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Social conflicts in *Dictyostelium discoideum* : a matter of scales

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Abstract

The 'social amoeba' *Dictyostelium discoideum*, where aggregation of genetically heterogeneous cells produces functional collective structures, epitomizes social conflicts associated with multicellular organization. 'Cheater' populations that have a higher chance – quantified by a positive spore bias – of surviving to the next generation when mixed with cooperators bear a selective advantage. Their spread is thus expected to undermine collective functions over evolutionary times. In this review, we discuss the two main approaches adopted to conceptualize social conflicts in *Dictyostelium discoideum*: describing social interactions as a property of cell populations (strains), or as a result of individual cell choices during the developmental process. These two points of view are often held equivalent and used interchangeably. While the population-level view grants more direct evolutionary inference, however, the cell-level interpretation reveals that such evolutionary predictions may be modified if mechanisms such as dependence on the environment, development and intrinsic unpredictability of cell fate choices are taken into account. We conclude by proposing a set of open questions that in our opinion lie at the core of a multi-scale description of aggregative life cycles, where the formulation of predictive evolutionary models would include cell-level mechanisms responsible for spore bias alongside population-level descriptors of multicellular organization.

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1 Introduction

Many unicellular organisms spend at least part of their lifetime within associations that have a functional role, as they allow their composing cells to resist stress, to be defended against predators, or to engage in collective behaviour. Multicellular organization has been integrated in life cycles, that in some cases alternate periods of growth as single cells, and phases - typically triggered by nutrient depletion - where initially sparse cells gather in more or less complex multicellular aggregates (Grosberg & Strathmann, 2007; Du et al., 2015). The transition from a chiefly unicellular life style to such aggregative life cycles occurred at least six times independently along the tree of life and in all major eukaryotic clades (Parfrey & Lahr, 2013; Du et al., 2015). Its repeated emergence suggests this form of multicellular organization is not the outcome of serendipity, but may reflect general organization principles (Grosberg & Strathmann, 2007; van Gestel & Tarnita, 2017; Arias Del Angel et al., 2020).

The social amoeba *Dictyostelium discoideum* has been widely used to identify such principles and to explore the action of selection on cellular collective organization. The evolutionarily stability of its multicellular life cycle, despite conflicts among cells that adopt different social strategies, makes it a model organism for addressing both the maintenance of cooperative behaviour (Strassmann & Queller, 2011; Medina et al., 2019) and the evolutionary emergence of new levels of organization (van Gestel & Tarnita, 2017).

D. discoideum's life cycle comprises a vegetative phase, where cells grow in isolation, and a collective social phase induced by starvation (Kessin, 2001). The multicellular phase starts with aggregation, when cells converge towards aggregating centers by chemotaxis guided by the gradient of a signalling molecule, cyclic adenosine monophosphate (cAMP) (Devreotes & Zigmond, 1988; Fisher et al., 1989). Eventually, most cells in the population belong to multicellular aggregates, or mounds, each composed of tens of thousands individual cells. Later, mounds elongate into slugs, chemotactic and phototactic worm-like structures with the ability to sense and move towards bright and dry environments, like the soil surface (Raper, 1940; Bonner et al., 1950). Here, slugs produce fruiting bodies that can be picked up by insects and dispersed (Smith et al., 2014). Starting from the mound stage at latest, cells proceed to differentiate into several tissues (Early et al., 1993; Kessin, 2001). Because of their prevalence and their ease of detection, most attention has been given to two cell types: spores, that seed the following generation, and stalk cells, that support the spore mass. Analogous to somatic cells in metazoans, stalk cells die.

Giving up one own's descendants to favour spore dispersal is considered the most extreme degree of altruistic behavior, and raises the question of the evolutionary stability of such arrangement (Strassmann & Queller, 2011). In 'paradigmatic' multicellular organisms with single-cell bottleneck followed by clonal growth, conflicts between different cell types (e.g. between normal and cancer cells (Aktipis et al., 2015)) can get resolved by purging entire cell lineages (Godfrey-Smith, 2009). Their disruptive effect is instead enhanced when multicellular aggregates are genetically heterogeneous (Buss, 1982). In *Dictyostelium*,

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different genotypes can coexist within a same fruiting body both in the wild and in the lab, indicating that this organism has found solutions to curb the effects on fitness of such conflicts and their evolutionary impact.

The first fundamental issue when considering the action of selection on multicellular organization is how to measure differential fitness between co-aggregating genotypes. In *Dictyostelium*, reproductive success can be evaluated at the end of the life cycle, when cells are terminally differentiated into spores or stalk. Spore cells are indeed the only fraction of the population that is able to survive long periods of starvation, and reproductive success hinges upon their production. Moreover, cells that die forming the stalk provide a clear advantage to spores. Due to these features, shared also by other organisms such as *Myxobacteria* (Velicer et al., 2000), genotypes that tend to form an increased fraction of spores when mixed in chimerae are commonly called 'cheaters'. Strains that – being found in lesser proportion in the spores – get exploited by virtue of their disproportional contribution to the stalk, are called instead 'cooperators'. Practically, social strategies are assessed in chimerae obtained by mixing, prior to aggregation, cells belonging to two different strains. Spore bias is then typically quantified as the percentage of spores of one strain in the spore pool, relative to the percentage of cells of that strain in the initial mix (Kuzdzal-Fick et al., 2010, 2011; Gilbert et al., 2007) (this assumes that the spore-to-stalk ratio within fruiting bodies is constant, but see (Buttery et al., 2009, 2010) for generalizations). All else being equal, then, a cheater strain will see its frequency increased in the population of vegetative cells ensuing from spore germination, thus in the following generations. In the domain of evolutionary biology, most attention has been devoted to understanding why in *Dictyostelium* selection of cheater strains does not doom collective function altogether.

The intuition that the advantage reaped by cheaters within one life cycle will result, if aggregation occurs over and again, in the long-term demise of cooperators matches well the formalism of evolutionary game theory (Hofbauer & Sigmund, 1998). Games that oppose cooperators and cheaters, such as the Prisoner's Dilemma or Public Good Games, typically predict that unbridled natural selection is expected to wipe out cooperation.

In this review, we step back and examine the observational bases of different conceptual models for social interactions in *Dictyostelium*, paying particular attention to the evolutionary expectations associated to the existence of cheaters. We first discuss the conditions for maintaining cooperative behaviour under the assumption that the outcome of interactions between strains are exclusively determined at the genetic level. We successively review the growing literature on non-genetic (chance, environment and social context) dependence of the developmental process, which ultimately produces a given partition of cells in spores or stalk. Although it is largely unknown how much such dependence can alter quantitatively the outcome of strain interactions, the possibly large evolutionary consequences of a variable relation between a genotype and spore bias motivates delving into the underlying cell-level mechanisms, which we discuss in the second part of the paper. Finally, we discuss possible solutions to describing cell

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behaviour on multiple spatial and temporal scales, and to selecting null and predictive models for the evolution of aggregative multicellular organisms.

2 Strategies of interacting strains

Spore bias is traditionally measured in chimerae where two different strains are mixed – often in equal proportions – at starvation, after which cells undergo only one more cell division. When strains are equivalent, thus, the fraction of spores belonging to one strain is expected to be equal to the proportion of that strain in the initial mix. Deviations from this 'neutral' composition of the spore mass quantify the degree of cheating of one with respect to another strain (Kuzdzal-Fick et al., 2010, 2011; Gilbert et al., 2007). Cheating behaviour is thus defined at the level of interacting populations of cells, connecting directly the genotype of the strain to the outcome of the social interactions. For instance, 'obligate cheaters' are genotypes, found in natural isolates or derived from lab strains, that have a positive spore bias when mixed with other strains, but that cannot develop alone (Buss, 1982; Kuzdzal-Fick et al., 2011; Ennis et al., 2000)). Such strains always have a reproductive advantage over different genotypes, however they cannot disperse if they meet a strain of their same kind.

The reproductive output in chimerae can thus be formalized as the payoff of a game opposing individual strains. Cheater genotypes exploit cooperator genotypes by enhancing their own representation in the following generation. Such a situation is represented by the Prisoner's Dilemma, a two-player game whose chief feature is that cheating is always the most rational option if the strategy of the partner is unknown, even though the best result is achieved when the two cooperate. Evolutionary game theory predicts that, after many rounds of the game (here, cycles of co-aggregation) in which players (here, strains with a fixed associated social strategy) meet at random, cooperators will be outnumbered by cheaters.

The problem of maintaining or evolving cooperation in two-players games has found several solutions in the general framework of game theory (Nowak, 2006). In the case of *Dictyostelium*, the most commonly invoked means of preventing the invasion of cheaters is kin selection, where high genetic relatedness is the key condition for cooperative behavior to be favoured by natural selection (W. E. Kerr, 1950; Strassmann & Queller, 2011). According to Hamilton's rule (Hamilton, 1964), in order for altruistic genes to increase in frequency, the level of genetic relatedness r between the cooperator and the recipient of the cooperative act must exceed c/b, where c is the cost paid by the cooperator and b is the benefit received by the recipient. Originally, the relatedness r in a population was defined, based on genetic identity by descent, as the probability that two random individuals share the same allele at one given social locus. Subsequently, other measures of social interaction bias towards individuals that carry the cooperative allele have been proposed as proxies for relatedness, most notably the frequency of cells of a given type in the population (Queller, 1994). More generally, cooperative behaviour is expected to spreads as long as coop-

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erative individuals have a sufficiently higher chance of interacting with other cooperators than with cheaters, thus assort positively, and this independent of identity by descent (Fletcher & Doebeli, 2009). Relatedness, and generally assortment, are population-level statistics, that describe the average behaviour of cells of a given genotype in the population. As well as strain interaction parameters, they can in principle vary in time, but are usually considered to be constant across multiple aggregation cycles. Under these assumptions, sociobiology maintains that strong relatedness explains the maintenance of cooperative social behaviour against the spread of cheating in aggregative multicellular organisms (Strassmann et al., 2000; Medina et al., 2019).

Genetic assortment between strains

Evidence of genetic assortment in *D. discoideum* populations both in natural and artificial environments has been put forward in support of the importance of kin selection. In natural populations, assortment was quantified based on genetic identity. Relatedness between strains was estimated by polymorphism in microsatellite sequences, even though these were not strictly located in genes responsible for social behaviour. These molecular studies found higher levels of relatedness within fruiting bodies compared to soil samples (Fortunato et al., 2003; Gilbert et al., 2007). In the laboratory, where chimerae of couples of strains are obtained in standardized conditions, one can quantitatively assess the dependence of cheating intensity on strain proximity. Mixing natural clones in 15 co-aggregations, Strassman and colleagues found a positive correlation between spore bias and genetic distance (Strassmann et al., 2000). Analysis of the composition of fruiting bodies, instead of the proportion of spores in the population, found that genetically distant strains segregate more than closer ones (E. A. Ostrowski et al., 2008). Similar observations realized in lab-created chimerae of *D. purpureum* and *D. giganteum* (Sathe et al., 2010) confirmed that strains of two species mix to varying degrees, with strains genetically farther apart often segregating in separate multicellular aggregates.

These studies support the idea that even though strains may be unable to completely exclude each other from groups, they can bias group composition so as to reduce genetic dissimilarity. However, the permanence of the social identity – as a cooperator or a cheater – of a given strain now hinges upon how aggregation takes place and on the extent to which cells partitioning into groups (what in physics would be called structure at the 'mesoscale') is controlled by genes.

Cell assortment, as measured by proxies such as genetic relatedness, can be achieved in multiple ways. First, it can be the consequence of 'passive' mechanisms, that do not require any particular adaptation for strain-specific recognition. Passive sources of assortment are thus most relevant for explaining how multicellular organization emerged from unicellular ancestors, before more sophisticated means of cell-cell signalling were set in place. Passive mechanisms include limited dispersal in a spatially extended environment, whereby populations are structured in clusters of genetically identical individuals (Hamilton,

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1964). Limited dispersal can for instance explain why a regional pool of species is not fully represented in single fruiting bodies that assemble from locally aggregated cells. Non-specific differences in physical properties, such as adhesion or motility can moreover result in non-uniform mixing and sustain cooperative behaviour even when cells are initially uniformly distributed in space (Garcia et al., 2014, 2015; Joshi et al., 2017; van Gestel & Nowak, 2016).

Second, high assortment can be achieved through active sorting that makes cells group preferentially with cells of the same genotype, a mechanism also known as 'kin discrimination'. *D. discoideum* possesses a number of specific genes involved with cell-cell adhesion that are expressed during both aggregation and development, and that are central to multicellular organization (Glöckner et al., 2016). In particular, the family of Tiger genes coding for trans-membrane proteins provides a lock-and-key mechanism for adhesion between cells that carry a same allele (Benabentos et al., 2009). Analogous to self *versus* non-self-recognition mediated by major histone compatibility loci, Tiger genes display a 40-fold elevation in genetic diversity compared to the rest of the genome (E. Ostrowski et al., 2015; Flowers et al., 2010; Benabentos et al., 2009). Such a high degree of polymorphism is consistent with the idea that recognition with high genetic resolution is essential to achieve efficient segregation between co-aggregating strains (E. A. Ostrowski, 2019). It is moreover considered as a signature of the selective advantages conferred by novel genetic variants of 'green beards',that prevent the invasion of cheaters faking the signals of cooperation by weakening the linkage between adhesion and cooperative behaviour. (Gruenheit et al., 2017). Since Tiger genes play also a role in auto-organization within chimeric aggregates (Gruenheit et al., 2017), however, it is not yet entirely clear at what stage – aggregate formation or multicellular development – they mainly affect the outcome of interactions between strains. The distinction is not futile, in that molecular mechanisms may be expected to provide a firmer basis to genetically-determined strategies if the bias is the outcome of multicellular, canalized, development, rather than being determined during aggregation, when strains encounter multiple sources of non-genetic variability.

Evolutionary dynamics of genotypes

In order to ascertain if the degree of assortment provided by a given mechanism is sufficient to explain the stability of cooperation in *Dictyostelium*, one would ideally like to check that Hamilton's rule applies quantitatively. A major obstacle to this is the difficulty of measuring the relevant parameters, first and foremost relatedness, without assuming a priori that they are constant for any given couple of strains. What can be done, instead, is to check that changes in strain frequencies on long time scales are consistent with observations realized on a single aggregation cycle.

Experimental evolution assays have been conducted by repeating cycles of aggregation and dispersal in conditions that are as close as possible to producing random cell encounters ('low relatedness' conditions) (Kuzdzal-Fick et al., 2011). Strains that increased in frequency in 30 cycles also produced a larger

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share of spores than the ancestral strain which was used to seed all the experimental lines. Estimation of the mutation rate from ('high relatedness') lines propagated clonally in a separate experiment moreover indicated that the change in frequency, estimated via a population genetics model, was not quantitatively compatible with random drift (Kuzdzal-Fick et al., 2011). It was therefore explained as a consequence of the selective advantage conferred by cheating. Exclusion of cooperators by cheater strains was however not observed. Other experimental evolution assays however did not support the hypothesis that selection always favours cheating strains. In an experiment involving mixtures of environmentally collected strains, 10 cycles of aggregation-dispersal were conducted starting in conditions of high and low cell density (Saxer et al., 2010). As expected, high density conditions associated to higher relatedness, and resulted in a smaller variability in the strains dominating the population. Unexpectedly, however, strains evolved at high relatedness were not cheated by the winner of the low relatedness treatment, as would have been predicted by the theory of kin selection.

Other than direct evolutionary experiments, methods from population genetics have been deployed in natural populations to reveal selection acting on cheating. The genomic signatures of 'social genes', *i.e.* genes preferentially expressed during the multicellular phase of the life cycle, display signs of rapid evolution (high rate of non-synonymous mutations) compared to the rest of the genome (Sucgang et al., 2011). This result, however, has been subsequently interpreted as the effect of diluted selection, occurring when the expression of social genes is temporally restricted to the multicellular phase of the life cycle (de Oliveira et al., 2019). When this effect is taken into account, previously reported differences in the level of polymorphism between pre-stalk and pre-spore genes (Noh et al., 2018) are no longer detected, making it impossible to conclude on the role of kin selection in shaping the evolution of social interactions in *D. discoideum*.

Association of a genotype – through its social behaviour – to its expected evolutionary consequences thus appears insufficient to explain the evolutionary dynamics of aggregative multicellular organization. Part of the problem may stem from representing multicellular function as the product of a game that opposes cooperating and cheating players, where these players are the strains co-aggregating in a chimera. If this view makes an immediate and enticing link to the Prisoner's Dilemma, that stands out as the null model for evolutionary predictions, it reposes on assumptions that are not routinely tested, such as the existence of 'strategies' that are genetically set and invariable.

In more mechanistic terms, one can also consider the population-level outcome of strain interactions as the effective description (at a macroscopic, population-level scale) of cell-level interactions among cooperator and cheater strains (Peña et al., 2014, 2015; Van Cleve, 2017). 'Interaction payoffs' for a given pair of strains now depend on population structure, that is dynamic and not purely established by genes. Therefore, there is no guarantee that they can be permanently associated to a given genotype, whose associated social role (if it is a cheater and how much it cheats) is bound to change during the evolution-

ary process. Perhaps more disturbingly, effective games may describe situations where cheaters do not beat cooperators over repeated random encounters, so that the existence of *Dictyostelium* strains that vary in spore allocation would not represent an evolutionary paradox. As we discuss later, for instance, if strains play an effective snowdrift game, the evolutionary stable strategy is a polymorphic state (Doebeli, 2004).

The question then poses of how is spore bias generated, and on to the extent of genetic control over the outcome of strain-level interactions. In the following, we discuss the experimental evidence that spore bias depends also on the physiological conditions and social environment experienced by cells, so that the genotype controls only partially the result of social interactions, with important implications as to what should be the null expectations for the evolution of aggregative multicellular life cycles.

3 Cell-level strategies

A central feature of aggregative multicellularity in *Dictyostelium* is that genetically identical cells differentiate into spores or stalk (see (Brown & Firtel, 1999) for a review of the underlying molecular processes). Viewed at the cellular level, cheating of one cell that is part of a binary chimera is then associated to a probability of becoming a spore higher than for cells of the other strain. This alternative point of view has implications in the way strategies are conceptualized. With the exception of few obligate cheater strains, that only form spores and can be thought of playing 'pure strategies', a cell-level strategy now reflects any single player's 'choice' between two alternative fates, one allowing survival, and the other leading to death.

A first way to formalize such choice is to consider that every strain is characterized by the probability that any of its cells will cooperate – forming the stalk – or cheat – becoming a spore. Such genetically-encoded strategy would not change in time. However, the outcome of interactions between a focal cell and multiple other cells will generally depend on the social structure of the population, notably the size and composition of multi-player groups (Gokhale & Traulsen, 2014; Peña et al., 2015). It can turn out that strains composed by cells that have a higher probability of cheating are unable to outcompete more cooperative strains (Matsuda & Harada, 1990; Hudson et al., 2002; Uchinomiya & Iwasa, 2013). A simple scenario when this happens is the so-called 'Simpson's paradox', reflecting the fact that, when individuals interact in groups, the difference in payoff of two strategies can have opposite sign if one considers single individuals within groups or the (weighted) average across all the population (Chuang et al., 2009). Applied to *Dictyostelium*, this means that even though strains that produce more spores are advantaged in every group, their overall spore production – averaged over aggregates of different composition – would be diminished by the poor performance of fruiting bodies dominated by cheaters. Thus, cell-level cheating would not translate into strain-level cheating.

Stepping back from the notion that cheating is a strain-level genetically-

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determined strategy, in this section we consider alternate conceptual models of how cellular fate is determined in chimerae, and their expected consequences on the evolutionary dynamics of strains that display positive spore bias (summarized in Table 1). In particular, we would like to stress that in these frameworks 'cheaters' – defined as usual through binary mixes of equal amounts of genetically different cells – are not expected to be systematically selectively favoured. This observation questions whether the existing classification of social behaviour is relevant for addressing the evolution of aggregative multicellularity. Moreover, it highlights the need for a better understanding of the mechanisms underpinning differences in spore production.

Conceptual model	Mechanism	Evolutionary consequences
Lottery	Phenotypic variation independent of the genotype (see Supp. Inform.)	Neutrality of cheating upon one aggregation
Bet-hedging	Unpredictable environmental variations affecting all cells	Neutrality of cheating on long times
Context-dependence	Frequency-dependent spore bias	Possible polymorphic evolutionary stable states

Table 1: Conceptual models for the evolution of cell-level behavioural strategies that do not lead to the unconditional evolutionary success of 'cheater' strains (as defined based on spore bias in a given environment).

Cellular 'lotteries'

Genes are at the basis of cellular behaviour and dictate how external inputs are translated into specific phenotypic states. However, the probability that single *Dictyostelium* cells turn into a spore or contribute to the stalk can be also affected by factors other than the genotype. Ample evidence exists that phenotypic heterogeneity, and in particular non-genetic differences among cells that were established before the beginning of the multicellular phase, can bias developmental fate (Chattwood & Thompson, 2011).

In the Supplementary Information, we review several experimental studies correlating the probability that isogenic cells develop into a spore with its phenotypic state before and during multicellular development. These investigations, summarized in Table S1 of the SI, reveal that decisions at the cellular level may reflect factors out of direct genetic control, such as the history of the cell during vegetative growth – notably the availability and quality of food – or the phase of the cell cycle at the moment of starvation. Notably, the relevant phenotypic traits of the cell may change depending on its social context, as we will discuss later. Although it is not yet clear how, during development, initially heterogeneous cellular phenotypic features are translated into settled social roles (we discuss a few hypotheses in the SI), weakening the causal relationship between

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a cell's social behaviour and its genes opens the door to establish alternative null models for the evolutionary dynamics.

Let us consider the extreme case where spore bias is determined independently of the cell's genotype, so that selection acts on purely phenotypic variation (Nanjundiah, 2019). Such scenario, represented by 'lottery' or 'musical chairs' conceptual models, has been invoked as a mechanism mitigating the success of cheating strains (Strassmann & Queller, 2011; Rainey, 2015).

Several factors affecting cell fate in monoclonal populations could contribute to loosening the link between the genotype of a cell and its probability of turning into a spore. A potential intrinsic source of unbiased phenotypic heterogeneity is the necessity of any cell to progress through the cell cycle. If, as discussed in the Supplementary Information for monoclonal populations, the cell cycle phase is not synchronized in the population, and it sets the probability of forming a spore, then the fate of any focal cell will be essentially determined by the time when starvation occurs. Like in a "musical chair" game, the moment when aggregation starts is out of one cell's direct control, making cell fate choice a stochastic decision independent of the genes. As long as cell cycle phase is uniformly distributed in the overall population, a cell indeed cannot predict what its phase is relative to cells of its own or another strain. Population-level observations that cultures can be synchronized by cold shock, release from stationary phase or treatment with drugs that block the cell cycle (Maeda, 1986; Araki & Maeda, 1995; Weijer & Duschl, 1984) indicate that cell cycles are generally desynchronized. This is also supported by a quantitative mathematical model of phase drift along lineages, indicating that cells loose rapidly synchronization in typical *D. discoideum* culture conditions (Gruenheit et al., 2018), even though they may not in other circumstances (Segota et al., 2014).

Unpredictability in cell-fate decision could moreover be the consequence of external rather than internal contingency: independent of the genotype, some cells may happen to be better fed than others after having encountered different amounts of food, or food of different quality. Such contingencies are expected to affect every cell in similar manner before aggregation starts. As a consequence, reproductive success would not be a heritable trait associated to any given genotype. Nanjundiah and co-workers proposed that the 'quality' of a cell when it faces starvation, established from a combination of genotype, environment and historical contingency, underpins the probability of developing into a spore (Zahavi et al., 2018). The stalk would be composed chiefly by cells that are anyways condemned by their poor nutritional status, while spores would comprise cells that have a higher chance of survival. In this perspective, not only cheating would not be expected to swipe through the population, but the conflicting nature itself of the interactions within the multicellular stage would be downsized.

Environmental fluctuations and bet-hedging

Although the weight of stochasticity relative to genetic determinism in cell fate determination is unknown, pure lottery models seem unrealistic, as cell fate

is certainly affected by genes. Evolutionary outcomes similar to lottery models are nonetheless obtained when the genetically-encoded probability of becoming a spore varies in time: spore bias can be predicted in any given environment based on the genotype, but the genotype's frequency in the long term depends on the sequence of conditions cells experience. Such contrast between short-term and evolutionary success is commonly encountered in microbial species, where multiple phenotypes – including those that appear maladapted to a specific environmental context – coexist within monoclonal populations (Ackermann, 2015; Grimbergen et al., 2015). Single-cell stochastic transitions between phenotypes with different adaptive value allow strains to cope with a varying environment by hedging their bets among several alternative behaviours (Kussell & Leibler, 2005). Instead of supposing, as in lottery models, that cell fate is independent of the genotype, bet-hedging models assume that all strains face the same type of reproductive uncertainty. Let us consider the previously discussed case of different cell quality (Zahavi et al., 2018). Even if cells of a given strain have a higher quality in one specific environment, such relative advantage may reverse in other environments. Averaging over multiple aggregation-dispersion cycles in variable conditions, different strains may end up having the same overall success.

These concepts have been specifically applied to study the evolution of the so-called 'loner' strategy, adopted by *Dictyostelium* cells that do not join at all multicellular aggregates. In games traditionally opposing cheating to cooperation, addition of such a strategy is sufficient to avert the tragedy of the commons (Hauert, 2002). In *Dictyostelium*, the loner strategy has been proposed as a way to prevent the invasion of cheaters (Dubravcic et al., 2014; Tarnita et al., 2015). The potential relevance of non-aggregated cells has been supported by experimental observations both on lab and wild strains. A sizeable fraction of cells is indeed invariably found outside aggregates. These cells are able to start vegetative growth faster than aggregated cells when nutrients are renewed shortly after aggregation, but they cannot survive long period of starvation (Dubravcic et al., 2014; Tarnita et al., 2015; Rossine et al., 2020). The partition of a population in loner and aggregated components was modelled as the consequence of a cell-level stochastic choice, where the genotype determines the probability of staying alone (Dubravcic et al., 2014; Tarnita et al., 2015; Martínez-García & Tarnita, 2016). Even if cell fate choice within aggregates is genotype-independent, different strains vary in spore production because of their differential contribution to aggregates. Numerical simulations showed that frequent replenishment of nutrients favours genotypes that have a larger fraction of solitary cells, whereas more aggregative types that commit to social behaviour have an advantage in times of famine. On longer time scales, environmental unpredictability and limited dispersal lead, independent of relatedness, to coexistence of multiple genotypes in spite of differences in their social behaviour.

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Cell-level response to social context

Phenotypic variability is not only influenced by extrinsic fluctuations that affect all cells equally. Even before multicellular groups can be clearly distinguished, the local environment of one cell is indeed dictated by other cells present within the same local neighbourhood. Similarly, in multicellular aggregates cells interact with each other through chemical signals (as reviewed in (Loomis, 2014)) and mechanical forces. Such local 'social' environment is particularly important to determine cell fate, thus strain dominance, in chimerae. When strategies are considered at the level of single cells, a manifestation of social context-dependence is that spore bias depends not only on the genotype, but also on how many cells belong to one or another of the co-aggregating strains.

Numerous studies indicate that frequency-dependent changes in spore bias is the rule rather than the exception in chimerae of both *D. discoideum* (Gilbert et al., 2007; Madgwick et al., 2018) and other dictyostelids (Sathe & Nanjundiah, 2018). Strains identified as cheaters by mixing equal amounts of cells of two genotypes thus have variable success against a cooperator counterpart when their relative proportions are changed. In particular, when they make up most of the population, the proportion of spores that a cheater strain produces may be upper bounded if the stalk/spore ratio is maintained. Moreover, in a chimera composed of a mutant that does not produce stalk cells (Buss, 1982) and a strain that develops normally, an increase in the proportion of cheater cells may produce disproportionately large, prone to collapse, spore heads and thus undercut the reproductive success of the cheater itself.

When frequency-dependence is taken into account in game-theoretical models for interacting strains, repeated rounds of co-aggregation can yield different evolutionary predictions, only a subset of which prospect cheating as winning strategy. For instance, if spore bias is positive when cheaters are rare and negative when they are common, as in the snowdrift game, the evolutionary dynamics will lead to regimes of coexistence of opposite social strategies. Though context-dependent cell behaviour is often neglected when evolutionary projections are based on strain-level dominance of genotypes, a few mechanisms involving density or frequency of cells have been recently considered in their population-level effects on spore bias.

As discussed earlier in this section, a possible source of indirect effects on the proportion of spores produced by one strain in a chimera is the partition between aggregated and non-aggregated cells. When exploring the mechanistic bases of this partitioning, the probability of being a loner was found to depend, other than on the genotype, on cell density and environmental factors such as the hardness of the agar substrate (Rossine et al., 2020). Such dependence on both the biotic and abiotic context was explained by a mathematical model where the cell decision to aggregate is stochastic and conditional on a locally established quorum. In a genetic chimera, the probability that one cell of a given strain aggregates therefore depends on the nature and the proportion of other co-aggregating strains. For instance, strains that tend to aggregate less can

still contribute to aggregation of another strain, and they do so more efficiently when they are more dense. The end result of cell self-organization in groups is then frequency dependence, which can sustain coexistence of multiple strains over evolutionary times.

Cells can also modify their behaviour within multicellular aggregates, in response to proportions of co-aggregating strains. Within slugs, for instance, the concentration of diffusive compounds was suggested to be the key mediator of cell-level frequency-dependent fate determination (Parkinson et al., 2011) (discussed in more detail in the SI). Responsiveness to diffusible stalk-inducing factors (e.g. DIF) in particular, but also their production, was indeed found to reflect the linear social hierarchy of strains previously established based on cheating ability (Buttery et al., 2010). When considering the mechanistic bases of cellular strategies, complex behavioural patterns – whereby strains would adjust their behaviour depending on the social partner – were therefore suggested to follow from simple principles of context-dependent decision-making, that naturally lead to frequency-dependent interactions (Matsuda & Harada, 1990; Hudson et al., 2002; Madgwick et al., 2018).

In conclusion, spore bias is a population-level manifestation of cell-level mechanisms that span ranges of genetic vs epigenetic determinism and that respond differently to the abiotic and biotic context. In pairwise interactions, the contribution of different factors with distinct and independent effects on strains fitness was quantified by an analysis of variance (Buttery et al., 2010). Variation in contribution to the spore head in binary chimerae of natural clones was partitioned in three components: indirect genetic effects of the social partner's genotype, direct effect of the strain's own genotype, and epistatic interactions between the genotypes of the two partners. The first component reflects the influence of the competing strain on the focal strain's social behavior. The others connect to cell-level behaviour in a monoclonal population and in a chimera (other than the previously mentioned strain-level effects), respectively. The strain genotype (*i.e* the second component) was found to explain 57.6% of the variation in spore production, thus dominating the two terms linked to social interactions between strains. The importance of epistasis (23%) moreover suggests that the social context is as important as strain-level effects (Buttery et al., 2010). Without a mechanistic model able to explain how the partition in these three orthogonal components is realized, and what is the origin of the epistatic effects, such statistical analysis is however of limited application to evolutionary studies involving other strains or conditions of aggregation. It nonetheless suggests that natural selection may select cooperative behaviours despite their apparent failure in pair-wise competition.

Discussion

In this review, we pointed out that the predicted evolutionary fate of strains that, in chimerae, produce more than their fair share of spores depends on how such 'cheating' is achieved and formalized. Central to this picture is the

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level at which social behaviour is assessed, and the extent to which it is rooted in the genotype – thus invariable on ecological time scales. We wish now to discuss conceptual and experimental approaches that we deem most promising in advancing knowledge of how multicellular organization in *Dictyostelium* got established and is maintained. Primarily, this requires identifying what are the material bases of conflicts within multicellular aggregates, so as to ascertain what strains, and in which circumstances, are expected to see their evolutionary success curtailed by the peculiar structure of aggregative multicellular life cycles.

Describing social behaviour at multiple scales

Connecting cell-level to collective-level behavior is a classic undertaking not only for evolutionary biology (Okasha, 2006), but also for mechanistic bottom-up approaches to tissue organization (Ladoux & Mège, 2017). Bottom-up approaches describing cell mechanics and movement aim at classifying behaviours that emerge from interactions of units with differential physical properties. They yielded important insights, for instance, on how cells sort within tissues (Beatrici & Brunnet, 2011; Steinberg, 2007), and in particular on differentiation in *Dictyostelium* (Maree & Hogeweg, 2001). Although they remain simplified representations, these models are easier to interface with cell-level observations and can provide explicit descriptions of the origin of biases in aggregate composition and in spatial distribution of cells, as well as of the evolution of collective functionality (Garcia et al., 2015; Guttal & Couzin, 2010; Joshi et al., 2017; van Gestel & Nowak, 2016; Staps et al., 2019; Colizzi et al., 2020). Their integration into general evolutionary frameworks is, however, less straightforward. It often relies on numerical simulation and poses the problem of how to estimate – let alone evolve – the large number of parameters involved in microscopic descriptions. Simple mechanistic approaches, on the other hand, are useful tools for exploring the multiplicity of existing life cycles beyond that of *Dictyostelium* and to evaluate the role of selection acting at different levels of biological organization (Rainey & De Monte, 2014; De Monte & Rainey, 2014; van Gestel & Tarnita, 2017).

Other approaches connecting cells and multicellular structures rely on representation of fitness at multiple levels to infer the evolutionary dynamics. Multilevel selection proposes that trade-offs between benefits and costs to the lower-level units can be scaled up to determine fitness at the collective level (Michod, 2007). Similarly to the sociobiological approach, that is based on translating individual-level costs and benefits into inclusive fitness as a property of a whole population (Gardner & West, 2014; B. Kerr & Godfrey-Smith, 2009), the statistical description of the outcome of interactions does not inform on the processes underlying population-level success. Though these approaches have the great advantage of permitting elegant generalizations and exploitation of tools developed for population genetics, the existence and magnitude of genetically-determined, individual fitness costs and benefits are not easy to assess without elucidating the mechanisms underlying population-level statistics.

Finding meaningful ways to connect cell- and collective-level properties in

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assemblies that contain a collection of genotypes and phenotypes, and such that cell-level traits result in the functionality of the ensemble, is a central problem also in more general settings, like microbial communities (Tarnita, 2018; Liautaud et al., 2019; Doulcier et al., 2020). There, evolution of system-level properties through mutations in traits affecting species interactions, some of which of mutualistic or cooperative nature, is considered possible despite – and maybe thanks to – the high diversity among interacting cells. Viewing evolution of muticellularity in *Dictyostelium*, as well as in other microbes that form genetically heterogeneous aggregates, as an instance of community-level evolution may be useful for explaining the first emergence of higher levels of organization.

Stochastic vs deterministic bases of behaviour

The second challenge for formalizing selective differences among *Dictyostelium* strains is to evaluate the importance of cell-level stochasticity and the extent to which this can be effectively captured by deterministic models. Advances in single-cell observation techniques revealed the ubiquity of cell-to-cell phenotypic variation, invisible to population-level measures (Altschuler & Wu, 2010). Intracellular fluctuations, for instance due to small numbers of transcription factors, combined with nonlinearities in gene regulation networks, are believed to be major determinants of phenotypic heterogeneity in microbes and beyond (Perkins & Swain, 2009; Balázsi et al., 2011; Norman et al., 2015) and are increasingly considered as key factors influencing their evolutionary dynamics (van Boxtel et al., 2017; Draghi, 2019). The presence, within a monoclonal cell population, of phenotypes that are maladapted to a given environment at any given time is explained by their long-term advantages. Indeed, in rapidly fluctuating environmental conditions, bet-hedging among alternative phenotypes confers an overall advantage (Kussell & Leibler, 2005; Grimbergen et al., 2015).

Stochastic choices are thought to be involved at different moments of the life cycle of *Dictyostelium*, with possible implications on the final differentiation in stalk and spores. At the onset of aggregation, in establishing aggregation centers (Gregor et al., 2010; Sgro et al., 2015). During aggregation, in the decisions whether to follow the cAMP gradient (Rossine et al., 2020). During development, in mixing of pre-spore and pre-stalk cells within a slug (Weijer, 1999). On the other hand, phenotypic heterogeneity can also result from deterministic sources, such as the distribution of cell cycle phase in asynchronously dividing cultures (Jang & Gomer, 2011; Gruenheit et al., 2018) or the spatial distribution of cell density (Vidal-Henriquez & Gholami, 2019).

The extent to which different sources of variability can be treated as equivalent, when one only considers their population-level collective effects, is an open question. Spiking gene expression, for instance, produces regular population-level oscillations if cells respond to an external forcing, and an average stable signal if integrated over the timescale of aggregation (Corrigan & Chubb, 2014). It has moreover been proposed that heterogeneity in gene expression, with possibly long-term consequences on cell fate, results from modulation of spiking frequency, that happens on very fast time scales compared to the devel-

opmental process. Distributed individual cell choices, either driven by stochastic fluctuations or by asynchronicity, might indeed average out and be effectively represented by deterministic equations (Antolović et al., 2017).

In evolutionary game theory, mixed strategies describe cases when players have a fixed probability of adopting alternative fixed behaviours. For sufficiently simple games, the evolutionary predictions of the deterministic 'mean field' equations are identical to the case when a corresponding fraction of the population adopts one of the strategies (Hofbauer & Sigmund, 1998). Even in more complicated situations, when players interact in groups, the evolution of behavioural frequencies can be described by effective macroscopic equations (Peña et al., 2015). Stochasticity is then encompassed by the same deterministic theoretical framework used for fixed strategies. What can be lost in this transition is however the relation between the microscopic definition of a social behaviour and its macroscopic – thus also evolutionary – characterization. Determining whether a microscopic behaviour, say a higher probability of forming spores, is going to lead to the expected demise of more cooperative variants requires knowledge of many other factors, including population structure, responsiveness and game synergy (Van Cleve, 2017), which are not easily assessed and are not guaranteed to remain constant during evolution.

Interplay of different time scales

Finally, a major obstacle to connecting individual-level stochastic behaviour and strain-level spore bias in *Dictyostelium* is that social and abiotic environments experienced by cells change on time scales comparable with the developmental process. In other words, the phenotypic state of one cell and that of the surrounding population can feed-back onto one another during one life cycle. Such feedback potentially allows cells to evaluate the composition of the aggregate and consequently adjust their developmental fate (turning into spores or stalk). Strain-level decisions would then be dictated by 'strategic' cell-level choices within one single generation rather than by long-term evolutionary processes (Madgwick et al., 2018). Recently, molecular tools have been used to start examining how such decision-making is implemented during the process of aggregation and development (Gruenheit et al., 2018; Nichols et al., 2020).

The third major conceptual challenge in improving evolutionary models is hence to describe context-dependence in a mechanistic fashion. Predictions of different such models may then be compared to experimental data and with each other, so as to pinpoint what biological features are essential and what can be neglected with respect to their evolutionary consequences. It is generally accepted that when the conditions experienced by a cell do not vary too fast, the optimal strategy for coping with fluctuations is sensing the environment and switching phenotype accordingly (Kussell & Leibler, 2005). Such kind of response can occur on a rapid time scale – especially if it involves metabolic rather than regulation changes – and provides an important source of phenotypic heterogeneity (Schreiber & Ackermann, 2020).

Particularly important for *Dictyostelium* are variations in the social envi-

ronment associated to its peculiar life cycle. The combination of short-term cell-level competition within clonal aggregates and long-term organization has been addressed in relation to the evolution of multicellular life cycles (Rainey & Kerr, 2010; Hochberg et al., 2008; Wolinsky & Libby, 2016). Phenotypes that would be classified as cheats in the social phase were pointed out to have other functions, such as allowing reproduction of the higher-level structure and division of labour. More generally, feedbacks between ecology and the resulting evolutionary dynamics essentially influence the fate of cheating (Weitz et al., 2016; Lion, 2018; Tilman et al., 2019). Traits that underpin conflicting strategies within the multicellular phase, but that also affect behaviour of isolated cells – for instance cell motility – have been shown to give rise to eco-evolutionary cycles akin to aggregative multicellular life cycles, where social 'cooperators' and 'cheaters' coexist (Miele & De Monte, 2021). This model predicts that selection for escalating social conflicts drives the emergence of a temporal alternation of solitary living and phases when social conflicts manifest within aggregates. Moreover, it proposes that a metapopulation structure may not be essential for maintaining strain diversity: evolution would lead to an effectively neutral regime, where exclusion of mutant strategies becomes progressively slower.

Quantifying the importance of eco-evolutionary feedbacks poses major experimental challenges, as it requires to follow individual cells and their environment throughout the developmental cycle. Methodological advances in high-resolution single-cell microscopy (Sgro et al., 2015) and in the use of molecular markers (Muramoto & Chubb, 2008) allow us nowadays to access the internal state of single cells at the same time as they undergo major rearrangements of their environmental context, paving the way to define models that integrate processes across spatial and temporal scales.

On the theoretical side, new models that explicitly describe, along with developmental choices, the self-organized population structure may illuminate on the ecological mechanisms underpinning evolutionary dynamics. Comparison with data would be possible beyond the resolution of population-level observables, thus achieving further integration of theory and observations.

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Supplementary Information

Link between phenotypic heterogeneity at the onset of aggregation and developmental fate in *Dictyostelium*.

Even in monoclonal populations, in which every cells share the exact same genotype, a combination of extrinsic and intrinsic stochastic factors causes cells to display phenotypic heterogeneity. In *Dictyostelium*, the effect of phenotypic differences can be conveniently assessed by mixing populations that differ in their preparation protocol and/or their physiological state. Spore bias induced by non-genetic factors can be measured, after marking one of the two subpopulations, exactly as discussed in section 1. The effects of non-genetic factors on social behaviour can thus be quantified by comparing the number of spores produced by each culture with the expectation from their proportions in the initial mix. In this document, we review evidence for the existence of multiple, and likely non-independent, sources of phenotypic bias (summarized in Table S1). Moreover, we discuss the possible mechanisms connecting phenotypic heterogeneity during vegetative growth to cell fate determination during development.

Cell phenotypes	**Positive correlates to spore bias**	**Reference(s)**
Glucose concentration	Cells being fed with extra glucose	(Leach et al., 1973)
Intracellular calcium	Low intracellular calcium	(Azhar et al., 1996; Kubohara et al., 2007)
Intracellular pH	High pH	(Kubohara et al., 2007)
Intracellular ATP	Low ATP	(Hiraoka et al., 2020)
Starvation timing	Earlier starvation before aggregation is started	(Kuzdzal-Fick et al., 2010)
Cell cycle progression	Late cell cycle phase	(Zada-Hames & Ashworth, 1978; Ohmori & Maeda, 1987; Gruenheit et al., 2018)
Sensitivity to DIF	Higher sensitivity to DIF	(Thompson & Kay, 2000b)
Cell motility	Slower cells (theoretical prediction)	(Bonner, 1957)

Table S1: Phenotypic factors affecting cell fate, relation between their value at the onset of aggregation in binary chimerae and spore bias.

Physiological state

Already 50 years ago, cultures grown on glucose were reported to have a positive spore bias when mixed with cells from a similar strain (carrying a marker

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mutation that does not affect development) grown in poorer medium (Leach et al., 1973). The quality of nutrients provided during vegetative growth has since then been confirmed to affect not only cell fate at the end of development (Takeuchi et al., 1986), but also the probability to join aggregates at all (Dubravcic et al., 2014). Similarly, cells at varying degrees of starvation show a differential tendency to become spores. Cultures that have been starved for four hours before aggregation have a positive spore bias when mixed with freshly harvested cells of the same strain (Kuzdzal-Fick et al., 2010).

Differences in quality and duration of feeding result in heterogeneity of the physiological state of the cell, which can bias later developmental stages. Cells whose intracellular pH was artificially decreased, for instance, were found to be biased towards the stalk pathway (Kubohara & Okamoto, 1994). Similarly, concentration of Ca^{2+}, bimodally distributed in freshly starved amoebae, has been correlated with spore bias: lower intracellular calcium concentration is associated to a higher probability to become spores (Azhar et al., 1996). Finally, it was recently reported that cells with higher concentration of ATP before aggregation maintain such differential throughout development and eventually produce stalk cells (Hiraoka et al., 2020).

In natural conditions, food location and quality, duration of starvation or intracellular concentrations are largely independent of the cell genotype. For instance, variations in the environmental concentration of folic acid, a chemoattractant produced by bacteria may result in heterogeneous intracellular calcium concentration (Yumura et al., 1996). Therefore, it is likely that the effects of physiological heterogeneity on spore bias evidenced in laboratory conditions are relevant for wild populations as well.

Cell cycle phase

In addition to environmental variability, phenotypic heterogeneity may also arise as a consequence of intrinsically variable cellular processes. Previously mentioned physiological conditions affecting cell fate biases, indeed, appear to be linked to one other through their relation with cell cycle phase. Cytosolic Ca^{2+} concentration (Azhar et al., 2001; Jang & Gomer, 2011) and intra-cellular pH (Aerts et al., 1985) have been shown to vary during the cell cycle. This is also the case for two factors that play a central role in cellular organization within the multicellular slug, whose effects we discuss below in greater detail: sensitivity to a family of diffusive compounds responsible for differentiation into stalk cells (DIF) (Thompson & Kay, 2000a) and cell motility (Walmod et al., 2004). The phase of advancement in the cell cycle could thus result in phenotypic heterogeneity within a monoclonal population, and influence the ultimate developmental choice of any given cell.

Numerous studies support the notion that cell cycle phase at the onset of aggregation influences spore bias. The correlation between cell cycle phase in synchronized cultures and the frequency in the spore pool has been known for forty years (Zada-Hames & Ashworth, 1978). Experiments using cell cycle

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inhibitors (Gomer & Ammann, 1996) or release from stationary phase (Weijer et al., 1984) as means to synchronize cell cultures confirmed that cell cycle position at starvation reflects into developmental cell fate. By using single-cell RNA-seq Thompson and co-workers recently provided a molecular characterization of such observations (Gruenheit et al., 2018). They analyzed the transcriptome of a monoclonal vegetative population of *D. discoideum* strain AX3 and identified more than 1600 genes that can be divided, based on their level of expression, in two clusters. One cluster is specifically expressed in cells that are in phase S/M, whereas the second is composed of genes expressed in late G2 phase cells. Then, using pre-spore and pre-stalk markers, they mapped cell cycle position to cell fate and showed that M/S phase cells mostly differentiate into stalk cells, whereas late G2 cells are enriched in spores. Consistently with a direct link between cell cycle phase and cell fate, the ratio of G2 to M/S phase cells in a population is around 4:1, which closely matches the ratio of spores/stalk cells within a fruiting body (Gruenheit et al., 2018).

Cell cycle phase effects on development led Maeda and colleagues to propose the existence of a checkpoint in the late G2 phase, where cells bifurcate between growth and differentiation (Maeda, 2011). In cultures synchronized by a cold shock (Ohmori & Maeda, 1987), indeed, cells starved in mid-G2 phase (before the checkpoint) initiate aggregation more rapidly than cells starved in late G2 phase, and are more likely to become spores.

The correlation between cell cycle advancement and developmental timing was further supported through PCA analysis on single-cell transcriptomic data (Antolović et al., 2019). As early as at the mound stage, cells display heterogeneity in developmental advancement. The principal components of such variability also capture differences in cell cycle stages. Cell cycle phase is thus considered to be a determinant factor - though minor in amplitude compared to overall changes in the transcriptome throughout development - in determining eventual developmental choices (Antolović et al., 2019).

A consequence of the correlation between cell cycle phase and developmental fate is that cell-level strategy – the probability that a cell becomes a spore – is determined by phase positioning relative to the population, thus potentially decorrelating genotype and behaviour. Consistent with this view is the capacity of cells to reprogram their development when their local environment is perturbed. For instance, if one part of a slug is experimentally removed, cell fate decision are reassessed (Raper, 1940). Similarly, once extracted from their social context by dissolving a slug into fresh medium, cells de-differentiate and resume unicellular growth (Soll & Waddell, 1975) in a way that is highly robust to mutations in developmental genes (Nichols et al., 2020).

The question is then: How can phenotypic differences established at the beginning of aggregation affect, much later, a cell's social behavior?

Cell phenotype through development

Several mechanisms are believed to be involved in transforming phenotypic differences at the beginning of multicellular development into divergent cell

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fates. Single-cell tracking (Houle et al., 1989; Araki & Maeda, 1995; Jang & Gomer, 2011; Gruenheit et al., 2018) and mathematical models (Maree & Hogeweg, 2001; Umeda & Inouye, 2004) indicated two main (non-exclusive) ways whereby cell fate gets established.

First, cells could be primed to respond differently to differentiation signals that are equally available to all cells within an aggregate. Among the signals exchanged by co-developing cells, Differentiation Induction Factors (DIF) affect cell fate by inducing differentiation into stalk (Kay et al., 1983; Jang & Gomer, 2011). While extracellular concentrations in the mound gets readily homogenized by diffusion and cell mixing, cells differ in their responsiveness to DIF (Chattwood & Thompson, 2011). This parameter is correlated with cell physiology at the onset of aggregation. For instance, cells fed on a medium containing glucose, as well as those in a late phase of the cell cycle exhibit a lower DIF responsiveness with respect to cells grown without glucose and those in an early cell cycle phase (Thompson & Kay, 2000a). Moreover, DIF responsiveness is also affected by heterogeneity in intracellular Ca^{2+} established before the multicellular phase. Of the two subpopulations with low and high Ca^{2+} content observed in freshly starved cultures, only the latter increases the uptake of extracellular Ca^{2+} upon stimulation with one molecule of the DIF family, DIF-1 (Azhar et al., 1997).

Second, the geometry of the aggregate could impose or reinforce patterns through direct cell-cell contacts or morphogen gradients. Positional information within the mound and the slug is associated to the cell's eventual developmental fate. Phenotypic heterogeneity at the onset of aggregation could hence bias terminal differentiation by influencing where a cell is located within multicellular aggregates. The correlation between cell position and cell fate appears to get established as soon as cells organize into streams by attaching head-to-tail during their migration towards the mound (Fujimori et al., 2019). Maeda suggested that cell positioning during aggregation plays a central role in connecting cell cycle phase and developmental fate (Maeda, 2011). When facing starvation, cells that have passed the checkpoint between growth and differentiation would stop dividing and act as autonomously pulsing aggregation centres (Wang et al., 1988). By attracting cells at other stages of the cell cycle, they would gain a head start in establishing their position in the mound (Maeda, 2011), and subsequently gather at the center of the aggregates, a position thought to be linked with pre-spore fate (Huang et al., 1997).

As well as in the mound, position along the slug axis is associated to different cell fates in the future fruiting body: cells at the back of the slug tend to turn into spores, whereas most of those at the front form the stalk. In a clonal population, cells may sort during slug migration on the basis of motility (Strandkvist et al., 2014) or adhesion (Houle et al., 1989). Even though the exact role of differential motility and adhesion in establishing positional information is not yet completely worked out, their involvement in cell fate determination was confirmed by a recent single-cell transcriptomic study. Genes involved in cell motility and, to a lesser extent, in cell-cell adhesion were indeed found to be up-regulated in pre-stalk relative to pre-spore cells, indicating a likely role of

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cell sorting in establishing tissue organization (Antolović et al., 2019). Both calcium concentration (Azhar et al., 1996) and pH (Van Duijn & Inouye, 1991) differences, moreover, can result in heterogeneity in cell motility, which is also known to vary with the phase of the cell cycle (Walmod et al., 2004). Analysis of a handful of trajectories suggests that, corresponding to bimodality in calcium concentration at the onset of development (Azhar et al., 1996), also cell motility could be bimodally distributed (Goury-Sistla et al., 2012). Already in 1957 John Tyler Bonner suggested that faster cells would position themselves to the front of the slug, thus becoming stalk with a higher probability (Bonner, 1957). Motility differences have moreover been recently related to the evolutionary emergence of aggregative multicellular life cycles (Miele & De Monte, 2021). However, for heterogeneity in motility at the onset of development to affect cell fate, it is necessary that motility differences are maintained after starvation, something for which there is mixed and indirect evidence. On one side, permanence could be associated to differentials in concentration of ATP (Hiraoka et al., 2020). This compound indeed is involved both in cytoskeleton-mediated cell contraction (Clarke & Baron, 1987) and is consistently higher in pre-stalk cells, that show enhanced speed and cAMP chemotaxis (Hiraoka et al., 2020). Observations of vegetative cells, on the other hand, show that motility can change relatively rapidly in time, and reflect the rate of encounters with other cells (d'Alessandro et al., 2018). Moreover, recent observations of cells from disaggregated slugs observed two sub-populations moving at different speed, but these did not correspond to pre-stalk and pre-spore sub-populations (Nichols et al., 2020).