Evolution of multicellular reproduction through co-option of ecological interactions

Alexandre P. Fernandes[a,✉], **Renske M. A. Vroomans**[a,b,1], **and Enrico Sandro Colizzi**[a,c,1,✉]

[a]Sainsbury Laboratory, University of Cambridge, 47 Bateman Street, Cambridge, UK CB2 1LR
[b]Department of Genetics, University of Cambridge, Downing Street, Cambridge, UK CB2 3EH
[c]Center INRIA de Lyon, 56 Boulevard Niels Bohr, Villeurbanne, France, 69100

During the transition to multicellularity, cells evolved novel regulatory mechanisms to coordinate cell division and differentiation, which enabled the emergence of group reproduction. These mechanisms were repurposed from molecular and cellular traits that once mediated interactions between single-celled organisms. However, it remains unclear how these traits were evolutionarily integrated to form the first developmental programs in multicellular life. To address this issue, we developed a spatially structured evolutionary model in which cells can migrate, divide, and adhere to their neighbors — behaviors common to most unicellular eukaryotes. When coupled to a selective pressure driven by food scarcity, the model reveals that the ecological context plays a central role in the evolution of multicellular reproduction. Depending on the spatial distribution of food in the environment, both unicellular and multicellular life cycles with diverse modes of reproduction could evolve. Among these were multicellular life cycles that reproduce through unicellular propagules, the most prevalent reproductive strategy in multicellular life, which emerged spontaneously as a dispersal strategy in some environments. We show that these propagules are genetically homologous to the lineage's unicellular ancestors, which were co-opted and repurposed as reproductive structures during evolution. Furthermore, after multicellular lineages with propagules evolved, they could colonize environments that were previously dominated by unicellular life. Altogether, our results show how ecological interactions between single cells can transform into developmental processes during the evolutionary transition towards multicellularity.

multicellularity | evolution | co-option | ecology | development

Correspondence: ***alexandre.porcherfernandes@slcu.cam.ac.uk or enrico-sandro.colizzi@inria.fr***

Significance statement

Reproduction is a universal feature of life. Yet, the evolution of multicellularity transformed it fundamentally: while single-celled organisms reproduce via cell division, reproduction in multicellular organisms is a complex process involving the coordination of many cells. How these new forms of multicellular reproduction first evolved is currently unknown. Using a computational model, we study how group reproduction emerges from the collective dynamics of individual cells. The model shows that unicellular ancestral life cycles can be repurposed as propagules used for reproduction in multicellular species, suggesting that genetic co-option is a key mechanism through which early development evolves.

Introduction

While unicellular organisms reproduce through cell division, multicellular reproduction is a collective process that requires coordination of cell division, collective behavior, and spatial organization to generate a new individual. Modern multicellular organisms have evolved a diverse array of reproductive strategies, which can be broadly divided into two main categories (1, 2): (i) unicellular propagation, where the organism reproduces by generating single-celled reproductive units, and (ii) multicellular propagation, where the organism divides into two multicellular groups of equal or unequal size. Unicellular propagation is abundant across the tree of life, including sexual reproduction through gametes and formation of unicellular vegetative propagules or spores (3, 4). Although not as common as unicellular propagation, multicellular propagation occurs in all major multicellular clades, including bacteria (5, 6), algae (7), fungi (8), plants (9, 10), and animals (11, 12) (see (13, 14) for comprehensive reviews of multicellular life cycles). Additionally, both forms of reproduction have been observed to evolve in wet-lab experiments (15–18).

But what drives the evolution of different reproductive strategies? Computational models have suggested that unicellular propagules reduce genetic conflicts between cells within the multicellular organism (19, 20) or slow down the accumulation of mutations (1), while experimental evolution supports their role in enabling higher-level individuality (21). Conversely, multicellular reproduction can be advantageous in cell clusters with rigid cell connections that limit cell division, enabling cell growth in regions previously constrained by the cluster's geometry (22). More broadly, the selective advantage of unicellular or multicellular replication may depend on how cell birth and death rates scale with group size (23) and on the effect of cooperation between cells in multicellular groups (24).

The ecological context also plays an important role in shaping the evolution of multicellular reproduction. Many organisms have evolved unicellular propagules, such as spores, pollen, or cysts, that can disperse passively via wind or water and are often more resistant to environmental stress than their multicellular progenitors. This makes them well-suited for survival in harsh environments and an effective means of dispersal. Models and experiments suggest that diverse life cycles, including those alternating between unicellular and multicellular stages, can evolve in response to fluctuat-

DRAFT

ing environments (25–28), or arise through self-organisation of multicellular groups that can reproduce autonomously due to adhesion interactions between cells (29). The predicted influence of the environment on the evolution of multicellular reproduction is also evident in nature, where seasonal fluctuations in nutrient availability regulate life cycle transitions in many eukaryotes. For example, nutrient scarcity triggers the multicellular phase in the life cycle of the slime mold *Dictyostelium discoideum*, whereas the Holomycota *Fonticula alba* forms collective feeding streams only when bacterial food is available (30). Conversely, the emerging multicellular group can itself alter the ecological landscape, affecting the evolutionary trajectories of cellular properties (31) and creating new ecological niches (32).

The ecological and genetic contexts have also played a role in the origin of multicellularity itself. As cells formed tighter associations during the transition to multicellularity, new ecological functions — e.g. predation avoidance (15, 18) or enhanced resource capture (33, 34) — selected for traits that increased group sizes, improved the spatial integrity of the cluster, and mediated collective responses to environmental fluctuations (1, 35, 36). Comparative phylogenetic studies show that these selective demands at the onset of multicellularity were largely met by coopting the pre-existing molecular toolkit (37, 38): proteins used for adhesion with the substrate or sensing environmental cues were repurposed for intercellular adhesion (39, 40), while cell-cycle and stress regulators became involved in cellular differentiation (41, 42). However, the contribution of the co-option of these ancestral modules to the evolution of development and reproduction at the onset of multicellularity remains unknown. Promising experimental results show that reproductive propagules can readily evolve after multicellularity, suggesting that they could have evolved via co-option of an ancestral unicellular life cycle (17).

To clarify how ecological pressures influence the spatial organization of cells during reproduction and how the co-option of ancestral modules enables novel ecological functions, we need a modeling framework that integrates the evolution of gene regulation with ecological interactions between cells and their environment (43, 44). Existing models, however, typically lack a spatially-explicit environment, abstract away genetic regulation of reproductive behavior, or restrict the evolution of reproductive strategies to a few, preselected options. To address this issue, we used a bottom-up approach to study the evolution of multicellular reproduction. We developed a model where a population of cells inhabits a structured environment and consumes resources to survive and replicate. We model a minimal set of cellular traits — intercellular adhesion, chemotaxis, and cell division — that are widespread across the eukaryotic tree of life (4). With this setup, we investigate how spatially structured ecological interactions among unicellular individuals give rise to reproduction at the onset of multicellularity. We characterize these developmental dynamics and show that they evolve by co-opting pre-existing life-cycles and are stabilized through an eco-evolutionary feedback.

Results

Model setup. To investigate the evolution of reproductive strategies in nascent multicellular organisms, we constructed a hybrid Cellular Potts Model (45–47) where a population of cells forages for food on two-dimensional space (Fig. 1A). Food is introduced at a constant rate into the environment as circular "patches", each composed of several food units. The food patches release a chemoattractant, creating a gradient around them that cells can follow to locate and consume resources, which are stored as metabolic reserves. If all the food in a food patch is consumed, it stops generating the chemoattractant signal, allowing cells to re-route to the next source of food. Cells have a constant metabolic rate that depletes their metabolic reserves. When the cell's reserves are exhausted, it dies and is removed from the lattice. We also include a small uniform probability of death due to other factors which are not modeled explicitly, such as metabolic stress.

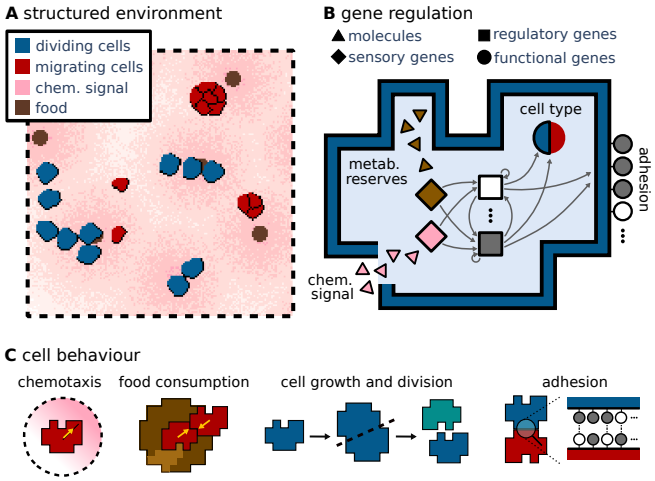

Fig. 1. Model setup. **A** Cells live in a structured environment where they forage for resources by following the chemoattractant signal that diffuses from patches of food. Here, a small section of the 2000x2000 lattice is shown. **B** Diagram of a cell's gene regulation. Cell behavior is controlled by a Boolean GRN that senses the amount of metabolic reserves accumulated in the cell and the strength of the chemoattractant signal. These inputs are processed by a layer of 18 regulatory genes that will determine the cell state (migrating or dividing) and the activation state of the 16 adhesion genes. Active genes are shown in gray, inactive in white. Arrows show the connections between genes in the GRN. **C** We model two cell states: migrating and dividing. Cells can accumulate metabolic reserves by consuming food if they share a lattice site with a food site and adhere to their neighbors when they express complementary combinations of adhesion proteins.

Cellular behavior is controlled by a Gene Regulatory Network (GRN) composed of sensory, regulatory, and functional genes (Fig. 1B). The sensory genes receive as input the concentration of the chemical signal at the cell's location and the amount of internal metabolic reserves. These inputs are then processed by a layer of regulatory genes that control the activation of the downstream functional genes. Functional genes dictate cell behavior. We model a minimal set of cellular behaviors commonly observed in microbial eukaryotes (48–51): cells can migrate following the chemical signal diffusing from food sources, consume food from the environment, divide, and adhere to their neighbors (Fig. 1C). The cell state — either migrating or dividing — is determined

by the output of the first functional gene. We model these two behaviors as mutually exclusive, following the observation that migration and division require conflicting use of the cytoskeleton in many eukaryotes (52–54). Migrating cells perform chemotaxis by biasing their movement towards the direction of the perceived chemical gradient (55), while dividing cells cannot actively migrate and grow over a fixed period before dividing into two daughter cells. When a cell divides, the parameters of the GRN of one daughter cell can mutate with a small probability, while the other cell inherits the parental parameters. Both daughter cells inherit half of the metabolic reserves of their mother. An additional set of sixteen functional genes encodes adhesion proteins that are expressed on the cell membrane and mediate adhesion between adjacent cells. These proteins are modeled as ligands and receptors, and adhesion strength (γ) between adjacent cells increases with the degree of complementarity between their ligands and receptors (for all the model details, see SI Appendix, Model description).

Together, these components create a structured dynamic environment in which individual cells must integrate internal and ecological cues to survive. The mutations to the GRN during cell division introduce genetic variation into the population. This variation, combined with differential survival and reproduction under ecological constraints, drives an evolutionary process without directly specifying its outcome, which is thus free to emerge spontaneously. In the following sections, we examine how this setup gives rise to diverse life cycles that can coordinate multicellular reproduction.

Evolution of diverse life cycles is mediated by food distribution. Using this setup, we ran 72 simulations each starting with a population of 800 cells. The cells start with a randomized GRN which can evolve throughout the simulation. Simulations ran for 10^8 time steps in one of six environmental conditions (EC 1-6) that differ in the heterogeneity of food distribution. We explore food heterogeneity systematically across ECs, by doubling food patch area and halving creation frequency between successive ECs, while holding total food influx constant (Table S1 and Fig. S1). EC 1 has a relatively even resource distribution, with many small, closely spaced food patches, whereas in EC 6 resources are concentrated into a few large, distant patches. At the end of each simulation, we characterized the dominant evolutionary outcome by extracting the last common ancestor of the population and analyzing its behavior through follow-up simulations without mutations (see Materials and methods, Non-evolutionary simulations).

Three main life cycles evolved across our simulations: a strictly unicellular life cycle, a strictly multicellular life cycle, and a mixed life cycle that is multicellular but regulates the formation of unicellular propagules (Fig. 2A and Videos S1-4, see Materials and methods, Categorisation of life cycles). In all cases, cells have evolved to migrate up the chemoattracting gradient when their metabolic reserves are low, and divide after consuming food from the environment. In the multicellular + unicellular propagules life cycle (Fig. 2A, blue), cells adhere to form multicellular clusters during migration, but stop adhering once they initiate division. These dividing cells are left behind as unicellular propagules, while the multicellular cluster continues migration following a new gradient after local resources are depleted. After dividing, the daughter cells of the unicellular propagule resume migration and re-activate adhesion genes, forming a newborn multicellular cluster that follows the chemoattracting gradient to find new resources, thus completing the life cycle.

Some strictly multicellular lineages also form propagules. In this case, the propagule is multicellular and is formed by dividing cells that downregulate adhesion to migrating cells, while still adhering to each other. We observe a spectrum of multicellular reproductive stratgies: at one end are lineages that genetically encode the formation of propagules by consistently downregulating adhesion between migrating and dividing cells (green in Fig. 2), while at the other end are lineages whose clusters divide rarely, and exclusively by phyisically tearing themselves apart when different parts of the cluster are attracted by separate food patches (yellow in Fig. 2).

Food heterogeneity strongly impacts the evolution of reproductive strategies in the model (Fig. 2B). Strictly unicellular strategies evolve most often when food is homogeneously distributed (EC 1-2), while high-adhesion multicellular strategies usually evolve when food distribution is heterogeneous (EC 5-6). Intermediate environments are characterized by the dominance of unicellular and multicellular propagules (EC 3-4). Lineages with different life cycles show contrasting adaptive functional characteristics that aid survival in the environments where they evolved. For example, high-adhesion multicellular lineages have larger cluster sizes when compared to strictly unicellular and propagule-forming lineages, since their cells are concentrated in a few clusters that rarely divide (Fig. 2C). These large multicellular clusters display collective chemotaxis, allowing them to migrate faster than small clusters or single cells. This happens because cells in a group can integrate spatial information about the patchy chemoattractant signal by pulling and pushing neighboring cells, allowing them to correct each other's trajectories towards the source of food (56). Collective migration, and thus stable multicellularity, becomes more advantageous the longer the distance cells must migrate to reach food, which explains why high-adhesion multicellular life cycles dominate the most heterogeneous environmental conditions (see SI Appendix, Selective advantages of multicellularity and unicellularity, Video S5, Fig. S2A and B).

Conversely, unicellular lineages disperse better through the environment when compared to multicellular lineages (Fig. 2D). Separating after division helps cells colonize and consume multiple food patches at once, which is especially advantageous when resources are split among many food patches in the most homogeneous environments (see SI Appendix, Selective advantages of multicellularity and unicellularity, Video S6, Fig. S2C and D).

Previous studies suggested that reproduction through single-celled units could have evolved as a mechanism to reduce genetic conflict in populations of clonal multicellular

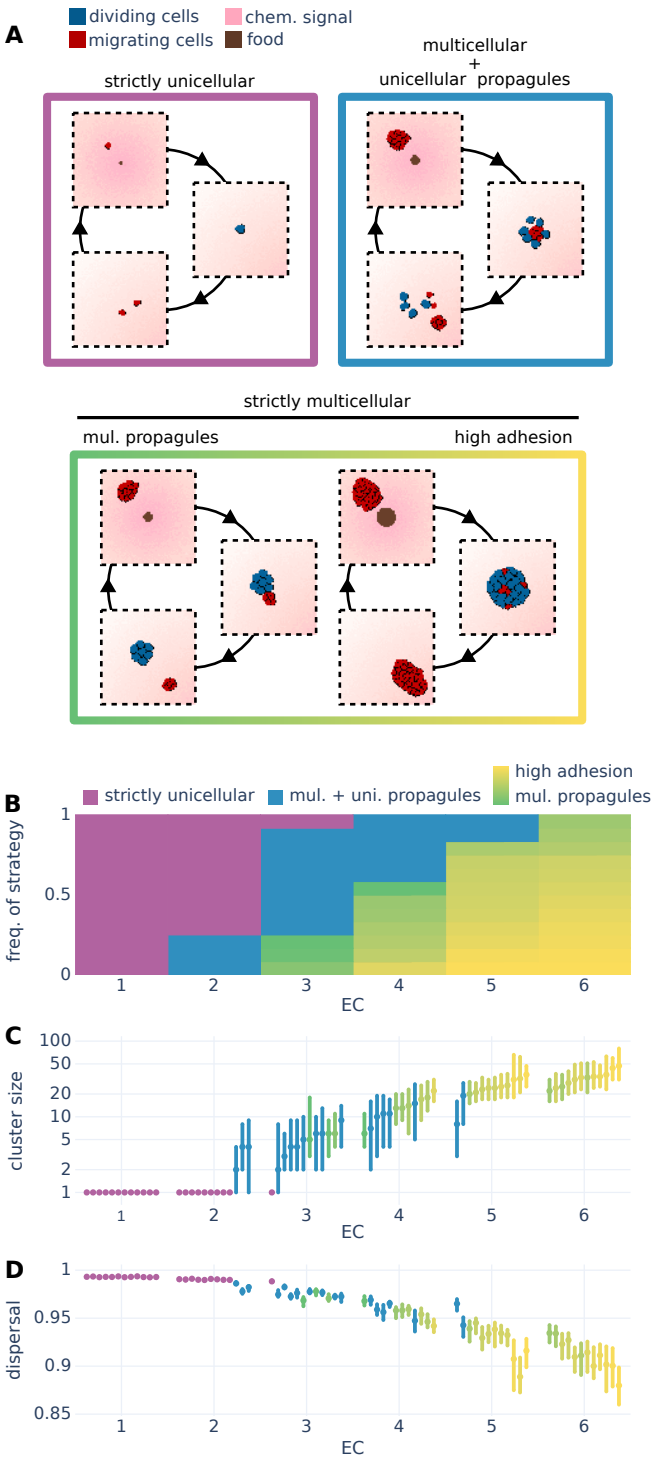

Fig. 2. Spatial heterogeneity of resources drives the evolution of complex multicellular life cycles. **A** Three categories of life cycles evolve in our simulations: strictly unicellular, multicellular + unicellular propagules, and strictly multicellular. Strictly multicellular strategies form clusters of dividing cells whose adhesion to the migrating group ranges from loose multicellular propagules that readily detach to highly adhesive groups that rarely split. **B** Distribution of evolved life cycles in different environmental conditions. **C** Cluster sizes and **D** dispersal of all evolved lineages (median and interquartile ranges, see Materials and methods, Measure of dispersal). Lineages were ordered by median cluster size.

organisms (1, 19). To determine whether this genetic factor could explain the evolution of single-celled propagules in our model, we analyzed the phylogeny of evolved cell populations from the simulations (Fig. S3). We found that the cell clusters of both strictly multicellular and propagule-forming populations are highly heterogeneous due to their aggregative nature, indicating that in this case the reduction of genetic conflicts does not play a role in the evolution of propagules. Instead, the main factor driving the evolution of reproduction via propagules seems to be ecological. In the intermediate environments where unicellular and multicellular propagules evolve, food patches are far apart enough that collective migration is advantageous, while being plentiful enough that dispersal is critical for patch colonisation. As a result, propagule-forming lineages have evolved a multicellular migrating phase that allows them to form clusters to benefit from group migration (Fig. 2C), together with the ability to make unicellular or multicellular propagules for reproduction and dispersal (Fig. 2D).

Development of reproductive strategies: evolved coordination of adhesion and cell state regulation. Because the formation of propagules detaching from migrating clusters emerged spontaneously in our model, we next characterize the regulatory mechanisms that make it possible. We started by quantifying adhesion strength between cells expressing different cell states across our simulations. This shows that propagule-formers coordinate the expression of their cell state and adhesion genes, leading to migrating cells that adhere strongly to other migrating cells but not to dividing cells (Fig. 3A, blue and green). The gene regulatory network achieves this coordination by evolving regulatory genes that co-regulate cell state and the expression profile of adhesion genes, resulting in genetic coupling between the two traits (Fig. S4A). Strictly unicellular and high-adhesion multicellular lineages also often co-regulate cell state and adhesion genes (Fig. S4B), but changes to the expression of adhesion proteins in these cases do not change the adhesion strength between cell states (Fig. 3A, and lineages I, II, IV and V in Fig. S4C).

We next clarify how the observed adhesion dynamics are regulated by the inputs received by the GRN. In all lineages, cells initiate division when their metabolic reserves exceed a specific value. Because this threshold is genetically encoded (Fig. S4), the timing of initiation of cell division varies greatly among lineages, but is very consistent within the cells belonging to each lineage (Fig. 3B). In Fig. 3C, we track a cell within a migrating cluster to show how food consumption activates the coordinated regulation of cell division and adhesion, leading to its transition into a propagule. The tracked cell (white border) initially adheres to its migrating cluster using one adhesion profile (step I, green). After feeding, the cell initiates division and expresses a non-adhering combination of adhesion proteins (II, purple), causing it to sort out from the cluster due to differential adhesion (46) and to detach as a propagule (III). After division, the two daughter cells revert to the original adhesion profile and migrate as a new two-celled cluster (IV) that colonizes a new food

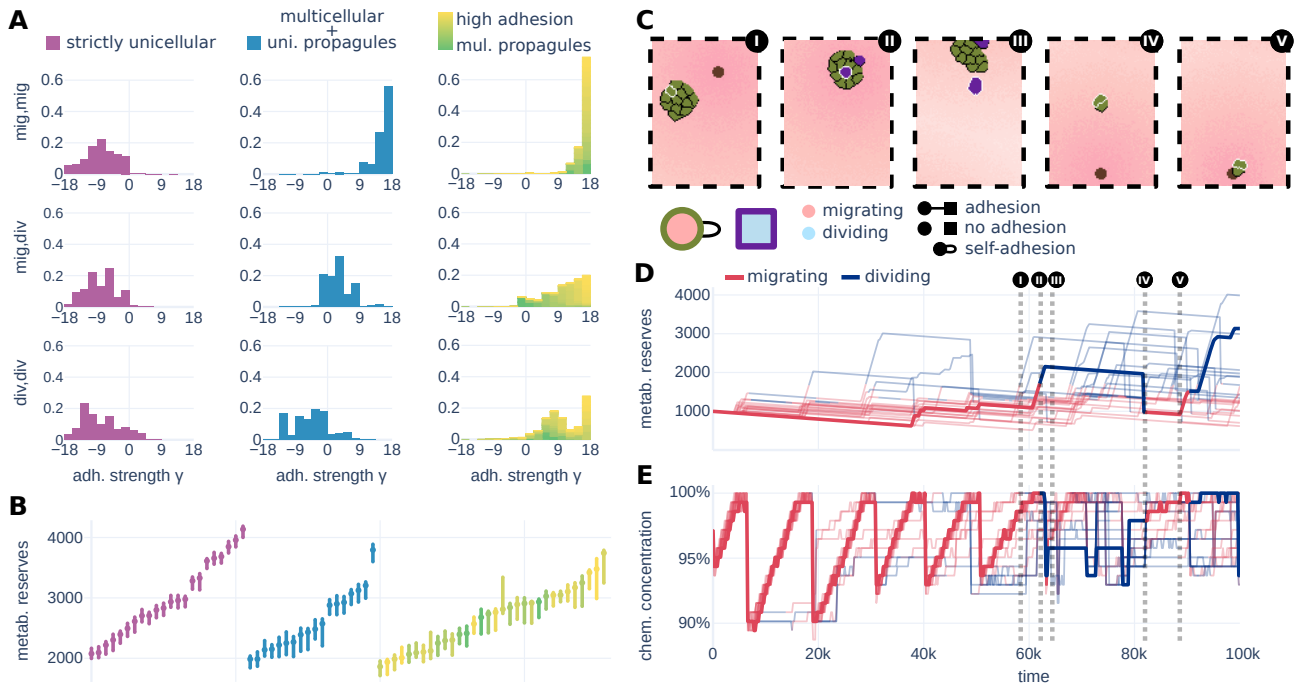

Fig. 3. Coordinated expression of cell state and adhesion genes leads to the formation of propagules. **A** Distribution of adhesion strengths (γ) measured across our simulations for cells expressing different pairings of cell states. Propagule-forming life cycles couple the regulation of cell state and adhesion proteins, leading to differential adhesion between pairs of cells according to their cell states (note: $\gamma \lesssim 0$ results in cells detaching from one another). **B** Metabolic reserves are used as a cue for division. We show the interquartile range of threshold values beyond which division is initiated for different lineages. Circles mark the medians. **C** Formation of a unicellular propagule. The color of the cells indicates which combination of adhesion proteins they express. Below the sequence of images, we show a diagram representing how strongly cells expressing each combination of adhesion proteins adhere to each other. The color inside the symbol shows whether the combination of adhesion proteins is expressed by cells during migration or division. A line connecting the symbols is drawn if cells expressing the two adhesion profiles adhere. Tracking **D** metabolic reserves, and **E** chemoattractant concentration in a subset of cells over time shows how the GRN uses these values to effect adhesion and behavior changes. The thicker line represents the trajectory of the cell highlighted in white in C.

patch (V). We show that the primary signal used for initiating propagule formation is the level of internal metabolic reserves, which cells use as a threshold to decide when to divide or migrate (Fig. 3D). The chemoattractant signal is used to further tune decision making, causing division to end early when at high concentrations (Fig. 3E, detailed in Fig. S5).

Widespread co-option drives the evolution of propagules. To investigate the evolutionary steps that lead to propagule formation, we extracted strictly unicellular populations from EC 1 and high-adhesion multicellular populations from EC 6 and let them evolve for an additional 10^8 time steps in EC 3, which selects for propagule formation according to our previous evolutionary experiments (Fig. 2). This allowed us to analyze the evolution of life cycles under controlled starting genetic landscapes, where all cells are initially unicellular or multicellular. We observed an increase in the adhesion strength between migrating cells ($\widetilde{\gamma}_{\text{mig,mig}}$) in lineages that were initially strictly unicellular (Fig. 4A, lineage 1), reflecting the establishment of a multicellular migrating phase. Since the adhesion strength between dividing cells and all other cells ($\widetilde{\gamma}_{\text{div}}$) remains low, the lineage is now able to form propagules. Conversely, lineages that were initially high-adhesion multicellular evolve lower $\widetilde{\gamma}_{\text{div}}$ while maintaining high $\widetilde{\gamma}_{\text{mig,mig}}$, indicating that they too have evolved propagule formation (Fig. 4A, lineage 2).

Following individual lineages reveals that propagule formation evolves via co-option of the lineage's ancestral life cycle. Cells in lineage 1 (Fig. 4B) initially display a strictly unicellular life cycle after being transferred from EC 1 to EC 3, with cells expressing a single adhesion profile (I). The first step towards forming propagules consists of evolving genetic coupling between cell state and adhesion proteins (II): the ancestral adhesion profile (green circle) is now expressed only during migration, while a new profile (purple square) is expressed during division. This change has no immediate functional effect, since the new adhesion profile does not result in intercellular adhesion. Nevertheless, it represents a preadaptation for the subsequent evolution of propagule formation. The critical step to the evolution of propagules (III) is the appearance of adhesion proteins that cause migrating cells to adhere to each other (dark red triangle), while the expression of the ancestral (green circle) adhesion proteins becomes associated with the dividing cell state. Because there is no adhesion between cells expressing these two adhesion profiles, dividing cells can separate from migrating clusters to form propagules. Once evolved, propagule formation takes over the unicellular population and fixes rapidly (Fig. 4C), highlighting its selective advantage in EC 3. In lineage 2, propagules also evolve via co-option, this time of an ancestral multicellular life cycle. The ancestral combination of adhesion proteins (dark blue diamond) is co-opted for the regulation of a migrating phase, followed by the evolution of novel combinations of adhesion proteins (pink triangle and yellow pentagon) that enable propagule formation (4B). The evolution of this trait in lineage 2 also leads to a fast selec-

tive sweep in the population (Fig. 4C). In Fig. S6, we show that unicellular propagules evolved via co-option in 5 out of 7 lineages that were originally strictly unicellular, and in 7 out of 8 lineages that were originally high-adhesion multicellular, according to our detection algorithm (see SI Appendix, Inference of co-option).

Strictly multicellular lineages seem to have evolved propagule formation more easily than strictly unicellular lineages when transferred to EC 3, evidenced by the fact that 11/12 multicellular lineages evolved a lower $\widetilde{\gamma}_{\text{div}}$, while only 7/12 strictly unicellular lineages evolved a positive $\widetilde{\gamma}_{\text{mig,mig}}$ in the same period of time (Fig. 4D and E). Presumably, this disparity in the number of lineages that evolve propagules is due to the difference in mutation availability between the two cases. Starting from a non-adhering state, most regulatory mutations do not lead to changes in adhesion, creating a large neutral plateau in phenotype space. Thus, strictly unicellular lineages may experience a long series of neutral mutations before reaching a combination that produces adhesion, at which point mutations affecting adhesion proteins become selective. Altogether, this suggests that the initial evolution of adhesion reshapes the accessible evolutionary pathways, facilitating the evolution of subsequent multicellular traits such as propagule formation.

Eco-evolutionary protection of multicellularity. To better understand how environment colonisation and competition could shape ecological dynamics at the onset of multicellularity, we ran a series of experiments where lineages with different life cycles invade ECs 1, 3 and 6 and compete with host lineages that evolved in these environments. (Fig. 5A). In most instances, the host lineages — which evolved in the environment where competition is taking place — outcompeted the invading lineages. However, invading lineages with a multicellular + unicellular propagule life cycle outcompeted host lineages in EC 1 in all replicas of the experiment. This prompted us to investigate whether multicellularity could represent a viable strategy in EC 1, despite not evolving there (Fig. 2).

After transferring strictly multicellular and multicellular + unicellular propagule lineages to EC 1 and letting these lineages evolve for 10^8 time steps, we observed that multicellularity persisted in the majority of the simulations (35/49, Fig. 5B), with all lineages either preserving or evolving a propagule-forming life cycle (Fig. S7A). Moreover, multicellularity was better protected in lineages that had originally evolved in environments similar to EC 1, as evidenced by the large number of lineages from ECs 5 and 6 that lost multicellularity after the transference. This suggests that protection of multicellularity may be contingent on certain phenotypic or genetic factors. For example, reaching the basin of attraction of the multicellular life cycles that perform well in EC 1 might be easier if the lineage can already make propagules or is pre-adapted to environments with a fast turnover of food patches.

In the symmetric experiment, where we transfer strictly unicellular and multicellular + unicellular propagule lineages to EC 6, the inverse pattern can be observed, with a unicellular phase being preserved more often in the ECs that are most different from EC 6 (ECs 1 and 2 in Fig. S7B and C), rather than similar. After being transferred to EC 6, the lineages that preserved a unicellular phase displayed poor fitness. This is also unlike the previous experiment, where the lineages that preserved a multicellular phase performed well in their new environment (compare Fig. S7D and E). Altogether, this suggests that lineages preserving a unicellular phase did not adapt well to EC 6, most likely because they struggled to navigate the phenotype space due to low mutational availability (similarly to the case shown in Fig. 4D and E).

Discussion

Understanding the origin of multicellular reproduction requires explaining how traits of individual cells were repurposed to organise development (43, 44). Here, we show that developmental regulation arises spontaneously through adhesion-mediated interactions between neighboring cells. In our computational framework, cells can evolve a gene regulatory network to interact with other cells and the environment, but we do not impose developmental rules or ecological responses as a selection criterion. This allows us to investigate the evolution of reproduction as a novel, emergent process (57). We find that both unicellular and multicellular modes of reproduction could evolve in our simulations, depending on the heterogeneity of the distribution of food in the environment. In intermediate environments, cell lineages evolved multicellular clusters that periodically release propagules. Propagules are beneficial in these environments because they improve dispersal, while the cluster from which they originate benefits from collective chemotaxis. We show that these lineages regulate the formation of propagules by coupling their cell state to specific expression profiles of adhesion proteins. This trait evolves through the co-option of ancestral strictly unicellular or strictly multicellular life cycles, which are encapsulated as a phase in the developmental cycle of the propagule-forming strategy.

Comparative genomic and phylogenetic studies have shown that the evolution of several multicellular traits involved the co-option of proteins that originally served different functions in their unicellular ancestors (37, 53, 58). In our simulations, multicellular reproduction evolved through the same process. The architecture of the GRN is fixed in our model, and no new regulatory machinery can evolve. Yet, multicellular reproduction still emerged via the co-option of modules of regulation of adhesion. Before the evolution of multicellularity, these modules mediated ecological interactions between single-celled organisms, which spread apart in the environment by expressing adhesion proteins with low complementarity. The same regulatory modules were then repurposed during the transition towards multicellularity to regulate the formation of propagules used for reproduction in a developmental context. Our model therefore highlights co-option as a mechanism through which properties of individual cells can be integrated into coordinated reproductive cycles featuring a unicellular propagule stage. These propagule-forming life cycles bear a striking similarity to

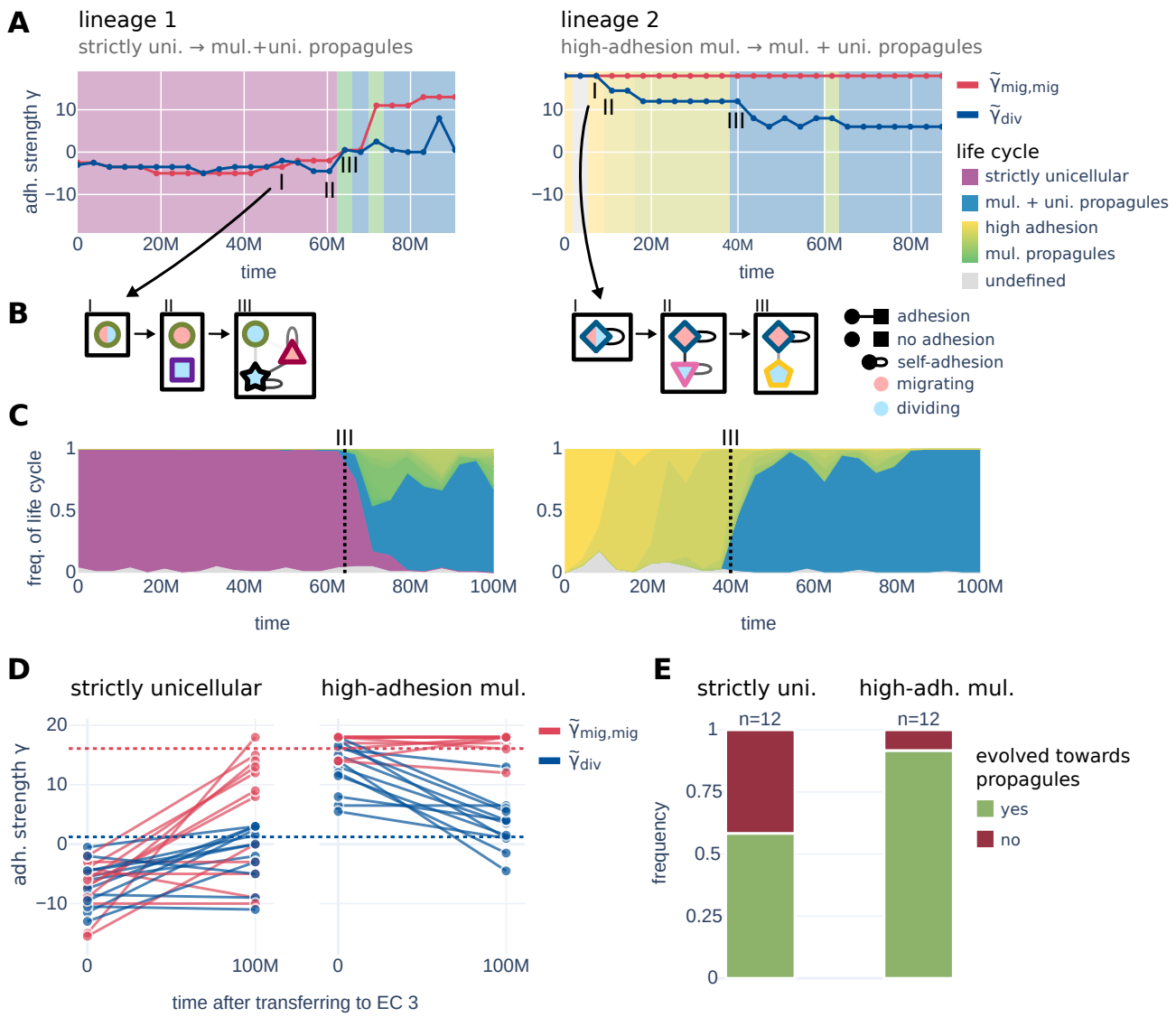

Fig. 4. Propagule formation evolves through the co-option of unicellular or multicellular life cycles. **A** Evolution of adhesion properties of a strictly unicellular lineage taken from EC 1 (lineage 1) and a strictly multicellular lineage taken from EC 6 (lineage 2) after their cells are transferred to an environment selecting for propagule formation (EC 3) for 10^8 time steps. The median adhesion strength between migrating cells $\widetilde{\gamma}_{\text{mig,mig}}$ and between dividing cells and all other cells $\widetilde{\gamma}_{\text{div}}$ is shown in red and blue, respectively, with the background color indicating the life cycle expressed by the lineage at that time (see Materials and methods, Categorisation of life cycles). **B** Evolution of regulation of adhesion proteins. Each unique combination of adhesion genes expressed by a lineage is represented by a unique color and symbol (same as Fig. 3C). The inner color of the symbols indicates whether that combination of adhesion genes is expressed during division or migration. A line connecting the symbols is drawn if cells expressing the two adhesion profiles adhere. **C** Distribution of life cycles among 100 cells taken from the simulations where lineages 1 and 2 originated. Time step III marks the first time propagule formation evolved in lineages 1 and 2. **D** Evolution of $\widetilde{\gamma}_{\text{mig,mig}}$ and $\widetilde{\gamma}_{\text{div}}$ of strictly unicellular lineages (left) and high-adhesion multicellular lineages (right) after they were transferred to EC 3. Dotted lines indicate the average value for the trait in populations endogenous to EC 3. **E** 7 out of the 12 lineages that started with a strictly unicellular life cycle (left) and 11 out of the 12 lineages that started with a high-adhesion life cycle (right) evolved towards making propagules (respectively by increasing their $\widetilde{\gamma}_{\text{mig,mig}}$ and decreasing their $\widetilde{\gamma}_{\text{div}}$, see subfigure D) after being transferred to EC 3.

those observed in previous experiments performed in *C. reinhardtii*, where the rapid evolution of propagules was suggested to be evidence of co-option (17). Our results corroborate this hypothesis, showing that co-option of ancestral life cycles for reproduction is not only possible, but evolutionarily likely. More broadly, our results suggest that co-option might represent a general solution to the emergence of collective traits, enabling the evolution of complex life cycles while avoiding the organisational costs associated with building more elaborate regulatory machinery.

The eco-evolutionary feedback between cells and sources of food in our model recapitulates real-life examples of ecology shaping the evolution of life cycles, and therefore provides a possible explanation for their evolution. Organisms that alternate between unicellular and multicellular lifestyles often rely on fluctuations of nutrient availability to regulate switches between these stages. Similarly to how propagule-forming lineages in our model migrate as a multicellular cluster during a feeding stage and then produce propagules after food is depleted, many amoebozoans and opisthokonts perform collective feeding when resources are abundant, followed by unicellular reproduction through single-celled

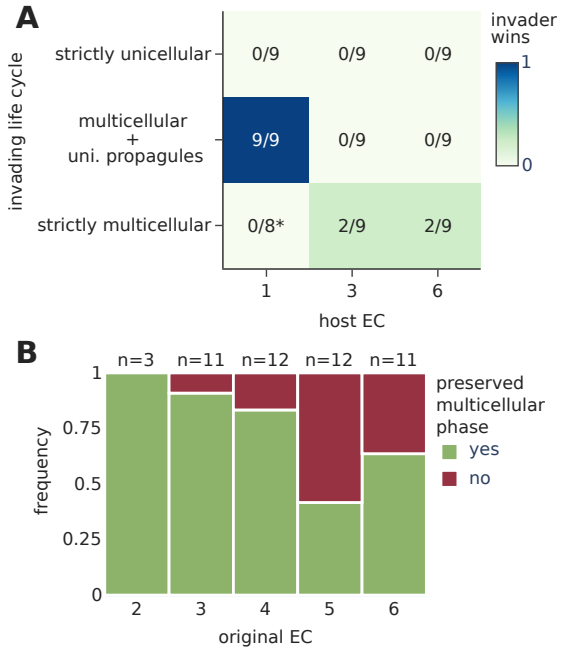

Fig. 5. Multicellularity is evolutionarily protected in EC 1. **A** Number of competitions where the invading strategy wins against the host strategy. We compete three invading lineages with different life cycles against three host lineages in ECs 1, 3, and 6. Lineages were selected based on their inferred fitness (see Materials and methods, Inference of fitness) in the environment hosting the competition. The asterisk marks one data point that we excluded because neither lineage outcompeted the other in the simulation. Mutations were disabled in these simulations to prevent further evolution. **B** Frequency of initially multicellular strategies that preserved a multicellular phase after being transferred to EC 1 for 10^8 time steps.

structures once food becomes scarce (14). For example, the holomycotan *Fonticula alba* forms cell collectives that feed on patches of bacteria and later differentiate into a sorocarp for reproduction through spores (30). Collective feeding followed by unicellular reproduction has also been described in Myxogastria (59–61), Labyrinthulomycota (62, 63), and choanoflagellates like *Salpingoeca rosetta* (64–66). Other choanoflagellates, e.g. *Choanoeca flexa*, instead use alternative environmental signals such as salinity to regulate a transition to the multicellular stage (67). Interestingly, this species forms multicellular groups both through aggregation and continued cell division, similarly to the cells in our simulations.

We showed that multicellularity could not initially evolve in environments with homogeneous resources (EC 1) when starting from random genomes. This may be due to unicellular strategies being more adaptive than early forms of multicellularity in these environments. Once lineages become unicellular, mutations that lead to intercellular adhesion become rare, making multicellularity less likely to evolve (Fig. 4D and E). However, this ecological interference between unicellularity and weak multicellularity could be avoided by allowing lineages to first evolve multicellularity and propagules in intermediate environments (EC 3), after which they could successfully colonize EC 1. Similar effects have been previously described in the context of protein evolution, where changes in the environment can bridge fitness valleys and enable further functional adaptation of a protein sequence (68–70). Our results suggest that environmental shifts can likewise promote the evolution of major innovations, such as multicellularity. The role of EC 3 in facilitating this transition is reminiscent of environmental scaffolding, where particular ecological contexts transiently support the emergence of new levels of organization (71–75).

In summary, we have shown that variation in the spatial distribution of resources in the environment promotes the evolution of diverse life cycles and modes of reproduction. Our model captures this constructive form of evolutionary innovation (57), showcasing how ecological interactions can be co-opted to regulate development at the onset of multicellularity.

Materials and methods

Our model consists of an evolving population of cells living in a two-dimensional arena. Cells must consume resources to survive and replicate. Resources are patchily distributed and release a chemical signal that cells can follow through chemotaxis. Each cell contains a GRN represented as a Boolean network. The expression of different genes in the GRN determines whether the cell is migrating or dividing and whether it adheres to neighboring cells.

Following the CPM formalism, we implement a Hamiltonian equation which describes the energy of the system. A Monte Carlo method is then used to minimise this energy by accepting or rejecting copy attempts between pairs of lattice sites. A full description of the model can be found in the SI Appendix, Model description. The source code of the model was built on top of the Tissue Simulation Toolkit framework (45) and can be found at https://github.com/aleferna12/regulation_evolution2. Parameters used for the simulations are available in Table S2.

Non-evolutionary simulations. After obtaining the results from our evolutionary simulations (as described in Results), we ran a set of non-evolutionary simulations where mutations are disabled. The purpose of these simulations was to build a large, fine-grained dataset that we could use to analyze functional traits of the life cycles that evolve in our model. We start each simulation with 100 cells cloned from an evolved lineage's most recent common ancestor (MRCA). The simulation then runs for 10^6 time steps while the cell population reaches steady state, after which we record the data used for the analyzes described below (over the course of an additional 10^6 time steps). Data points are recorded every 20,000 time steps.

Categorisation of life cycles. We categorise the life cycles evolved in the simulations into three groups: strictly unicellular, multicellular + unicellular propagules, and strictly multicellular. To classify a lineage's life cycle, we first record all unique combinations of adhesion proteins expressed by its cells in a non-evolutionary simulation (see Materials and methods, Non-evolutionary simulations), storing them in the vector E. We also store the frequency with which each combination of adhesion genes was expressed by the cells according to their cell state in the vectors F^{mig} and F^{div}, such that

F_i^{mig} is the frequency with which a cell in the simulation is both migrating and expressing E_i. We then calculate the median adhesion strength between cells, given their cell states:

$$\widetilde{\gamma}_{t_1,t_2} = \text{wmed}(A, W)$$

$$A = [\gamma(E_i, E_j) \mid 0 \leq i, j < |E|]$$

$$W = [F_i^{t_1} \times F_j^{t_2} \mid 0 \leq i, j < |E|]$$

where t_1 and t_2 are cell states (either migrating, dividing, or all, in which case $F^{\text{all}} = F^{\text{mig}} + F^{\text{div}}$), $\text{wmed}(A, W)$ gives the median of the vector of adhesion strengths A weighted by the vector of expression frequencies W, and $\gamma(E_i, E_j)$ is the adhesion strength calculated by comparing the vectors of expression of adhesion proteins E_i and E_j expressed by cells with sigmas s_i and s_j respectively, such that $\gamma(E_i, E_j) = \gamma(s_i, s_j)$ (see SI Appendix, Cell adhesion).

This gives us six combinations of adhesion strength $\widetilde{\gamma}$ values between the different cell states: $\widetilde{\gamma}_{\text{mig,mig}}$, $\widetilde{\gamma}_{\text{mig,div}}$, $\widetilde{\gamma}_{\text{mig,all}}$, $\widetilde{\gamma}_{\text{div,div}}$, $\widetilde{\gamma}_{\text{div,all}}$, and $\widetilde{\gamma}_{\text{all,all}}$. We usually abbreviate $\widetilde{\gamma}_{t,\text{all}}$ to simply $\widetilde{\gamma}_t$, for example $\widetilde{\gamma}_{\text{div,all}} = \widetilde{\gamma}_{\text{div}}$ in the text and figures. We then classify a life cycle according to the following decision tree: if migrating cells do not adhere to other migrating cells (i.e. $\widetilde{\gamma}_{\text{mig,mig}} \leq 0$), we classify the life cycle as strictly unicellular. Else, if dividing cells do not adhere to other dividing cells (i.e. $\widetilde{\gamma}_{\text{div,div}} \leq 0$), we classify the life cycle as multicellular + unicellular propagules. Otherwise, we classify the life cycle as strictly multicellular, and use $\widetilde{\gamma}_{\text{div}}$ to differentiate between lineages that often form propagules ($\widetilde{\gamma}_{\text{div}} \approx 0$, green in Fig. 2) and those that never or rarely do ($\widetilde{\gamma}_{\text{div}} \gg 0$, yellow in Fig. 2).

In Fig. 4, we examine the evolution of propagule formation across 10^8 time steps. Because the evolutionary simulation contains only a few cells of the lineage of interest, we cannot directly extract enough data to run the analysis as described above. Instead, to calculate the γ values displayed in Fig. 4, we artificially feed the GRN of the lineage's ancestor with inputs experienced by other cells in the simulation, and compare the GRN's outputs with data from the population in real-time during evolution. When a life cycle cannot be reliably identified with this method, we conservatively label it "undetermined" in the figure.

Measure of dispersal. To measure the dispersal of a lineage in its environment, we periodically record the positions of all cells in the lattice in a non-evolutionary simulation (see Materials and methods, Non-evolutionary simulations). We then perform a standard quadrat analysis using a quadrat size of 100x100 lattice sites. We define the dispersal of the population at a time step t as:

$$d_t = 1 - \frac{\text{var}(Q_{\text{p}})}{\overline{Q}_{\text{p}}}$$

$$Q_{\text{p}} = \{q/N_t \mid q \in Q\}$$

where $\overline{Q}_{\text{p}}$ is the mean of the set containing the proportion of cells in each quadrat Q_{p}, N_t is the population size at time t, and Q is a set containing the number of cells on each quadrat at time step t. This ensures that our dispersal metric d_t lies in the interval $(0, 1]$, where ≈ 0 represents the situation where all cells are contained in a single quadrat and 1 represents the situation where each quadrat contains the same number of cells. Finally, we define the dispersal of the lineage as the mean of all of its recorded d_t (measured every 20,000 time steps in non-evolutionary simulations, see Materials and methods, Non-evolutionary simulations).

Inference of fitness. We infer the fitness of a population by measuring how much food it has consumed from its environment at steady state (when the rate of food consumption and the influx rate of food reach equilibrium). Populations that can empty the environment of food before reaching steady state are considered to have fitness $\overline{f} = 1$, while those that require the lattice to be full to survive have fitness $\overline{f} = 0$. $\overline{f}$ is calculated by taking the mean of the time series of f_t, measured every 20,000 time steps in non-evolutionary simulations (see Materials and methods, Non-evolutionary simulations), where $f_t = 1 - \beta_t/\Gamma$, with β_t being the number of food units on Λ_{food} at time step t and Γ being the maximum amount of food supported on the lattice.

ACKNOWLEDGEMENTS

This work was supported by grants from the Gatsby Charitable Foundation (G112566) to R.M.A.V. and from the Cambridge Trust to A.P.F. We thank members of the Sainsbury Laboratory for helpful discussions and suggestions.

Bibliography

1. Richard K. Grosberg and Richard R. Strathmann. The Evolution of Multicellularity: A Minor Major Transition? *Annual Review of Ecology, Evolution, and Systematics*, 38(Volume 38, 2007):621–654, December 2007. ISSN 1543-592X, 1545-2069. doi: 10.1146/annurev.ecolsys.36.102403.114735. Publisher: Annual Reviews.
2. Yuanxiao Gao, Arne Traulsen, and Yuriy Pichugin. Interacting cells driving the evolution of multicellular life cycles. *PLOS Computational Biology*, 15(5):e1006987, May 2019. ISSN 1553-7358. doi: 10.1371/journal.pcbi.1006987.
3. R. K. Grosberg and R. R. Strathmann. One cell, two cell, red cell, blue cell: The persistence of a unicellular stage in multicellular life histories. *Trends in Ecology & Evolution*, 13(3): 112–116, March 1998. ISSN 0169-5347. doi: 10.1016/S0169-5347(97)01313-X.
4. Yoshinori Kawabe, Qingyou Du, Christina Schilde, and Pauline Schaap. Evolution of multicellularity in Dictyostelia. *The International journal of developmental biology*, 63(8-9-10): 359–369, January 2019. ISSN 0214-6282. doi: 10.1387/ijdb.190108ps.
5. Anja Kamp, Hans Røy, and Heide N. Schulz-Vogt. Video-supported Analysis of Beggiatoa Filament Growth, Breakage, and Movement. *Microbial Ecology*, 56(3):484–491, October 2008. ISSN 1432-184X. doi: 10.1007/s00248-008-9367-x.
6. František Adamec, David Kaftan, and Ladislav Nedbal. Stress-Induced Filament Fragmentation of Calothrix Elenkinii (cyanobacteria) Is Facilitated by Death of High-Fluorescence Cells. *Journal of Phycology*, 41(4):835–839, 2005. ISSN 1529-8817. doi: 10.1111/j.1529-8817.2005.00104.x. _eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1529-8817.2005.00104.x.
7. Ester Cecere, Antonella Petrocelli, and Marc Verlaque. Vegetative reproduction by multicellular propagules in Rhodophyta: an overview. *Marine Ecology*, 32(4):419–437, 2011. ISSN 1439-0485. doi: 10.1111/j.1439-0485.2011.00448.x. _eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1439-0485.2011.00448.x.
8. Jean-Luc Jany and Teresa E. Pawlowska. Multinucleate Spores Contribute to Evolutionary Longevity of Asexual Glomeromycota. *The American Naturalist*, 175(4):424–435, April 2010. ISSN 0003-0147. doi: 10.1086/650725. Publisher: The University of Chicago Press.
9. Helena M. P. Garcês, Connie E. M. Champagne, Brad T. Townsley, Soomin Park, Rui Malhó, Maria C. Pedroso, John J. Harada, and Neelima R. Sinha. Evolution of asexual reproduction in leaves of the genus Kalanchoë. *Proceedings of the National Academy of Sciences*, 104 (39):15578–15583, September 2007. doi: 10.1073/pnas.0704105104. Publisher: Proceedings of the National Academy of Sciences.
10. Li-Min Zhang, Yu Jin, Si-Mei Yao, Ning-Fei Lei, Jin-Song Chen, Qian Zhang, and Fei-Hai Yu. Growth and Morphological Responses of Duckweed to Clonal Fragmentation, Nutrient Availability, and Population Density. *Frontiers in Plant Science*, 11, May 2020. ISSN 1664-462X. doi: 10.3389/fpls.2020.00618. Publisher: Frontiers.
11. Matthew Clements, Kennedy Wolfe, Kit Schwartz, and Maria Byrne. Forever fissiparous: asexual propagation and stable demography in a tropical and geographically isolated asterinid sea star. *Marine Biology*, 166(6):69, May 2019. ISSN 1432-1793. doi: 10.1007/s00227-019-3518-0.
12. Bernd Schierwater, Hans-Jürgen Osigus, Tjard Bergmann, Neil W. Blackstone, Heike Hadrys, Jens Hauslage, Patrick O. Humbert, Kai Kamm, Marc Kvansakul,

Kathrin Wysocki, and Rob DeSalle. The enigmatic Placozoa part 1: Exploring evolutionary controversies and poor ecological knowledge. *BioEssays*, 43 (10):2100080, 2021. ISSN 1521-1878. doi: 10.1002/bies.202100080. _eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1002/bies.202100080.

13. Ilya A. Gavrilov-Zimin. Ancient reproductive modes and criteria of multicellularity. *Comparative Cytogenetics*, 17:195–238, October 2023. ISSN 1993-078X, 1993-0771. doi: 10.3897/compcytogen.17.109671.
14. Łukasz Lamża. Diversity of 'simple' multicellular eukaryotes: 45 independent cases and six types of multicellularity. *Biological Reviews*, 98(6):2188–2209, December 2023. ISSN 1464-7931, 1469-185X. doi: 10.1111/brv.13001.
15. Martin E. Boraas, Dianne B. Seale, and Joseph E. Boxhorn. Phagotrophy by a flagellate selects for colonial prey: A possible origin of multicellularity. *Evolutionary Ecology*, 12(2): 153–164, February 1998. ISSN 0269-7653, 1573-8477. doi: 10.1023/A:1006527528063.
16. William C. Ratcliff, R. Ford Denison, Mark Borrello, and Michael Travisano. Experimental evolution of multicellularity. *Proceedings of the National Academy of Sciences*, 109(5): 1595–1600, January 2012. ISSN 0027-8424, 1091-6490. doi: 10.1073/pnas.1115323109.
17. William C. Ratcliff, Matthew D. Herron, Kathryn Howell, Jennifer T. Pentz, Frank Rosenzweig, and Michael Travisano. Experimental evolution of an alternating uni- and multicellular life cycle in Chlamydomonas reinhardtii. *Nature Communications*, 4(1):2742, November 2013. ISSN 2041-1723. doi: 10.1038/ncomms3742. Publisher: Nature Publishing Group.
18. Matthew D. Herron, Joshua M. Borin, Jacob C. Boswell, Jillian Walker, I.-Chen Kimberly Chen, Charles A. Knox, Margrethe Boyd, Frank Rosenzweig, and William C. Ratcliff. De novo origins of multicellularity in response to predation. *Scientific Reports*, 9(1):2328, February 2019. ISSN 2045-2322. doi: 10.1038/s41598-019-39558-8. Publisher: Nature Publishing Group.
19. Denis Roze and Richard E. Michod. Mutation, Multilevel Selection, and the Evolution of Propagule Size during the Origin of Multicellularity. *The American Naturalist*, 158(6):638–654, December 2001. ISSN 0003-0147. doi: 10.1086/323590. Publisher: The University of Chicago Press.
20. István Oszoli and István Zachar. Group-selection via aggregative propagule-formation enables cooperative multicellularity in an individual based, spatial model. *PLOS Computational Biology*, 20(5):e1012107, May 2024. ISSN 1553-7358. doi: 10.1371/journal.pcbi.1012107.
21. Katrin Hammerschmidt, Caroline J. Rose, Benjamin Kerr, and Paul B. Rainey. Life cycles, fitness decoupling and the evolution of multicellularity. *Nature*, 515(7525):75–79, November 2014. ISSN 1476-4687. doi: 10.1038/nature13884. Publisher: Nature Publishing Group.
22. Eric Libby, William Ratcliff, Michael Travisano, and Ben Kerr. Geometry Shapes Evolution of Early Multicellularity. *PLOS Computational Biology*, 10(9):e1003803, September 2014. ISSN 1553-7358. doi: 10.1371/journal.pcbi.1003803. Publisher: Public Library of Science.
23. Yuriy Pichugin, Jorge Peña, Paul B. Rainey, and Arne Traulsen. Fragmentation modes and the evolution of life cycles. *PLOS Computational Biology*, 13(11):e1005860, November 2017. ISSN 1553-7358. doi: 10.1371/journal.pcbi.1005860. Publisher: Public Library of Science.
24. Yuanxiao Gao, Yuriy Pichugin, Chaitanya S. Gokhale, and Arne Traulsen. Evolution of reproductive strategies in incipient multicellularity. *Journal of The Royal Society Interface*, 19(188):20210716, March 2022. doi: 10.1098/rsif.2021.0716. Publisher: Royal Society.
25. Merlijn Staps, Jordi van Gestel, and Corina E. Tarnita. Emergence of diverse life cycles and life histories at the origin of multicellularity. *Nature Ecology & Evolution 2019 3:8*, 3 (8):1197–1205, July 2019. ISSN 2397-334X. doi: 10.1038/s41559-019-0940-0. Publisher: Nature Publishing Group.
26. Yuriy Pichugin, Hye Jin Park, and Arne Traulsen. Evolution of simple multicellular life cycles in dynamic environments. *Journal of The Royal Society Interface*, 16(154):20190054, May 2019. doi: 10.1098/rsif.2019.0054. Publisher: Royal Society.
27. Julien Barrere, Piyush Nanda, and Andrew W. Murray. Alternating selection for dispersal and multicellularity favors regulated life cycles. *Current Biology*, 33(9):1809–1817.e3, May 2023. ISSN 0960-9822. doi: 10.1016/j.cub.2023.03.031.
28. Eric Libby and Paul B. Rainey. Eco-Evolutionary Feedback and the Tuning of Proto-Developmental Life Cycles. *PLoS ONE*, 8(12):e82274, December 2013. ISSN 1932-6203. doi: 10.1371/journal.pone.0082274.
29. Jordi van Gestel and Martin A. Nowak. Phenotypic Heterogeneity and the Evolution of Bacterial Life Cycles. *PLOS Computational Biology*, 12(2):e1004764, February 2016. ISSN 1553-7358. doi: 10.1371/journal.pcbi.1004764. Publisher: Public Library of Science.
30. Christopher Toret, Andrea Picco, Micaela Boiero-Sanders, Alphee Michelot, and Marko Kaksonen. The cellular slime mold Fonticula alba forms a dynamic, multicellular collective while feeding on bacteria. *Current Biology*, 32(9):1961–1973.e4, May 2022. ISSN 0960-9822. doi: 10.1016/j.cub.2022.03.018. Publisher: Elsevier.
31. Renske M. A. Vroomans and Enrico Sandro Colizzi. Evolution of selfish multicellularity: collective organisation of individual spatio-temporal regulatory strategies. *BMC Ecology and Evolution*, 23(1):35, July 2023. ISSN 2730-7182. doi: 10.1186/s12862-023-02133-x.
32. Maria Kalambokidis and Michael Travisano. Multispecies interactions shape the transition to multicellularity. *Proceedings of the Royal Society B: Biological Sciences*, 290(2007): 20231055, September 2023. doi: 10.1098/rspb.2023.1055. Publisher: Royal Society.
33. John H Koschwanez, Kevin R Foster, and Andrew W Murray. Improved use of a public good selects for the evolution of undifferentiated multicellularity. *eLife*, 2:e00367, April 2013. ISSN 2050-084X. doi: 10.7554/eLife.00367.
34. Luke L. M. Heaton, Nick S. Jones, and Mark D. Fricker. A mechanistic explanation of the transition to simple multicellularity in fungi. *Nature Communications*, 11(1):2594, May 2020. ISSN 2041-1723. doi: 10.1038/s41467-020-16072-4.
35. Kai Tong, G Ozan Bozdag, and William C Ratcliff. Selective drivers of simple multicellularity. *Current Opinion in Microbiology*, 67:102141, June 2022. ISSN 1369-5274. doi: 10.1016/j.mib.2022.102141.
36. Pina Marotta, Antonella Ruggiero, and Gust Bilcke. Editorial: Unicellular organisms as an evolutionary snapshot toward multicellularity. *Frontiers in Cell and Developmental Biology*, 11, August 2023. ISSN 2296-634X. doi: 10.3389/fcell.2023.1254636. Publisher: Frontiers.
37. Thibaut Brunet and Nicole King. The Origin of Animal Multicellularity and Cell Differentiation. *Developmental Cell*, 43(2):124–140, October 2017. ISSN 15345807. doi: 10.1016/j.devcel.2017.09.016.
38. Arnau Sebé-Pedrós, Bernard M. Degnan, and Iñaki Ruiz-Trillo. The origin of Metazoa: a unicellular perspective. *Nature Reviews Genetics*, 18(8):498–512, August 2017. ISSN 1471-0056, 1471-0064. doi: 10.1038/nrg.2017.21.
39. Monika Abedin and Nicole King. Diverse evolutionary paths to cell adhesion. *Trends in cell biology*, 20(12):734–742, December 2010. ISSN 0962-8924. doi: 10.1016/j.tcb.2010.08.002.
40. Paul S. Murray and Ronen Zaidel-Bar. Pre-metazoan origins and evolution of the cadherin adhesome. *Biology Open*, 3(12):1183–1195, November 2014. ISSN 2046-6390. doi: 10.1242/bio.20149761.
41. Erik R. Hanschen, Tara N. Marriage, Patrick J. Ferris, Takashi Hamaji, Atsushi Toyoda, Asao Fujiyama, Rafik Neme, Hideki Noguchi, Yohei Minakuchi, Masahiro Suzuki, Hiroko Kawai-Toyooka, David R. Smith, Halle Sparks, Jaden Anderson, Robert Bakarić, Victor Luria, Amir Karger, Marc W. Kirschner, Pierre M. Durand, Richard E. Michod, Hisayoshi Nozaki, and Bradley J. S. C. Olson. The Gonium pectorale genome demonstrates co-option of cell cycle regulation during the evolution of multicellularity. *Nature Communications*, 7(1):11370, April 2016. ISSN 2041-1723. doi: 10.1038/ncomms11370. Publisher: Nature Publishing Group.
42. Alan C. Love and Günter P. Wagner. Co-option of stress mechanisms in the origin of evolutionary novelties. *Evolution*, 76(3):394–413, 2022. ISSN 1558-5646. doi: 10.1111/evo.14421. _eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1111/evo.14421.
43. Jordi van Gestel and Corina E. Tarnita. On the origin of biological construction, with a focus on multicellularity. *Proceedings of the National Academy of Sciences*, 114(42):11018–11026, October 2017. doi: 10.1073/pnas.1704631114. Publisher: Proceedings of the National Academy of Sciences.
44. Pierrick Bourrat, Peter Takacs, Guilhem Doulcier, Matthew C. Nitschke, Andrew J. Black, Katrin Hammerschmidt, and Paul B. Rainey. Individuality Through Ecology: Rethinking the Evolution of Complex Life From an Externalist Perspective. *Ecology and Evolution*, 14(12):e70661, 2024. ISSN 2045-7758. doi: 10.1002/ece3.70661. _eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1002/ece3.70661.
45. Josephine T. Daub and Roeland M.H. Merks. Cell-based computational modeling of vascular morphogenesis using Tissue Simulation Toolkit. *Methods in Molecular Biology*, 1214: 67–127, 2015. ISSN 10643745. doi: 10.1007/978-1-4939-1462-3_6. Publisher: Humana Press Inc.
46. François Graner and James A. Glazier. Simulation of biological cell sorting using a two-dimensional extended Potts model. *Physical Review Letters*, 69(13):2013–2016, September 1992. doi: 10.1103/PhysRevLett.69.2013. Publisher: American Physical Society.
47. James A. Glazier and François Graner. Simulation of the differential adhesion driven rearrangement of biological cells. *Physical Review E*, 47(3):2128–2154, March 1993. doi: 10.1103/PhysRevE.47.2128. Publisher: American Physical Society.
48. James G. Umen. Volvox and volvocine green algae. *EvoDevo*, 11(1):13, July 2020. ISSN 2041-9139. doi: 10.1186/s13227-020-00158-7.
49. Núria Ros-Rocher, Alberto Pérez-Posada, Michelle M. Leger, and Iñaki Ruiz-Trillo. The origin of animals: an ancestral reconstruction of the unicellular-to-multicellular transition. *Open Biology*, 11(2), February 2021. ISSN 20462441. doi: 10.1098/RSOB.200359. Publisher: The Royal Society.
50. Qingyou Du, Yoshinori Kawabe, Christina Schilde, Zhi-hui Chen, and Pauline Schaap. The Evolution of Aggregative Multicellularity and Cell–Cell Communication in the Dictyostelia. *Journal of Molecular Biology*, 427(23):3722–3733, November 2015. ISSN 00222836. doi: 10.1016/j.jmb.2015.08.008.
51. Sanea Sheikh, Cheng-Jie Fu, Matthew W. Brown, and Sandra L. Baldauf. The Acrasis kona genome and developmental transcriptomes reveal deep origins of eukaryotic multicellular pathways. *Nature Communications*, 15(1):10197, November 2024. ISSN 2041-1723. doi: 10.1038/s41467-024-54029-z. Publisher: Nature Publishing Group.
52. David L. Kirk. *Volvox: A Search for the Molecular and Genetic Origins of Multicellularity and Cellular Differentiation*. Cambridge University Press, 1998. ISBN 978-0-521-45207-6. Google-Books-ID: 1MdJS2086usC.
53. Nicole King. The Unicellular Ancestry of Animal Development. *Developmental Cell*, 7(3): 313–325, September 2004. ISSN 15345807. doi: 10.1016/j.devcel.2004.08.010.
54. Marine Olivetta, Chandni Bhickta, Nicolas Chiaruttini, John Burns, and Omaya Dudin. A multicellular developmental program in a close animal relative. *Nature*, 635:1–8, November 2024. ISSN 1476-4687. doi: 10.1038/s41586-024-08115-3. Publisher: Nature Publishing Group.
55. Joost B. Beltman, Athanasius F.M. Marée, Jennifer N. Lynch, Mark J. Miller, and Rob J. de Boer. Lymph node topology dictates T cell migration behavior. *Journal of Experimental Medicine*, 204(4):771–780, March 2007. ISSN 0022-1007. doi: 10.1084/jem.20061278.
56. Enrico Sandro Colizzi, Renske M.A. Vroomans, and Roeland M.H. Merks. Evolution of multicellularity by collective integration of spatial information. *eLife*, 9:1–57, October 2020. ISSN 2050084X. doi: 10.7554/ELIFE.56349. Publisher: eLife Sciences Publications Ltd.
57. Enrico Sandro Colizzi, Paulien Hogeweg, and Renske M.A. Vroomans. Modelling the evolution of novelty: a review. *Essays in Biochemistry*, 66(6):727–735, December 2022. ISSN 0071-1365, 1744-1358. doi: 10.1042/ebc20220069. Publisher: Portland Press Ltd.
58. Nicole King, M. Jody Westbrook, Susan L. Young, Alan Kuo, Monika Abedin, Jarrod Chapman, Stephen Fairclough, Uffe Hellsten, Yoh Isogai, Ivica Letunic, Michael Marr, David Pincus, Nicholas Putnam, Antonis Rokas, Kevin J. Wright, Richard Zuzow, William Dirks, Matthew Good, David Goodstein, Derek Lemons, Wanqing Li, Jessica B. Lyons, Andrea Morris, Scott Nichols, Daniel J. Richter, Asaf Salamov, Jgi Sequencing, Peer Bork, Wendell A. Lim, Gerard Manning, W. Todd Miller, William McGinnis, Harris Shapiro, Robert Tjian, Igor V. Grigoriev, and Daniel Rokhsar. The genome of the choanoflagellate Monosiga brevicollis and the origin of metazoans. *Nature*, 451(7180):783–788, February 2008. ISSN 0028-0836, 1476-4687. doi: 10.1038/nature06617.
59. Steven L. Stephenson. Past and Ongoing Field-Based Studies of Myxomycetes. *Microorganisms*, 11(9):2283, September 2023. ISSN 2076-2607. doi: 10.3390/microorganisms11092283.
60. Sydney E Everhart and Harold W Keller. Life history strategies of corticolous myxomycetes: the life cycle, plasmodial types, fruiting bodies, and taxonomic orders. *Fungal Diversity*, 29 (1):1–16, 2008.
61. Pauline Schaap, Israel Barrantes, Pat Minx, Narie Sasaki, Roger W. Anderson, Marianne Bénard, Kyle K. Biggar, Nicolas E. Buchler, Ralf Bundschuh, Xiao Chen, Catrina

Fronick, Lucinda Fulton, Georg Golderer, Niels Jahn, Volker Knoop, Laura F. Landweber, Chrystelle Maric, Dennis Miller, Angelika A. Noegel, Rob Peace, Gérard Pierron, Taeko Sasaki, Mareike Schallenberg-Rüdinger, Michael Schleicher, Reema Singh, Thomas Spaller, Kenneth B. Storey, Takamasa Suzuki, Chad Tomlinson, John J. Tyson, Wesley C. Warren, Ernst R. Werner, Gabriele Werner-Felmayer, Richard K. Wilson, Thomas Winckler, Jonatha M. Gott, Gernot Glöckner, and Wolfgang Marwan. The Physarum polycephalum Genome Reveals Extensive Use of Prokaryotic Two-Component and Metazoan-Type Tyrosine Kinase Signaling. *Genome Biology and Evolution*, 8(1):109–125, January 2016. ISSN 1759-6653. doi: 10.1093/gbe/evv237.

62. Labyrinthulomycota. In John M. Archibald, Alastair G.B. Simpson, and Claudio H. Slamovits, editors, *Handbook of the Protists*, pages 507–542. Springer International Publishing, Cham, 2017. ISBN 978-3-319-28147-6 978-3-319-28149-0. doi: 10.1007/978-3-319-28149-0.
63. Izumi Iwata, Kei Kimura, Yuji Tomaru, Taizo Motomura, Kanae Koike, Kazuhiko Koike, and Daiske Honda. Bothrosome Formation in *Schizochytrium aggregatum* (Labyrinthulomycetes, Stramenopiles) during Zoospore Settlement. *Protist*, 168(2):206–219, April 2017. ISSN 1434-4610. doi: 10.1016/j.protis.2016.12.002.
64. Stephen R Fairclough, Zehua Chen, Eric Kramer, Qiandong Zeng, Sarah Young, Hugh M Robertson, Emina Begovic, Daniel J Richter, Carsten Russ, M Jody Westbrook, Gerard Manning, B Franz Lang, Brian Haas, Chad Nusbaum, and Nicole King. Premetazoan genome evolution and the regulation of cell differentiation in the choanoflagellate Salpingoeca rosetta. *Genome Biology*, 14(2):R15, 2013. ISSN 1465-6906. doi: 10.1186/gb-2013-14-2-r15.
65. Rosanna A. Alegado, Laura W. Brown, Shugeng Cao, Renee K. Dermenjian, Richard Zuzow, Stephen R. Fairclough, Jon Clardy, and Nicole King. A bacterial sulfonolipid triggers multicellular development in the closest living relatives of animals. *eLife*, 1:e00013, October 2012. ISSN 2050-084X. doi: 10.7554/eLife.00013.
66. Stephen R. Fairclough, Mark J. Dayel, and Nicole King. Multicellular development in a choanoflagellate. *Current Biology*, 20(20):R875–R876, October 2010. ISSN 09609822. doi: 10.1016/j.cub.2010.09.014.
67. Núria Ros-Rocher, Josean Reyes-Rivera, Uzuki Horo, Chantal Combredet, Yeganeh Foroughijabbari, Ben T. Larson, Maxwell C. Coyle, Erik A. T. Houtepen, Mark J. A. Vermeij, Jacob L. Steenwyk, and Thibaut Brunet. Clonal-aggregative multicellularity entrained by salinity in one of the closest relatives of animals, October 2024. Pages: 2024.03.25.586565 Section: New Results.
68. Marjon G. J. de Vos, Alexandre Dawid, Vanda Sunderlikova, and Sander J. Tans. Breaking evolutionary constraint with a tradeoff ratchet. *Proceedings of the National Academy of Sciences*, 112(48):14906–14911, December 2015. doi: 10.1073/pnas.1510282112. Company: National Academy of Sciences Distributor: National Academy of Sciences Institution: National Academy of Sciences Label: National Academy of Sciences Publisher: Proceedings of the National Academy of Sciences.
69. Marjon G. J. de Vos, Frank J. Poelwijk, Nico Battich, Joseph D. T. Ndika, and Sander J. Tans. Environmental Dependence of Genetic Constraint. *PLOS Genetics*, 9(6):e1003580, June 2013. ISSN 1553-7404. doi: 10.1371/journal.pgen.1003580. Publisher: Public Library of Science.
70. Barrett Steinberg and Marc Ostermeier. Environmental changes bridge evolutionary valleys. *Science Advances*, 2(1):e1500921, January 2016. doi: 10.1126/sciadv.1500921. Publisher: American Association for the Advancement of Science.
71. Guilhem Doulcier, Peter Takacs, Katrin Hammerschmidt, and Pierrick Bourrat. Stability of ecologically scaffolded traits during evolutionary transitions in individuality. *Nature Communications*, 15(1):6566, August 2024. ISSN 2041-1723. doi: 10.1038/s41467-024-50625-1. Publisher: Nature Publishing Group.
72. Andrew J. Black, Pierrick Bourrat, and Paul B. Rainey. Ecological scaffolding and the evolution of individuality. *Nature Ecology & Evolution*, 4(3):426–436, March 2020. ISSN 2397-334X. doi: 10.1038/s41559-019-1086-9. Publisher: Nature Publishing Group.
73. Caroline J. Rose, Katrin Hammerschmidt, Yuriy Pichugin, and Paul B. Rainey. Metapopulation structure and the evolutionary transition to multicellularity. *Ecology Letters*, 23(9):1380–1390, 2020. ISSN 1461-0248. doi: 10.1111/ele.13570. _eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1111/ele.13570.
74. Walter Veit. Scaffolding Natural Selection. *Biological Theory*, 17(2):163–180, June 2022. ISSN 1555-5550. doi: 10.1007/s13752-021-00387-6.
75. Adriano Bonforti and Ricard Solé. Unicellular–multicellular evolutionary branching driven by resource limitations. *Journal of the Royal Society Interface*, 19(191):20220018, June 2022. ISSN 1742-5689. doi: 10.1098/rsif.2022.0018.