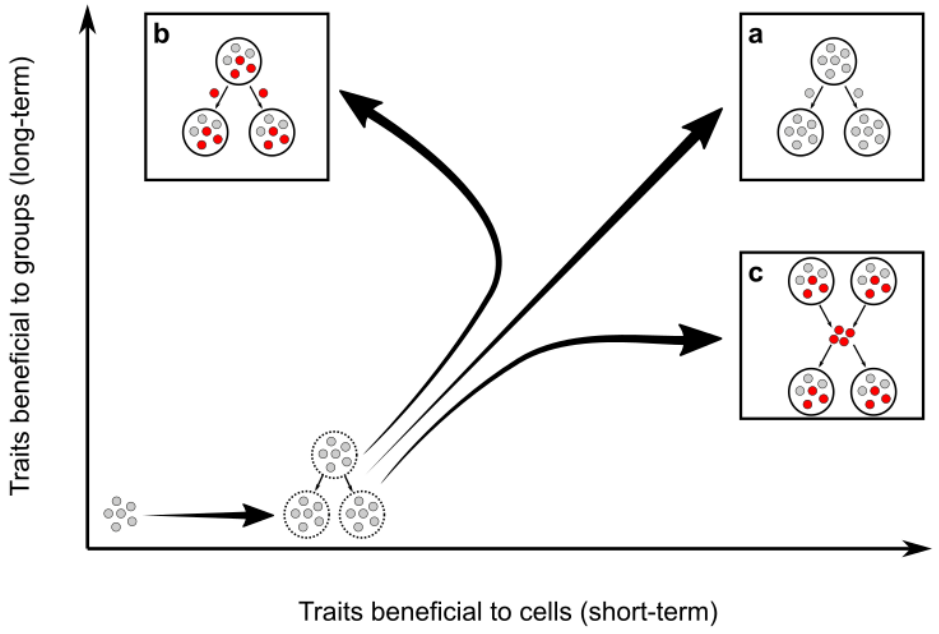

Figure 8. The origins of life cycles and the notion of fitness decoupling. The mode of group reproduction impacts the relationship between two levels of selection: the cell level (relative to the free-living state), and that of the emerging group. Free-living cells (bottom left corner) may gain some benefit by living in groups. If these groups reproduce, there exists the possibility of a Darwinian process at the level of the group, and the emergence of traits and associated fitness at this 'higher' level. Box (a) illustrates an example of a group that reproduces by fragmentation. This mode of reproduction represents a case of 'coupled' fitness at the two levels; group fitness is merely a by-product of the fitness of the constituent parts. Larger groups contain more particles 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. The 'higher level' simply represents lower-level population structure rather than a true higher level of individuality. Selection of traits that benefit cells within groups will also increase the fitness of the groups and visa versa. Such traits are not emergent properties of the group and do not contribute to an increase in complexity. Needed for the emergence of a true higher level of individuality is selection of traits that evolve independently of cell growth rate. One possibility emerges when we consider a group reproduction stage involving a second phenotype (b). The moment two cell types contribute to group function and reproduction, there exists a two-phase life cycle (a life cycle involving soma and a germ line). Traits that enhance the operation of the life cycle itself can evolve independently of, or even in opposition to, the growth rate of cells in either of the two phases of the life cycle. This is the idea behind the concept of fitness decoupling thought to occur during the early stages of an evolutionary transition in individuality. It is nonsensical to attempt to tease apart the two levels of selection in extant multicellular organisms, however, if the free-living ancestral cells were available, it would be possible to measure the process of fitness decoupling during the very early stages of an evolutionary transition while there still exists the possibility of quantifying the growth of cells within the emerging multicellular individual. This is the approach we have used previously when contrasting life cycles analogous to (a) and (b)[9]. A third

possibility explored in the present study considers the impact of ecology - that is, the relative emphasis of interactions within and between groups – on the fitness spectrum of the two levels. Contrast the life cycle illustrated in Box (b) (Non-Mixed Ecology) with an identical two-phase life cycle that incorporates competition during the propagule phase of emerging multicellular groups (Box (c), Mixed Ecology). While the existence of a germ line can effect 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.

Groups in the Non-Mixed Ecology could multiply to occupy new niches only upon the death of another 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 (Figure 4e), which facilitated the almost guaranteed detection of a new cell type. The emphasis on persistence over cell number in this structured environment is akin to a *K*-selected ecological framework[29]. In contrast, mixing populations prior to dispersal created an environment resembling an *r*-selected ecology in which cells from all groups within each meta-population competed for all niches in the next generation[29]. Groups in the two ecologies experienced identical environments during maturation, however in the dispersal phase of the Mixed Ecology the most numerous WS cell type from all eight groups in the population was selected to seed the next generation. The resulting selection for increased WS cell density was therefore crucial for groups to prevail under such ecological pressure. Whereas the *r*-selected ecology experienced by populations in the Mixed Ecology placed the emphasis of selection on cell numbers, the *K*-selected Non-Mixed groups were released from the pressure for fast growth, providing an opportunity for innovation.

The two ecologies in this study are distinguished by the partitioning of variation across the meta-populations. Competition drives natural selection, and variation is crucial for competition. It follows then that the distribution of variation within and between groups determines the relative intensity of natural selection within and between groups. The importance of a bottleneck in multicellular life cycles has been attributed to its effect on the distribution of variation. A bottleneck redistributes variation (innovation) from within the parent to between its offspring[13], allowing selection to operate on different innovations between higher-level individuals. While

both ecologies here experience a single-celled bottleneck during the life cycle, the mixing stage negated this effect because it prevents the redistribution of variation among offspring; on the contrary, all post-mixing offspring are identical.

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 clear 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, of 'paradigm' 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 to withstand a less structured ecology. In other words, a structured environment can provide the ecological scaffolding to support their persistence during the initial period of evolution in which complex adaptations can arise and prevail over selection solely for growth rate. Upon removal of the scaffold, such features, such as boundaries that demarcate groups, allow groups to continue to function as evolutionary individuals in a less structured environment.

Upon the emergence of a degree of integration, groups are likely to withstand a higher level of within-group conflict. Extant multicellular organisms tolerate varying degrees of cell-level selection, as evidenced by the diverse modes of multicellular reproduction that incorporate intense competition at the gamete level. Many plants, for example, engage in synchronous seed dispersal – a life cycle not unlike that depicted in Figure 8c. Cancer is a classic example of lower-level selection subsisting in multicellular organisms that is largely contained by selection at the higher level (cancers generally arise later in life, after reproduction[30]). In polyandrous animals, sexual selection also occurs at two levels: a higher level with competition between individuals for mating, and a lower level with competition between sperm for fertilization of eggs within female genital tracts. This lower level has often been shown to account for a large fraction of total variance in male fitness (and hence of the opportunity for selection); for example, 46% in red jungle fowl[31], or 40% in snails[32]. Competition between units of the lower level (i.e., germ cells) is extreme in many aquatic invertebrates during broadcast spawning. Here, the animals (higher level) never meet as sperm and eggs (lower level) are released into the water column, where competition for fertilization takes place.

Contrast the innovation made possible by evolution in a *K*-selected environment with extant multicellular assemblages that have evolved in less physically structured environments. True slime molds (Myxomycetes), and social Myxobacteria, for example, exhibit rather sophisticated features such as 'wolf-pack feeding' that allow cells to benefit from group-living[33]. In contrast, cellular slime molds such as the Dictyostelids can form multicellular fruiting bodies when their food supply is exhausted[34]. All of these groups 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 ')[35]. 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[30,36,37]), and selection on individuals within group-structured populations (where traits costly to individuals are the focus of attention[5,38-40]). 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[40]. 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.

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[41], and propagated through ten generations of evolution (one generation equated to one WS-SM-WS life cycle[9]. Further details are described in Figure 1 and in Hammerschmidt *et. al.*[9]. 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.

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[42].

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 Ecology, and representative clone (nested within Ecology). 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.

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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

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