Quantitative evolutionary analysis of the life cycle of social amoebae

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Abstract

Social amoebae are eukaryotic organisms that inhabit soil of almost every climate zone. They are remarkable for their switch from unicellularity to multicellularity as an adaptation to starvation. When starved, millions of single cells aggregate and form a multicellular fruiting body, which contains reproductive spore cells and dead stalk cells, which help in spore dispersion. This costly behavior made social amoebae a model system for addressing major questions of the evolution of cooperation and multicellularity. In this study we look at three different aspects of social amoebae behavior; aggregation, non-aggregation and competition, and ask how they contribute to our understanding of cooperation in social amoebae and microbial systems in general.

We explored the known but neglected observation that, upon starvation, not all cells aggregate and engage in multicellular development. We describe phenotypically and genetically non-aggregating cell proportion in *D. discoideum* species. Both aggregating and non-aggregating strategy are costly or beneficial depending on duration of starvation. With our computational model we propose that partitioning the population into unicellular and multicellular states is adaptive in fluctuating environments with unpredicted duration of starvation periods. Social amoebae may therefore lie at the intersection of cooperation and bet-hedging.

In the second part, we provide a new framework for addressing the contrasting observations of high genetic diversity in natural populations of social amoebae and experimentally suggested low diversity-high relatedness required for cooperation. We propose that complex life cycle of social amoebae provides multiple competition points that can possibly play an important role in maintaining diversity and cooperation. We explore this experimentally and computationally by looking at competition over the whole life cycle between 6 natural isolates of *D. discoideum*. Our simulation model indicates that competition at different stages of the life cycle can lead to exclusion of "social winners". Though we failed to explain strain coexistence. Although preliminary, our results emphasize the importance of integrating species ecology in cooperative studies.

Finally, we focus on a new aggregation dynamics in *P. pallidum* species observed in our lab. Aggregation is a population level process during which population gets divided into numerous subpopulations/aggregates that face selection independently. Such population partitioning can have strong evolutionary consequences on cooperation that have not yet been explored experimentally. We describe the population dynamics qualitatively and propose several quantitative measurements of population partitioning into aggregates. Our preliminary results suggest that there is a preference for aggregates of certain size, but there is no spatial organization of aggregates.

Résumé (français)

Les amibes sociales sont des organismes eucaryotes présents dans le sol de presque toutes les zones climatiques. Ils sont remarquables pour leur passage d'un état unicellulaire à un état multicellulaire en réponse à la carence en nutriments. En période de carence, des millions de cellules forment des agrégats qui constituent chacun un nouvel organisme multicellulaire, contenant des spores, cellules reproductives, et des cellules de tige, cellules mortes qui favorisent la dispersion des spores. Ce comportement, de par le coût payé par les cellules de tige, a permis d'utiliser les amibes sociales en tant que système-modèle pour aborder des questions majeures de l'évolution de la coopération et de la multicellularité. Dans cette étude, nous examinons trois aspects différents du comportement des amibes sociales; agrégation, non-agrégation et compétition, et nous analysons comment ces aspects contribuent à notre compréhension de la coopération chez les amibes et systèmes microbiens en général.

Nous avons exploré le fait bien connu mais négligé qu'en phase de carence nutritive, une fraction des cellules ne participent pas à la formation des agrégats pas et ne sont pas engagées dans le développement multicellulaire. Nous décrivons les facteurs phénotypiques et génétiques qui déterminent la fraction de cellules hors-agrégats chez D. discoideum. Les deux stratégies, d'agrégation et de non-agrégation, sont coûteuses ou bénéfiques d'un point de vue évolutif selon la durée de la phase de carence. Nous avons développé un modèle pour simuler ce processus. Nous proposons que le partitionnement de la population dans des états unicellulaire et multicellulaire est adaptative dans des environnements fluctuants avec une durée imprévisible des périodes de carence nutritive. Les amibes sociales sont donc situées à l'intersection de deux thèmes émergents en évolution microbienne, la coopération et le "placement des paris".

Dans la deuxième partie, nous proposons un nouveau cadre pour aborder les observations a priori contradictoires de la diversité génétique dans les populations naturelles d'amibes sociales et une faible diversité nécessaire pour la coopération. Nous proposons que le cycle de vie complexe des amibes sociales fournit plusieurs points de compétition qui peut servir à la fois comme stabilisateur de la diversité et de la coopération. Nous explorons cette hypothèse expérimentalement avec un modèle en analysant la compétition entre 6 isolats naturels de D. discoideum. Notre simulation-modèle indique que la compétition à différents stades du cycle de vie peut conduire à l'exclusion des "gagnants sociaux". Toutefois nous n'avons pas réussi à expliquer la coexistence à long terme de souches génétiquement distinctes. Bien que préliminaires, nos résultats soulignent l'importance d'intégrer l'écologie des espèces dans les études de coopération microbienne.

Enfin, nous nous concentrons sur une nouvelle dynamique d'agrégation chez P. pallidum observée dans notre laboratoire. L'agrégation est un processus au niveau de la population au cours duquel la population se divise en nombreuses sous-populations (agrégats) qui font face à la sélection de manière indépendante. Un tel fractionnement de la population peut avoir de fortes conséquences évolutives du point de vue de la

coopération qui n'ont pas encore été explorées expérimentalement. Nous décrivons la dynamique des populations qualitativement et proposons plusieurs mesures quantitatives de partitionnement de lapopulation en agrégats. Nos résultats préliminaires suggèrent qu'il existe une préférence pour les agrégats d'une certaine taille, mais qu'il n'existe aucune organisation spatiale des agrégats.

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An enormous thanks to my mom, dad, my sister Asja and my boyfriend Mathieu for making me have an equilibrated life full of friendship, love, compassion, support, smiles, long walks in the mountains and long days at the beach.

Finally, thanks to my lab mates and friends for contributing to this equilibrium. Science is fun but it is love that makes us alive!

“The most exciting phrase to hear in science, the one that heralds the most discoveries, is not "Eureka!" (I found it!) but 'That's funny!" ”

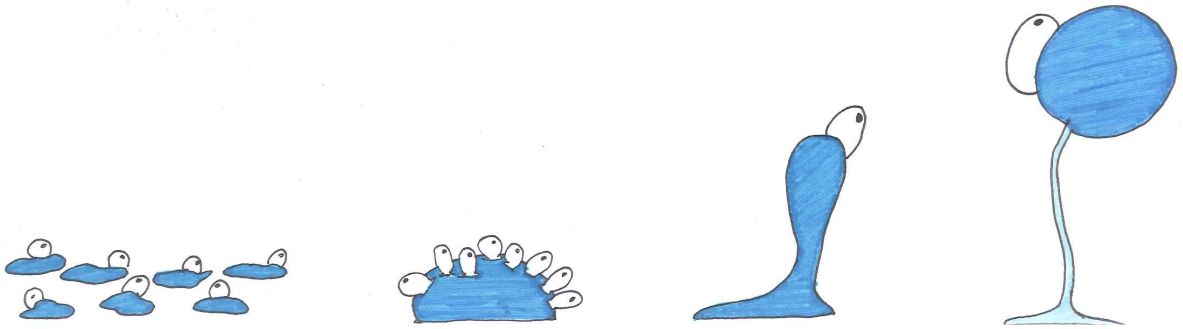
Isaac Asimov

“There are only two ways to live your life. One is as though nothing is a miracle. The other is as though everything is a miracle.”

Albert Einstein

“Logic will get you from A to Z; imagination will get you everywhere.”

Albert Einstein



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Chapter 1 General Introduction

This PhD is the result of my exploration of various topics in evolutionary biology. Personally, I tend do have a rather dispersed mind; meaning I easily get interested by different topics. At the same time my two supervisors tend to be of a similar kind. Together, we took this PhD as a possibility to explore different questions that reunited our curiosity. The main axes of our research were 1) the use of social amoebae as source of inspiration and model organisms, 2) the population level perspective to questions we asked. Within this in mind we explored the following subjects: Chapter 1: population partitioning into aggregating and non-aggregating cells as a possible bet-hedging mechanism; Chapter 2: how the complexity of social amoebae life cycle regulates population genetic diversity and cooperative conflicts; and Chapter 3: dynamics of aggregation and population level organization. I will discuss on population level behaviors and social amoebae main axes of our research in general introduction and introduce each subject in more detail at the beginning of each Chapter.

POPULATION-LEVEL BHAVIOURS AND EFFECTS

Traditionally in biology, and especially in evolutionary biology, we are focused on genes and individuals, on genotypes and phenotypes. However, there are many behaviors for which we need to look at populations to truly understand them.

Figure 1 - 1 Examples of population level behaviours. A) Termites are capable of building massive nest because of highly social interactive networks between individuals. Termite nest in Kakadu National Park, Anustralia[1]. B) Coordinated bird flock flight emerges as a result of simple between individual bird interactions[2]. C) Multicellular social amoebae *D.discoideum* slugs are phototactic although single amoebae cells show no light directed movement. [3]

[1]http://www.123rf.com/photo_7444374_massive-cathedral-termite-mounds-nasutitermes-triodae--kakadu-national-park-northern-territory-austr.html, [2]http://fuza.ru/marvelous/14906-zagadki-prirody-chernoe-solnce-danii.html, [3]http://ucsdnews.ucsd.edu/graphics/images/2004/dictyslug_lg.jpg

Population level perspective – making sense of individual behaviors

Certain individual behaviors do not make sense unless observed at the level of population. In this thesis we have worked on two such examples: cooperative (in Chapter 4) and bet-hedging behaviors (in Chapter 3). We will explain briefly both behaviors and further details will be introduced in corresponding chapters.

Cooperation can be defined as "a behavior that benefits another individual (the recipient) and which is maintained (at least partially) because of its beneficial effect on the recipient" (West et al. 2006). Therefore, by default cooperation is a population level phenomenon that results from individual interactions. In addition, cooperative behaviors are often costly for a single individual but beneficial when group level behavior is taken into account (ex. indirect fitness benefits). So while it is maladaptive for ant worker to be sterile if looked in isolation, it makes sense for an ant colony to have lots of workers investing in foraging and maintenance and having just one queen that reproduces a lot (Seeley 1997). In the same sense, in our model organism, social amoeba, we cannot explain why some cells differentiate into dead, stalk cells, by just looking at stalk cells. We need to understand their relationship to reproductive spores, with whom they together forma a group, a fruiting body (Nanjundiah & Sathe 2011) (see General Introduction: Social amoebae).

Another phenomenon that transcends individuals is bet-hedging (Chapter 3). When bet-hedging individuals display behaviors probabilistically. As a result, different individuals display different behaviors at one time point. This is an adaptation to unpredictable and changing environment in which there is no one behavior that is the most fit (Meyers & Bull 2002; Simons 2011). This does not make sense if looking at the level of an individual, because an individual may be displaying a behavior that is not adapted to the environment. But, it makes sense at the level of the population because the population is displaying a range of behaviors making it always adapted to the environment. Therefore, while the individual is not the most fit, the population is. This has been shown in bacteria that change between fast growing/antibiotic sensitive and slow growing/antibiotic resistance states (Balaban et al. 2004), *B. subtilis* expressing sporulating and non-sporulating state (Veening et al. 2008), plants seeds that germinate at different time points (Simons 2009), and many others.

Emergent behaviors

Certain systems perform complex population level behaviors that arise from a set of simple individual level interactions. Flight of bird flock or movements of schools of fish are typical such examples (Figure 1–1B). When in group birds interact and modify their flight according to a simple set of interactions (Young et al. 2013), the result of which is a coordinated movement of the entire flock. These behaviors are called emergent, because they emerge at a level of population; single individual is not capable of producing them. As Aristotle expressed it: *"The whole is greater than the sum of its parts."*

Other examples include ant colony organization as a result of simple ant interactions (Figure 1-1A) (Gordon 1996). Complex embryogenesis and differentiation results from simple cell-cell interactions (Edelman et al. 2010; Robertson et al. 2007). A range of behaviors that are population density dependent are called "quorum sensing". In these behaviors single cell behavior is initiated by threshold level of a certain extracellular signal. The threshold level is attained by increasing the number of individuals that secrete the signal. Therefore, a certain population density is needed for the behavior to be triggered, single cells cannot initiate them on their own. Some examples include bioluminescence of bacteria *Vibrio fischeri* (Milton 2006), *P. aeruginosa* biofilm formation (Shrout et al. 2006) and similar ideas on group decision-making in nest choice in social insects (Seeley et al. 2006).

In social amoebae, the model organism used in this study, has several emergent behaviors. Aggregation of millions of cells is triggered by a starvation signal, that is regulated by cell density (see General Introduction: Social amoebae). No matter how starved the cells are, only above a certain cell density will they actually aggregate (a quorum sensing behavior). We will focus more on aggregation as a population level phenomenon in Chapter 5. Another emergent behavior in social amoebae is group phototaxis. Single cells are not phototactic, but a group of aggregating cells, that forms a worm-like structure called slug, is phototactic (Figure 1-1C) (Miura 2000). Although not part of this PhD thesis, we also studied the group-level properties of phototaxis, in particular how the size of the slug affects phototactic movement.

Individual and population level interactions

We have presented how individuals interact to create new, population-level behaviors. We will now discuss how these two levels interact. How individuals affect population-level properties and how the population level properties affect individuals. The dilution effect in disease transmission is a very good example. Transmission of a disease depends on individual interactions and susceptibility of interacting individuals. Hence, disease transmission between individuals will depend on population composition. As a result, a susceptible individual has high probability of being infected when in population of susceptible individuals and low probability of getting infected when in population of resistant individuals (LoGiudice et al. 2003; Keesing et al. 2006). Other frequency-dependent examples are Batesian mimicry in snakes (Pfennig et al. 2001), color-pattern rareness in guppies (Olendorf et al. 2006) and sex ratios (Smith 1980). Similar effects include density-dependent behaviors; one's survival is dependent on the density of other individuals in the same territory (a population). For example, an increase in density can cause increase in competition, which may decrease survival (Svensson et al. 2001; Agnew et al. 2002). In aposematic species, an increase in the density of aposematic individuals can cause increased learning and avoidance by predators and therefore increase survival (Mappes et al. 2005). In social amoebae this interplay between individual and population level is interesting because different strains strongly

interact when forming multicellular fruiting body (Kaushik et al. 2005; Buttery et al. 2009). As we discuss in Chapter 4 genetic composition of the group can have important effects on stabilization of cooperation.

Population level perspective in our work

A phenotype is a result of interactions between a genotype and environment. This environment can involve abiotic factors, such as temperature, biotic interactions with other species, like parasitism, and it can also involve the presence of other individuals, its social environment. Here, we have shown how our social environment affects what we do, how we do it and why we do it. All of this emphasizes the need to look at the whole (population) to understand the behavior and dynamics of its parts (individuals). Vice versa the behavior of the parts can only be understood by considering the behavior of the whole. During this thesis we have explored different population level behaviors in social amoebae system.

In Chapter 3 we explore the existence of two cell states with respect to aggregation: aggregated and non-aggregated cells. From individual and population level perspective we ask what are the cost and benefits of both phenotypes, and propose a bet-hedging-like population level adaptation.

In Chapter 4 we ask how competitive interactions at the level of individuals and populations affect population diversity and stabilize cooperation. In the Annex of Chapter 2 we discuss on relationships between the two levels and how important evolutionary feedbacks between them may be. We further discuss on the notions of levels of selection and associated debates when looking at evolution at higher - levels.

In Chapter 5 we focus on aggregation in amoebae *P. pallidum.* Aggregation by itself is an emergent behavior that results out of quorum sensing synchronization of cell signaling. We explore the special case of dynamical, multiple-step aggregation. We further look at relationship between multiple-step aggregation and population level aggregate organization.

SOCIAL AMOEBAE

Organism

Social amoebae are unicellular eukaryotic organisms that belong to the group of amoeboid protozoa. They are characteristic for their constantly changing "amoeboid" shape caused by the cell movement technique. Cells move by projecting pseudopodia, extensions of cell plasma and membrane, that later contract by the use of actin and myosin network. These "shapeless" cells have a size of 10-20μm in diameter for *D.discoideum* and vary among species (Bonner & Frascella 1953). In nature amoebae feed on bacteria and divide asexually through mitosis every 2-4h (our results Chapter 4, Figure 4-5). Laboratory strains are able to grow on defined axenic medium with approximate doubling time of 8-12h (personal observation, (Watts & Ashworth 1970)). They got their name "social" from a multicellular behavior that is observed when cells starve (Figure 1-4).

Ecology

Social amoebae inhabit forest soils and associated leaf litter and animal dung (Swanson et al. 1999; Bonner & Lamont 2005). In the soil amoebae feed on bacteria and are itself a food source of soil nematodes and worms (Figure 1-2). This makes them important players in the regulation of soil communities. Dispersal is an important element in social amoebae, affecting territory occupation, species interactions and survival. They are mainly dispersed in a form of dormant spores. Dispersion of spores happens in the similar manner as dispersion of many plant seeds; various animals (from nematodes and arthropods to birds and small mammals) pick up the amoebae spores while feeding on soil. Spores go throughout the digestive tract without being degraded and are deposited at another place together with animal dung (Sathe et al. 2010). Transport by air and water are additional logical candidates although they have never been proven. Dispersing spores get to places with new food sources. Genetic analysis of fine scale soil samples and single fruiting body analysis have shown that within a species different strains often interact and co-aggregate (Fortunato, Strassmann, et al. 2003; Sathe et al. 2010; Buss 1982; Ketcham & Eisenberg 1989), although monoclonal patches have also been isolated (Gilbert et al. 2007; Gilbert et al. 2009). This opens space for competitive interactions, the issue we focus on in Chapter 4. High dispersal and niche sharing also facilitates mixing of different species. Species interact mainly while in vegetative phase (Horn 1971; Eisenberg et al. 1989; Ketcham et al. 1988). Between species co-aggregation is possible (Jack et al. 2008), but rather rare due to between species incompatibilities for aggregation due to different use of chemo-attracting molecules, developmental differences (Bonner & Adams 1958) and possibly specie specific cell-cell adhesion (Hirose, R Benabentos, et al. 2011).

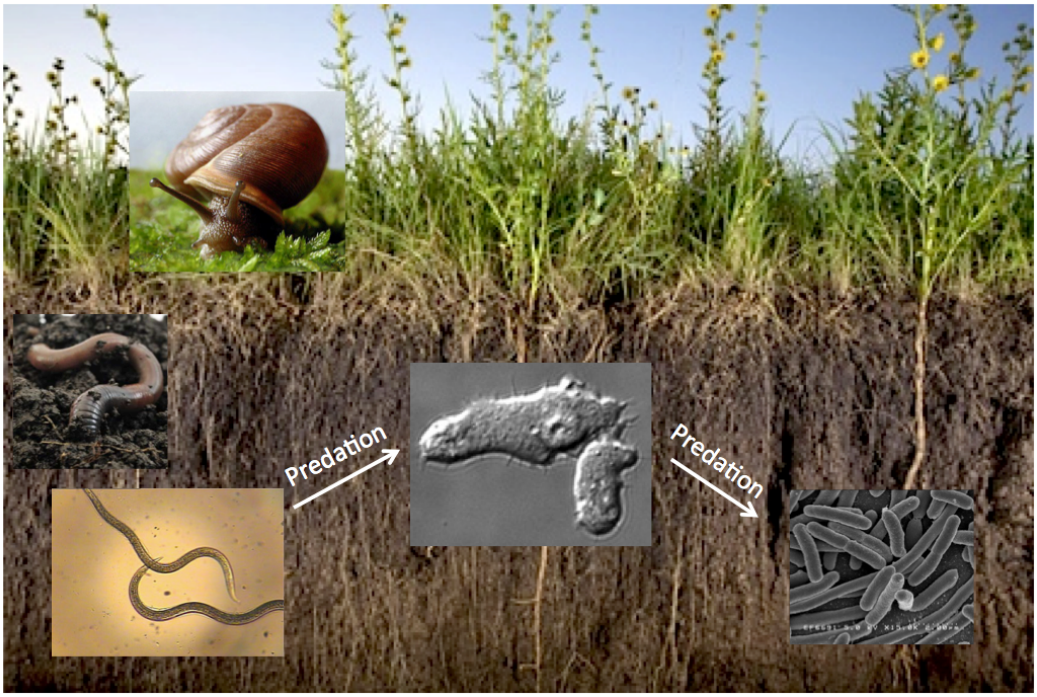


Figure 1 - 2 Social amoebae habitat: they live in the soil and associated leaf litter where they are predated by nematodes and they feed on bacteria.

Spatial distribution

Geographically, social amoebae can be found in wide range of habitats going from the tundra in the north, to temperate and tropical forests (Figure 1-3A) (Swanson et al. 1999). There is a clear relationship between species diversity and latitude and altitude increase (Figure 1-3B). The lower the latitude (close to tropics) and altitude (close to plain) the greater is species diversity, while moving to northern part of hemisphere and mountain altitudes the number of species decreases (Cavender 1973). This seems to be mainly caused by climate stability and species dispersal. Seasonal changes in temperature and humidity, as low winter temperatures and hot and dry summer conditions, causes high mortality rate of both cells and spores. These are latter on replaced by new species when conditions are favorable. On the other hand constant conditions in the tropics favor more abundant and constant species community. This goes in hand with Hubbell's theory of neutral diversity that states that stochastic processes such as immigration, emigration and extinction are the main causes that shape species diversity (Hubbell 2001).

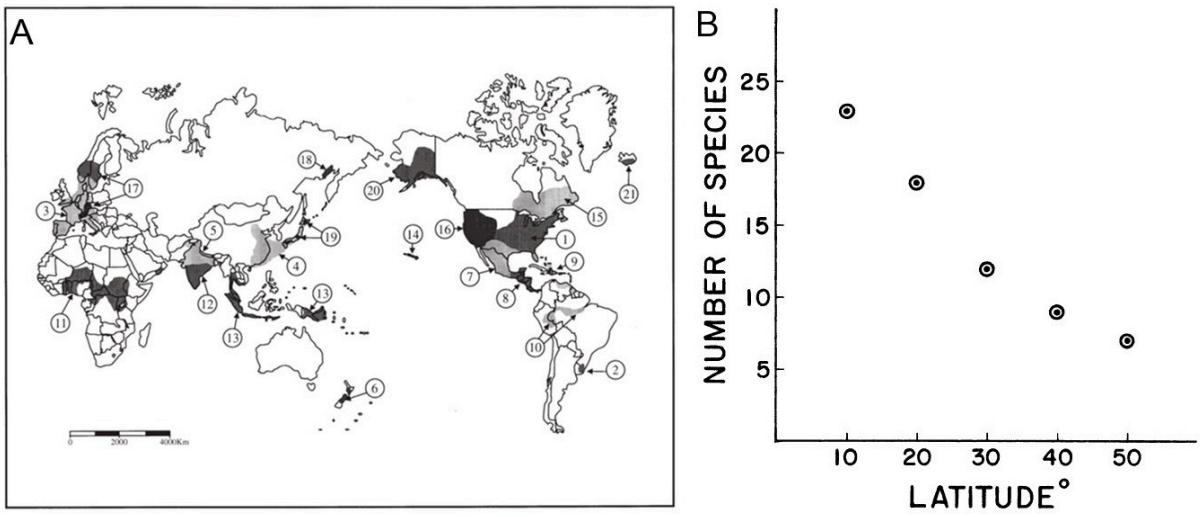


Figure 1 - 3 Geographical distribution of social amoebae. A) Social amoebae inhabit a wide range of habitat from tundra in the north to tropic in the south (Swanson et al. 1999), B) Species abundance decreases with increase in latitude (Cavender 1973).

Life cycles - Survival strategies

If food was an infinite source, social amoebae would divide indefinitely in the single cell stage. But food is not infinite and what makes these organisms so interesting is what happens after the food is gone. Figure 1-4 shows the *D. discoideum* life cycle in the presence and absence of food, with vegetative/dividing stage being just a small part of the life of these amoebae. It becomes clear that life cycle of social amoebae is a complex of multiple survival strategies that range from asexual to sexual and unicellular to multicellular scenarios. There are 3 main strategies that have evolved mainly as an adaptation to starvation: 1) single cell dormancy 2) sexual reproduction and dormancy and 3) social cycle and dormancy (Figure 1-4). All 3 strategies at one stage include production of dormant states that can sustain long starvation periods. What makes it interesting is different ways that they used to get there.

Microcyst

The "simplest" survival strategy is the formation of single cell microcyst. In certain species when starved each cell gets encapsulated in cellulose coat and undergoes changes that prepare it for dormancy. The conditions of microcyst formation are poorly understood, but there is a requirement for starvation and high osmotic pressure (Kessin 2001). These conditions are difficult to be recreated in the lab, making microcyst formation a poorly described process and it is not known to what extent this strategy is used. It is not found in *D. discoideum*, but has been confirmed in *P. pallidum* and *D. mucoroides* (Kessin 2001).

Macrocyst – sexual life cycle

Another strategy is formation of macrocyst. Macrocyst formation is a form of sexual reproduction (sexual cycle in Figure 1-4). It requires two cells with different mating types that fuse to form a giant cell with single nuclei. The giant cell secrets a cAMP signal that attracts surrounding amoebae. Attracted amoebae are than used as an energy source and get cannibalistically ingested by the giant cell. The energy is used to form cellulose wall and the maturation of macrocyst. The macrocyst later goes through meiotic and mitotic divisions that produce sexual offspring (O'Day & Keszei 2011). In the lab macrocyst formation is initiated by a mix of starvation and high humidity conditions. Poor mating rate in the lab conditions makes it not a frequently studied mechanism. Studies on genetic variation or ribosomal DNA and linkage disequilibrium analysis between natural isolates of *D. purpureu* and *D.discoideum* have indicated high occurrence of sexual life cycle in the wild (Mehdiabadi et al. 2009; Flowers et al. 2010). On the other hand similar studies with *D. giganteum* reveled very low genetic differentiation and no phylogenetic structure (Mehdiabadi et al. 2010). The occurrence and importance of sexual life cycle probably varies between species and is dependent on environmental conditions. Except for survival, sexual life cycle may play important role in strain mixing, generating new genetic combinations (Mehdabadi 2010).

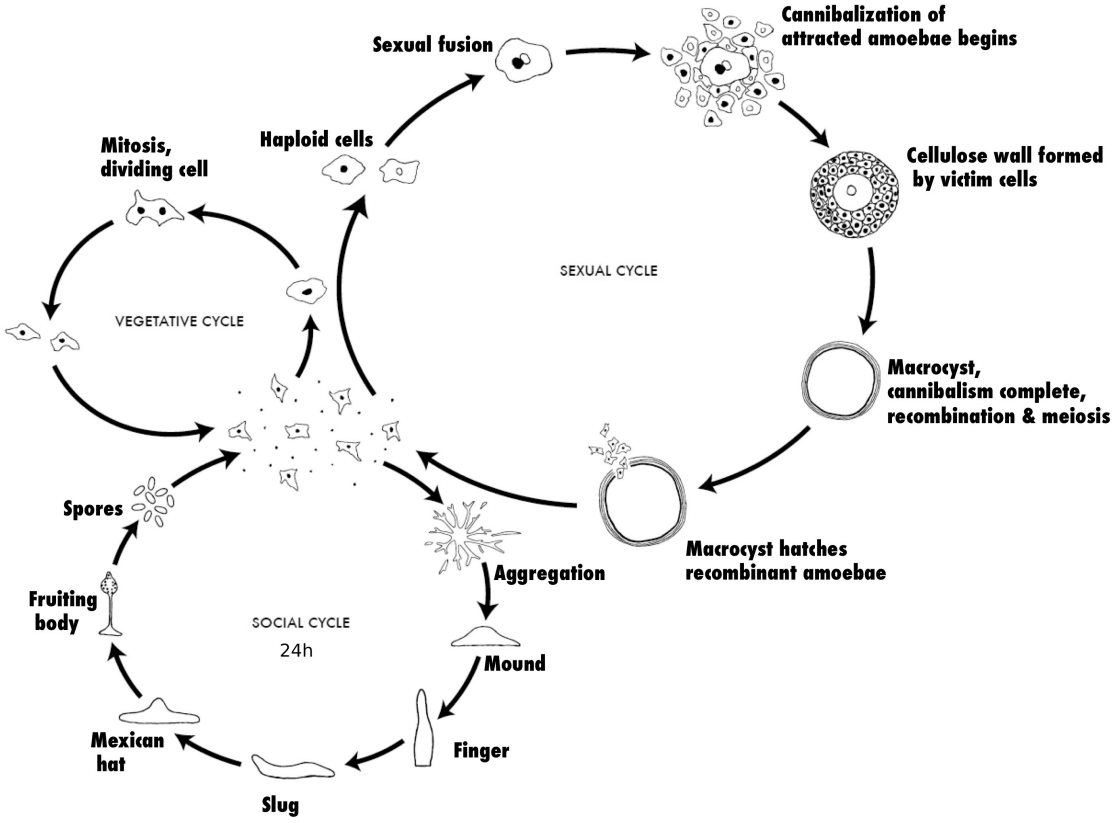


Figure 1 - 4 Life cycle of *D.discoideum*. In the vegetative stage single cells consume bacteria and divide asexually by mitosis. When all the food is consumed cells can enter in sexual or social cycle. (http://dictybase.org/Multimedia/DdLifeCycles/index.html)

Social life cycle

The most complex and probably most recently evolved is the social strategy (see Figure 1-4). It is a population level response to starvation that positions social amoebae at the edge of unicellularity and multicellularity. When starved, streams of 10^2-10^6 cells aggregate and form aggregation centers. Each aggregate differentiates into a worm - like multicellular organism called slug. In certain species, like *D. discoideum*, slugs are migratory stages that serve to displace the cells to areas with food and to the soil surface where dispersion is facilitated. This is facilitated by slug's ability to sense light, chemo-attractants, humidity and temperature and modify its movement according to it. After a period of migration the slug stops moving and forms a sessile fruiting body. The fruiting body is a mushroom - like structure with a spore mass sitting on top of a stalk. 70-80% of the cells in fruiting body are spores that can germinate again as single cells upon contact with fresh nutrients. The other 20-30% of the cells are stalk cells that produce an extracellular matrix to form the stalk structure that promotes the dispersion of spores. High energetic costs of stalk production cause these cells to die when development has finished. The whole cycle, from single starved cells to mature fruiting

body, takes around 24h. During this time cells cannot divide. Even if the food becomes available, once the late aggregation stage is reached, cells cannot revert to vegetative growing state, they stay committed in the development until its end (Katoh et al. 2007; Shaffer 1961). As we explore further in Chapter 1 this further increases the costs of cooperation. At the end of development spores get dispersed further by water or animals (Sathe et al. 2010). Under suitable conditions they germinate into vegetative amoebae cells.

Cost and benefits of each strategy

Each of the 3 strategies can be seen as an evolutionary balance of costs and benefits, although no evidence in the literature has been provided. All strategies form a dormant resistant structure in the form of microcysts, or spores. Microcyst provides single cell dormancy with no population costs. But because the cells are left on the ground there is reduced cell dispersion. On the other hand the social life cycle has the population costs due to the death of stalk forming cells, but gives benefits from increased dispersion by slug migration and stalk formation. The sexual life cycle also has population level costs due to digestion of neighboring cells for formation of resistant macrocyst. It provides benefits in form of production of offspring with new genetic combinations. To what extent each strategy is used in the wild is not known. Because of the difficulty to induce microcyst and sexual reproduction in the lab it is not known to what extend it is spread among social amoebae. Finally, the choice will probably depend on wide set of ecological parameters.

Social life cycle - Morphogenesis and differentiation

Starvation and population aggregation

The social cycle is the most well known one that gave the descriptive name to the group, the social amoebae. Because of its simple inducibility in the lab it is the most studied one. The most well described cycle is the one of *D.discoideum*. This extraordinary behavior involves grouping of millions of amoebae cells into a new multicellular organism. It is triggered by starvation signals inside the cells when all the bacteria have been consumed. One of the first signals is the production and secretion of cAMP. Cells emit and are chemotacticly attracted to synchronized pulses of cAMP (Gregor et al. 2010; Gerisch & Wick 1975; Shaffer 1975). As a consequence streams of cells move towards the emitting center that becomes the center of aggregation (Figure 1-5A). During this period cells simultaneously go through changes in cytoskeleton, adhesion (cell-cell adhesion increases), motility and a complete turnover in gene expression profiles that evolve during the whole process of morphogenesis (Kessin 2001).

Aggregate maturation and cell differentiation

The aggregate is a group 10^2-10^6 cells embedded in an extracellular sheet made of cellulose and glycoproteins. The sheet serves as protection against predation (Kessin et

al. 1996), and gives structural support during the whole development (Kessin 2001). Once in an aggregate cell gene expression changes and multicellular development begins. This period is marked by cell differentiation and group morphogenesis. One of the most important changes is cell differentiation into two cell types, prespore and prestalk cells. Cell fate is decided by the end of aggregation. Two main factors in cell differentiation are cAMP and differentiation inducing factor DIF. cAMP regulates cell differentiation by activating a cascade of genes that activate and repress prestalk and prespore gene experission. DIF on the other hand acts directly by activating prestalk genes and suppressing prespore genes (reviewed in (Aubry & Firtel 1999)). In the aggregate cells are mixed and all sense the same environment, there is not positional effect or concentration gradient that decides which cell will become spore or stalk. Measurement of DIF sensitivity at single cell level showed that cells respond to DIF signal in an off/on manner and that there is high between - cell variability to DIF response (Figure 1-5B) (Stevense et al. 2010). Therefore non-genetic cell differences cause some cells to be less sensitive to DIF and become prespore cell or to be more sensitive to DIF signal and become prestalk cells. Causes of non-genetic cells differences have been a subject of many studies. In Box 1 we give an overview on how phenotypic differences between cells could affect their fate. Cell differentiation at the end aggregate stage is followed by slug formation and cell sorting out. That is, prestalk cells come to the front of the slug leaving the anterior part to prespore cells (Bonner 1959; Tasaka & Takeuchi 1979). It seems that and interplay between differential adhesion and cAMP chemotaxis plays a key role in this sorting out (reviewed in (Kay & Thompson 2009)). Once sorted out positional effects act adjust and maintain cell fate. Exact mechanism responsible for such maintaninance of cell fate are still not known. It is know that when anterior prestalk or posterior prespore region of the slug is removed, the remaining region regenerates the missing part through cell reorganization and rediferentiation (Sakai 1973). It has been proposed that inhibitory molecules such as *racin* and DIF, secreted by prespore cells, act as inhibitors of prestalk cell conversion to prespore cells (Shaulsky & Loomis 1993; Kay et al. 1999).

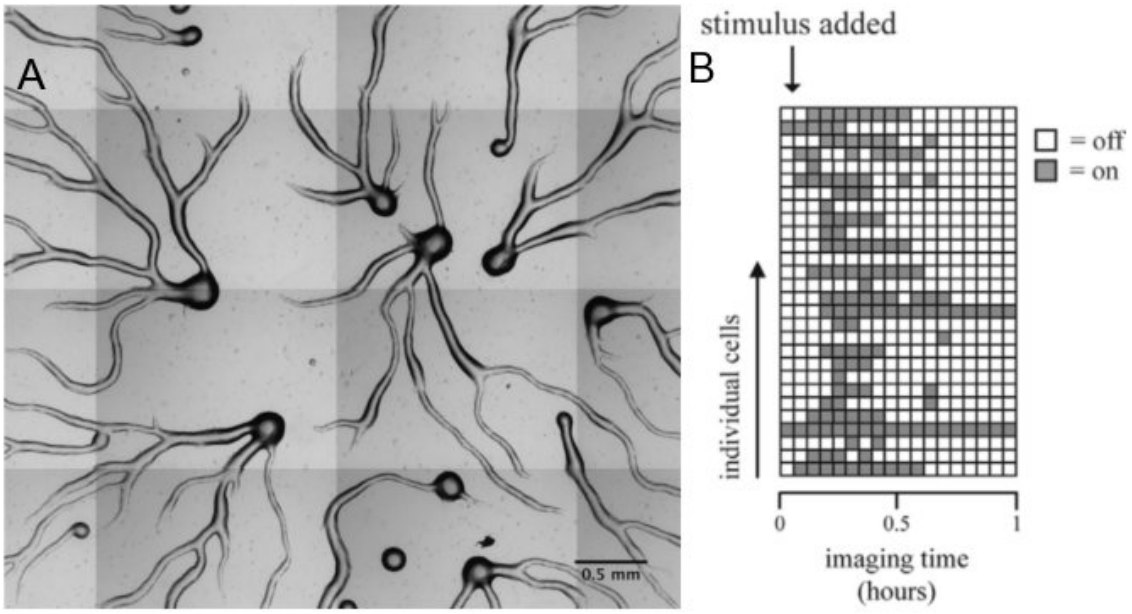


Figure 1 - 5 Development of social amoebae is marked by aggregation and cell differentiation. A) Development starts with streams of cells chemotacticly moving towards the aggregation center. B) Cell differentiation is partially regulated by variable cell response to external DIF stimulus. A group of cells have been stimulated by 100 nM DIF/5 mMcAMP stimulus. Response of each cell has been recorded through time; one cell corresponds to one horizontal line. "on" defines a period when transcription of prestalk gene *ecmA* is visible, response to DIF stimulus. "off" defines a period of no response to DIF (non prestalk gene expression) From (Stevense et al. 2010)

Box 1 Non-genetic cell differences and cell fates

Nutritional state

Some 30 years before (Stevense et al. 2010) actually showed between-cell differential response, researchers started questioning if non-genetic differences can affect cell fate. The main idea was that if we could choose which cells to put in spores and which into stalk the most beneficial would be that the cells that are in "good" state become spores and the ones that are in "bad" state become stalk. To check this, they grew cells in rich (high glucose) and poor (low glucose) medium, mixed them before aggregation and looked which cells went preferentially into spores. Indeed, cell that were better nourished (grown in glucose rich medium) preferentially developed into spores (Leach et al. 1973; Castillo et al. 2011). Even when not mixed, cells grown in glucose rich medium produce fruiting bodies with more spores (up to 90% cells form spores, compared to 80% in cells grown in medium with no glucose) (Forman & Garrod 1977). Molecular studies show that cells grown in medium without glucose are more responsive to external DIF (Thompson & Kay 2000). This is in line with the fact that DIF induces differentiation of stalk genes.

Cell cycle

But even the cells that are not exposed to different diets show preferential differentiation. Cell cycle stage at the beginning of starvation is another source of heterogeneity that affects cell fate. Several studies demonstrated that cells from G2 phase are more enriched in spores and cells from S phase are more enriched in stalk (Gomer & Firtel 1987; Weijer et al. 1984; Mcdonald & Durstonf 1984). Natural *Dictyostelium* populations are asynchronous. The ratio of cells in G2 and S phase in these populations turns out to be around 4:1, which is close to the ratio of spore and stalk cells 80%:20%. Finally, cells from early stage of cell cycle are more sensitive to DIF that those from late stage(Thompson & Kay 2000).

Calcium level

Intracellular Ca^{2+} level is another physiological indicator of cell differences. Namely, when a population of starved amoebae was labeled with Ca^{2+} specific dye they sorted out in two groups; the population with high Ca^{2+} level and low Ca^{2+} level. When mapped in the slug cells having high concentrations of Ca^{2+} preferentially go into anterior prestalk region and cells with low levels of Ca^{2+} to anterior prespore region(Azhar et al. 1996; Saran et al. 1994). Now, how does this related to our previous knowledge of cell differentiation? Ca^{2+} is important in regulating numerous signal transduction pathways that use calcium. This is mainly achieved through calcium binding proteins, like calmoduline. Many of these pathways play an important role in cell cycle progression, making Ca^{2+} an important cell cycle regulator(Whitaker & Patel 1990; MEANS 1994; Takuwa et al. 1995). There is a clear relationship between intracellular Ca^{2+} concentration and cell cycle (Azhar et al. 2001). Also, DIF raises cellular Ca^{2+} level making cell more sensitive to the signal(Schaap et al. 1996). Therefore, the heterogeneities in Ca^{2+} are the result of Ca^{2+} utilization during cell cycle. Different concentrations of Ca^{2+} are linked to different responsiveness to the DIF signal that leads to differences in stalk to spore differentiation.

Slug and fruiting body formation

Slugs have cylindrical morphology and are 0.5-2mm long and around 0.1mm wide (Miura 2000). The main "function" of the slug is its ability to move in response to the gradients of light, temperature, humidity and pH. Directed movement enables slugs to move towards soil surface where there are better conditions for spore dispersal. They move between 0.3 and 2 mm/h with speed increasing with slug size (Bonner 1995). The movement is guided by the anterior zone of prestalk cells that use cAMP waves to coordinate the slug movement (Miura 2000). Evetualy, the slug stops moving and differentiates into a mushroom-like structure called fruiting body. Prestalk cells secrete an extracellular matrix that forms the stalk structure that raises the spore cells. Therefore, although they die at the end, stalk cells are important for both slug movement and spore dispersion.

Evolution and phylogeny

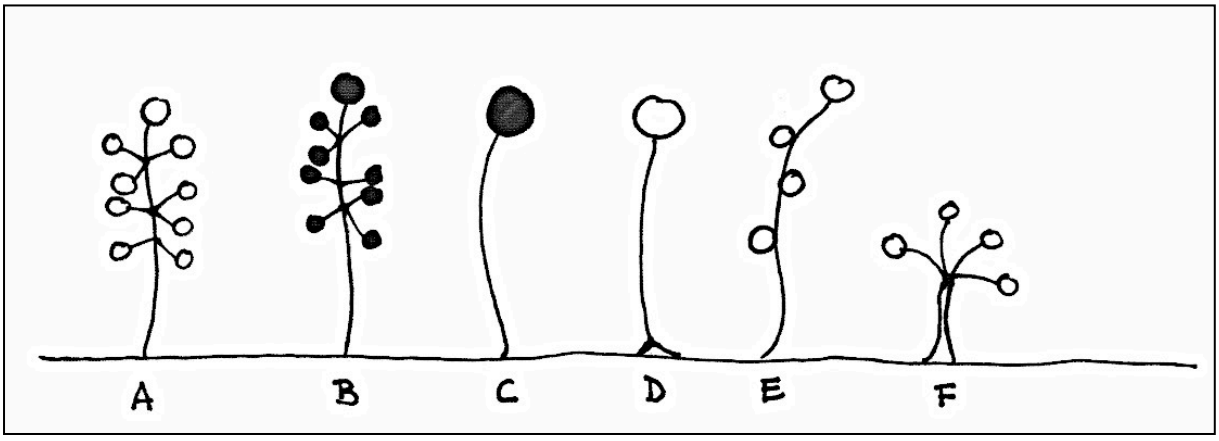


Figure 1 - 6 Fruiting bodies of different species of *Dictyostelis*. A) *P. pallidum*, B) *P. violaceum*, C) *D. purpureum*, D) *D. discoideum*, E) *D. rosarium* and F) *D. polycephalum*. Size of the fruiting bodies is not in real scale.

Social amoebae probably have evolved before the separation of animal and fungal clades but not before the separation of plants (Baldauf 1997). Although most is known about *D. discoideum* and *P. pallidum* species, they actually make a group of about 150 species. As shown in Figure 1-6 and Figure 1-7, species of social amoebae differ in their life cycles, aggregation, slug stages, fruiting body morphology and so on (Schaap 2007). Despite these differences, cell level functions and behaviors of all species resemble each other (Heidel et al. 2011; Sucgang et al. 2011). Genome structure is very similar and some genes can be exchanged between species using molecular methods without high consequences. We can divide them into two major groups; *Acrasids* and *Dictyostelids* (Kaushik & Nanjundiah 2003; Olive 1902). The main difference between the two is the lack of clear cell differentiation in *Acrasids*. While in all *Dictyostelids* fruiting body formation is associated with clear differentiation of cells into viable spore cells and dead

stalk cells. In *Acrasids* the stalk is either formed by secretion of extracellular matrix by spores, or by formation of stalk by live cells. Cell aggregation remains the common step in both groups. The importance of aggregation is its role in dispersion. Forming aggregates allows cells to move over larger distances with bigger speed and it elevates cells from the ground by forming the stalk. While role of aggregation is clear it is less obvious why in *Dictyostelids* a fraction of the population dies, when stalk can equally be formed solely of extracellular matrix as in *Acrasids*. Although there is no direct evidence (Kaushik & Nanjundiah 2003) speculate that the two strategies are a balance of cost and benefits of having small stalk composed of extracellular matrix (1mm fruiting bodies in *Acrasids*) or forming high, solid stalk but with the cost of cell death (1-5mm high fruiting body in *Dictyostelids*). Since stalk is important for dispersal the higher the stalk the further you can be dispersed. They further speculate that another important advantage of a tall and stable stalk is that it is capable of supporting larger amount of spores. In *Dictyostelids* aggregates differentiate into slugs that enhance cell dispersion even more. Given the assumption that dispersion is a measure of reproductive success the choice of investing part of the population to form stalk will be preferred if it increases the dispersion rate. This cost to benefits trade offs resemble to the cost and benefits between different survival strategies discussed above.

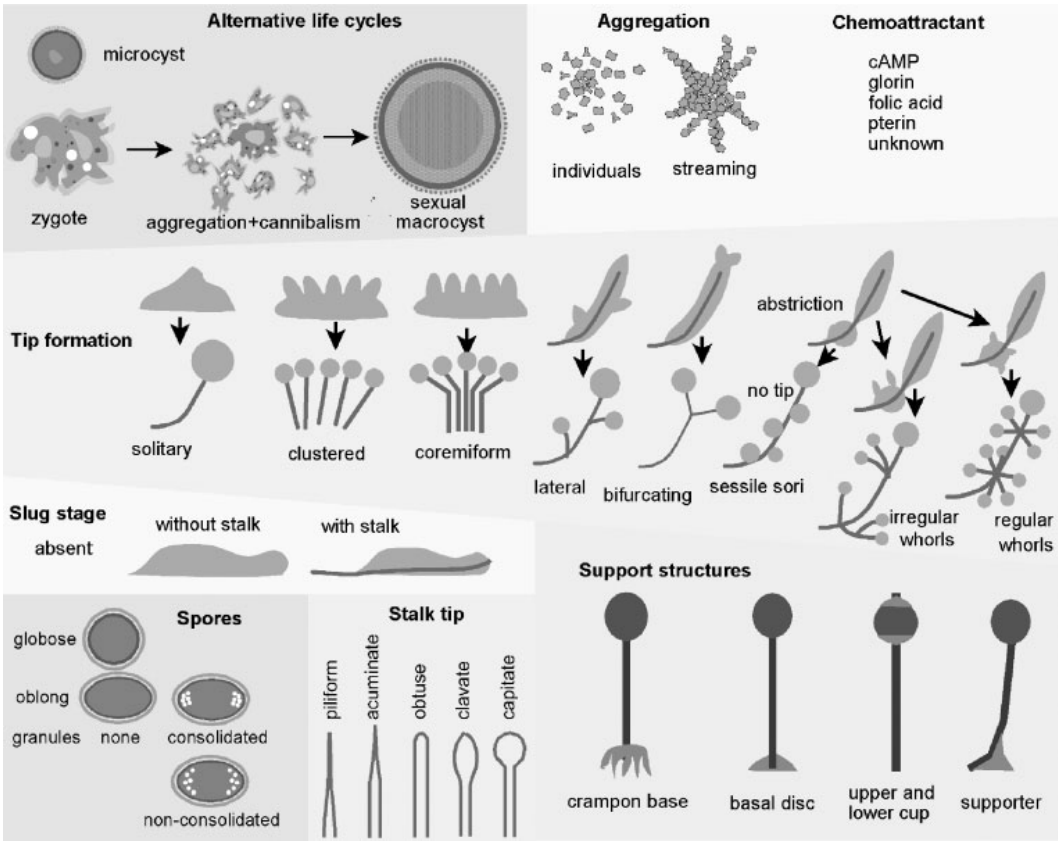


Figure 1 - 7 "Phenotypic variation in the social amoebas. *"Cartoon representation of morphological and behavioral variation at the cellular and organismal level in social amoeba species."* From (Schaap 2007)

Biological model

During the second half of the 20th century, the social amoebae went from being jet another microorganism to one of the most popular unicellular model organism in biological studies. This was primarily due to the facts that; it is eukaryotic and haploid, unicellular, easy to cultivate in the lab, grows fast in the lab (doubling time 2-8h), genetic manipulations are rather easy and has short (24h) multicellular phase. Today, *D.discoideum* is a popular system for studies of cytoskeleton organization, cell movement and adhesion, phagocytosis, chemotxis, signaling pathways, cell differentiation, developmental pathways and genes, host-pathogen interactions, cooperation studies and many more (Kessin 2001).

Experimental possibilities

Dictybase

There is a web based research community called "dictybase" that seeks to integrate all research groups, publications and experimental techniques (www.dictybase.com). It is also an easy to use and universal depository center for all social amoebae natural isolates, wild-type strains and mutants as well as plasmid constructs. This greatly facilitates the use of *D. discoidem* in laboratory studies and exchanges between labs.

Genetics

The most well-studied species is *D.discoideum*, who we also used in our study. It has a 34Mb genome spread over 6 chromosomes. The genome is highly AT-rich (cite Kessin). It has been fully sequenced and contains many homologue of human genes. Natural isolates often contain extra-chromosomal plasmids, some of which have been transformed into plasmid vectors (Firtel et al. 1985; Noegel et al. 1985). Our results show that this can sometimes limit the transformation of natural isolates with an additional laboratory plasmid (Chapter 2: Materials and Methods and Chapter 4). The Genomes of other species, *P. pallidum, D. purpureum* and *D. fasciculatum* are in the process of sequencing. All genome data is freely available on-line in the dictybase.

The fact that the genome is haploid and known makes the system very good for genetic studies and between-species gene comparisons. A long list of mutant strains is already generated and easily available through dictybase. These include single or double gene mutants in almost all developmental, signaling, metabolic pathways and many others. In our research we extensively used the availability of wide range of natural isolates, wild-type and mutant strains from the dictybase.

Genetic diversity studies often use microsatellites primers for identifying genetically different clones within a soil samples (Fortunato, Strassmann, et al. 2003). As discussed further in Chapter 4, such studies give quantitative information on clone mixing, competition and spatial distribution (Saxer et al. 2010).

Molecular biology

D. discoideum is primarily used as a molecular biology model organism. This means that a wide range of optimized molecular techniques is available. Cell transformation with extra-chromosomal plasmids is well-established and easy to work with (Materials and Methods). By changing the promoter region, we can have an ubiquitous (Levi et al. 2000) or cell-type specific (spores/stalk) (developed in our lab by C.Nizak and S.Kamat) expression of genes and fluorescent cell markers (ex. GFP). Such constructs give valuable information on development and stalk/spore differentiation.
Extra-chromosomal plasmids are also used in creation of mutant genotypes. Homologous recombination is usually used to disrupt genes. This has generated a list of single gene mutants that are ready to use. Libraries of random genomic insertions are made using Restriction enzyme mediated integration (REMI). Phenotypes of these random mutants often lead to discoveries of gene involvement in specific developmental pathways, such as defector phenotypes discussed in Chapter 4 (Ennis et al. 2000).

Microscopy

Microscopy techniques are used for both individual-cell and population-multicellular based studies. Of our special interest is the use of live-cell imaging and fluorescent microscopy during the development. These techniques are interesting because they allow us to localize and track cells in live. Live-cell imaging has mainly been used for cell movement and chemotaxis during both vegetative and developmental phase (Dormann & Weijer 2006). Fluorescence microscopy is used in *D.discoideum* studies of developmental gene expression, cytoskeleton organization, programmed cell death and other topics (Bretschneider et al. 2002; Parkinson et al. 2011; Cornillon et al. 1994). For example, cell localization in front or back of the slug tells us whether a cell has acquired prespore or prestalk fate (Thompson & Kay 2000). Quantitative estimations of spore to stalk cell ratio have however been based on counting cells at the onset of starvation and spores at the end of the development. Similarly, competitive assays are done by differentially staining genetic strains and quantifying strain enrichment into spores. As we explain further in Chapter 1 and 2 this provides only indirect estimation of the numbers of stalk cells and does not take into account the non-aggregating cells and non-germinating spores. As emphasized in Chapter 3, non-aggregating cells may be an important population level response and there quantification is important for both population survival (Chapter 3) and strain competition studies (Chapter 4). In our lab we have developed a microscopy set up for more precise characterization of *D.discoideum* life cycle that quantifies both non-aggregating cells and spore cells (Materials and Methods). For this we combined; i) fluorescence staining with GFP based markers, ii) low cell ration of fluorescent cells for single cell localization inspired from (Dormann et al. 1997) and iii) live-cell imaging setup for cell tracking during the whole life cycle inspired from (Houchmandzadeh 2008). The right combination of plasmid type, promoter, antibiotic marker and transformation technique, gives an almost 100% population fluorescence. In Chapter 4 we show how such single cell tracking technique can be used for quantitative measurements of non-aggregating cells. We can further imagine using it for estimating aggregate size or single cell movements in the slug.

Chapter 2 Materials and Methods

EXPERIMENTAL PROCEDURES

** Mark indicates a technique developed and/or optimized during this PhD.

Strains and Culturing

D. disocideum laboratory strains and culturing

Dictyostelium discoideum axenic strains used in the study were AX3, DH1, *phg2*, *pdsA*, and *carA*. All the strains were cultured in HL5 medium at 22°C if not mentioned otherwise. In experiments on nutritional effect we used: FM minimal medium (Formedium), NS and NS with 85mM glucose medium (glucose added after autoclaving) (Garrod & Ashworth 1972). See medium composition in Medium and Buffers.

Natural isolates and culturing

Dictyostelium discoideum natural isolates used in the study were 34.1, 28.1, 105.1, 63.2, 85.2 and 98.1 isolated from North Caroline by (Francis & Eisenberg 1993). *Polysphondylium pallidum* strain used was CK-8 (dictybase strain ID: DBS0236805). All strains were cultured by plating cells, or spores, on SM/5 agar plates (9cm diameter) with 200µL of overnight *K. aerogenes* culture.

Heat killed bacterial culture **

Bacterial strain used in the study was *Klebsiella aerogenes* (Aa). We developed a protocol for killing bacteria at high temperature, "heat killed bacteria". Heat killed bacterial cultures were prepared by centrifuging 50 mL of overnight LB cultures at 4°C, 5000g for 10min and diluting the pellet in 1mL KK2 buffer. To heat kill the bacteria the suspension was incubated for 20 minutes at 80°C. The bacteria were stored at -20°C. Similar protocols, often including autoclaving of bacterial culture, are used by other research groups (Fey et al. 2007; Gaudet et al. 2008).

Transformation and Dyeing

Cell transformation

GFP and RFP expressing amoebae were obtained by transforming cells with pTX-GFP (Levi et al. 2000) or pTX-RFP plasmids (constructed in our lab) (Figure 2-1). The GFP (or RFP) gene was put under ubiquitously expressing promoter actin15. Standard electroporation procedure was optimized in the lab by my supervisor Clement Nizak and co-workers prior to my arrival in the lab. Cells were grown in 75cm^2 flasks until dense but not confluent (usually 1 day before confluency). The medium was changed 4-

6h before transformation. For transformation cells were re-suspended in 10mL of ice-cold HL5 and kept on ice for 30min. Cells were centrifuged for 5min, 500g at 4°C. Supernatant was re-suspended in 800µl of electroporation buffer and transferred into ice cold 4mm electroporation cuvettes containing 30µg of plasmid DNA. Cells were electroporated at 0.85 kV and 25 mF twice, waiting for 5 s between pulses. Cells were transferred from the cuvette to 75cm^2 flask with HL5. The next day, transformants were selected with 5µg/ml G418 (SIGMA). The concentration of G418 was gradually increased to 20µg/ml G418 over 1-2 weeks. Transformed strains were maintained at this concentration of G418, yielding GFP and RFP-expressing cell lines that were analyzed by flow cytometry to confirm their unimodal cellular fluorescence distribution (>99% of fluorescent cells upon analysis of 10^6 cells).

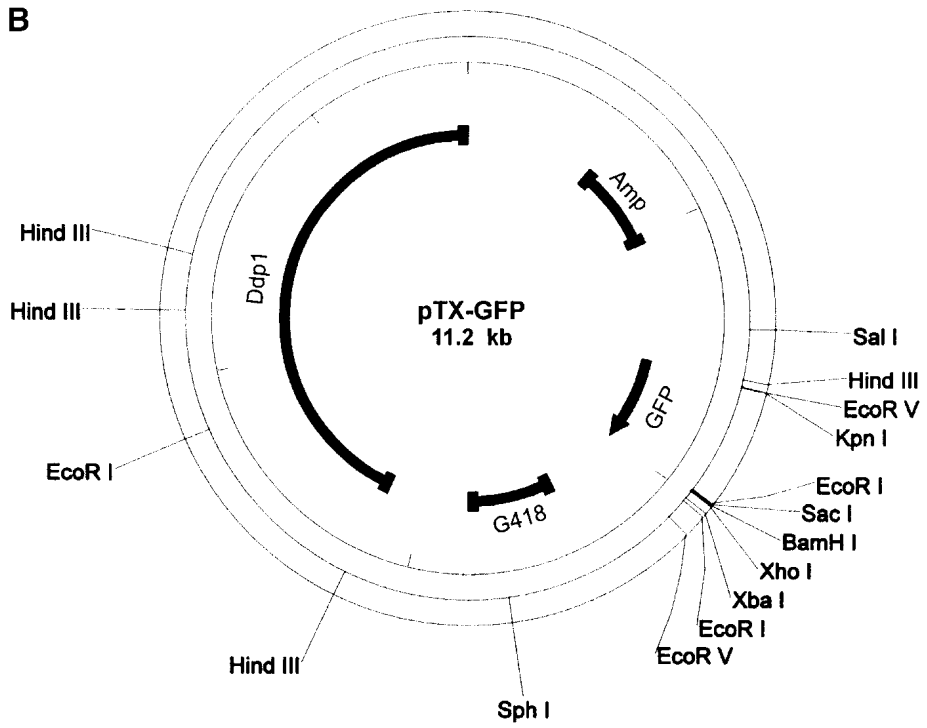


Figure 2 - 1 pTX-GFP plasmid construct used in our study. From (Levi et al. 2000)

Transforming *D.discoideum* natural isolates **

No standardized cell transformation protocol for natural isolates of *D.discoideum* exists. We have tried to develop our own protocol. Here are details of the protocol and obtained results.

Protocol: Natural isolate strain was grown on SM/5 plates with *K. aerogenes* or in flasks with SorC and heat killed bacteria as food source (10ml of SorC + 300µl of heat killed bacteria). In case of liquid cultures in SorC medium was changed several hours before transformation (10ml SorC + 250µl of heat killed bacteria). In both cases exponentially

growing cells were re-suspended is ice cold SorC and let sit for 30min on ice. Suspension was centrifuged 3 times on 500g, 5min, 4°C after which standard transformation protocol was performed. At the end cells were transferred to HL5 medium with streptomycin. Next day the medium was changed to a) SorC + 200µl heat killed bacteria + streptomycin + G418 (7.5µg/ml) or b) SorC agar plates with 7.5µg/ml G18 and 200µl heat killed bacteria. SorCan heat killed bacteria were used to prevent bacterial division that causes the consumption of selective G418 antibiotic.
The transformation was performed with self replicating plasmids pTX-GFP and pTX-RFP and integrating plasmid V18 GFP.
Results: transformation showed no success rate. In some cases isolated colonies that were resistant to G418 were observed. In every case they would not multiply and die within several days.

Cell staining with chemical dyes

Invitrogen CellTracker Probes Red CMTPX and Green CMFDA are commonly used for staining *D.discoideum* cells. We worked according to previously established protocols (Nizak et al. 2007; Buttery et al. 2009). Working solution was 10mM dye re-suspended in DMSO. Cells were washed and re-suspended in ice colds phosphate buffer. If natural isolates were used they were re-suspended in phosphate buffer and centrifuged 3 times at 300g, 5min, 4°C. Cells were counted and re-suspended to $1x10^4$cells/µl. CellTracker dye was thawed quickly and a dye was added to a final concentration of: Red dye- 25µM for 30min, or 10µM for 1h, and Green dye - 50µM for 30min. Cells were incubated in the dark with gentle shaking for time indicated for each concentration. After incubation excess dye was removed by centrifuging the cells 2-3 times in ice-cold phosphate buffer. Cells were observed under the microscope for their fluorescence.
When only part of the population was dyed, the "non-dyed" population was treated the same way as dyeing one. The only exception being that the cells were not incubated with the dye but with the same concentration of DMSO.
Results: The dyeing was successful for both *D.discoideum* and *P. pallidum* natural isolates and *D. discoideum* AX3 strain. In all cases dyeing was not sufficiently strong for tracking single cell fluorescent, but population fluorescence was strong enough. In addition dye would bleach fast making it difficult to track non-aggregated cells over time. When natural isolates were used bacteria that were still left in suspension and that were dyed together with cells were a big source of noise. This made it difficult to distinguish the background from single fluorescent cells.

Aggregation

D. discoideum starvation experiment **

Cells were subjected to two different starvation conditions: sudden and gradual starvation. Sudden starvation is a standard starvation protocol used in all *D.discoideum*

and *P. pallidum* studies (Fey et al. 2007). We established gradual starvation protocols in liquid and on bacteria during the period of this PhD.

Sudden starvation: If not mentioned otherwise sudden starvation was used as a standard plating protocol: When cell culture was near confluency, medium with antibiotics was replaced with antibiotic free medium. No difference was found when cells from exponential growth phase (before confluence) were used. After 4-6h cells were washed out of nutrient medium and centrifuged in KK2 buffer at 500g for 5 min. The pellet was re-suspended in KK2 buffer to the concentration of $1x10^5$cells/µL. For density dependent aggregation experiment cells were re-suspended to the concentration of $1x10^3$, $1x10^4$, $5x10^4$,$1x10^5$ or 5-7.5$x10^5$ cells/µL. Green and red fluorescent cells were mixed in ratios indicated in Image analysis section. 30µl of suspension was plated on 6cm plates filled with 2mLof 2% Phytagel (SIGMA). In case of pairwise mixtures, strains grown in different medium or genetically different strains, the ratio of two strains was 1:1.

Gradual starvation in liquid: the cells were collected 1-2 days after reaching confluency in HL5. Cell washing and plating was done as in sudden starvation experiment described above.

Gradual starvation on bacteria: another way of slowly starving the cells is to plate them with bacteria and to let them deplete the food source as in natural conditions. Two types of plating were done: homogenous and heterogeneous plating. In both cases RFP-expressing AX3 and GFP-expressing AX3 cells were grown in HL5 medium with 20µg/mL G418. When confluent cells were re-suspended in KK2 buffer and centrifuged at 500g for 5min. The cell pellet was re-suspended in KK2 to the concentration of $1x10^5$cells/µL. Green and red fluorescent cells were mixed in ratios indicated in Image analysis section. For heterogeneous plating 200µL of heat-killed bacteria was mixed with 100µl of cell suspension. The mixture was spread on a 6cm plate with 2mL of 2% Phytagel. This gave rise to heterogeneous distribution of cells and bacteria (Sup. Figure S2). For homogenous plating 100µl of heat-killed bacteria were mixed with 100µl of cell suspension. A 100µl drop was plated on a 6cm plate with 2ml of 2% Phytagel and let to dry under the hood. This gave a very homogeneous cell distribution (Sup. Figure S3-2). In both cases, cells fed for ~8h on heat-killed bacteria before the beginning of starvation, and thus divided at most twice after plating. The density of cells at the onset of starvation (measured via a similar method as the one for measuring the non-aggregating cell fraction, see below) was comparable to that of cells processed according to the sudden starvation protocol.

P. pallidum aggregation

Starvation induced aggregation was observed by: i) plating cells on nutrient free agar or ii) plating cells on bacterial plates and letting them deplete food gradually.

Plating on nutrient free agar: cells were grown on SM/5 plates with *K. aerogenes* as food source. Cells were washed of in ice cold KK2 and centrifuged 3 times on 500g, 4°C to remove access bacteria. Bacteria free cells were plated on phytagel plates at density

around $1x10^5$ cells/µl. Under these conditions cells start aggregating within several hours.
Plating cells on bacterial plates: cells were prepared as above. Cells were plated on 5ml SM/5 plates with 100µl overnight *K. aerogenes* culture.

Image Acquisition

Time-laps microscopy **

The 6 cm diameter Petri dish was imaged on an automated inverted microscope setup duplicated from (Houchmandzadeh 2008). The set up was constructed by Clement Nizak prior to this PhD. The setup was made of: OlympusIX70 inverted microscope, Photometrics CoolSNAP HQ^2 CCD camera, Zeiss NHBO 100 microscope illuminating system, Thorlabs SH05 shutter, Thorlabs TSC001 shutter controller, and 2.5x-5x-10x-20x objectives (5x was used for all experiments shown here). Images were acquired in WinView/32 and the whole setup was controlled by custom-made visual basic software. The setup allows Petri dish scanning at regular time intervals, with phase contrast and fluorescence image acquisition for each image at all time points. An area of around 1cm x 1cm was scanned every 15-30 min for *P. pallidum* aggregation and 1h-2h for non-aggregated cells experiments, with 5x or 2.5x objective. A mosaic image is reconstructed by combining all the images of contiguous areas of the Petri dish at a given time point by a custom-made macro using Image J software.

Image Analysis

All images were analyzed in Image J. Image J is an open source, public domain, Java-based image processing program. Program and various plugins can be found at official website: http://rsbweb.nih.gov/ij/

Non-aggregated cells **

Mixing a small percentage of red fluorescent cells in a population of green fluorescent cells allowed us to get the image single cells as single red fluorescent dots (Fig 2-2). This a experimental approach was inspired from studies of cell motion within aggregates (Dormann et al. 1997). The approach was developed by my supervisor Clement Nizak and co-workers prior to beginning of my PhD and optimized during the course of my PhD. We optimized the red to green cell ratios depending on plated cell density: for experiments with $1x10^5$and 5-$7.5x10^5$ cells/µL0.25-0.5% of RFP cells was mixed with 99.5-99.75% GFP cells, for $1x10^4$ cells/µL 1% of RFP cells were used and for $1x10^3$ cells/µL 2% RFP cells were used. For pairwise mixtures the ratio was made as following: 50% of strain A in GFP was mixed with 49.75% of strain B in GFP and 0.25% of strain B in RFP. Reciprocal mixing was done as well, for example 0.25% of GFP cells were mixed with 99.75% of RFP cells. The choice of fluorescent signal had no effect on cell behavior.

Images were acquired by time-laps fluorescence microscopy. All the images were analyzed in Image J by custom-made macro made by us. The analysis consisted in counting fluorescent dots before and after aggregation. For each experiment 1000 – 10 000 dots/cells were monitored. Dead cells were excluded from counting by looking at cell displacement as an indicator of cell viability. Two fluorescent images taken 1-2h apart were overlapped and cells that showed no displacement were counted and subtracted from the overall non-aggregating population.
The density of red dots (RFP-expressing cells) was used to estimate cell density at the onset of starvation in all experiments. Cell density was comparable at the onset of starvation for all starvation protocols used.

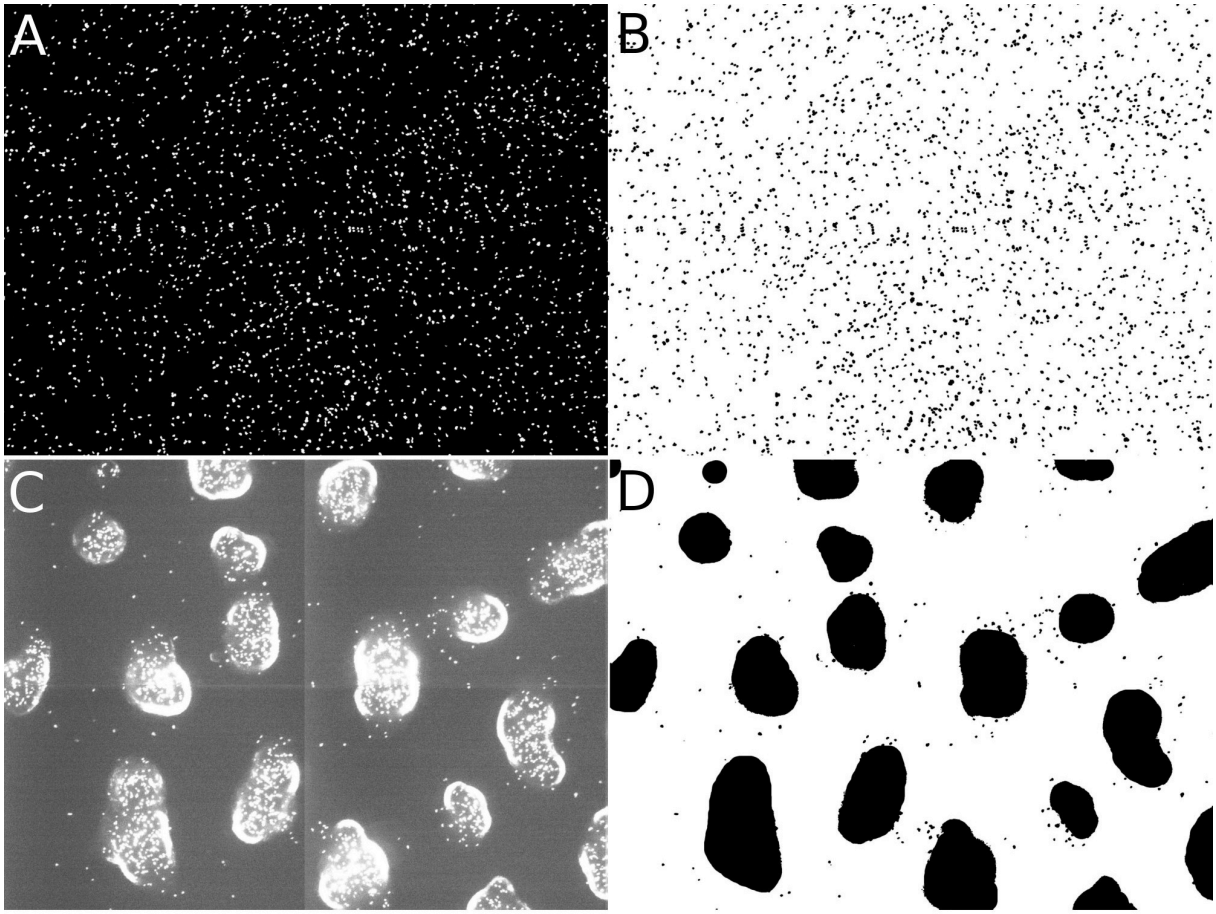


Figure 2 - 2 Image analysis of *D. discoideum* non-aggregating cells. Fluorescent images (A and C) are treated in Image J to get a binary image with fluorescent cells as black dots (B and D). Number of dots before and after aggregation is counted using Analyze Particles command.

P. pallidum aggregation **

All images were analyzed in Image J. Phase contrast images were converted to a binary image of white background and black aggregates. This was done by manually setting the image threshold. Image was cleared of background noise by using Despeckle and Remove Outliers commands, and manually. The process demanded a great deal of manual work for most mid-aggregation steps when a real aggregate boundary was

difficult to estimate based on phase contrast alone. Final working image is show in Figure 2-3.

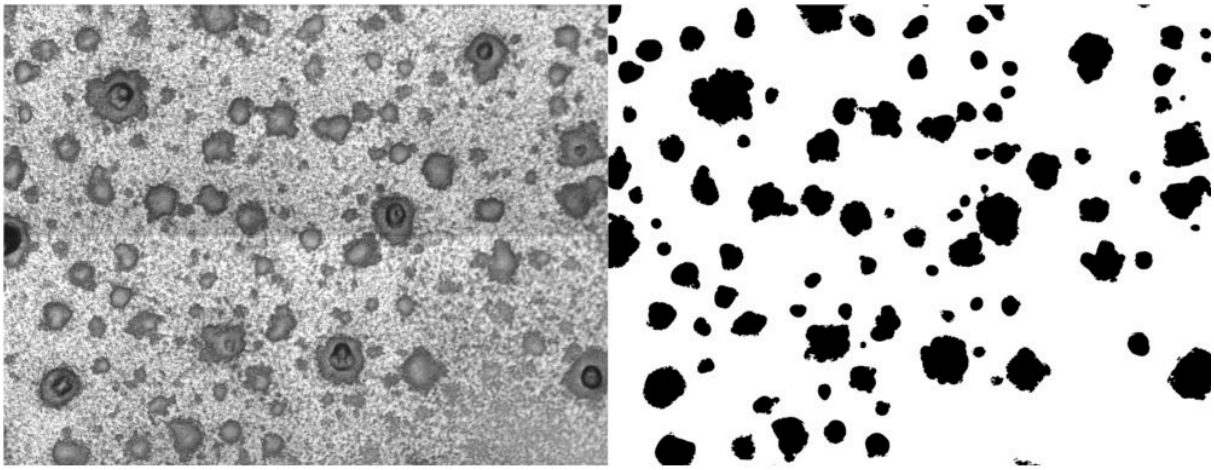


Figure 2 - 3 Image analysis of aggregation in *P. pallidum*. A) phase contrast image and B) binary image ready for analysis.

Two-point correlation

To estimate spatial structure of aggregates we used two point correlation function. We used an Image J plugin built by Johannes Schindelin, free to download at http://wbgn013.biozentrum.uni-wuerzburg.de/ImageJ/two-point-correlation.html. The plugin is based on the paper: J. G. Berryman and S. C. Blair, *Use of digital image analysis to estimate fluid permeability of porous materials I. Application of two-point correlation functions* (Berryman & Blair 1986). The function calculates the correlation of finding two points (pixels of radius r) at distance d. The program uses FFT transformation to accelerate the calculation. Original images were too big for analysis, therefore before analysis images were scaled 0.1 times its original scale.

Aggregate size

Aggregate size was calculated in Image J using Analyze particles command. Aggregate size is represented as aggregate area in pixels.

Strain Specific Life Cycle Properties

Growth rate on bacterial plates **

Standard growth rate measurements for D.discoideum are done in liquid culture. To measure growth rate on bacterial plates we developed our own experimental procedure. Experiment was done with 6 natural isolates; 34.1, 28.1, 105.1, 63.2, 85.2 and 98.1. Spores were plated on SM/5 plates with 200µL of overnight *K. aerogenes* culture. Spores germinated into cells and cells started dividing. 15-20h after plating spores cells were removed from the plates by washing the plates in ice-cold KK2 buffer. Suspension was

centrifuged 3 times in ice cold KK2 for 5min, 300g to remove bacteria so we could count the cell concentration. $1x10^5$ cells were re-suspended in 500µL of overnight *K. aerogenes* and plated on 15cm petri dish with SM/5 agar. 16-20h after plating we started to measure cell growth. Growth was measured for 3 independent plates/time point every 2h during 8h. For each measurement cells were removed by scraping the cells from the plate in ice-cold KK2 buffer to prevent cell division. Suspension was centrifuged 3 times for 5min, 300g on 4°C to remove bacteria. Cells were counted using haemocytometer. Growth curve was represented as log2 of the cell number over time. Growth rate was calculated as slope of the linear regression of the growth curve.

Sporulation efficiency

Sporulation efficiency tests the "efficiency" of a cell to become a spore. It is calculated as a ratio of N_{spores}/N_{scells}. It is a common procedure used in many studies (Buttery et al. 2009; Fortunato, Queller, et al. 2003). We used the same procedure as in their studies with minor adaptations (centrifugation force and timing before cell plating)
Spores were plated on SM/5 pates with *K. aerogenes.* Spores germinated and a fresh lawn of cells was collected. Cells were scraped from plate in ice-cold KK2 buffer. Suspension was centrifuged 3 times on 300g, 4°C to remove bacteria. Cells were re-suspended in KK2 buffer to a final concentration of $1x10^5$ cell/µl. Cells were plated on 2% phytagel plates or filter papers. For plating on phytagel plates 1mL ($1x10^8$ cells) was plated and spread on 6 cm plate. The plate was left to dry under the hood. For plating on filters, cells were left for 2-3h in phosphate buffer in order to consume any bacteria left and to finish division. Cells we counted and re-suspended to final concentration $1x10^5$ cell/µl. 30µl ($3x10^6$ cells) was plated as a drop on filter. Plates were kept in the incubator for 2 days for fruiting bodies to form. Plates were washed in SORC with 0.1% TWEEN. Spore concentration was counted with the hemocytometer.

Germination efficiency

<u>On bacterial plates</u>
Germination efficiency protocol was adopted from previous studies (Castillo et al. 2011; Jack et al. 2008).
Experiment was done with 6 natural isolates; 34.1, 28.1, 105.1, 63.2, 85.2 and 98.1. Spores were plated on 9cm SM/5 plates with 200µl of overnight *K. aerogenes* culture. Spores germinated, cells divided, consumed bacterial food and when starved formed new spores. Fresh spores were re-suspended in ice-cold HL5 with 0.1% TWEEN and vortexed. HL5 was used instead of phosphate buffer cause spores would attach to surface of tube when in suspension with phosphate buffer. 100 spores were plated with 500µl of overnight *K. aerogenes* culture and plated on 14cm petri dish with SM/5 agar. After 3 days we counted number of formed plaques. Germination efficiency was counted as $N_{spores}/N_{plaques}$. For each strain experiment was repeated 7-9 times with 3 replicas per measurement.

In liquid **

Experiment was done with all 6 *D.discoideum* natural isolates. Germination was tested in liquid SORC and HL5. There are no to us know studies that tested germination of natural isolate sin liquid cultures. We have therefore developed our own protocol.

In SORC (lq): spores were plated in liquid suspension of 5ml SORC and 400µL heat killed bacteria. Spore germination was monitored under the microscope.

IN HL5: spores were plated in HL5 with 10% fetal bovine serum. Spore germination was monitored under the microscope.

Cell viability

Cell movement as a measurement of cell viability **

A protocol for studying cell viability as a function of cell movement was developed during this PhD.

Cell movement was used as an indicator of cell viability. AX3 cells were grown in HL5 medium. Cells in exponential phase were washed in ice-cold MCPB buffer (1.42g Na_2HPO_4, 1.36g KH_2PO_4, 0.19g $MgCl_2$, 0.03g $CaCl_2$, 1L water, pH = 6.5, filtered) and centrifuged at 500g for 5min. In order to remove all traces of HL5, the procedure was repeated 3 times, each time diluting the pellet in fresh MCPB. $2x10^5$ cells were plated on nutrient-free Phytagel plate. Plating cells at low concentration disabled cell aggregation. Cell position was recorded at t=0 and t=30min, by taking a phase contrast image with 10x or 20x objective. Displacement of 200 cells was examined manually by eye, and number of dead/static and alive/moving cells was recorded. This was repeated every 24h until all the population was not moving. Experiments were performed twice in duplicate.

Dead/live cell staining

Standard protocols of measuring cell viability in *D. discoideum* use colorful chemical dyes that stain differentially dead and live cells (Giusti et al. 2008; Cornillon et al. 1994). The basic principle is that cell membrane of dead cells is more permeable, therefore allowing the entrance of external dyes. This gives stained dead cells and non-stained alive cells. The most common used dies are trypan blue and propidium iodide. For both dies we adapted protocols from previous studies (Giusti et al. 2008).

Trypan blue (TB): Trypan blue colors dead cells in blue while live cells rest colorless. This is because of the mentioned membrane impermeability of live cells to the dye. Sigma 0.4% solution was used. Chemical is carcinogenic so gloves were used during experiment. 0.1ml of TB was mixed with 0.4ml of cell solution (in phosphate buffer) and let sit for 5min in the incubator. Cells were observed under the microscope. In *D. discoideum* the chemical is used but it is know to give poor staining. In my case the staining was too poor for unbiased quantification.

Propidium iodide (PI): PI is membrane impermeable and cannot penetrate through the membrane of viable cells, but goes through the membrane of dead cells. The result is red fluorescence of dead cells and no fluorescence of live cells. Molecular Probes propidium iodide was used. Chemical is carcinogenic so gloves were used during experiment. The

stock of 1.5mM PI in water was used. Working solution was 4µM PI. Cells on petri dish were washed in phosphate buffer. 5ml of working solution was poured into plate and let sit in the dark at 22°C for 10min. PI suspension was removed and cells were washed 1-2 times in phosphate buffer. Cells were directly observed under the microscope. In our case the fluorescence signal was visible but difficult to quantify. The possible reason is not a complete match between absorbance and emission profiles of my filter and PI spectrum.

Statistical Analysis

Statistical analysis was performed in R. Significant difference between the samples was calculated using Welch two sample t test function in R (t.test(x,y)). To test among groups differences we used one way ANOVA test in R, using oneway.test() function.

Standard Techniques

Freezing amoebae cells

Grow cells in 75ml cell culture flask with HL5 medium. When confluent, re-suspend in ice cold HL5 and centrifuge for 5min, 500g, 4°C. Re-suspend the pellet in 0.75mL of cell freezing medium–DMSO (Sigma) (quantity for one75cm^2 flask) and transfer to freezing vials. Place in ice-cold isopropanol box. Put at -80°C for one day. Next day transfer to liquid nitrogen.

Freezing amoebae spores

Scrape spores from the agar plate into 15ml or 50ml falcon tube using ice-coldSorC or KK2 buffer. Vortex to free spores from sorus. Centrifuge for 5min at 500g, 4°C. Re-suspend the pellet in 1.5mL of cell freezing medium–DMSO (Sigma) (quantity for one 9cm petri plate). Put 0.75ml in single freezing vials. Place in ice-cold isopropanol box. Put at -80°C for one day. Next day transfer to liquid nitrogen.

Freezing bacteria

Centrifuge 50ml of overnight bacterial culture at 5000g, 10min, 4°C. Re-suspend pellet with 1-2ml of 50% glycerol. Put at -18°C. Next day transfer to -80°C.

Thawing cells

Vial with frozen cells was let to thaw slowly in water. Thawed cells were quickly removed from the vial (because DMSO-freezing media damages the cells) and mixed with 10ml of ice cold HL5. Suspension was centrifuged on 500g, 5min, 4°C. The pellet was re-suspended in 5ml of room temperature HL5 and transferred to 25cm^2 flask. The next day the medium was changed and if needed antibiotic was changed (G418).

Thawing spores

Frozen spores were taken from liquid nitrogen. Small amount of spores were scraped from the surface and re-suspended in 200µl on overnight bacterial culture. The suspension was plated on SM/5 plates (9cm petri dish). Vial with frozen spores was quickly returned to liquid nitrogen.

Transforming bacteria with pTX RFP and pTX GFP plasmid

E.coli DH10 B strain was used to prepared competent cells. Cells were stored at -80°C. For one transformation one eppendorf tube of competent cells (120µl) was thawed slowly on ice. 10ng of plasmid DNA was added to the tubed and mixed gently by tapping the tube with the finger. The DNA+bacteria suspension was let to sit on ice for 10min. The tube was transferred to 42°C for 45sec and than on ice for 2min. 1ml of LB was added and let for 1h at 37°C. 0.5ml of suspension was mixed with 50ml LB and ampicillin over night.

MEDIA AND BUFFERS

All media and buffers were prepared as indicated by dictybase (http://dictybase.org/)

HL5
5 g peptone
5 g tryptone
10 g glucose
5 g yeast extract
0.35 g $Na_2HPO_4*7H_2O$
0.35 g KH_2PO_4
1L water

Adjust pH to pH 6.4 - 6.7
Autoclave
Keep medium at 4°C and in the dark (fridge)

FM minimal medium
Ready to use mix from ForMedium

NS
(Garrod& Ashworth, 1972)
14.3 peptone
7.15 yeast extract
0.641 g $Na_2HPO_4*2H_2O$
0.49 KH2PO4
1L water

Adjust pH to pH 6.7
Autoclave

NS+85mM Glu: glucose was added after autoclaving to NS medium.
3.68mL of 40% glucose for 100ml of NS medium

SM/5 plates
2 g glucose
2 g peptone
0.2 g yeast extract
0.1 g $MgSO_4$ (or 0.2 g $MgSO_4*7H_2O$)
1.9 g KH_2PO_4
1.0 g K_2HPO_4
15 g agar
1L water

Adjust pH to 6.5 ± 0.1
Autoclave

KK2
2.2g KH_2PO_4
0.7g K_2HPO_4
1L water
Autoclave

SOR and SORC (Sorensen's buffer)
Sor (4L)
8.0 g KH_2PO_4
1.16 g Na_2HPO_4 (or 2.2 g $Na_2HPO_4.7H_2O$)
pH should be 6.0 ± 0.1

SorC (Sor with 50 μM Ca++)
Add 4 ml 50 mM $CaCl_2$ (or 0.2 ml 1 M $CaCl_2$) to 4 liters Sor
Autoclave

Sandrine's MCPB buffer
1.42 g Na_2HPO_4
1.36g KH_2PO_4
0.19g $MgCl_2$
0.03g $CaCl_2$
1L water
pH=6,5
Filtered

2% phytagel (100ml)
2g phytagel
100ml SORC

Phytagel is difficult to dissolve. Heat gently in the microwave and stir. If "overcooked" ameba will not aggregate.
Do not need to autoclave.

MODEL

Model **

The model represents *D. discoideum* life cycle with interchanging growth and starvation periods. During the growth phase individuals grow according to a logistic equation (1) with growth rate λ and carrying capacity $K = N_{\max}$

$$\frac{dN}{dt} = \lambda N\left(1 - \frac{N}{K}\right) \quad (1)$$

We assume that food runs out when population has reached maximum density *K.* At this point starvation period T starts. The population splits into an aggregating ($N_{\text{agg}} = \alpha N$) and a non-aggregating ($N_{\text{non-agg}} = (1-\alpha)N$) fraction according to the aggregation factor α. Aggregating cells become spore and stalk cells with the proportion of spore cells given by sporulation efficiency s, so $N_{\text{spores}} = sN_{\text{agg}}$. During the starvation period spores are dormant; their growth and mortality rate are assumed to be zero. When conditions become favorable again, spores germinate with germination efficiency g and start dividing, but only after a fixed development time *D*. During the starvation period the non-aggregating cells do not divide and are subjected to mortality with instantaneous mortality rate μ, and their dynamics are governed by

The advantage that non-aggregating cells have is a head start when conditions improve, as spores produced by aggregating cells need costly time to develop. By the time the

$$\frac{dN_{\text{non-agg}}}{dt} = -\mu N_{\text{non-agg}}$$

latter start growing, the descendants of the non-aggregating cells may have the opportunity to use up a sizable portion of the resources that have become available. Here, we assume that no more spores can germinate than the remaining carrying capacity allows.

Non-aggregating cells (Chapter 3)

Analytical expressions developed by Minus van Baalen

As a first step in understanding the relative benefits of aggregation and non-aggregation consider the fates of cells of either type at the moment starvation sets in. A non-aggregating cell stops reproducing but is subject to mortality so when conditions become favorable again, T time units later, it has a probability $e^{-\mu T}$ of surviving the starvation period. Working out the fate of aggregating cells is simple: it has a probability sg to become a germinating spore when conditions improve. An aggregating cell thus has a fitness equivalent of

$$W_{\text{agg}} = sg$$

As discussed, germination involves a time cost: during a time D its surviving non-aggregating competitors can start reproducing, giving the latter an extra reproduction bonus (a period of logistic growth), giving a fitness equivalent of

$$W_{\text{non-agg}} = e^{-\mu T} \frac{e^{\lambda D}}{1 + \frac{n_0}{K}(e^{\lambda D} - 1)}$$

where n_0 is the number of surviving non-aggregating cells.
The expected fitness (descendants by the time conditions improve) of a cell that has a propensity α to aggregate can thus be expressed as

$$W = \alpha W_{\text{agg}} + (1 - \alpha) W_{\text{non-agg}}$$

This result suggests that (if the duration of the starvation period is fixed) it is either profitable to join an aggregation (if $W_{\text{agg}} > W_{\text{non-agg}}$) or to stay solitary (if $W_{\text{agg}} < W_{\text{non-agg}}$): a bet-hedging strategy is not favored. However, this result does not take into account the frequency dependence that acts on the fitness of non-aggregating cells. That is, if many cells aggregate the number of surviving non-aggregating cells (n_0) will be low, boosting the profitability of remaining solitary. If many cells remain solitary, on the other hand, n_0 will be high, reducing the profitability of remaining solitary. Whether this frequency dependence results in population heterogeneity cannot be stated right away and other methods are necessary. The same is true when the environment, and in particular the starvation period T is variable and unpredictable.

In order to study potential benefits of producing both aggregating and non-aggregating cells, strains with different aggregation factors α were put in competition using a multi-strain variant of the above-described model. The population is made of i strains each with α=0 (all cells aggregate), 0.1, 0.2, … 1 (none of the cells aggregate). All strains had the same growth rate λ = 0.38, mortality rate μ = 0.002 for t ≤ 165h, and μ = 0.053 for t > 140h (approximation of experimental mortality curve, see Figure 3-6C), sporulation efficiency s = 0.8 and germination efficiency g = 0.63. All values are based on experimental measurements and are the average of measurements obtained for 6 natural isolates from Table 2-1. Competition was carried out in two types of conditions, either constant or varying starvation periods T. In the case of varying starvation periods, the duration of starvation was randomly chosen from a uniform distribution $U(x,y)$ at the end of every growth period. Population size was taken as an estimate of strain fitness. At the end of every growth cycle, the number of alive and growing individuals $N(t)$ is plotted. In the case of varying starvation periods, the geometric mean over 100 simulations is plotted.

Strain competition (Chapter 4)

Simulation model was used to perform competition between our 6 natural isolate over the whole life cycle. Strains were competed at 3 stages: growth, sporulation and germination. Since we could not measure the fraction of non-aggregating cells of each clone, we assumed that aggregation rate is 1 (all cells aggregate).
Competitive parameters growth rate λ_i, sporulation efficiency s_i and germination efficiency g_i are measured in previous experiments (Table 2-1). Since we were unable to measure sporulation efficiency for all strains, in the model we used the data from Buttery et al, 2009. Their results show the unrealistic sporulation efficiency for strains 34.1, 28.1 and 105.1; more than 100% of cells become spores. In the sporulation section I discussed the probable sources of this error. In the model it was not possible to use this data. I have therefore approximately decided that for strains 34.1 and 28.1 the sporulation efficiency is 0.97 0.98 and 0.95. For other strains measured sporulation efficiency from Buttery et al, 2009 was used. The simulation starts with population mixed of equal cell ratios for all 6 strains. Strains are let to compete over many growth-sporulation-germination-growth cycles. At each cycle I assume perfect mixing and strain co-aggregation. The model assumes no interactions between strains. This is a simplified assumption that we know is not always true. Several studies have shown that interactions are frequency depended (Buttery et al. 2009) and strain depended (Kaushik et al. 2005). The complexity of these interactions is still too high for it to be modeled for multi strain interactions. For example, it has been showed that sporulation efficiency of strain A changes when A is in monoclonal population, A is in pairwise mixture with B and A in mixture with B and C (Kaushik et al. 2005). We have therefore decided to use the interaction free model because: 1) it allows use to understand the importance of each competitive parameter, 2) the complexity of 6 strain interactions is not necessarily explainable by the use of 2 or 3 clone interactions.

Strain	Growth / h	Sporulation efficiency	Germination efficiency
34.1	0.30	0.98	0.54
28.1	0.35	0.97	0.59
105.1	0.35	0.95	0.68
63.2	0.33	0.91	0.66
85.2	0.33	0.76	0.63
98.1	0.27	0.51	0.73

Table 2 - 1 Experimentally measured parameters used in the model of competition between natural isolates.

Chapter 3 Population Partitioning Between Unicellular and Multicellular Strategies in Social Amoebae *D. discoideum*

Article submitted for publication

ABSTRACT

Social amoebae of *Dictyostelium discoideum* are widely studied for their multicellular development program as a response to starvation and constitute a model of choice in microbial cooperation studies. Aggregates of up to 10^6 cells form fruiting bodies containing two cell types: (i) ~80% of reproductive spores, and (ii) ~20% of dead stalk cells that promote spore dispersion. But not all cells aggregate and take part in this cooperative behavior. A part of the population ignores the aggregation signal and continues behaving as single cells, possibly avoiding the costs of cooperation and taking advantage of incoming nutrients. We have developed a new cell-tracking technique based on time-laps fluorescence microscopy and image processing. This enabled us to quantify population partitioning into social/aggregating and unsocial/non-aggregating cells. In realistic starvation conditions, up to 15% of cells do not aggregate, which makes this third cell fate a significant component of the population-level response of social amoebae to starvation. Non-aggregating cells have an advantage over cells in aggregates since they can resume growth earlier upon arrival of new nutrients, but they have a shorter lifespan under prolonged starvation. We find that phenotypic heterogeneities linked to cell nutritional state bias the representation of cells in the aggregating vs non-aggregating fractions. Next, we report that the fraction of non-aggregating cells depends on genetic factors that regulate the timing of starvation, signal sensing efficiency and aggregation efficiency. In addition, interactions between clones in mixtures of non-isogenic cells affect the partitioning of each clone into both fractions. We further test in a model the evolutionary significance of the non-aggregating cell fraction. The partitioning of cells into aggregating and non-aggregating fractions is optimal in fluctuating environments with an unpredictable duration of starvation periods.

INTRODUCTION

Every organism has a set of optimal conditions that maximizes its growth and survival. Yet, its living environment constantly deviates from these conditions. In some cases individuals can adapt to changes by sensing the environment and modifying their phenotypes accordingly, which is known as phenotypic plasticity (Stearns 1989). However, if the sensing mechanism is too costly, phenotypic plasticity may not be optimal even in the presence of environmental variation. Differentiation on a stochastic basis into different phenotypic states adapted to different environments, also known as risk spreading or bet-hedging, has also been proposed as an adaptation to environmental variation (Kussell et al. 2005; Acar et al. 2008; Rivoire & Leibler 2010). Dormant states have often been described as such bet-hedging strategies. Examples span from plant seed dormancy (Simons 2009), arthropod diapauses (Hopper 1999) to bacterial sporulation (Veening et al. 2008). For entering and exiting the dormant state, cells or organisms depend on environmental cues. Yet, these cues are not always reliable indicators of the future environment. Therefore, in such unpredictable environments it pays off for a plant to have its seeds germinating stochastically at different time scales to insure that at least some of them will germinate at the time that is beneficial for its growth (Cohen 1966).

Here we focus on the dormancy of the cellular slime mold *Dictyostelium discoideum* as an adaptation to nutritional stress. *D. discoideum* amoebae live in soil where they feed on bacteria and divide mitotically. When starved, cells enter into the dormant social phase of the life cycle. Up to 10^6 cells aggregate to form a multicellular organism that goes through a slug stage followed by the formation of a fruiting body. The slug is a motile, chemotactic and phototactic worm-like structure that senses and moves towards environments that are favorable for dispersion, germination and cell proliferation. The fruiting body is a sessile mushroom-like structure with the spore mass sitting on top of a stalk. Dormant spores can survive for months in the absence of food, and germinate into single cells upon dispersion towards nutritive areas. The stalk lifts the spores from the ground, which helps spore dispersion. Cells in the stalk, which represent ~20% of the total cell population, die owing to the metabolic cost of making up the stalk (Kessin 2001). Its social behavior has made *D. discoideum* a very popular system for studying altruism, cheating and cooperation (Kaushik & Nanjundiah 2003; Strassmann & Queller 2011), but not all aspects of its population-level adaptation to stress have been studied. Our main motivation was to study a previously known but neglected fact that not all cells aggregate upon starvation. We have thus revisited the *D. discoideum* population-level response to nutritional stress by focusing on the aggregation stage. Incomplete aggregation would have strong evolutionary significance. Aggregation is costly due to the death of stalk forming cells and the irreversible arrest of cell division until the end of development (Katoh et al. 2007). Cells that do not aggregate do not pay these costs and may have the advantage of resuming growth immediately upon arrival of new nutrients.

If conditions improve quickly, non-aggregating cells thus may have an important advantage and thus constitute an adaptive response. While often considered as an experimental error or insignificant, we asked whether the fraction of non-aggregating cells constitutes an important component of the population-level response.

In this study we present the first attempt to describe the *D. discoideum* response to starvation stress as a population partitioning into two states: aggregating and non-aggregating. We do this by focusing on two major points: (i) mechanistic (phenotypic and genotypic) sources of population partitioning and (ii) its fitness benefits and evolutionary adaptation. In microbial systems it has been shown that cell states such as cell cycle phase, nutritional state or age are sources of phenotypic heterogeneities (Avery 2006; Veening et al. 2008). On the other hand, different genetic backgrounds could give rise to different rates of heterogeneity, giving insights into the underlying molecular mechanisms. Here we develop a new quantitative live cell microscopy technique to analyze the effects of cell nutritional state, genetic background and environmental organization on population partitioning between aggregating and non-aggregating cells. In addition, we propose a model based on experimentally determined parameters to illustrate potential evolutionary benefits of population partitioning in fluctuating environments.

RESULTS

Not all cells aggregate

When we plated a population of genetically identical axenic cells of *D. discoideum* on nutrient-free substrates at 3 x 10^4 – 10^6 cells/cm^2 density range (Hashimoto et al. 1975), we observed that some cells aggregate while others remain outside of aggregates (Figure 3-1). A possible explanation is that the cells that did not aggregate are simply dead cells. Movie S1 shows that non-aggregating cells are actively moving, alive cells that are intermixed with aggregating cells at the onset of starvation, thus ruling out this possibility. It could also be that these cells have acquired mutation that makes them unable to aggregate. We rule out this possibility by showing that a population generated from germinating spore cells that have passed through just 3-5 divisions reproduces the same population partitioning (Figure 3-7 D). Another explanation may be that partial aggregation is an artifact of laboratory adapted axenic strain that is not found in natural isolates. In Sup. Figure S3-1 we show that the same population partitioning is found in natural isolates. Population partitioning into aggregating and non-aggregating cells is therefore a process occurring in both axenic strains and wild isolates of social amoebae. The non-aggregating cells we report here are clearly distinct from cells left in slug traces (Kuzdzal-Fick et al. 2006) since the former are never aggregating as we have shown in Movie S1. For the same reason, non-aggregating cells are also clearly distinct from the

immune-like cells identified in a previous study (Chen et al. 2007). The motility of the non-aggregating single cells we observe also rules out the possibility that these cells are sporulation without aggregating, as in single cell encystation that has been reported for other *Dictyostelium* species but not so far in *D. discoideum* (Kessin 2001).

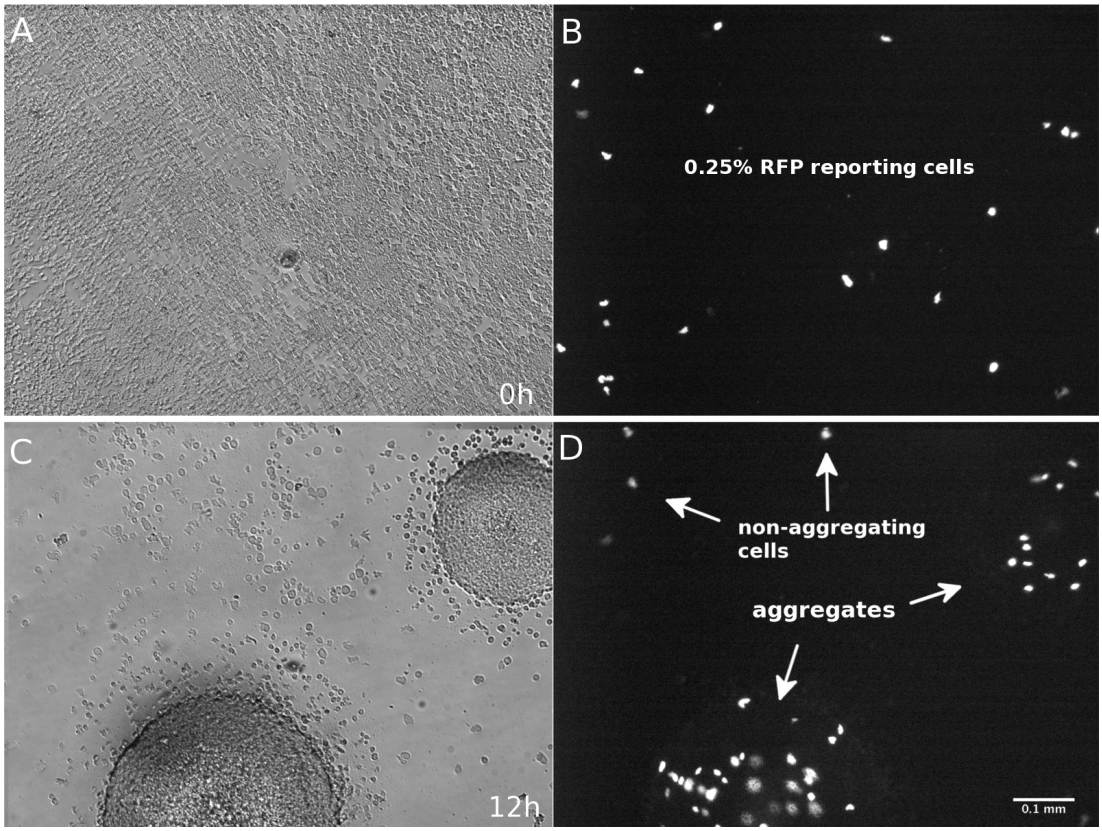


Figure 3 - 1 Upon starvation, a *D. discoideum* population partitions into aggregating and non-aggregating cells. AX3 cells were plated on nutrient free-agar and imaged before (A, B) and after (C, D) aggregation. B and D are fluorescent images with 0.25% of AX3 RFP cells (single dots) within a population of AX3 GFP cells. The percentage of non-aggregating cells was estimated as the ratio of dots counted outside aggregates after aggregation and dots counted before aggregation.

New microscopy technique for quantifying non-aggregating cells

To analyze quantitatively this process, we have developed a technique to track single cell behavior at each time point of the life cycle. Inspired from studies of cell motion within aggregates (Dormann et al. 1997), a small proportion (0.25%-2%) of RFP-expressing reporting cells was mixed with GFP-expressing cells, and RFP cells were tracked (see Chapter 2 Materials and Methods:). In the fluorescence image single RFP cells appear as single red dots surrounded by undistinguishable GFP cells (Figure 3-1B). Since cell division ceases during starvation, tracking RFP-expressing single cells allowed us to determine the relative numbers of aggregating vs non-aggregating cells, and thus

quantitatively describes the population partitioning into aggregating and non-aggregating cells. Previous techniques based on counting cells at the onset of starvation and germinating/colony-forming spores provide only indirect estimation of the numbers of stalk cells, non-aggregating cells, or non-germinating spores. In contrast, our strategy provides a direct estimation of the numbers of cells at the onset of starvation and aggregating vs non-aggregating cells. Our automated microscopy setup is similar to the one used in a previous study (Houchmandzadeh 2008). We scan and image an area of 5 cm^2 every 10 min for 24 h, allowing us to record the dynamics of the response of large populations (millions of cells) at the single cell resolution.

Phenotypic plasticity affects population partitioning

Onset of starvation and population heterogeneities

When cells of the AX3 wild-type axenic strain are grown in liquid rich medium (HL5) and subsequently plated on nutrient-free substrate, 2.51±0.6% of the population does not aggregate. This standard starvation protocol consists in the sudden transition from exponential growth in rich medium to starvation on nutrient-free agar. However, in natural conditions starvation is probably a much more gradual process of food depletion. We analyzed how different starvation processes can affect population partitioning (Figure 3-2A) at the same cell density range at the onset of starvation. We compared suddenly starved exponentially growing cells, starved stationary phase cells (1-2 days after confluency), and cells grown on bacterial plates that slowly deplete the food source, the latter being the most realistic starvation process with respect to natural conditions. While stationary phase cells show no significant difference compared to exponentially growing cells, cells feeding on bacteria and thus gradually starving showed a 3-fold increase in the proportion of non-aggregating cells, 6.3±3.17% ($p = 0.027$) in the case of a homogenous bacterial lawn as food source. Gradual starvation on bacterial plates most likely increases heterogeneities in comparison with standard starvation protocols. We supposed that this was due to cell-to-cell differences in the timing of starvation. Some cells would start aggregating while others are not yet fully starved, therefore less/not sensitive to the aggregation signal. Increasing further heterogeneities during cell plating should thus increase further the non-aggregating cell fraction. In the case of a heterogeneous bacterial lawn as food source, the fraction of non-aggregating cells increases to 13%±1.79% ($p =0.004$). A possible explanation is that highly heterogeneous cell plating creates areas with different cell densities within a lawn of bacteria (Sup. Figure S3-2C, D). Areas with high cell densities deplete bacteria faster and start starving and aggregating quicker, while cells in low cell density areas still have nutrients surrounding them and they are not sensitive to aggregation signal when the former sense starvation. In homogenous bacterial lawns, cells and bacteria are evenly distributed favoring more homogenous and synchronous onset of starvation across the population (Sup. Figure S3-2). We hypothesize that differences at the onset of starvation result in a cell fate bias towards one phenotype or the other (as previously proposed in

the case of stalk vs spore differentiation in aggregates (Nanjundiah & Bhogle 1995). To analyze these effects in the most reproducible and controllable manner, all following experiments were performed following the standard sudden starvation protocol (plating on nutrient-free agar) applied to cells grown in various well-defined conditions, with known genetic backgrounds, mixed at precise ratios and plated at controlled cell densities.

Nutritional effects

Nutritional state affects whether a cell will becomes a spore or a stalk (Leach et al. 1973). Cells grown on rich medium (NS medium with 85mM glucose) are enriched in spores while cells grown in poorer medium (NS medium lacking glucose) are enriched in the stalk (which we have also observed, see Sup. Figure S3-4). We thus asked whether nutritional state is a main determinant of the aggregating vs non-aggregating cell fates. We grew AX3 cells in media differing in nutrient content and analyzed whether they are differentially enriched in the non-aggregating state (Figure 3-2B). Four different media were tested: HL5 rich medium, FM minimal medium, NS with 85mM glucose (NS Glu) and NS medium. AX3 cells grown on FM minimal medium showed a significant two-fold increase in the fraction of non-aggregating cells, 5.85±1.9% ($p<0.01$), with respect to HL5-grown cells (2.51±0.6%). In addition, cells grown on NS Glu medium showed a small but significant decrease in non-aggregating cells (1.47±0.31%, $p<0.01$) compared to HL5 grown cells (2.51±0.6%). However, we observed that cells grown in NS medium did not differ from cells grown in NS with glucose in terms of non-aggregating cell fraction, making the role of glucose difficult to interpret.

Nutritional effects and cell interactions

Cells in different nutritional states have different aggregation rates on their own. We next examined how cells in different nutritional states interact in mixtures in order to analyze how introducing population nutritional state heterogeneity affects population partitioning. Pairwise mixtures of FM-grown cells with HL5-grown cells and NS-grown cells with NS Glu-grown cells were tested. Cells grown in NS or NS Glu that did not differ when alone showed no difference in behavior when in mixtures (Figure 3-2C) (one way ANOVA $F=1.54$, $p=0.27$). On the other hand cells grown in FM were enriched 3 times more in the non-aggregating cell fraction when in mixture with HL5-grown cells, 15.4±7.12%, than on their own, 5.85% (Figure 3-2D). HL5-grown cells did not change their behavior when in mixture with FM-grown cells. As a control we monitored contribution to spores for both mixtures. As previously shown, cells grown in rich medium were enriched in spores in both NS Glu:NS and HL5:FM mixtures (Sup. Figure S3-4).

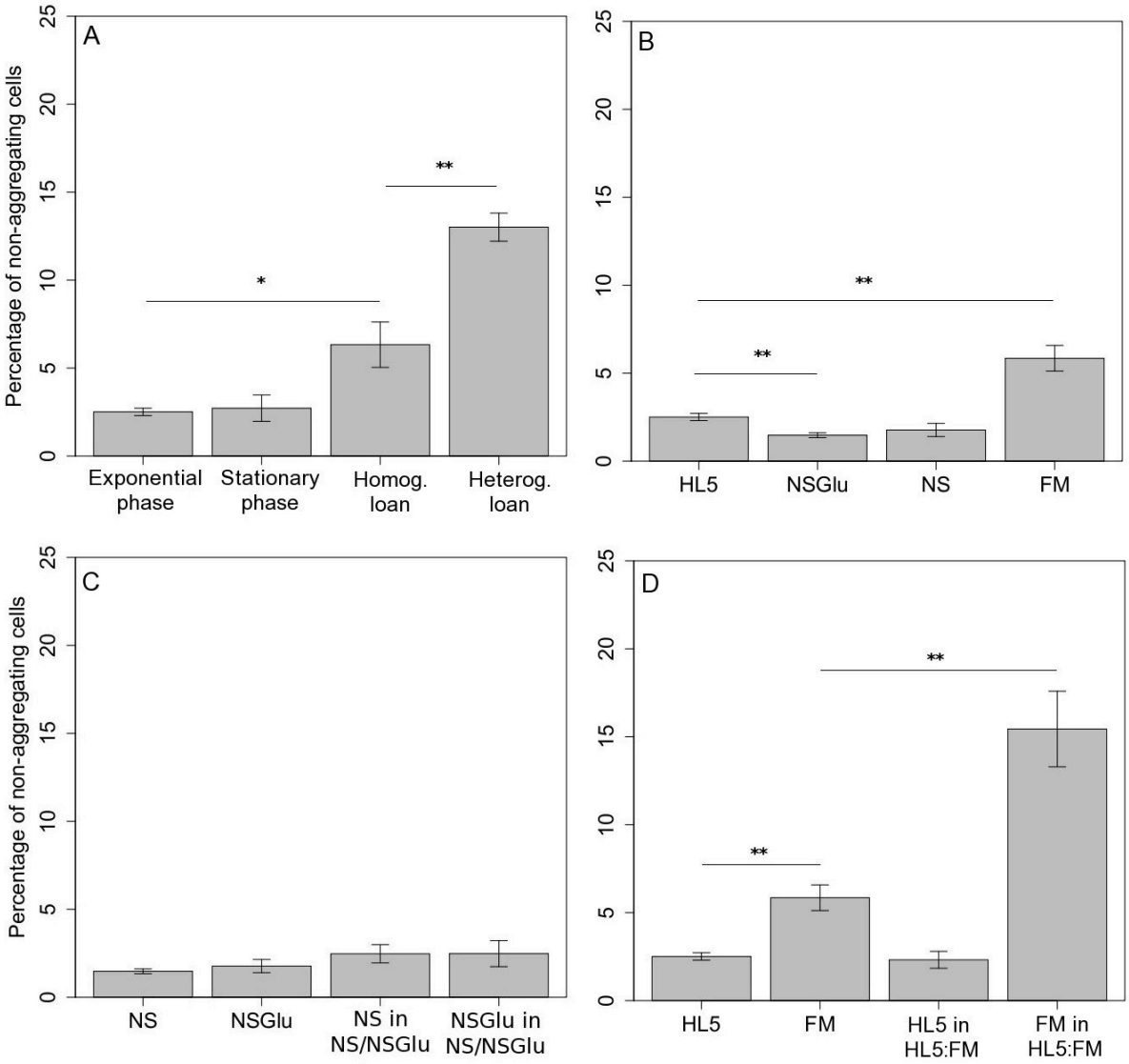


Figure 3 - 2 Starvation conditions, nutritional state and population partitioning. The percentage of non-aggregated cells (at initial density $3x10^6$ cells/cm^2) was measured for different cell states. **A) Effect of starvation conditions**. AX3 RFP and GFP cells were starved suddenly at exponential phase or at stationary phase, or gradually on homogenous bacterial lawns or on heterogeneous bacterial lawns. Gradually starved cells aggregate less than cells submitted to standard but less realistic sudden starvation protocols. **B) Effect of nutritional state.** AX3 cells were grown on HL5 rich medium, FM minimal medium, NS with 85mM Glucose (NSGlu) or NS medium, and subsequently plated on nutrient-free agar. Cells in the lowest nutritional state (FM) aggregate significantly less than cells fed with rich medium. **Interactions between cells in different nutritional states.** AX3 cells grown on: C) NS or NSGlu and D) HL5 or FM were plated either on their own or in 1:1 mixtures on nutrient-free agar. For example, HL5inFM = HL5 cells monitored in 1:1 mixtures, and FMinHL5 = FM cells monitored in 1:1 mixtures. In mixtures with HL5-grown cells, FM-grown cells aggregate even less than on their own, while HL5-grown cells aggregate equally in the presence of FM-grown cells as on their own.

We conclude that nutritional state distinguishes non-aggregating cells from aggregating cells, and that interactions between cells according to their nutritional state biases further partitioning between aggregating and non-aggregation cell fates. Cells grown on low nutrient medium have higher chances of becoming non-aggregated cells than cells grown on rich medium. The fact that NS-grown cells displayed the same behavior as NS Glu-grown and HL5-grown cells is probably because cells were relatively well fed in all three cases and not much affected by the absence of glucose (Garrod & Ashworth 1972). On the other hand FM-grown cells showed smaller cell size, slower growth and lower inner cell density indicating that they were affected by growth in poor medium (our unpublished observation). We can speculate that poorly fed FM-grown cells have low energy reserves, and that they consequently invest less into energetically costly multicellular development and thus aggregate less. The fact that, in mixture with HL5-grown cells, FM-grown cells showed an even lower rate of aggregation indicates the effect of cell-cell interactions during aggregation. No difference in the timing of aggregation was seen between FM- and HL5-grown cells. Therefore, cell nutritional state rather than aggregation timing was the cause of the differences in the fraction of non-aggregating cells.

Genetics of population partitioning

After exploring nutritional state effects, we tested whether different genetic backgrounds can lead to different population partitioning. In Figure 3-3 we show that two axenic strains, DH1 and AX3, significantly differ in the fraction of non-aggregating cells (t-test, $p=0.0008$). The DH1 strain showed 13.4% (±2.8%) of non-aggregating cells, which is five times higher than for the AX3 strain (2.5%±0.6%). This shows that the non-aggregating cell fraction depends on the genetic background and varies significantly between axenic wild-type strains.

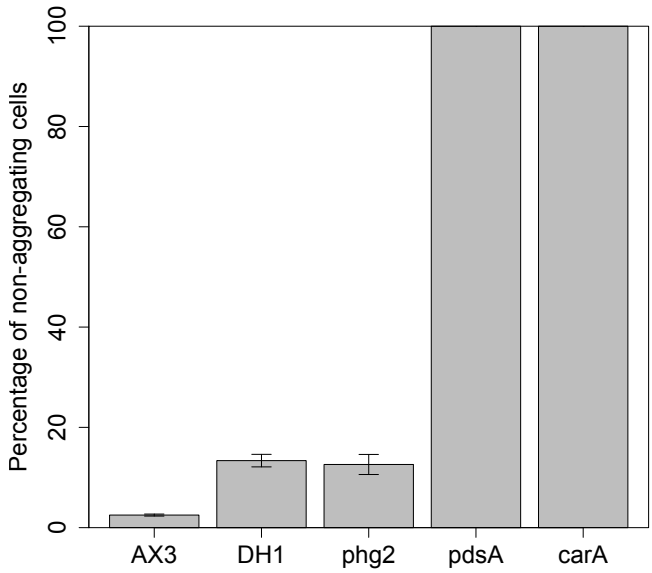
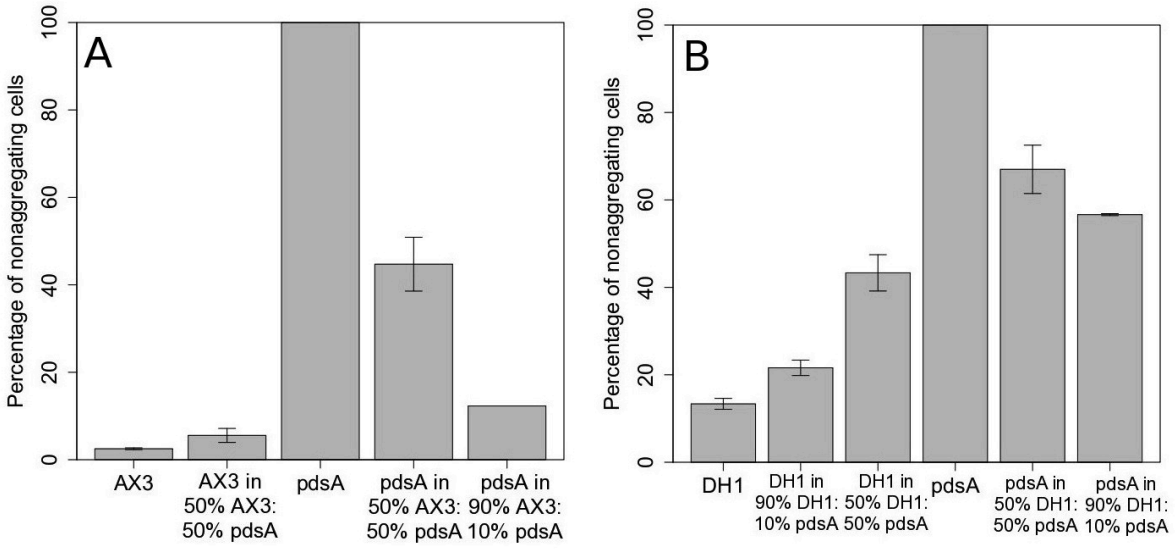


Figure 3 - 3 Genetic effects on population partitioning. The percentage of non-aggregated cells (at density $3x10^6$ cells/cm^2) was measured for genetically different wild-type strains (AX3 and DH1) and single-gene mutants (*phg2*, *pdsA*, *carA*) alone. Overall, cell genotype determines the fraction of aggregating cells

Genetic effects linked to cAMP-phosphodiesterase production

Following these results, we explored which genetic mechanisms may be involved in population partitioning. For this, we tested strains with single gene mutations in aggregation pathways. We used two mutants defective in signal sensing: 1) *carA,* a mutant in cAMP receptor protein cAR1, which is essential for binding of cAMP molecule responsible for aggregation, and 2) *pdsA*, a mutant in cAMP-phosphodiesterase (PDE), which removes cAMP from its cAR1 receptor making it again sensitive to the aggregation signal (Kessin 2001). As previously reported, when plated on nutrient-free agar, both strains showed no aggregation at all (Figure 3-3) (Caterinas et al. 1994; Sucgang et al. 1997). This showed that single gene mutations have a drastic effect on population partitioning. It is known that the presence of wild-type cells in *pdsA* mutant cell aggregates can rescue the non-aggregating *pdsA* phenotype (non-cell autonomous). Wild-type cells produce cAMP-phosphodiesterase, which gets secreted in the environment or stays bound to the cell membrane (Malchow et al. 1972; Lacombe et al. 1986). Mutant *pdsA* cells can use the secreted phosphodiesterase and become again excitable by the aggregation signal (Sucgang et al. 1997). We thus varied the ratio of wild-type cells (AX3 or DH1) in mixtures with mutant *pdsA* cells from 10% to 90% and observed how it affects aggregation of *pdsA* mutant and wild type strains. For both DH1:*pdsA* and AX3:pdsA mixtures, increasing the ratio of wild type cells decreased the proportion of *pdsA* non-aggregating cells (Figure 3-4), as expected. “Sharing” of cAMP-

PDE came at a cost for DH1 strain; the fraction of non-aggregating cells for DH1 increased in mixtures with *pdsA* (Figure 3-4B). The fact that cAMP-PDE "sharing" had less effects on AX3 aggregation and caused better aggregation of *pdsA* strain (Figure 3-4A) suggests that AX3 secretes more PDE protein than DH1. More generally, we propose that expression levels of cAMP-phosphodiesterase may tune the non-aggregated cell fraction. Low concentration of cAMP-phosphodiesterase increases the fraction of non-aggregating cells while increasing the concentration makes cells sense the cAMP signal better and leads to increased aggregation.



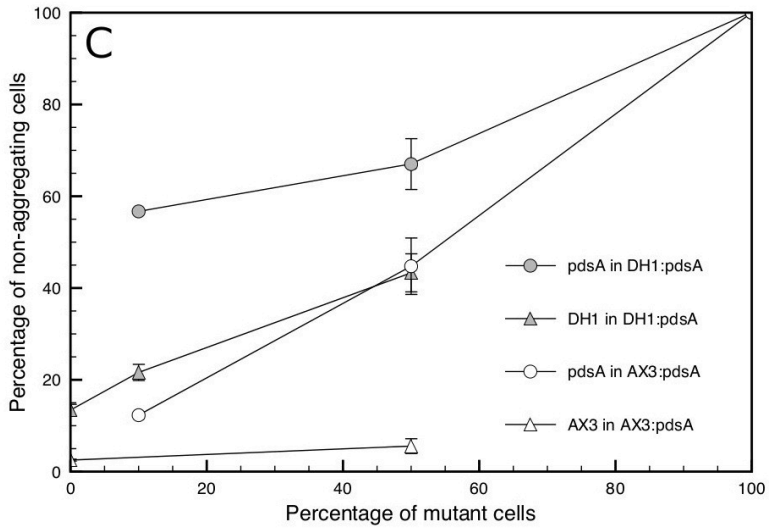


Figure 3 - 4 Effect of cAMP-phospohodiesterase on population partitioning. The percentage of non-aggregated cells (at density $3x10^6$ cells/cm^2) was measured for genetically different wild-type strains (AX3 and DH1) and cAMP-phosphodiesterase mutant *pdsA*. Measurements were done for each clone alone and in mixtures: A) AX3 : *pdsA* and B) DH1 : *pdsA*. Fraction of *pdsA* cells in the mixtures was varied form 10%, 50% and 90%. Since pdsA mutant cannot produce cAMP-phoshodiesterase, by varying *pdsA* cell fraction we indirectly varied the concentration of extracellular cAMP-phosphodiesterase enzyme.

Genetic effects of phg2 mutant

We found that differences in starvation sensing affect the partitioning between aggregating and non-aggregating fractions (Figure 3-2A). The *phg2* mutant strain has been shown to have early onset of starvation compared to its parental strain DH1 due to a higher nutrient starvation sensing threshold (Cherix et al. 2006). We used this single gene mutant to test the effect of the nutrition starvation-sensing threshold on partitioning. In addition the *phg2* gene codes for a serine/threonine kinase regulating cell substrate adhesion, actin cytoskeleton organization and motility (Gebbie et al. 2004). When tested alone *phg2* produced a similar fraction of non-aggregated cells when compared to its parental strain DH1, 12.6%±4.3% (t-test p=0.7), that is five-fold higher than that of AX3 (Figure 3-5). We further tested the behavior of *phg2* in mixtures with wild-type strains DH1 and AX3 (1:1 mixtures of AX3:*phg2* and DH1:*phg2*). Mixing at 1:1 had antagonistic effect and led to an increase of the non-aggregating cell fraction for *phg2* and its DH1 parent (Figure 3-5B). In the case of AX3:*phg2* 1:1 mixtures, *phg2* aggregated also less than when on its own, while AX3 aggregated equally well as when on its own (Figure 3-5A). We again demonstrate that in mixtures, strains mutually affect each other's behavior. The *phg2* mutant aggregates less in mixtures with wild-type cells than on its own, even in 1:1 mixtures with its parent wild-type strain that has a similar aggregation fraction on its own. Differences in starvation sensing and/or dysfunctional cytoskeleton organization and motility could explain the lower propensity of *phg2* cells for aggregation. The reduced aggregation of phg2 reduces the aggregation fraction of its parent DH1 but not that of AX3, showing like in the case of *pdsA* described above that DH1 is more affected than AX3 by interactions with mutant cells in mixtures. No significant difference in aggregation timing was seen between *phg2* and DH1 or AX3 strain.

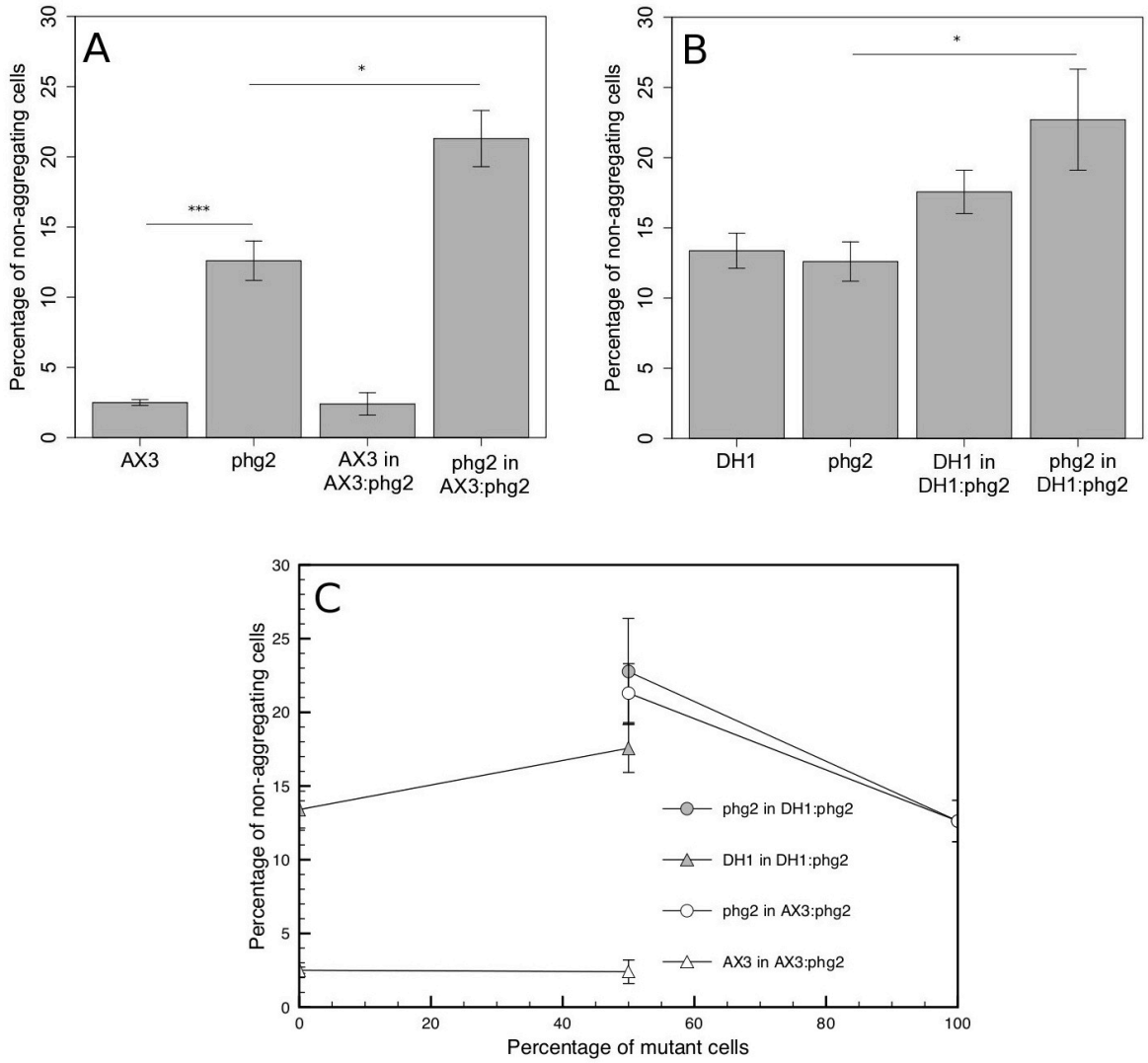


Figure 3 - 5 Genetic effects of *phg2* mutant. The percentage of non-aggregated cells (at density $3x10^6$ cells/cm^2) was measured for genetically different wild-type strains (AX3 and DH1) and *phg2* mutant. Measurements were done for each clone alone and in 1:1 mixtures: A) AX3 : *phg2* and B) DH1 : *phg2*.

Individual-level costs and benefits of the non-aggregating cell fraction

We have shown that upon starvation *D. discoideum* cell populations partition into cells that aggregate and cells that do not aggregate, and that non-genetic and genetic cell characteristics affect cell fates. We next analyze evolutionary consequences of this population partitioning. To do this we examined fitness costs and benefits of each phenotype on individual and population level.

Non-aggregating cells take advantage of early nutrient arrival

Once in an aggregate a cell is irreversibly committed to the multicellular development program (Katoh et al. 2007). During the 24h of development, cells cannot divide even if nutrients become available. Therefore, if food becomes available during the developmental period, non-aggregating cells may have an advantage over aggregating cells by immediately resuming growth. We tested whether non-aggregating cells are indeed capable of resuming growth upon arrival of nutrients. A bacterial suspension was added to a starving *D. discoideum* population during the course of development. At this point aggregates were at the slug stage and non-aggregating cells in surrounding environment had direct access to food (Figure 3-6A). In Figure 4B and Movie S3-2 we show that non-aggregating cells are capable of resuming cell division directly after arrival of nutrients, while slugs (formed of non-dividing aggregated cells) continue moving through the bacterial lawn and form fruiting bodies. We can also see that by the time fruiting bodies are formed, non-aggregating cells have already consumed high amount of nutrients, which will probably affect spore fitness by limiting the resources available for spore germination and proliferation (Movie S3-2).

Mortality of non-aggregating cells

Non-aggregating cells are motile and do not seem to enter a dormant state like spores do, making them likely to be much less fit than spores during prolonged starvation. We therefore determined the lifetime of individual AX3 cells in the absence of food source (on agar plates). Cell movement was used as an indicator of cell viability, with cells that did not change their position after 30 min considered as dead. In Figure 3-6C we show that the cell mortality curve can roughly be divided into 2 parts, with a low mortality period during the first 140-160 hours, followed by a high mortality period. Similar starvation induced mortality curves and long term cell survival have been shown by others for different wild-type and mutant strains (Otto et al. 2004). They demonstrated that, in the absence of food, cells survive through autophagy, degrading their own cytoplasmic components and organelles. It is thus likely that the relatively low cell mortality rate during first 140h-160h in our experiment is due to cell autophagy. The subsequent increase in mortality suggests that cells have degraded most of the inner cell components and autophagy can no longer serve as a mode of survival.

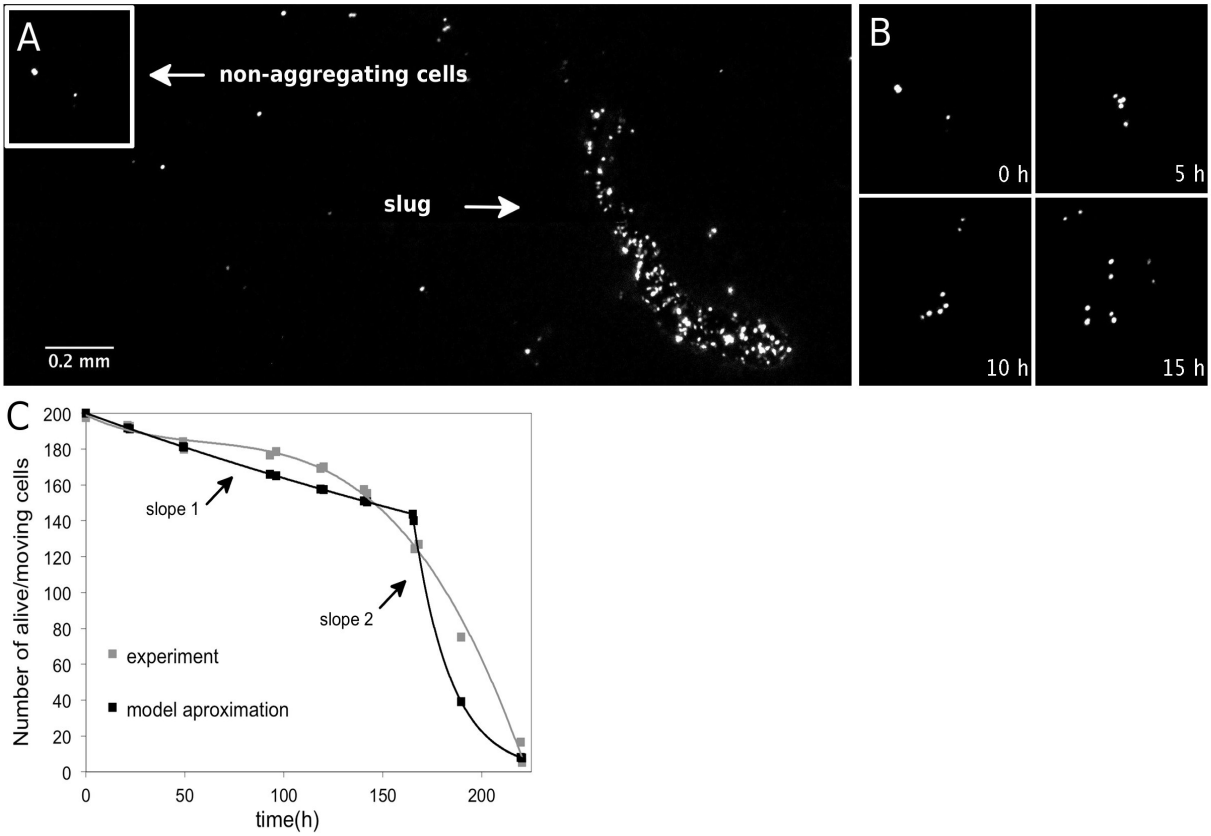


Figure 3 - 6 Non-aggregating cell growth on new incoming nutrients, and survival upon starvation. 18h after plating cells on nutrient-free agar aggregating cells have formed slugs while non-aggregating cells are starving. Nutrition in form of dead bacterial culture was added at this point. A) Fluorescent image of slugs and non-aggregating cells at the time of new nutrient supply. B) Inset from A showing a non-aggregating cell that resumes dividing upon addition of new nutrients, while aggregating cells are embedded in development. **C) Mortality of non-aggregating cells during starvation.** AX3 cells were washed in MCPB buffer and plated on nutrient-free agar. Cell movement was used as an indicator of cell viability. Experimental data are plotted as grey dots, showing an initial slow mortality until 165h followed by a higher mortality rate after 165h. The black curve is a fit based on two successive exponential decays (before and after 165h) used to model mortality in our simulations.

Cell history and cell fate

We further ask how deterministic is this population partitioning. In other words, can the same population partitioning be reproduced by starting from just aggregating or just non-aggregating cells? This is important for: i) ruling out the genetic differences between aggregating and non-aggregating cells and ii) showing the effect of epigenetic inheritance of cell fate. In Figure 3-7A-C and Movie S3-3 we show that when non-aggregating cells are de novo fed on bacteria they are capable of aggregating and developing into a fruiting body. This shows that aggregating cells are not mutant cells that cannot aggregate. It is possible that some cells in the non-aggregating cell area come

from cells left in slug traces. It known that these cells are capable of consuming the bacteria and dividing. We assume that cells from slug traces represent minority of cells in non-aggregating cell area and can not be responsible for aggregation observed. Further on in Figure 3-7D we show that a population generated from germinated spore cells (that has divided only 3-5 times) when plated on nutrient free agar, repartitions into aggregating and non-aggregating cells. Population generated from just spore cells shows lower non-aggregating cell percentage. This indicated the possibility of cell fate memory or epigenetic inheritance which makes aggregating cell more prone on aggregating again. These are still very preliminary results and the experiment needs to be repeated to confirm the effect. Overall high percentage of non-aggregated cells (13%) is probably due to changes in experimental protocol. Before plating cells were grown on Hl5 medium with bacteria in order to facilitate spore germination. Although cells were washed of nutrients it is possible that low amount of bacteria were left among cells, this possibly created differences in cell starvation states and raised non-aggregating cell percentage (as shown in Figure 3-2A).

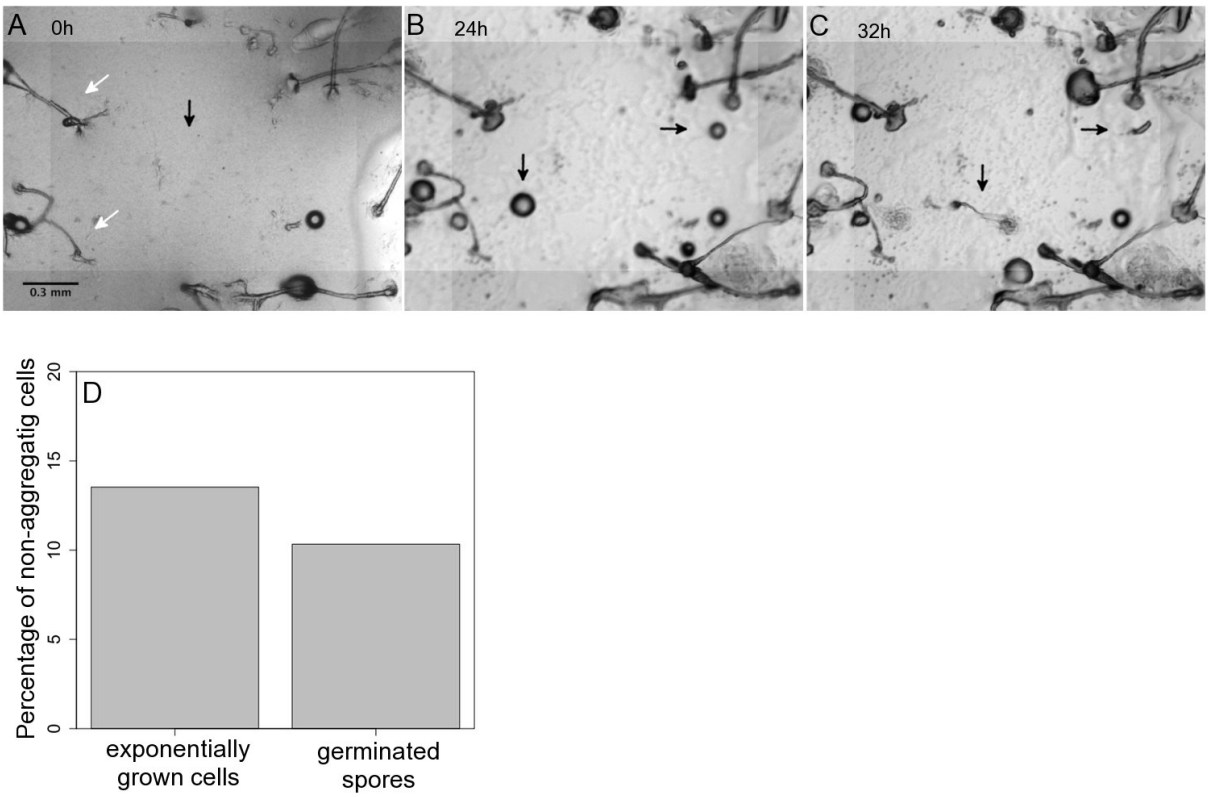


Figure 3 - 7 Population partitioning is the result of epigenetic and not genetic between cell differences. A-C Non-aggregating cells aggregate. A) After the aggregation had finished and fruiting bodies started to form (white arrows) bacteria were added. Black arrow points to the area with non-aggregating cells. B-C) Once bacteria are consumed non-aggregating cells form an aggregate that further on develops into a fruiting body (black arrow). **D) Population of germinated spore cells repartitions into aggregating and non-aggregating cells.** Percentage of non-aggregating cells when population of exponentialy growing cells or germinated spores is plated on nutrient-free agar.

Model: evolutionary framework

To test how phenotypic partitioning affects population fitness we developed a mathematical model that mimics the *D. discoideum* life cycle. We asked whether particular non-aggregation rates are selected in fluctuating environments having different, constant or variable, starvation duration and frequency. The model was defined as follows. Not all cells aggregate (Figure 3-1), cells that do not aggregate die according to the mortality curve from Figure 3-6C, non-aggregating cells are capable of resuming growth upon arrival of bacteria (Figure 3-6A and B, Movie S3-2), once in aggregates cells do not divide and are committed to multicellular development until the end (Katoh et al. 2007). All the parameters used in the model, such as growth rate, sporulation efficiency and germination efficiency were measured experimentally (see Suppl. Info.). Since aggregation is an adaptation to starvation and since the duration of starvation affects costs and benefits of each phenotype (mortality, growth), we tested how the duration of starvation determines the optimal non-aggregating rate. We competed 11 strains differing in their non-aggregating cell fractions. Investment into non-aggregating cells ranged from all cells aggregate (value 1) to none of the cells aggregate (value 0) and was fixed for each strain during the whole competition. Strains could not change their non-aggregation rate. For the sake of simplicity, we did not take into account interactions between strains that may increase or decrease aggregation rates, even though our own results demonstrated that such interactions do occur.

Simulation results

In Figure 5A and B we show that under constant starvation periods there are two stable strategies, no aggregation for starvation periods under 170h, and complete aggregation for longer starvation periods. The crossover occurs at 170h due to the increase in mortality rate after 165h (Figure 3-6C). Since natural environments are rarely so stable, with only long or only short starvation periods, we tested competition in environments with fluctuating, long (>170h) and short (<170h) starvation periods. We find that population partitioning into both aggregating and non-aggregating cells gives the highest (geometric) fitness benefits in these fluctuating conditions (Figure 3-8 C and D). The results also show that different fluctuations in starvation duration select for different non-aggregating rates. This is in agreement with other models and experiments that showed that optimal population response depends on the rate of environmental fluctuations (Kussell et al. 2005; Kussell & Leibler 2005; Acar et al. 2008).

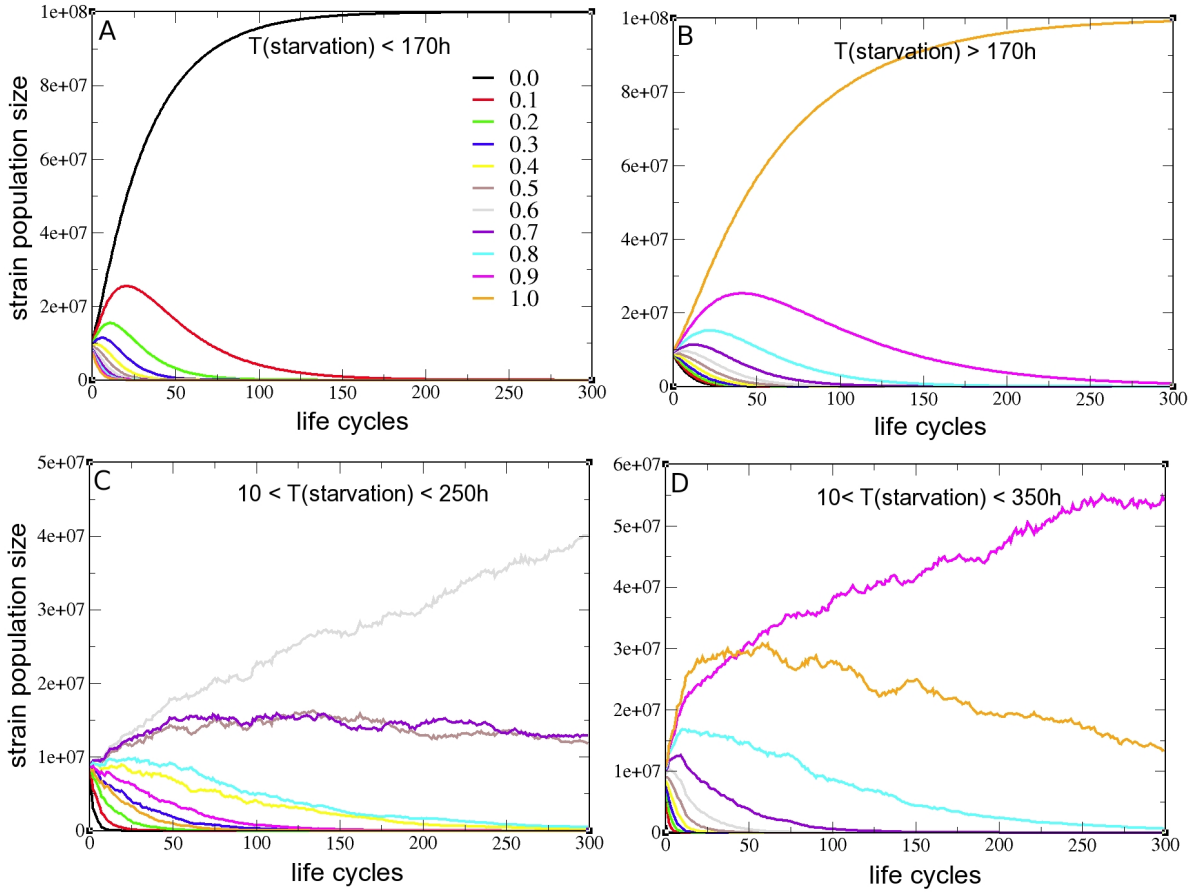


Figure 3 - 8 Population partitioning is advantageous in fluctuating environments. 11 strains with different fixed investments into non-aggregating cells were competed under different starvation conditions. Strain investment into non-aggregating cells varies from 0 to 1, with 1 corresponding to complete aggregation and 0to no aggregation. The duration of the starvation period was varied from <170h (A), >170h (B), randomly taken between 10h and 250h (C), randomly taken between 10h and 350h (D). For systematically long (>170h, B) and short (<170h, A) durations of starvation, strains with 100% aggregation and 0% aggregation take over respectively. For random starvation duration, a particular aggregation rate is selected, for instance 0.6 for 10h<T<250h (C) and 0.9 for 10h<T<350h (D), and thus the superimposition of both strategies is the optimal response.

DISCUSSION

Population partitioning into aggregating and non-aggregating cells

We report that upon starvation stress a population of *Dictyostelium discoideum* amoebae partitions into three major cell fates, and not just two: (i) non-aggregating cells; and aggregating cells that differentiate into (ii) dormant spores whose dispersion is promoted by (iii) dead stalk cells. We have measured the fraction of non-aggregating cells and found that it amounts to up to 15% of the total population in realistic starvation conditions. This is much higher than the 2-3% of non-aggregating cells that result in the standard sudden starvation protocols, and shows that it is important to mimic natural conditions. Non-aggregating cells are alive (Movie S1) and non-mutated cells (Figure 3-7D) that occur in both axenic strain and natural isolates (Figure S3-1). We have thus demonstrated that the non-aggregating cell fraction in natural starvation conditions constitutes a significant component of the population-level starvation response, at least of the order of the stalk cell subpopulation. For our detailed analysis of genetic and non-genetic contributions, we have nevertheless employed the standard sudden starvation protocol to ensure a full control over cell population composition and nutritional state, even though this protocol tends to minimize the non-aggregating cell fraction.

Phenotypic-Environmental effects

In isogenic populations, we show that partitioning depends on phenotypic heterogeneities linked to cell nutritional state (Figure 3-2). This is a previously reported determinant of the differentiation between spore and stalk cell fate in aggregates (Leach et al. 1973), together with intracellular Ca^{2+} levels (Azhar et al. 1996) and cell cycle phase (Gomer & Firtel 1987). Decreased aggregation in cells with low nutritional status correlates with lower investment into energetically costly aggregation. The nutritional state-dependent partitioning of the social amoebae population is reminiscent of previous studies reporting non-genetic population heterogeneities in *E. coli* persistor strains (Balaban et al. 2004), *P. fluorescens* colony morphology (Beaumont et al. 2009), *B. subtilis* sporulation (Veening et al. 2008) and many others.

Genetic effects

On the other hand, different genetic backgrounds can give rise to different levels of heterogeneity (Levy et al. 2012), giving insights into the underlying molecular mechanisms. We demonstrate that different wild type strains show different non-aggregating cell fractions (Figure 3-3). This has important implications when drawing a parallel with natural conditions. Distinct strains in nature may show different aggregation fractions leading to competition between different aggregation strategies, as we explore in our model in Figure 3-8. Further, our results on single-gene mutants underlie possible mechanistic differences between aggregated and non-aggregated cells.

We propose that genetic and phenotypic factors that regulate the timing of starvation (Figure 3-2A), cell nutritional state (Figure 3-2B and D), signal sensing efficiency (*cAR1* and *pdsA* mutants in Figure 3-3 and 3-4) and aggregation efficiency (cell motility and adhesion defects in *phg2* mutant Figure 3-5) determine whether a cell adopts the aggregating or non-aggregating phenotype. Differences in genes expression levels are a known source of phenotypic heterogeneities; *comK* in *B. subtilis* cell competence (Smits et al. 2005) , *spoA* in *B. subtilis* sporulation (Veening et al. 2008)**,** *S. cerevisiae* FLO-depended phenotype (Halme et al. 2004). It would be very interesting to monitor the same for early developmental genes, expressed at the beginning of aggregation, to see if distinct expression levels correlate with aggregating and non-aggregating cell states. Genes that control the efficiency of aggregation such as *cAR1* and *pdsA* are potential candidates.

Cell x cell interactions

Moreover, our results on interactions between mutant cells and wild type cells in mixtures show that partitioning of social amoebae populations is a complex process, and that competition between genotypes with different aggregation rates is non-linear. In other words, the behavior of strains in mixtures is not the mere linear superposition of their behaviors when taken separately on their own, which is reminiscent of the well-documented behavior of strains in mixtures during sporulation experiments (Kaushik et al. 2005; Buttery et al. 2009; Buttery et al. 2010).

Evolutionary consequences of population partitioning

Different phenotypes are often associated with different fitness cost and benefits. In our case, dormant spores survive for months without nutrients but take advantage of incoming food with a delay in comparison to non-aggregating cells. This lag corresponds to the duration of multicellular development and germination, i.e. up to 30 h, that is up to 8 times the single cell division time. Therefore, non-aggregating cells may divide up to 8 times in case nutrients are present soon after the beginning of multicellular development, while aggregating sporulating cells do not divide until the end of germination (Figure 3-6A and B). This confers a considerable evolutionary advantage to non-aggregating cells in such situations (2^8=256-fold). Our model explores the long term, evolutionary consequences of these effects on the competition between clones with different aggregation rates in fluctuating environments. We find that the aggregation rate is under selection in fluctuating environments and that the optimal rate depends on the fluctuations in starvation duration and frequency.

<u>Similarities with bet-hedging behaviors</u>

Strategies in which different phenotypes may show differential fitness advantages in different environments are often called bet-hedging, and have been shown to be adaptive in fluctuating environments (Kussell et al. 2005; Acar et al. 2008; Beaumont et al. 2009; Stearns 2000). In plants, the success of germination depends on rain precipitation. Since precipitations are unpredictable and variable, the diversification of

germination timings within season was predicted and demonstrated (Simons 2009). Similar examples include mosquito egg hatching (Khatchikian et al. 2010), copepods egg diapause (Hairston & Olds 1984), phenotypic switching in *S. cerevisiae* (Acar et al. 2008), persistor phenotype in *E. coli* (Kussell et al. 2005) and many others (Simons 2011). *B. subtilis* behavior resembles the most to what we report in *D. discoideum*. Upon starvation the population of *B. subtilis* partitions into sporulating and non-sporulating cells. Non-sporulating vegetative cells postpone their sporulation by consuming secondary metabolites and cannibalizing on each other, and have the advantage of immediate growth upon arrival of nutrients (González-Pastor et al. 2003; Veening et al. 2008). In *D. discoideum* aggregation is required for sporulation. Since sporulation is beneficial only if the duration of starvation is long enough (Figure 3-8), and since cells cannot *a priori* sense the duration of starvation, population diversification should be the optimal response. This is exactly what we get with our model in Figure 3-8. We therefore propose that partitioning between non-aggregating and aggregating cells is a form of bet-hedging in environments with unpredictable durations of starvation.

Consequences of population partitioning on cooperation

Cooperation in social amoeba and 3 cell fates

Consequently, our results have implications for cooperation studies using social amoebae as a model system. Studies on mixtures of non-isogenic cells show that some genetic clones bias their ratio into spores. Accordingly clones associated with phenotypes enriched into the spore mass were qualified as cheaters, and phenotypes underrepresented in the spores as altruists (Strassmann et al. 2000; Dao et al. 2000). Genetic clones, either wild isolates or single gene mutants isolated from genetic screens (Ennis et al. 2000; Santorelli et al. 2008; Santorelli et al. 2013), have been ranked in terms of "cheating" according to pairwise competition experiments between two genetic clones going through one round of sporulation. However, the behavior of a mixture of more than two clones going through a series of growth and sporulation cycles cannot be entirely explained based on this ranking (Saxer et al. 2010). The whole life cycle needs to be taken into account, as competition occurs between strains not only during sporulation within aggregates but also at other steps such as unicellular growth, with complex trade-offs (Nanjundiah & Sathe 2011). Here we characterize in this respect the aggregation step of the life cycle, and we show that the up to now neglected non-aggregating cell fraction constitutes a significant component of the population-level starvation response. This fraction is different for different genetic clones, it is at least of the order of the stalk cell subpopulation and interactions between clones do affect this fraction. Therefore, all three cell fates need to be taken into account when defining a clone's behavior when alone and in mixtures. We propose to decompose social amoebae behavior into social investment (aggregation vs non-aggregation) and altruistic investment (spore vs stalk in aggregates). Instead of classifying phenotypes as just altruistic and cheaters we will maybe find social cheaters (high aggregation efficiency

but low investment into stalk), asocial altruists (low aggregation efficiency and high investment into stalk), asocial cheaters (low aggregation efficiency and low investment in the stalk) etc.

Probabilistic cooperation

Population partitioning can also be interpreted as probabilistic expression of social behavior. Genetic and non-genetic mechanisms regulate the probability with which a cell acquires a social/aggregating phenotype. It has been shown that such probabilistic expressions of social phenotype may be strong anti-cheating strategies and play an important role in stabilizing cooperation (Hauert, Monte, et al. 2002; Garcia & De Monte 2013). The results presented here reinforce the notion that one should allow individuals to `opt out' of a social interaction to gain a more complete understanding, as has been argued for some time by game theoreticians (Batali & Kitcher 1995). For instance, allowing individuals to opt out of a social interaction may lead to evolutionary cycles (Hauert, De Monte, et al. 2002; Hauert et al. 2007). Our results show that environmental stochasticity, affecting relative fitness of social and asocial individuals may also favor opting out of at least a part of the population. It will be important to investigate further the role of population partitioning into aggregating/social and non-aggregating/asocial phenotypes on the stabilization of cooperation.

CONCLUSION

Using a new quantitative microscopy-based technique, we report that *Dictyostelium* social amoebae populations respond to starvation stress by partitioning into multicellular aggregates and unicellular, non-aggregating cells that have a head start in case of early nutrient arrival. Fraction of non-aggregating cells is determined by: genetic factors, environmental factors and cell-cell interactions. Based on our model simulation, we propose that population partitioning between multicellular/cooperative and unicellular responses to stress is optimal in fluctuating environments with variable starvation duration and frequency. *Dictyostelium* social amoebae thus possibly lies at the intersection of two key concepts in evolutionary microbiology, cooperation and bet-hedging, and define a unique model system to explore this new frontier.

PERSPECTIVES

Until know there was no scientific interest in cells that do not aggregate. Being the first to explore something is always great, but also leaves us with almost infinite number of unanswered questions, hypothesis and proofs. We are very conscious of all the weaknesses and lacking “proofs” and are doing further experiments as I am writing.

This work consits of two main axes: i) mechanistic one, which asks what genetic and environmental factors differ aggregating cells from non-aggregation, and ii) evolutionary one, which looks at adaptivness of population partitioning.

i) Our results suggest that factors such as starvation level, nutritional state, signal sensing ability and cell movement affect whether a cell will aggregate or not. *phg2* mutant that has lower starvation threshold to its parental strain DH1 did not show predicted increase in non-aggregating cells. Since different starvation thresholds were reported on NS/10 medium and we used non-nutrient medium, we need to test again non-aggregating cell fraction of both *phg2* and DH1 strain on NS/10 agar plates. We are also working with Bahram Houchmandzadeh from our lab in Grenoble on mathematical models of how different starvation levels could give rise to aggregating and non-aggregating cells.

Phenotypically, it would be interesting to look at the effect of intracellular levels of Ca^{2+} and cell cycle phase, that are know to affect spore to stalk cell fate, on aggregating to non-aggregating cell fate.

ii) In order to clearly demonstrate that population partitioning is a bet-hedging like strategy we need to show that aggregating cells when isolated and plated alone give rise to same fraction of aggregating vs non-aggregating cells. We are currently performing this experiment with spore cells that can easily be isolated. Unfortunately, the same is difficult to prove for non-aggregating cells, because it is difficult to imagine isolation of 20 μm cells in the forest of 0.5 cm fruiting bodies. Already the results using aggregating/spore cells to reinitiate population partitioning would give good evidence for potential bet-hedging like strategy.

Our mortality curve does not measure directly cell mortality. It would be good to have a more precise mortality curve by using cell viability markers. We have already tried using those but with low reporting signal. New stocks of cell viability kits should be used to retest the signal quality.

On the more long term, our model predictions need to be tested experimentally. Ideally two strains differing only in non-aggregating cell fraction (same growth rate, sporulation efficiency, germination efficiency) would be competed under varying periods of starvation. For the moment it is difficult to have strains differing only in non-aggregating cell fraction. Other possibility is to compete mutant strain with no aggregation and wilt type strain with small fraction of non-aggregating cells. By varying starvation periods we can test the success of one strain over the other. Interesting experiments have been performed on social bacteria *Myxococcus xanthus*, that has very similar life cycle to social amoebae. There non-aggregating/asocial strain has been evolved from aggregating/social after 1000 generations in nutrient rich liquid culture (Velicer et al. 1998). This showed that when food is constantly available loss of sociality is possible. It would be interesting to test this on social amoebae under constant nutrient or short starvation period conditions.

SUPPLEMENTARY FIGURES AND MOVIES

Supplementary Movies

Sup. Movie S3-1. Partitioning of *Dictyostelium* populations under starvation stress into aggregating vs non-aggregating cells. 0.25% or RFP-expressing AX3 cells mixed with 99.75% of GFP-expressing cells were plated according to the standard "sudden" starvation experimental protocol. After aggregates form, most single RFP-expressing cells are found in aggregates, and a minority of them are found outside of aggregates even though aggregating and non-aggregating cells were intermixed at the onset of starvation. Both phase contrast (left) and fluorescent (right) images show that non-aggregating cells are alive and motile. Counting single RFP-cells before aggregation and cells that are outside of aggregates after aggregation provides a direct estimate of aggregating and non-aggregating cell numbers.

Sup. Movie S3-2. Non-aggregating cells are capable of resuming growth immediately upon food arrival while aggregating cells are embedded in development. 18h after plating cells on nutrient-free agar, aggregating cells have formed slugs while non-aggregating cells are starving. Nutrition in form of dead bacteria was added at this point. Multicellular development goes on until the formation of fruiting bodies because cells in aggregates are irreversibly committed to development even in the presence of food. In contrast, non-aggregating cells feed on bacteria and divide several times. At the end of fruiting body formation, non-aggregating cells have already consumed most of the bacteria.

Sup. Movie S3-3. Non-aggregating cells are capable of aggregating. After the aggregation had finished and fruiting bodies started to form bacteria were added, T=0h. Once bacteria have been consumed non-aggregating cells aggregate and further on develop into a fruiting body.

Supplementary Figures

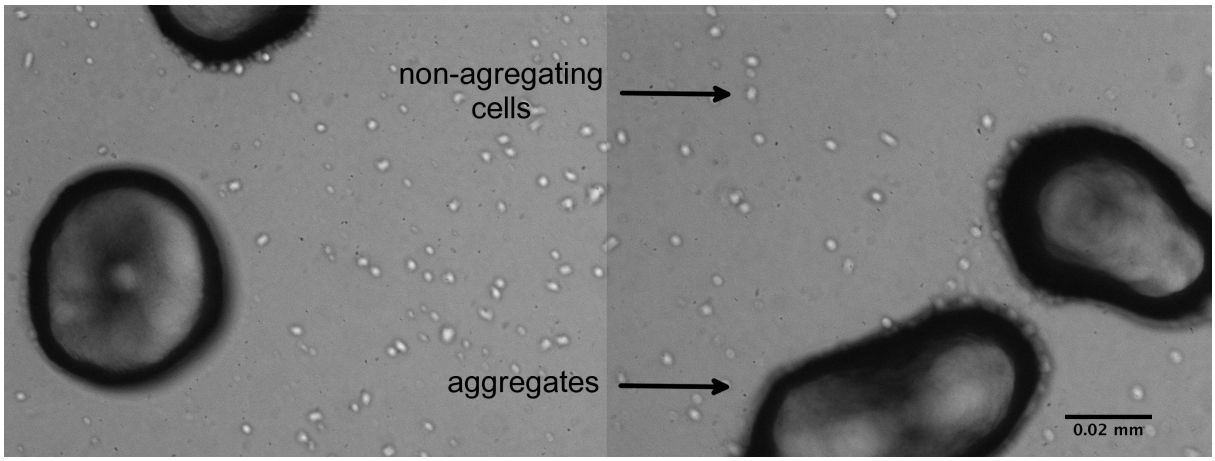


Figure S 3 - 1 Population partitioning in wild isolate population. Cells from wild isolate NC 28.1 (Francis & Eisenberg 1993) were grown on bacteria and plated on nutrient-free agar according to the standard "sudden" starvation protocol. Wild isolate populations partition into aggregating and non-aggregating cells as laboratory strains do.

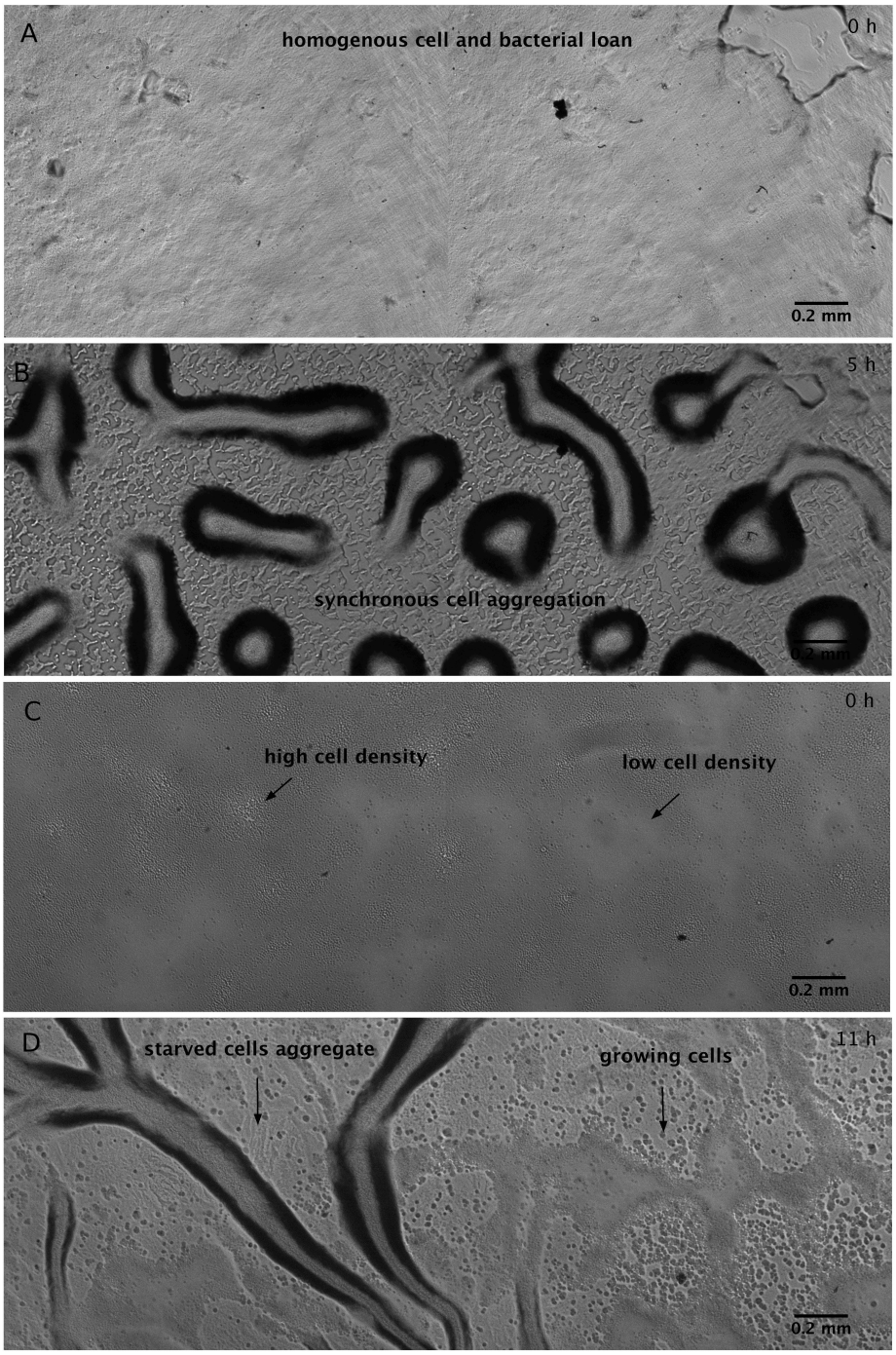


Figure S 3 - 2 Cell aggregation on homogenous and heterogeneous bacterial lawn.
AX3 cells were plated mixed with bacterial suspensions and plated on nutrient-free agar either homogenously (A) or heterogeneously (C). Homogenous plating yields a synchronous timing of starvation and aggregation over the whole plate (B). Heterogeneous plating yields a non-uniform timing of starvation and aggregation, with aggregates forming in some areas while cells are growing in other areas (D) of the same plate.

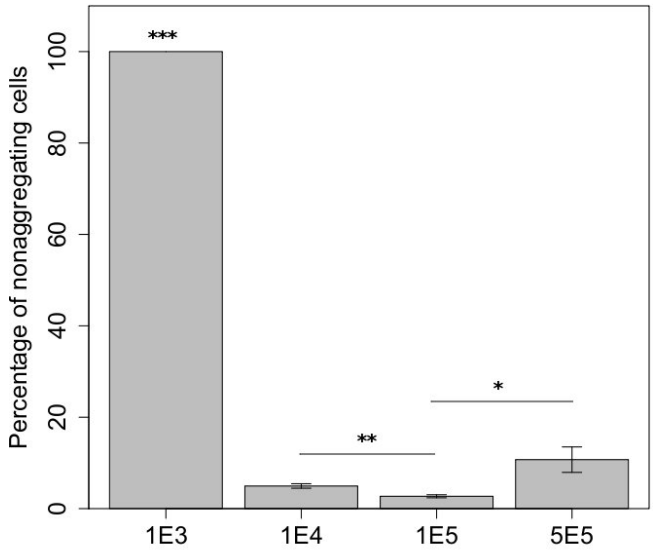


Figure S 3 - 4 Effect of cell density on non-aggregating cell fraction. AX3 cells were plated at densities, $1x10^3$, $1x10^4$, $1x10^5$ and $5x10^5$ cells/µl under sudden starvation protocol.

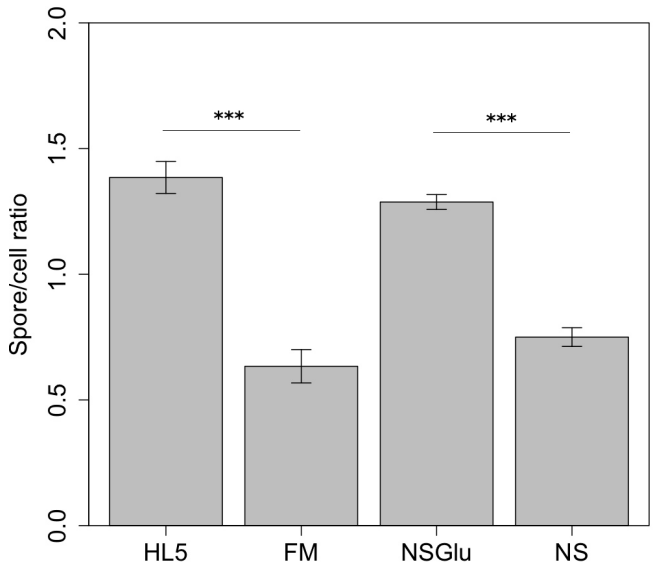


Figure S 3 - 3 Nutritional state of the cells biases spore/stalk differentiation in aggregates. RFP-expressing AX3 and GFP-expressing AX3 cells were grown on one of 4 different media: HL5 (rich medium), FM (minimal medium), NS Glu (rich medium with glucose) and NS (rich medium without glucose). Cells grown on these media were mixed at 1:1 ratio for HL5:FM and NSGlu:NS mixtures and plated at $1x10^5$ cell/µl concentration on filter papers. The ratio of spore percentage over plated cell percentage of each cell population in the 1:1 mixture indicates if one population is getting enriched into spores with respect to the other one. For spore/cells ratios = 1 both populations contribute equally to spores. If spore/cells ratio > 1, the population is enriched in spores, and if spore/cells ratio < 1, the population is underrepresented in the spores.

Acknowledgements

The *carA* and *pdsA* mutants were kindly provided by Kerry Ann Sheppard at the Dicty Stock Center of Northwestern University in Chicago, and the *phg2* mutant by Anna Marchetti and Pierre Cosson at the CMU in Geneva. We thank Vidyanand Nanjundiah, Santosh Sathe, Bahram Houchmandzadeh, Silvia de Monte, Sandrine Adiba, Madhu Priya and Natale Scaramozzino for many helpful comments during the course of this work. We are indebted to Shalmali Kamat during her internship at LIPhy Grenoble as well as Zak Frentz and Juliette BenArous at the Laboratory of Living Matter of Stanislas Leibler at Rockefeller University for their contributions during pilot experiments. We acknowledge Stanislas Leibler and all members of the Laboratory of Living Matter for many stimulating discussions and their invaluable support.

APPENDIX: For economists: Adaptation to uncertainty in biology and economy

As an AXA Research Fellow I was invited to present our work on social amoebae to a group of leading French economists and entrepreneurs. My task was to relate biological solutions to uncertainty and fluctuations to economical ones. This is a short text that resulted out of this talk at the AXA organized conference "Stress en entreprise". I had big fun doing this!

How does nature deal with stress and risk?
Or
How do bacteria play poker?

"*Q'y a-t-il de commun entre l'ecologie, le systeme economique, l'entreprise, la ville, l'organisme, la cellule? Rien si on les examine avec l'instrument habituel, l'approche analytique. Mais beaucoup, si on faire ressortir les grandes regles d'organisation et de regulation de tous ces systemes."*

Joel de Rosnay, Le Macroscope, 1975

So what principles do biology and economy share? The main principle that drives the both disciplines is the maximization of profit in economy or fitness in case of biological organisms. If one organism produces more offspring it will out-compete and maybe even extinct the other one. If one company has higher profit than the other one it will expand and take over the market leading to market monopolization. The difference is in terminology we use, not in processes that are happening. Thinking in these terms we can ask how do organisms like plants, animals and even bacteria deal with stressful situations and can we learn something out of it?

Very often stress and risk is caused by decision-making in imperfect environment. For example, investing into a startup without knowing how market will respond to it. How does this relate to organisms? What is a financial market to an economist an environment is to organisms. When a dessert plant seed needs to decide weather it will germinate or not it is making the same decision as an agriculturist that needs to choose the timing of wheat sowing or an economist deciding the timing for an investment. If the seed germinates but there is not enough rain in the future days the dessert plant seed will die, the agriculturist will have low wheat production and economist will have low investment return. So how to decide when to germinate knowing that environment is uncertain and you have limited amount of information about the future?

Human strategies are mainly based on prediction. We do not know how the market will look in one year, so we build complex mathematical models to predict it. But nature does not have big computers that predict future. That's why it has developed an array of

strategies that deal with the change and uncertainty. The basic principle behind all of them is « you fight change with change ». If you are a swan it means that you will migrate to the south when its starts being too cold. If you are a chameleon you will develop the ability to change colors. If you are a tree you will make sure than your seeds do not germinate at the same time. And if you are an immune systems you will produce around 10^6 different antibodies, hoping that a least one of them will work against the pathogen

Our work is focused on understanding stress management in amoebae *Dictyostelium discoideum*. These amoebae are unicellular organisms that live under the soil where they eat bacteria. When there is no food anymore, the famine period arrives. It can last 10h or 10days, the amoebas do not know. They have therefore developed 2 strategies to overcome these deadly periods. The social strategy that is successful if the famine is long and the non-social strategy that pays off if famine period is short. Since they do not know how long the famine will last, one part of the population plays one strategy and the other part the other one. What they are doing is no different that betting. They invest into both strategies and hope that one will work.

Environment is a constantly changing battlefield. To deal with these changes animals, plants, fungi and bacteria have developed strategies to deal with stress and risks. Some bet, some mutate, some cheat, some socialize. At the end is it really so different from what we do? Looking into other living systems enables us to discover other means of adaptation, their costs and benefits. And than maybe we can learn some things out of them. At the end they are the successful result of millions years long evolution.

Chapter 4 How Life Cycle Complexity Affects Genetic Diversity and Cooperation in Social Amoebae *D. discoideum*

Work in progress….

ABSTRACT

Laboratory experiments and theoretical models often explain cooperation as a consequence of high relatedness. Microbial social systems often do not meet these criteria as they are characterized by high clone mixing and high genetic diversity. Social amoebae are a typical example. Here we address this issue by looking at how the complexity of the social amoebae life cycle affects population genetic diversity and cooperation. We measured competitive performance at different stages of the life cycle for 6 natural isolates of *D. discoideum*. We show that strains indeed perform differently at different stages of the life cycle; some strains are good at sporulation but bad at germination, others are fast growers but bad germinators and so on. We further performed simulations to test the outcome of competition between strains. Our competitive model shows that competitive outcome depends on the overall performance of each strain. Interestingly, the strain that performed the best at cooperation (best sporulator) loses due to its low competitive performance at other stages of the life cycle. We suggest that the complex life cycle, with multiple competition points, can serve as a mechanism of eliminating defectors from the system and stabilizing cooperation. Our model failed to reproduce strain coexistence. Other competitive parameters and interactions between strains are thus needed in order to explain population genetic diversity.

INTRODUCTION

I have always been interested by interactions. Weather it is between individuals, populations or species, competitive, cooperative, predator-prey, parasitic or mutualistic one. Biological interactions are the basis of almost all behaviors observed in the nature and are one of the most important drivers of evolution. Just looking at the range of outcomes that are influenced or mediated by interactions makes one want to be a biologist and understand them even further. Interactions between ants are sufficiently amazing for a lifetime, but then they even grow fungal gardens. Than they are symbiotic food markets in mycorhize, or arms race between newts and snakes in creating the best poison and defeating the best poison, there are bacteria making biofilms, exchanging DNA and cannibalizing on each other and the list is endless. I have probably missed some of the most interesting ones. In the broader scale, at some point it all comes down to diversity. Understanding how community of species interacts and co-exists, why is there N number species and not N±x. Having this interactions fascinated mindset, during my PhD I wanted to explore the interactions between strains in to me new species D.discoideum. This organism has a fascinating life cycle that offers many opportunities to compete and cooperate. The interplay between these two fundamentally opposite processes made it an interesting system. How do you compete and cooperate at the same time? How diversity evolves if it increases competition?

Genetic diversity

Genetic diversity results from error prone DNA replication, chromosome recombination and heterozigosity (diploid species). As a consequence offspring do not carry the same genetic information as their parents. These changes may or may not have an effect on the individual phenotype, which can further have effect on fitness. In many cases genetic changes are neutral and individuals show no differences in fitness. In a minority of cases the changes confer a positive or negative effect on fitness. Population will thus consist of genetically different individuals, some of which represent different phenotypes that may or may not have different fitness (Figure 4-1). We can describe the population as a set of genotypes, each with frequency p_i, and fitness advantage s_i, with s_i being neutral, positive or negative. Population genetic model predict that over time each genotype gets extinct or fixed with certain fixation probability π_f. Fixation probability depends on population size and selective advantage of the genotype. This can be summarized so that $\pi_f \approx \frac{1}{N} + \frac{s}{2}$ (Houchmandzadeh n.d.). Meaning that in the neutral case all genotypes have equal fixation frequency of $\pi_f \approx \frac{1}{N}$. In the non-neutral case a genotype with a positive fitness advantage *s* will have increased fixation probability (Figure 4-2). In the theory of evolution, the first one is classified as neutral theory of evolution (Kimura 1984), while the second case represents the effect of natural selection (Darwin 1859). Although it is recognized that both forces shape the evolution, importance of one over the other is still

an open debate. The main debate being the probability for a newly arising mutation to be beneficial or neutral, and therefore the probability of one force or the other to shape the evolution. What is not debated is that adaptive traits are mainly selected by natural selection, while all the other genetic differences are mainly caused by random fixations of neutral mutations.

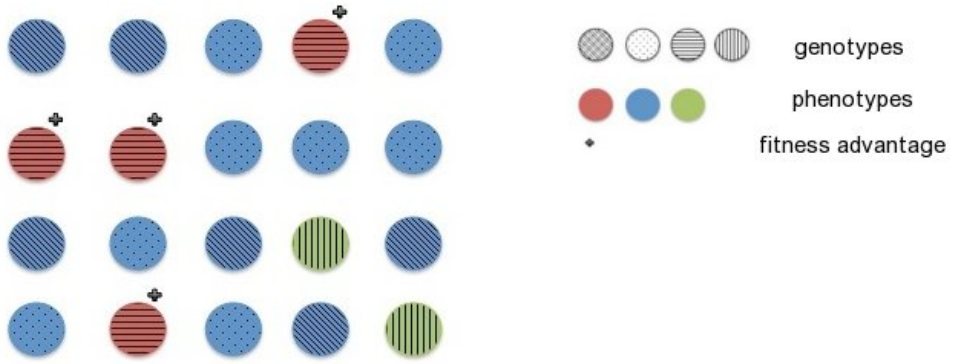


Figure 4 - 1 Population genetic diversity. Genetic mutations and recombination cause individuals to have different genotypes. Different genotypes give sometimes produce the same phenotype but often there are no differences. Different phenotypes may or may not give fitness advantage to an individual. The drawing represents a population of individuals (single circle) with certain genotypes and phenotypes.

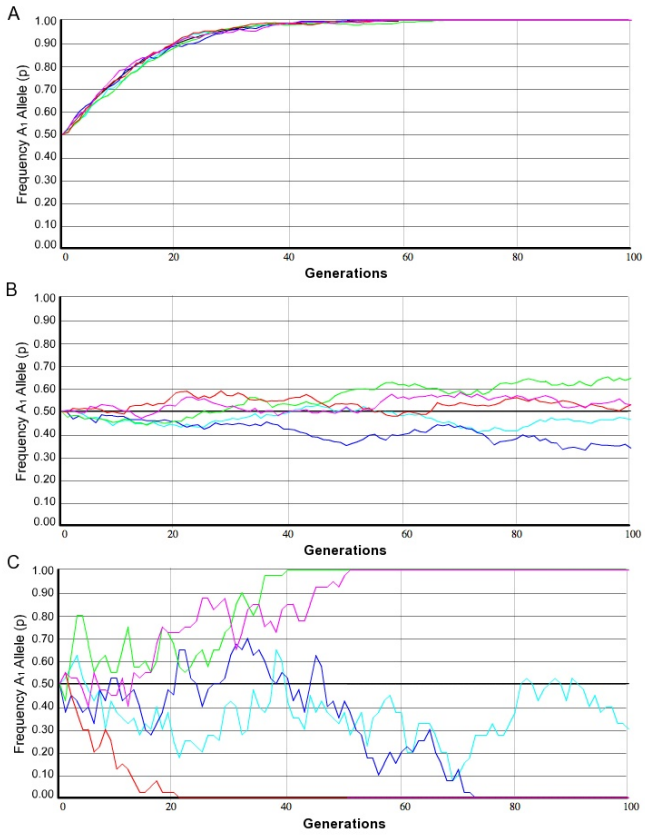


Figure 4 - 2 Hardy-Weinberg simulation of changes in frequency of allele A1. Pop size = 1000, initial frequency of allele A1 = 0.5, A) Natural selection in big populations: N=1000, fitness, s(A1A1)=1, s(A1A2)=0.9, s(A2A2)=0.8, B) Neutral evolution with big population size: N=1000, s(A1A1)=s(A1A2)=s(A2A2)=1, C) Neutral evolution in small populations: N=20. Each line presents one simulation outcome. Simulation preformed from *http://www.radford.edu/~rsheehy/Gen_flash/popgen/*

Genetic diversity in cooperative systems

Genetic diversity and genotype fixation in cooperative systems require special attention. This comes from 1) the frequency-dependent fitness benefits of cooperative phenotypes, decreased fitness when low in frequency and 2) the susceptibility of cooperative systems to invasion by "defector"/uncooperative phenotypes. The first problem is the problem of the origin of cooperative behaviors while the second ones deals with their stability. I will continue by focusing more on the problem of stability of cooperative behaviors (although the two are strongly connected).

Cooperative systems are prone to invasion by defecting individuals

As we have seen, when different genotypes have different fitness advantages this leads to fixation of the genotype with highest fitness advantage (Figure 4-2A). This may destroy social behavior, when a selfish new genotype confers fitness advantage for itself but not for the group. Within groups individuals perform actions beneficial for the

group, that are costly for the individual, and actions beneficial for the individual. This strategy is beneficial when everybody invests in the common good. Individuals investing less in the common good and more in their individual interest are called defectors or cheaters and will have will have fitness advantage. This is because they take advantage of the group, while not paying the costs of investing in the group. Therefore, a group of cooperative individuals is constantly under a treat of invasion by defector individuals. This has been shown both theoretically and experimentally for different cooperative systems (Dao et al. 2000; Chuang et al. 2010; Maynard Smith 1976). Several theories propose how cooperative systems resist the invasion of defector. They can be grouped into three important categories: kin selection, reciprocal altruism and multilevel selection.

Kin selection

Kin selection, proposed by W.D. Hamilton, is based on the observation that cooperative acts are often not shared with everybody but only with close relatives (Hamilton 1964a; Hamilton 1964b). Hamilton realized that if fitness represents number of genetic copies individual produces, than fitness is the sum of the individual's offspring (direct fitness) and its relatives offspring (indirect fitness). Hamilton called the total fitness inclusive fitness. Therefore, cooperative acts (that decrease direct fitness) are beneficial if directed towards relatives (increases inclusive fitness). Mathematically this can be written as $r \times B > C$, with r stands for relatedness between individuals, B for the benefits of receiving help and C for the costs of helping others. Kin selection predicts that cooperative system should be high in relatedness. Since genetic diversity within a group decreases relatedness between individuals, cooperative systems should also be low in genetic diversity. This is indeed characteristic of many social systems; many multicellular organisms are clonal, social insect colonies are highly related, in birds and mammals help is usually directed towards related individuals (West 2002; Griffin & West 2003).

Reciprocal altruism

Another way of excluding defectors is by reciprocal altruism: cooperate only with other cooperative individuals (Trivers 2006). Here genetic diversity can be high as long as everybody is sharing the same cooperative phenotype. This explains cooperation in systems where relatedness is low, such as humans (Fehr & Fischbacher 2003) and some bat behaviors (Wilkinson 1984). From gene-centered point, reciprocal altruism can be viewed as gene-gene interactions, where one should cooperate only with individuals having the same set of cooperative genes (Hamilton 1964a). Dawkins called this the green-beard effect, evoking to cartoon representation of "green-beard" genes that codes for phenotypic presence of green beard. Individuals having green beards should be able to recognize green beard in other individuals and behave differently (more cooperatively) towards them (Dawkins 1976). Such genes were shown to exist in yeast (Smukalla et al. 2008) and ants (Keller & Ross 1998).

Multilevel selection

Multilevel selection looks at cooperation as result of both individual and group level adaptation (Damuth & Heisler 1988). Multilevel selection considers that individuals interact within a group (individual level) and that groups interact between groups (group level). Damuth and Heisler (1988) recognizes two different cases of cooperation: MSL1 and MSL2. In both MSL1 and MSL2 individual fitness is the number of individual offspring an individual produces. Group fitness is what differs the two models. In MSL1 group fitness defined by the number of individuals a group contains, and it is proportional to average individual fitness. In MSL2 group fitness is defined as number of offspring group it produces and it does not need to be proportional to average individual fitness. Both mechanisms can operate at the same time; for example bigger groups may divide and create more offspring groups. A nice reflection on the topic an on importance of both cases is made by (Okasha 2005). Some work on group selection models (MSL1) has provided conditions under which group selection should be favored: competition between groups outweighs within group competition, benefits are maximized and cost are minimized, and when there is a decrease in genetic variance within group and an increase between groups (West et al. 2007). The last argument is very similar to kin selection requirements. Kin and group selection (MSL1) have shown to be mathematically the same and are just 2 different ways of looking at the same problem. MSL2 has been proposed as a very useful model in explaining emerging collectives such as major transitions in evolution (gene to chromosome, chromosome to cell, cell to multicellular organism) (Smith & Szathmary 1995).

Kin selection, reciprocal altruism and multilevel selection offer explanations for evolution and maintenance of cooperation. Still, how different genotypes interact inside groups, weather defectors and co-operators can coexist, what maintains and what reduces genetic diversity within groups are open questions.

Social amoebae: competition and cooperation

Social amoebae are interesting for studying the evolution of cooperation because they show high genetic diversity in wild populations. Different strains consume the same food source, grow, co-aggregate, get dispersed and germinate in the novel habitat. Dispersion, as well as interactions between strains are probably creating coexistence of different strains. Little is known about the nature of these interactions and both negative competitive and positive mutualistic interactions probably occur (Kaushik et al. 2005; Buttery et al. 2010; Buttery et al. 2009).

Due to their interesting social cycle, interactions between strains have almost entirely been studied during the social phase. The main competitive step during the social phase is investment in spores and in stalk cells. When in mixture the clone that contributes the most to the spores and the least to the stalk will have the highest fitness (Figure 4-3).

While in monoclonal population cell phenotypic differences determine cell differentiation (Box 1, General Introduction), it is shown that in polyclonal populations genetic background can influence cell fate (Santorelli et al. 2008). As a consequence cooperation in social amoebae is highly prone to invasion by individuals that invest more in spores and less in the stalk. Due to sterility of stalk cells and "hitch hiking" of defector individuals in spore mass, we could expect that stalk costs are compensated by indirect fitness benefits that favor reproduction of kin spores. This kind of thinking has lead several research groups to the idea that kin selection should operate in this system. Ever since a range of studies has focused on negative cooperate/cheat interactions and kin based anti cheating strategies during one step of the life cycle, the sporulation step.

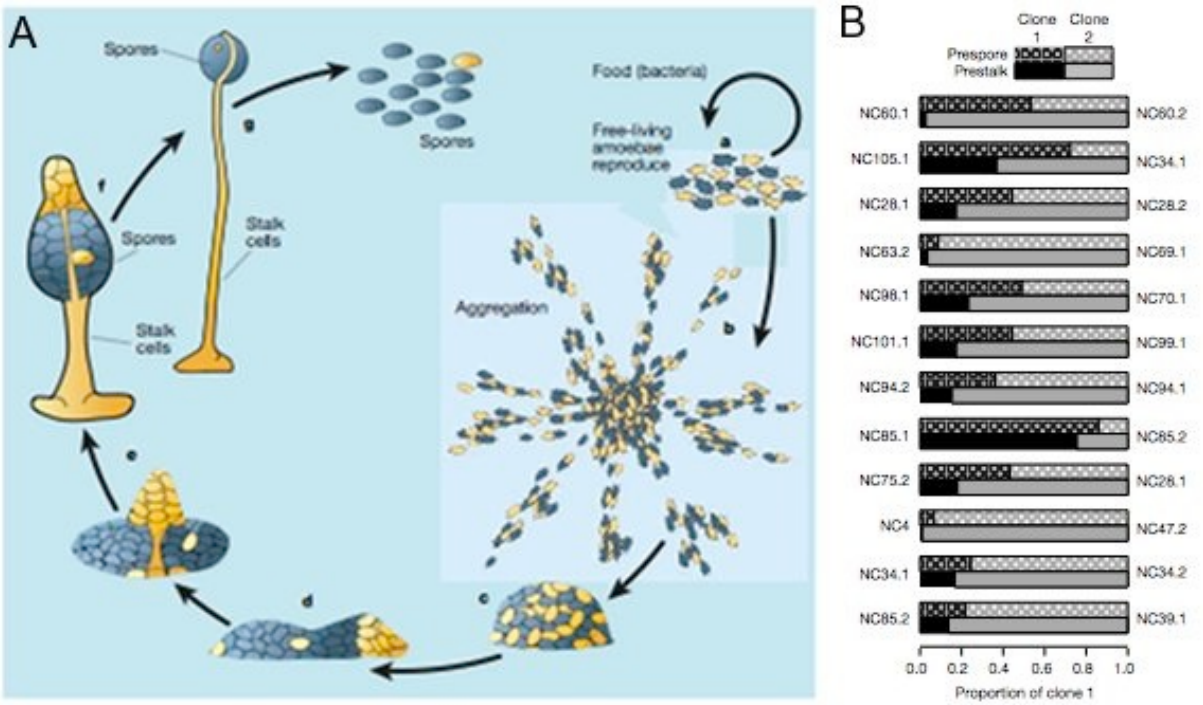


Figure 4 - 3 Defecting through spore bias. A) A cartoon representation of defecting through spore bias. Blue and yellow represent two strains. The two strains start aggregation with 1:1 ratio. Blue strain invests more to the spore mass, while yellow strain invests more to the stalk mass. As a result blue strain will get enriched into the spores and when the spore will germinate the ratio of the two strains in vegetative state will be biased towords blue strain (Kessin 2000). B) Results with pairwise 1:1 mixtures of natural isolates show that, as represented in the cartoon, srains do not contribute equally to spore and stalk mass (Strassmann et al. 2000).

Genetic predispositions for defecting/cheating

Several studies have shown that defectors are easily produced in the lab by random insertion mutagenesis methods (REMI) (Santorelli et al. 2013; Santorelli et al. 2008; Dao et al. 2000). This indicates that there is a high genetic potential for defecting. Analysis of spore or prestalk/prespore cell proportion in pairwise mixtures of natural isolates also showed strain specific disproportional contribution to spores (Figure 4-3B) (Strassmann et al. 2000; Fortunato, Queller, et al. 2003). In all these studies a defector is defined as a strain that when in mixture with other strain contributes more to spore population. This by itself was shown to be a biased approach because some strains form more spores even when alone, meaning that, when in mixture they are not defecting but simply contributing the same spore fraction as when alone (Buttery et al. 2009). More accurate definition of a defector strain is the one whose contribution to the spores is greater when in mixture than when alone (Buttery et al. 2009). Therefore, defecting is probably overestimated in the studies lacking the control for spore investment in monocultures.

Mechanism of defection

Several studies have explored the mechanisms by which a strain can defect. All studies agree that the result of defecting is an increase in spore proportion. This can be achieved through different pathways: 1) an predisposition to develop more prespore or fewer prestalk cells, 2) a defect in maintaining initial cell fate (*chtC* defective in maintaining prestalk cell fate (Khare & Shaulsky 2010)), or 3) an ability to affect cell fate of competing clone (*chtA* and *chtC* strain induce wild-type cells to become prestalk cels, while *chtB* strain decreases prespore gene expression in wild-type cells (Khare & Shaulsky 2010; Ennis et al. 2000; Santorelli et al. 2013)). A mix of defecting pathways was also found (Khare & Shaulsky 2010; Buttery et al. 2009). Other studies suggested that defecting can be obligate (a strain cannot aggregate when alone) or facultative (strain aggregates normally when alone, but forms more spores when in mixture). Obligate defectors reduce the stability of cooperation, where as facultative defectors lead to a decrease in genetic diversity through competitive exclusion of more cooperative strains.

Kin directed preferential aggregation in social amoebae

To explain the maintenance of cooperation in this highly defector prone system other studies have searched for kin selection mechanisms. By looking at spore allocation within a single fruiting body it was shown that when in mixture some strains prefer to aggregate with kin (Mehdiabadi et al. 2006). The required kin recognition possibly happens through self/non-self recognition of cell surface proteins that cause differential cell-to-cell adhesion. Genetic mutants and between strain gene exchange of *lagB1* and *lagC1* genes, coding for cell surface proteins, showed that amoebae cells with the same pair of genes are homogeneously mixed in aggregate. Amoebae cell that do not have a genetically identical pair of *lagB1* ad *lagC1* genes co-aggregate but sort out during

aggregation (Hirose, R Benabentos, et al. 2011; Benabentos et al. 2009). Pairwise mixtures of strains with increasing genetic distance were tested to see how sorting out is affected by genetic distance. Analysis of the spore proportion in these mixtures showed a highly variable but significant tendency of increased investment in spores sorting with increased strain genetic distance (Ostrowski et al. 2008). Altogether these studies showed that self-recognition system exists in social amoebae and that this can increase within fruiting body genetic relatedness by strain sorting out during aggregation.

In nature population are genetically diverse and co-aggregate

In the meantime studies on natural populations kept demonstrating that in nature genetic diversity is high, over small and large spatial scales (Buss 1982; Ketcham & Eisenberg 1989; Fortunato, Strassmann, et al. 2003). Genetic sequencing of spores from natural samples of fruiting bodies of *D. giganteum* and *D. purpureum* species showed that single fruiting body can contains up to 10 different clones (Sathe et al. 2010). This confirmed that in nature aggregates are highly heterogenous and that they co-aggregate. Finally, studies on more than two strain mixtures demonstrated that strain spore allocation in pairwise interactions may change when third clone is added (Kaushik et al. 2005). This showed that social amoebae behavior may be highly dependent on its social environment (other strains in the mixture), which results in complex and non-linear strain interactions.

Sum-up of current data

Preferential aggregation with kin exists (Hirose, R Benabentos, et al. 2011), but clones still mix (Sathe et al. 2010). Pleiotrophy in some cases is a way of excluding defectors (Foster et al. 2004), but other examples prove that defectors still go to fixation (Dao et al. 2000). In nature we find more frequently polyclonal (Fortunato, Strassmann, et al. 2003; Sathe et al. 2010) than monoclonal patches (Gilbert et al. 2007; Gilbert et al. 2009). Finally, a recent study has showed the limitations in using spore bias as sole measure of strain competitive success. (Saxer et al. 2010) demonstrated that spore bias alone cannot explain dynamics in the polyclonal populations. Thus, new approaches are needed to understand coexistence of clones in social amoebas.

Our approach

We approach this system more globally by looking at the entire life cycle. Our main goal is to see how the complexity of the life cycle regulates genetic diversity and cooperator-defector interactions.

Social amoebae have a complex life cycle composed of unicellular/vegetative state, where cells grow and divide, and social state, where cells aggregate and differentiate into spores and stalk cells. The above-mentioned studies focused on the social part of the life cycle and tried to explain competition and diversity based on the ability of a clone to form spores. While this is an important part of social amoebae life cycle it is only a part of it. Different strains also interact and compete outside of the aggregate, competing for resources, for instance. Thus, to understand genetic diversity and defector-cooperator dynamics we may need to look also at competition at different stages of the life cycle (Figure 4-4D). We propose to do this by identifying competition points throughout the life cycle and for each point measuring clone competitive ability. We further perform clone competition simulations to see what effect multiple stages of competition may have on strain diversity. We focus on 4 measurable competition points: growth rate, non-aggregating cells, sporulation efficiency and germination efficiency. We measure these for 6 natural isolates in monoclonal populations. To be consistent with previous studies on spore bias we chose to work with 6 isolates from North Caroline that had been used previously (Buttery et al. 2009; Fortunato, Queller, et al. 2003). The isolates were isolated from a $1km^2$ area in North Caroline, USA (Francis, D. and Eisenberg 1993). Spore allocation was tested for all pairwise mixtures of these 6 clones. This resulted in a single clone linear dominance hierarchy: strain A invests the most in spores in all mixtures, strain B invests the most in spores in all mixtures except when with A and so on (Figure 4-4A)(Fortunato, Queller, et al. 2003). This spore bias, when in mixture is, just a results of certain clones producing more spores even when alone (Figure 4-4B) (Buttery et al. 2009). The same study proposed different linear dominance based on clones "ability to cheat by self-promotion and social coercion" (Figure 4-4C). Both studies suggest a single clone dominance and thus cannot explain clone coexistence. We try to extend these studies by measuring, in addition, growth rate, non-aggregating cells proportion and germination efficiency (Figure 4-4D).

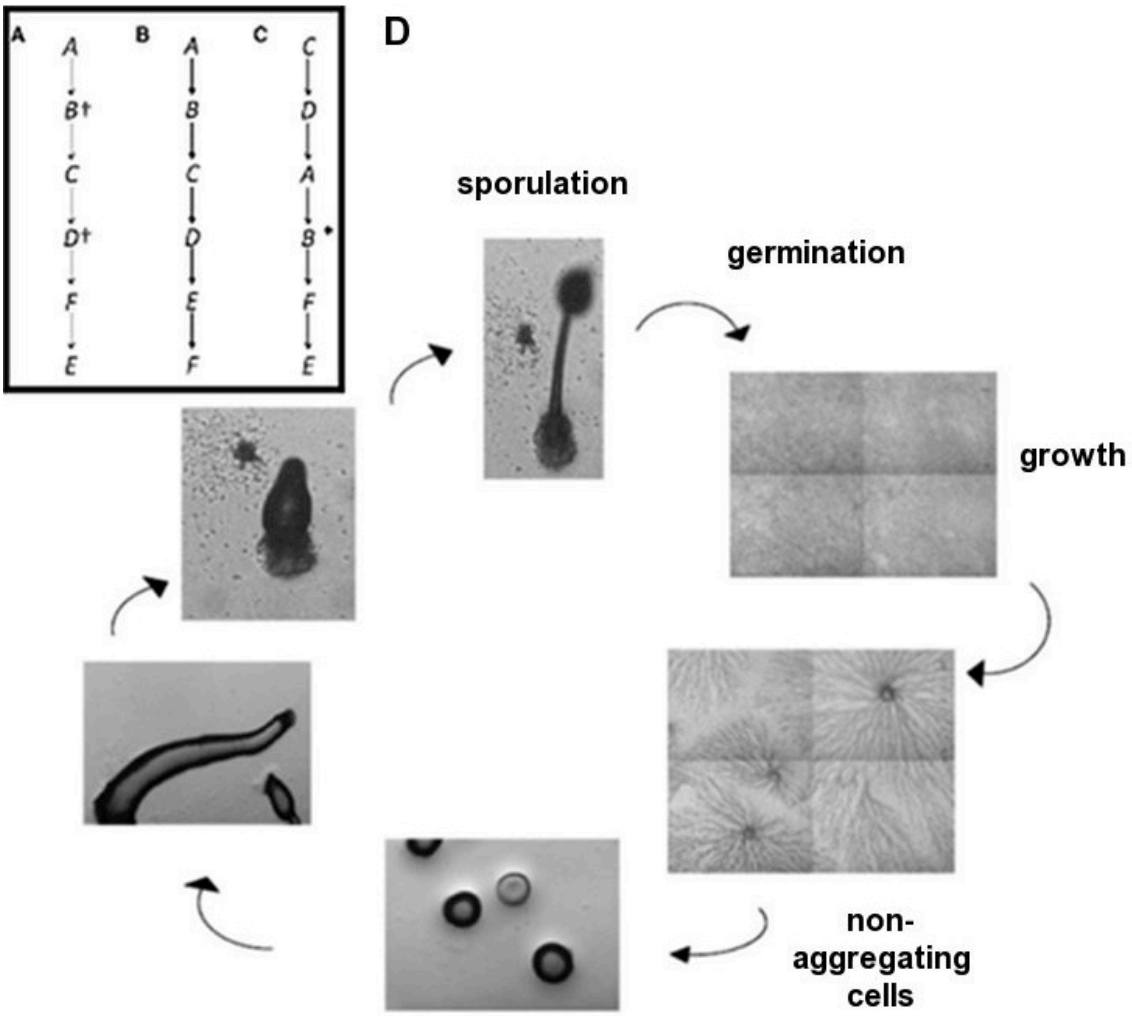


Figure 4 - 4 Our proposition for understanding strain interactions in *D. discoideum*. A), B) and C) are results from Buttery et al., 2009 that show linear dominance in spore investment for 6 natural isolates (represented by letters A-F). Hierarchy based on: A) spore investment of each clone when in mixture, B) spore investment for each clone when alone, C) change in spore investment when in mixture. D) We study clonal interaction at the level of entire life cycle. To do this for each clone alone we measure sporulation efficiency, germination efficiency, growth rate and investment in non-aggregating cells.

RESULTS

Competitive interactions are important for understanding species diversity. We therefore begin by defining points in the life cycle of *D. discoideum* at which competition can occurs: growth rate, proportion of non-aggregated cells, sporulation efficiency and germination efficiency (Figure 4-4D). Strain competition at the vegetative stage is defined by its maximum growth rate. Strain investment into non-aggregating cells defines its ability to compete in fluctuating environment (Chapter 3). Strains compete for entering into the viable spore mass by sporulation efficiency. Once the food becomes available spores compete for germination via germination efficiency. We measure each of these parameters for 6 natural isolates; 34.1, 28.1, 105.1, 63.2, 85.2, 98.1. These strains were isolated from the soil sample in Little Bus Gap, North Carolina, USA by (Francis, D. and Eisenberg 1993). Samples 26-75 were collected from a 100m x 50m area, while samples 76-105 were collected from the scattered points within the radius of 1500m. Minimal distance between samples was 1m. Genetic difference between the strains was not measured; different names represent solely different areas from which they were isolated. We start with the simplest case and measure parameters for monoclonal populations of each strain.

Growth rate

Maximum growth rate was measured by plating cells on SM/5 plates with *K. aerogens*. Cell number per plate was calculated every 2h (15-17h after plating). Overall, strains showed considerable variation in growth rates (Figure 4-5). Strains 28.1 and 105.1 had the highest growth rates, 0.35 h^{-1}, while strain 98.1 had the lowest growth rate of 0.27 h^{-1}. Average growth rate of all strains was 0.32 generation/hour. Only one other study measured strain differences in growth rate, but in *P. pallidum* species, and also have found strain differences in growth rate (Ketcham & Eisenberg 1989). This indicates that strains often differ in their growth and that is important to measure it for understanding competition.

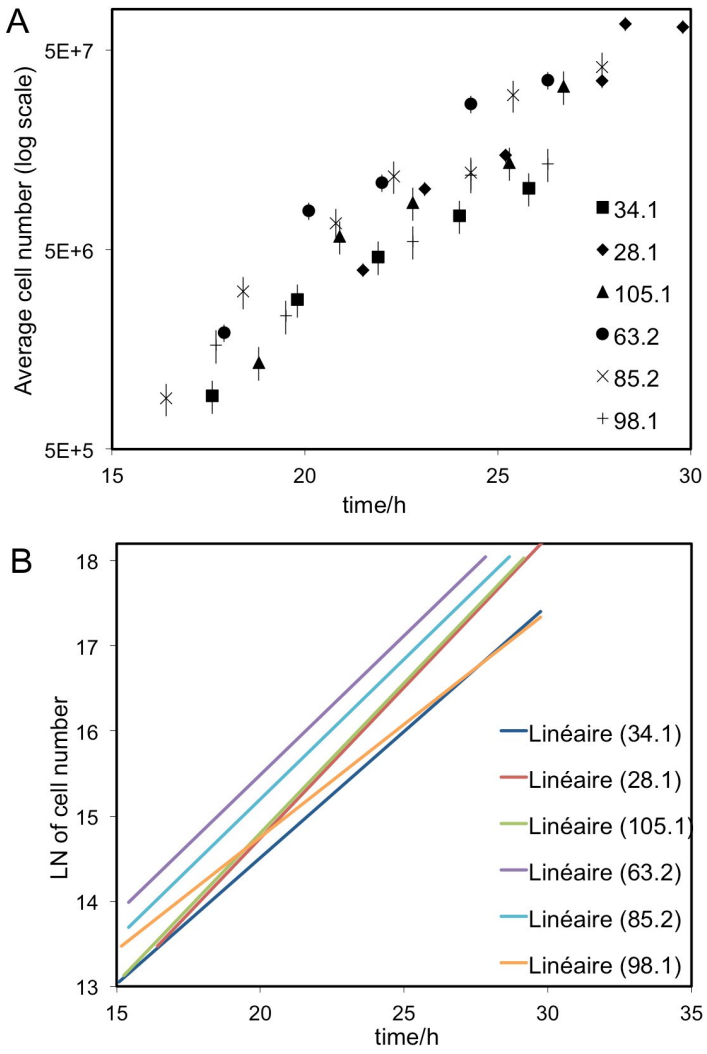


Figure 4 - 5 *D. discoideum* growth rate *on K. aerogenes* bacteria. Growth was measured for 6 natural isolates, 34.1, 28.1, 105.1, 63.2, 85.2, 98.1. A) Average cell number over time. Error bars represent standard error as percentage of population size. B) Linear regression of Ln of cell number over time. The slope of the regression equals to the strain growth rate.

Non-aggregating cells

Next competition step is the investment into aggregating vs non-aggregating cells. In Chapter 3 we discussed the importance of non-aggregating cells proportion. In Chapter 3, Sup. Figure S3-1 we showed that natural populations also partition differently into aggregating and non-aggregating cells. It was therefore very interesting to measure the fraction of non-aggregating cells of our natural isolates. To do this we first needed to develop a protocol for inserting a fluorescence signal into the cells of natural isolates. This can be done in two ways; by inserting plasmid with a fluorescent gene in the cell (as done for the laboratory strains in Chapter 3), or by dyeing the cells with fluorescent chemicals that penetrate through the membrane and make cells fluorescent. Both of the techniques are frequently used with laboratory strains of *D. discoideum*, but no protocol for natural isolates exists.

Fluorescent cell dyes

Several studies used CellTracker Probes Red CMTPX and Green CMFDA from Sigma to stain natural isolates (Buttery et al. 2009; Ostrowski et al. 2008). Using the same dyes and same protocol we managed to stain the cells but their fluorescence was too weak and too noisy (due to fluorescent bacteria in the background) for single cell fluorescence measurements. In addition dyes showed fast bleaching, which did not allow us to track single cells sufficiently long over the aggregation phase (around 10h). Therefore, dyeing cells with CellTracker Probes was not suited for measuring the proportion of non-aggregated.

Cell transformation with plasmid DNA having fluorescent protein

We further tested the cell transformation with plasmid carrying the fluorescence gene. Inserting external DNA into natural isolates is challenging and no standard protocols exist. The difficulty is caused by 1) plasmid instability due to potential presence of natural plasmids within the cells (Noegel et al. 1985), and 2) selective antibiotic consumed by bacteria. A few studies succeeded in transforming *P. pallidum* (Fey et al. 1995) and *D.discoideum* natural isolates (Hirose, Rocio Benabentos, et al. 2011). Inspired by these studies we tried to optimize a standard *Dictyostelium* electroporation protocol for North Carolina natural isolates (Chapter 2: Material and Methods, Transformation of *D.discoideum* natural isolates). None of the protocols gave a successful transformation. In some cases isolated colonies that were resistant to G418 were observed but plasmid was soon lost probably due to competition with natural plasmids.

Thus, we did not manage to obtain a measurable fluorescence signal for tracking single cells of natural isolates. No measurements for this competitive stage are therefore available (Materials and Methods for further details).

Sporulation efficiency

Sporulation efficiency is the most measured competitive ability. It defines the proportion of the population that forms spores, $N_{spores}/N_{cells\ plated}$. Cells that aggregated have two possible fates: as viable spore cells or as dead stalk cells. This has very high fitness cost for stalk cells and makes this step a very important competition point.

Case 1: measurement on gel

Previous studies measured sporulation efficiency for our 6 natural isolates (Figure 4-6C) (Buttery et al. 2009). They showed that strains do not invest equally in spore quantity, with the highest sporulation efficiency for strain 34.1, 1.44 (144% of cells become spores) and lowest sporulation for strain 98.1, 0.51 (51% of the cells become spores). These results are puzzling, as standard sporulation efficiency measured for laboratory strains is around 80%. Measured 140% clearely is an unrealistic estimator. What it probably means is that after plating cells continue dividing. Therefore plated cell number is not effective cell number at the time of aggregation. The differences in sporulation efficiency measured by Buttery et al are maybe just artifacts of different aggregation timings of different strains; with strain 98.1 aggregating instantly after cell division (no time for division) and strain 34.1 dividing and then aggregating. Nevertheless, we first tried to reproduce previous results and proceeded according to their protocol. The first difficulty was that we obtained aggregation but not sporulation for strains 34.1, 98.1 and occasionally 105.1. These strains develop normal fruiting bodies when allowed to develop on bacterial plates, but when plated on phytagel or SorC plates the development is arrested at the aggregation stage. Neil Buttery, in personal communication, confirmed the presence of occasional non-sporulating phenotype for strain 98.1 but not for strains 34.1 and 105.1. Other strains showed a similar hierarchy but lower sporulation efficiency than in previously published results (Figure 4-6A). The lower sporulation efficiency in strain 105.1 is probably due to poorer aggregation on agar since it failed to sporulate several times and when sporulating showed fewer fruiting bodies. The lower sporulation efficiency may be due to faster aggregation timing/lower division time as suggested previously.

Case 2: measurements of filter papers

To overcome the non-sporulating phenotype on the gel, that by some clones produced, we attempted to measure sporulation efficiency on filters (Figure 4-6B). In addition, to avoid overestimated sporulation efficiency due to continued cell division, cells were left for 2-3h in phosphate buffer before plating. This gave them time to consume bacteria left in the surroundings and to finish cell division. Unfortunately, filter plating did not change the non-sporulating phenotype of strains 34.1 and 98.1 and in addition it caused 105.1 to lose the sporulation completely (additional time on phosphate buffer was not the cause of this). Interestingly, plating on filters completely changed sporulation efficiency hierarchy among other strains (Figure 4-6B). Leaving cells for a few hours in

phosphate buffer did not have the expected effect of decreasing cell division time. On the contrary all strains showed sporulation efficiency above 100%, meaning that cells continued division after plating. These results suggest that sporulation efficiency is highly dependent on the aggregation surface, that strains 34.1 and 98.1 may need presence of bacteria to aggregate and that incubating cells in the non-nutrient buffer is not always an effective way to diminish cell division before aggregation.

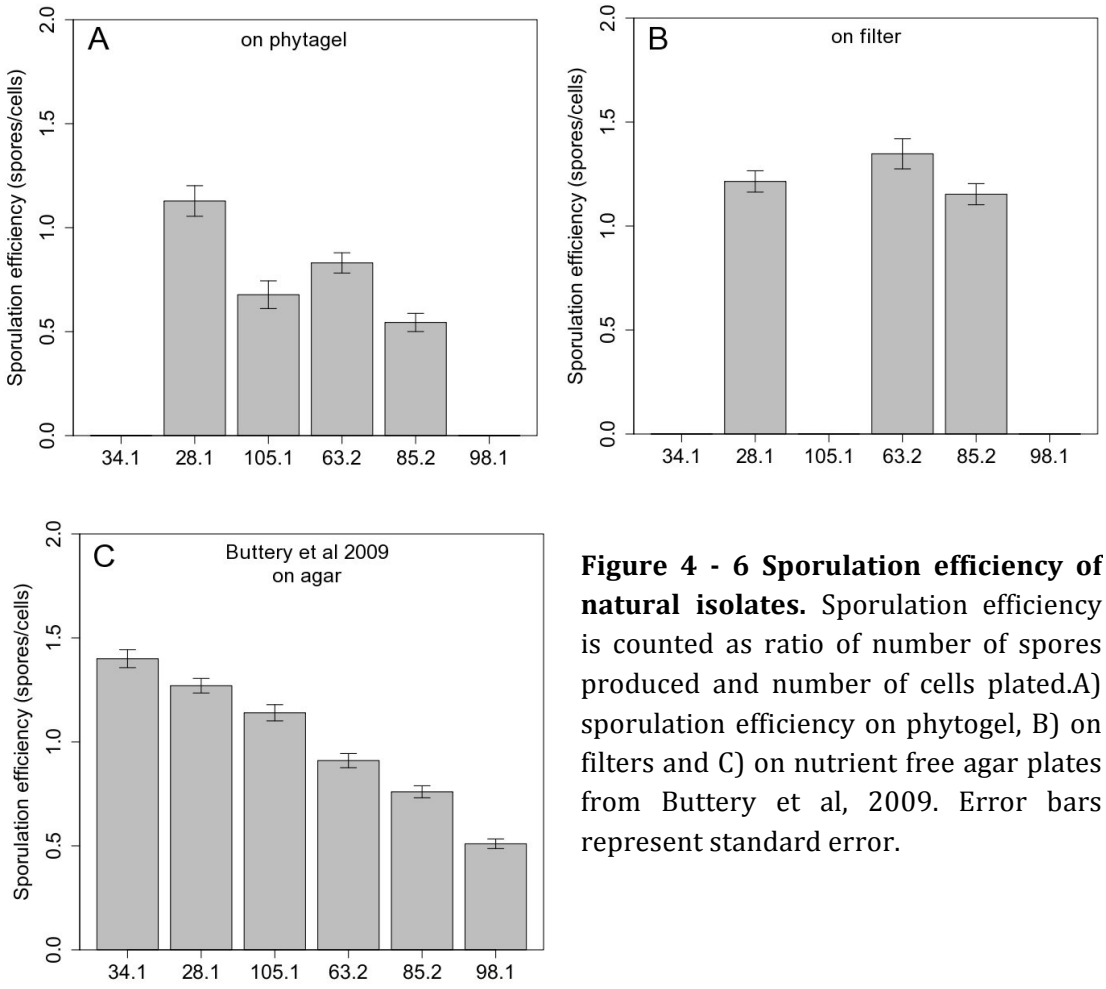


Figure 4 - 6 Sporulation efficiency of natural isolates. Sporulation efficiency is counted as ratio of number of spores produced and number of cells plated.A) sporulation efficiency on phytogel, B) on filters and C) on nutrient free agar plates from Buttery et al, 2009. Error bars represent standard error.

Germination efficiency

Spores germinate in the presence of food. Germinating fast and efficiently will give a head-start advantage to the strain. We measured germination efficiency as the proportion of spores that give viable dividing cells, $N_{viable\ cells}/N_{spores}$. This was measured by counting number of plaque forming units on bacterial plates. Each plaque forming unit represents one spore that germinated and started to divide and consume bacteria in its surrounding. Previous studies, on other strains, showed that not all spores germinate, resulting in a germination efficiency < 1 (Castillo et al. 2011; Jack et al. 2008). It was therefore interesting to assess how well our strains perform at germination. Strains showed differences in germination, within the range of 50-80% of the spores germinating (Figure 4-7). Germination efficiency order differed drastically to the growth and sporulation. For example, strain 98.1, which was the worst sporulator, turned out to be the best germinator.

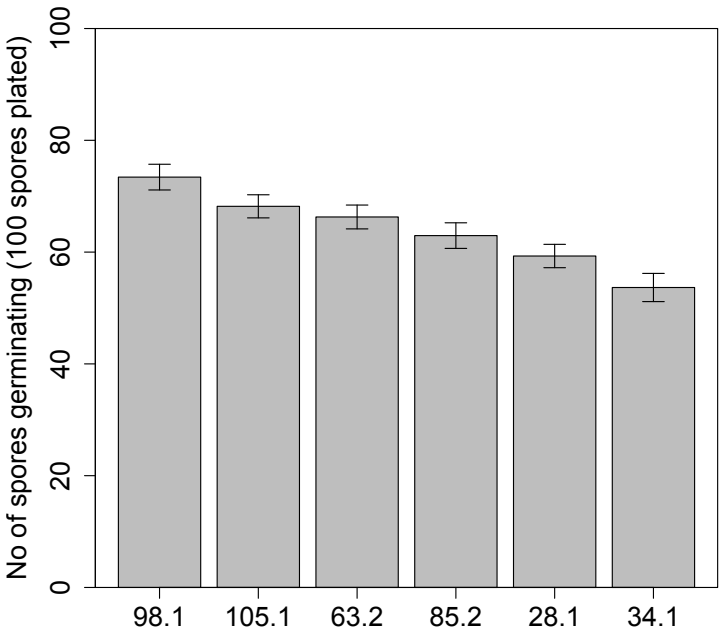


Figure 4 - 7 Germination efficiency of *D. discoideum* natural isolates. Bars represent number of spores germinating out of 100 spores plated. Error bars represnet standard errors.

Competition Model

Interplay of previously measured strain specific parameters

In previous experiments we measured how well each strain performs at each step of the life cycle. This showed that strains perform differently at different stages at the life cycle; strain 34.1 was the best sporulator according to Buttery et al, 2009, but it turned out to be a slow grower and a poor germinator, strain 28.1 is a good sporulator and grower but is a poor germinator, strain 105.1 is an good sporulator, fast grower and good germinator, and so on. This suggests that in the nature there is a potential for complex network of competitive interactions that depend on environmental conditions. We have therefore developed a mathematical model that simulates competition between strains.

Description of the model

For more details see Chapter 2: Materials and Methods – Model.
The model represents the social life cycle of *D. discoideum*, vegetative cells grow with rate λ_i for each strain i. When population reaches maximal population size (carrying capacity) $K = N_{max}$ starvation is induced and cells aggregate. Since we were unable to measure percentage of non-aggregating cells for natural isolates, the model assumes 100% aggregation success; there are no non-aggregated cells. Aggregated cells sporulate with sporulation efficiency s_i and germinate with germination efficiency g_i. Competitive parameters λ_i, s_i and g_i are as measured in previous experiments. The simulation starts with population with equal cell proportions for all 6 strains. Strains are then made to compete over many growth-sporulation-germination-growth cycles. At each cycle we assume perfect mixing and strain co-aggregation. The model assumes no interactions between strains; implaying that presence of one strain does not modify the behavior of an other strain. As discussed in Chapter 2: Materials and Methods and Chapter 4: Discussion, this is a simplified assumption that we know is not always true (Buttery et al. 2009; Kaushik et al. 2005) but serves as a starting point. We decided to use the interaction-free model because: 1) it helps to understand the importance of the competitive parameters, 2) it was shown that adding a third strain changes two-strain interactions (Kaushik et al. 2005). We do not know to which point the complexity of 6 strain interactions can be represented by lower complexity interactions, such as pairwise interactions.

Model results

Figure 4-8 represents the outcome of competition between 6 natural isolates. Figure 4-8A represents the cases where competition occurs only at the level of sporulation, all the other parameters being identical for all 6 strains. Unsurprisingly, strain 34.1, which invests most into the spores goes then to fixation. Figure 4-8B gives results of competition at the level of the entire life cycle. In this case strain 34.1 goes extinct, due to its low growth and germination rates. The strain that goes to fixation under these

conditions is strain 105.1. This is due to its fast division time, high sporulation and germination efficiency. Overall, the model shows that competitive interactions do not depend solely on strain contribution to spores and that in order to understand the outcome of competition at all stages of life cycle need to be taken into account.
Our simulations did not predict coexistence. This is not surprising given our assumptions that strains do not interact. The observation that such coexistence (diversity) occurs in nature implies that our model is not complete, and that some form of interaction should be incorporated.

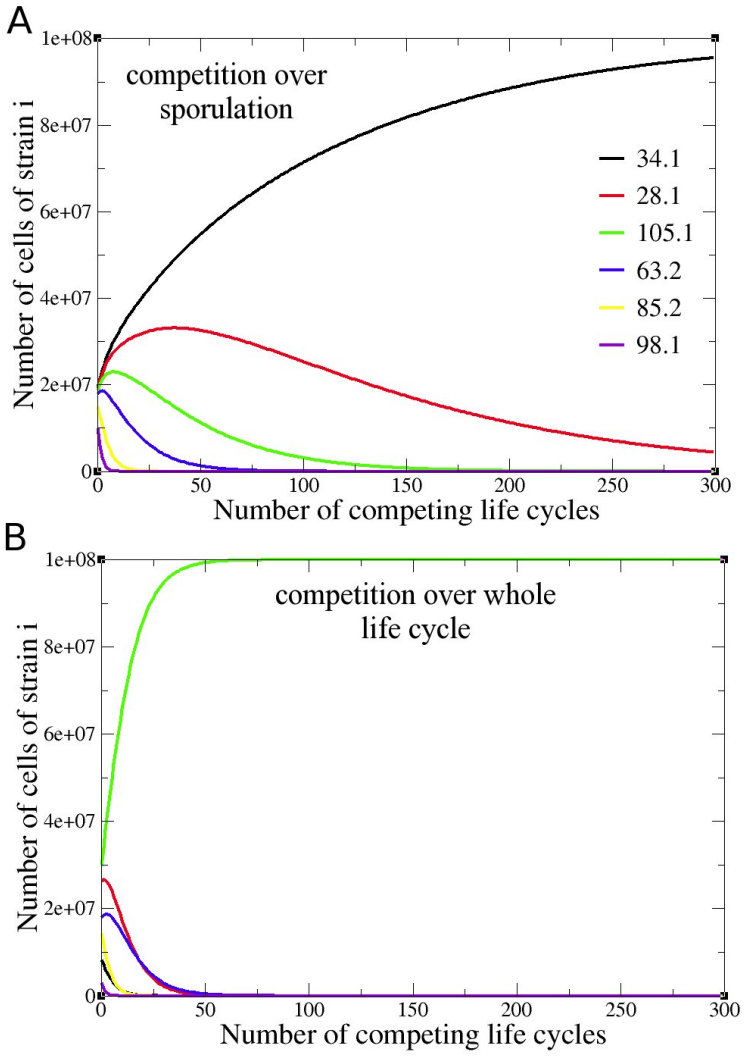


Figure 4 - 8 Competition model. The dynamics of 6 natural isolates over 300 life cycles. A) Competition just at the level of sporulation efficiency, all the other parameters are equal for all the strains. B) Competition at multiple levels of life cycle. All parameters were experimentally measured.

Environmental sources of phenotypic variability

There are many additional parameters that could affect competitive phenotype. As shown, changing aggregation surface had an important effect on aggregation and sporulation efficiency. Other examples may be, that different bacterial strains affect growth rate, that strains may respond differently to changes in humidity and pH, that there is be different resistance to parasites and so on. We tried many things that made us reflect on the phenotypic sensitivity to external conditions. Table 4-1 presents a small sum up of differences that were caused by small changes in external conditions. One example is illustrated in Figure 4-9; when in liquid culture with bacteria strain 34.1 does not germinate and does not grow, while strain 85.2 both germinates and grows successful. We present these observations to stress the limits of laboratory studies in understanding diversity in the wild. In nature all of these parameters and many more may play an important role in strain competitive interactions.

Condition/Strain	**34.1**	**28.1**	**105.1**	**63.2**	**85.2**	**98.1**
Sporulation on filter	-	+	-	+	+	-
Sporulation on phytagel	-	+	+	+	+	-
Sporulation on gel with bacteria	+	+	+	+	+	+
Germination in HL5 + 10% fetal serum bovine	-	+	+ (low)	+	+	+
Germination in SORC (lq) with dead *K.aerogenes* culture	-	+	+	+	+	-
Growth in SORC (lq) + dead *K.aerogenes* culture	-	+	-	+	+	-

Table 4 - 1 Strain phenotype is sensitive to environmental conditions. Testing growth, sporulation and germination of 6 natural isolate under different conditions. (+) means positive sporulation, germination and growth and (-) means no sporulation, germination and growth.

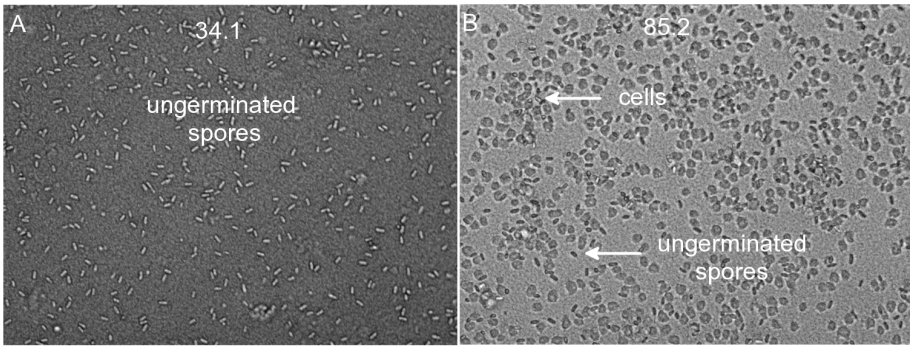


Figure 4 - 9 Differences in spore germination in liquid SorC buffer with bacteria. Spore germination of strains 34.1 and 85.2 in 2.5ml SorC(lq) with 400µl of dead bacteria as food source.

DISCUSSION

Life cycle complexity, competition and cooperation

Social amoebae display complex life cycle(s) that provide possibilities for multiple competition points. This makes the life cycle a possible player in the maintenance of genetic diversity and cooperation. Here, we tested this hypothesis by measuring competitive parameters of 6 natural isolates of *D. discoideum* at several stages of the life cycle. We further performed simulations to test the competitive outcome between these strains. Our results show that strains perform differently at different stages of the life cycle (Figure 4-5, Figure 4-6 and Figure 4-7). Some strains turn out to be good at sporulation but bad at germination, others are fast growers but bad germinators and so on. Our competitive model showed that strains competition at different stages of the life cycle is important for final competitive outcome. Overall strain performance, rather than just strain performance at sporulation, is a measure of strain success (Figure 4-8).

Life cycle complexity as stabilizer of cooperation

This overall performance is especially important for cooperator-defector competition that has attracted much attention in the literature. Previous studies focused only on one component of the life cycle, the strain investment in spore cells, concluding that the strain that invests the most to the spores will pass most of the "offspring"/viable cells to the next generation and therefore have the highest fitness. This reasoning makes one to suspect that defector individuals that do not invest into the group beneficial stalk, but mainly invest into the self beneficial spores, can easily invade (Santorelli et al. 2008; Santorelli et al. 2013; Khare & Shaulsky 2010; Ennis et al. 2000). If fitness equals spore production the question is how the social cycle is stabilized, how social individuals do resist the invasion of defectors. The main mechanism proposed, by "sporulation maximizing competition" is aggregation with highly related individuals, inspired by kin selection theory (Mehdiabadi et al. 2006; Kuzdzal-Fick et al. 2011). However, this turned to be of limited use in natural populations of social amoebae; as these show high strain mixing and great diversity (Fortunato, Strassmann, et al. 2003; Sathe et al. 2010).
Here we propose a new framework that takes into account highly mixed and genetically diverse populations and life cycle complexity. By considering the system at the level of life cycle we treat cooperation and genetic diversity as one and not two separate problems. In this case winning at one step does not automatically mean winning at another one as well. With our theoretical model we show that the strain 34.1 that invests most of its cells into spores, finally looses to the strain 105.1 when competition is over the entire life cycle (Figure 4-8). The strain 34.1 is not a cheater strain, but just a strain that even when alone has the high sporulation efficiency. We can further imagine similar exclusion effects with cheater strains. If cheater strains perform well at the competition for spores, but are less good growers or germinators, over the entire life cycle they could be excluded nevertheless. Thus, competition at multiple stages of the

life cycle is expected to affect the outcome of competition. Although preliminary, these results emphasize the importance of integrating species ecology in our understanding of defector-cooperator interactions in natural populations.

Strain coexistence and genetic diversity

Our model did not predict strain coexistence. It is important to note, however, that our goal was not to explain diversity as it occurs in the nature, but rather to explore the role of complex life cycle in stabilizing diversity and cooperation. This point merits some discussion. Although compared to previous studies, we take the analysis of competition a step further, our framework still represents a simplified vision of nature. There are many elements that we did not take into account and that may be important for strain coexistence. Some of these are: 1) other competition opportunities, 2) additional sources of phenotypic diversity, 3) interactions between strains and species, 4) dispersal and 5) unclear coexistence of these strains in the nature.

1) We managed to measure only some competitive mechanisms, while others fitness components such as the proportion of non-aggregated cells are still unknown. Understanding the ensemble of all competition mechanisms is needed to truly predict the outcome of competition.

2) Another point is the phenotypes may be extremely sensitive to external conditions, as suggested by Table 4-1. While we were only able to measure growth rate at one temperature, one humidity and on one bacterial strain, in nature, all of these conditions vary continuously and may affect competition between different strains in social amoebae (Horn 1971; Eisenberg et al. 1989).

3) Up till now we have considered that the presence of other strains does not alter a strain's behavior that is no interaction model. This is a simplified assumption that we know is not always true. Several studies showed that interactions are frequency dependent (Buttery et al. 2009) and strain dependent, specific genotype-genotype interactions (Kaushik et al. 2005; Buttery et al. 2010). Not much is known about the nature of these interactions and both negative and positive effects have been reported (Kaushik et al. 2005; Buttery et al. 2009). To what extent these interactions affect strain coexistence is still unknown and left to speculation. Except interacting and competing among themselves strains live in ecological community where they potentially interact with other social amoebae species (Landolt et al. 2006; Shim, Kew-Cheo 1998). Manipulation of species density and bacterial food source in soil samples showed that these social amoebae species compete for the same food source and that they effect each others densities (Horn 1971; Eisenberg et al. 1989). Some social amoebae species, such as *D. caveatum,* feed on other species of social amoebae which can additionally affect both strain and species diversity (Nizak et al. 2007). It may be that the whole ecosystem together, bacteria as food source, other species of social amoebae and their natural predators, regulates genetic diversity in the soil. We discuss these points in greater extent below in Chapter 4 - Appendix.

4) Dispersal is another important life-history component that we did not include in our model. Microbial communities are known to have high dispersal rates. This

increases gene flow and together with sexual reproduction and recombination can even further increase genetic diversity (Finlay 2002; Martiny et al. 2006). In social amoebae, costly fruiting bodies are assumed to have evolved to facilitate spore dispersal. Thus, dispersal is thought to be an important fitness component that can also serve as a generator of diversity (Bonner 2008). Birds and higher mammals provide long-range dispersal for social amoebae spores (Sathe et al. 2010; Suthers 1985; Stephenson & Landolt 1992), while small arthropods and predating nematodes disperse spores over shorter distances (Huss 1989). Three studies have closely looked at paleogeography, phylogeny and sexual reproduction in social amoebas. They unfortunately led to incoherent results; studies on *D. giganteum* and *D. discoideum* species support high dispersal, high mixing, low genetic structure and sexual reproduction among genetically distant clones (Mehdiabadi et al. 2010; Flowers et al. 2010), whereas a study of *D. purpureum* reports genetically structured populations with reproductive isolation between distant clones (Mehdiabadi et al. 2009). Although, it is still not clear to what extent dispersal and gene flow exchange contribute to strain diversity and coexistence they are sure important for social amoeba ecology and should not be forgotten.

5) Finally, we need to acknowledge that it is not known if these strains do indeed coexist at small spatial scales; they have been isolated at different places within a 1500m wide area (Francis, D. and Eisenberg 1993).

Germination efficiency –questions and speculations

Going beyond competitive interactions, our results led to other questions. One intriguing result is the high percentage of non-germinating spores. It is not obvious, certainly not from the evolutionary perspective, why some cells would go all the way to produce spores that do not germinate. According to our results a quite high percentage of spores may fail to germinate, between 30 and 30% (Figure 4-7). If we add these non-germinating cells to the population of dead stalk cells (20%) it makes aggregation a very costly behavior. It could be possible that our low germination efficiency is just an experimental artifact and in natural conditions all spores germinate. Another possibility is that there may indeed be biological explanations. It all resembles in many ways seed germination in plants. When favorable conditions arrive plant seeds germinate, but often not all seeds germinate at the same time. This variation in timing of germination has been shown to be an adaptation to fluctuating environments (Simons 2009). Variation may prevent the massive failure of reproductive output and assure that at least some part of the population will germinate at the right time. The way we measured germination efficiency in *D. discoideum* tells us how many spores germinate at one time point. But, as in plants, this may not mean that other spores will not germinate at different times or under different condictions. It would be very interesting to test if similar variation in germination timing exists among *D. discoideum* spores. Possible population level benefits of this behavior would be decreased competition between

individuals (West et al. 2002) and/or optimizing population exposure to predation and environmental changes as in plants.

PERSPECTIVES

The most interesting follow-up on this work would be to study long-term competition experiment in a laboratory set up. This would allow us to truly test model predictions and relevance of complexity of the life cycle for maintenance of diversity and cooperation. Microsatellite loci have already been used for identification of our strains (Fortunato, Queller, et al. 2003), and microsatellite based analysis of multiple strain competition has already been performed by others on different strains (Saxer et al. 2010). Another interesting competition experiment would be to perform competition over the whole life cycle between a known defector strain like *chtA* or *csaA* and its parental strain. These clones are known to have a competitive advantage during the sporulation phase, but is this sufficient to outcompete cooperative parental strain?

APPENDIX: Diversity and Individuality

Studying one thing often leads to another, and another, and another until you reach the bottom and you are left only with few, skinny, basic principles that you never thought of questioning because they are The Truth and The Law of everything you know. Then you start questioning even those and things start to be really interesting.
This Appendix is an overview of questions that came to me while studying genetic diversity in social amoebae. I talk abouth benefits of being diverse, chimerism and mosaicism and concepts such as Individuality and Levels of selection.

Costs and benefits of being diverse

Studying diversity from competition point of view evokes ideas of exclusion, dominance, wining of the best, defection, fight for co-existence and so on. To truly understand diversity other, sometimes opposing, ideas need to be equally taken into account. These ones include mutualism, benefits of co-existence and positive interactions. Although this was out of our studies scope, the united vision of both of these perspectives is needed for true reflection on genetic diversity. There are 2 ways of looking at diversity: between individuals and within individual. In this study we touched on both concepts; strain interactions at the level of the life cycle and within a multicellular fruiting body.

Diversity at the level of populations and ecosystems affects fitness

For the moment we have focused on how individual interactions affect individuals. In order to understand certain benefits of diversity we need to ask; how individual interactions affect higher-level entities, populations and ecosystems. This has been widely explored in agriculture, conservation ecology and climate change. Results have repeatedly pointed out on the positive effects of diversity on stability and homeostasis of populations and ecosystems (Ives & Carpenter 2007; a R. Hughes et al. 2008; Tilman et al. 2006). Stability is measured by looking at invasibility, variability, resistance, return rate to equilibrium and alternative stable states (Ives & Carpenter 2007). Therefore, diversity positively regulates all of these aspects within a population. The causes range from higher resistance to diseases of genetically diverse populations (Zhu et al. 2000), decreased invasibilty by alien species due to the higher probability of containing less invasible phenotype (Hodgson et al. 2002; Ruijven et al. 2003), increased ecosystem productivity due to regulation of insect communities (Johnson et al. 2006) and so on. In social amoebae no studies measured the effect of genetic diversity on population stability. We can only imagine the population level benefits of genetic diversity on resistance to pathogens or return rate to equilibrium after changes in pH or humidity.

Chimerism and mosaicism: within individual diversity

Chimeras and mosaicism

We started by individuals, we then climbed to populations and we will now go all the way down to within individual. It's just that individual is not any more what it used to be. It shifted from being vegetative cell to being a multicellular slug/fruiting body. This multicellular stage raises questions on how diversity is regulated within a single organism. In social amoebae millions of cells aggregate. Very often these cells are not genetically identical; up to 9 different strains have been found within a single fruiting body (Sathe et al. 2010). As we have seen, this genetic heterogeneity destabilizes the group through internal conflicts, such as competition through spore bias, and can disintegrate the group through fixation of defecting individuals. Nevertheless, intraorganismal genetic heterogeneity (IGH) is present in almost every organism we have looked at (Rinkevich 2000; Pineda-Krch & Lehtilä 2004). In bacteria *Myxoccocus xanthus* whose life cycle resembles to social amoebae one, genetically different strains aggregate (Velicer & Vos 2009), in red algae spores from different genotypes merge and form a heterogeneous algae (Santelices et al. 2003), in tunicate *Botryllus* multiple colonies fuse to form a single one (Rinkevich 2005), and in vertebrates (humans including) individual heterogeneities arise through cell mutations or cell exchange during pregnancy (Nelson 2002; Rinkevich 2000). Weather it is a fusion of different individuals (chimerism) or cell mutations (mosaicism) the end result is a genetically heterogeneous individual. Now that we know that IGH is not an exception to the rule it becomes interesting to question the costs and benefits it brings. Main costs of IGH are already discussed cell parasitism/defection/cheating (reviewed in (Pineda-Krch & Lehtilä 2004)).

Benefits of being chimeric

Interestingly chimerism does not bring only cost. Benefits of chimerism include increased survival and growth in algae (Santelices 2001), better tolerance to environmental variability in tunicate *Botryllus* (Rinkevich 2005) and asynchronous flowering in figs (Thomson et al. 1991). For social insect colonies, extensive literature has demonstrated that colony genetic heterogeneity reduces parasite transmission – through differential parasite resistance of different genotypes (Shykoff, Jacqui A. and Schmid-Hempel 1991), increased foraging rate and colony growth (Mattila & Seeley 2007), influences division of labor (Smith et al. 2008) and overall contributes to colony homeostasis (Oldroyd & Fewell 2007). Finally, diverse bacterial biofilms of *P. flourescens* have decreased invasion rate of defecting individuals (Brockhurst et al. 2010). In *D. discoideum* chimeric slugs bring benefits of increased size (Foster et al. 2002). This is simply due to the increase in size due to non-exclusion of genetically different cells upon aggregation. Fitness advantage of increased size comes through increase in dispersion through increased slug speed and taller stalk. Therefore, although monoclonal slugs move faster than chimeric ones of the same size, chimeric ones make bigger slugs that compensate for the slower speed (Castillo et al. 2005).

All these examples illustrate the wide range of benefits caused by genetic heterogeneity. As often done in evolutionary biology, we can only hypothesis that trade-offs between the costs and the benefits will shape the diversity at both population and individual level. It is interesting to think that decreased genetic diversity had been very important during the origins of social groups and multicellularity (W. O. H. Hughes et al. 2008), while once the group integrity had been established diversity evolves as an adaptation for better coping with changing environment.

Individuality and levels of selection

What is an individual?

When discussing within-individual genetic diversity we gave examples of multicellular organism such as humans, social insects and bacterial biofilms. Is it all right to call a bee colony an individual? Is it all right to call vegetative amoebae cell an individual and at the same time call amoebae slug an individual? What is an individual? Genetically heterogeneous organism and organism at the border of unicellularity and multicellularity make us pose these questions. There is no definition of what an individual is and there are hundreds of them at the same time (check (Strassmann & Queller 2010) for a list of different definitions). Conventionally an individual is defined as "reproductive, physiologically united, autonomous, genetically homogenous and unique entity, which is also the main unit of selection" (Pineda-Krch & Lehtilä 2004). This definition is based the way we think about organism but not on what they truly may be. As pointed out by (Santelices 1999) chimeras, mosaicism and superorganism are *"exceptions that have become too numerous to be regarded only as exceptions."*
Several studies have shown and emphasized that a new way of thinking about individuals is needed for a new definition to be made (Pineda-Krch & Lehtilä 2004; Rinkevich 2000; Clarke 2010; Santelices 1999; Pradeu 2010; Folse & Roughgarden 2010). (Folse & Roughgarden 2010) propose and interesting set of criteria for defining an individual and provide extensive discussion on how these are applied to living organisms. These criteria are: "*1) alignment of the fitness interests of the parts to ensure cooperation, 2) interdependence of the parts on one another for reproduction, such that the whole reproduces itself to create a similar whole entity with heritable fitness, and 3) functional integration and coordination demonstrating adaptation at the level of the whole."* I find that these criteria catch the essence of what an individual is. In addition they provide a complementary framework, rather than exclusion, for many previously stated definitions. *Alignment of fitness* requires no or little conflicts of lower level entities. This is often achieved through genetic homogeneity: unicellular bottlenecks in multicellular development and self/non-self recognition systems. This brings up interesting theories on importance of immune system, as very sophisticated self/non-self recognition, in evolution and stabilization of individuality (Pradeu 2010). From this perspective genetic heterogeneity can be a problem, because it increases conflicts. As we have seen this may be true in some cases but does not need to pose a problem in others,

where heterogeneity increases performance. Therefore, genetic homogeneity is seen more as a tool for aligning fitness interests and not as a requirement for individuality. *Interdependence for reproduction* such that a whole reproduces itself. This is mainly achieved through division of labor, germ-soma division being the most basic one. Division of labor is important because by itself transfers the fitness from lower to higher level entity (Michod 2007). *Functional unity* with all components working together as "*pieces of a machine*" enables the appearance of adaptations at higher levels that are absent at lower levels. Presence of such adaptations means that selection indeed acts at the level of the higher entity. These 3 criteria give us freer, but more accurate definition of individuality in which exceptions such as ant colonies, social amoebae slugs and chimerical algae become parts of the rule. An interesting debate is still going on about modular organism such s trees, fungi and colonial invertebrates (spongy, corals, ascidians) (Folse & Roughgarden 2010; Clarke 2010).

Levels and units of selection

The question of individuality is almost inseparable form the question of levels of selection. Often we hear or read: Evolution acts at the level of individuals. But if there is no definition of individuality how can we state that selection acts at the level of one? If we do not agree on what individual is, we cannot agree on how evolution operates. This has lead to many historical discussions such as the one on kin selection and group selection (discussed in Introduction). Which gets absurd once we agree that they are both talking about the same thing but are using different definitions of individuality. Even if we agree about what an individual is, the level of selection debate is still not over. Over the history of evolution different levels of selection have been proposed: selection acts at the level of genes (Dawkins 1976), cells, individuals (Darwin 1859), groups (Wilson 1975), species (Eldredge & Gould 1972). All these possibilities come from the basic requirements for something to become selective: replication, variability and differential fitness, and we can find all of them for all levels proposed. Many have proposed a unified, rather than divided, framework of all of these theories (Okasha 2010; Rinkevich 2000; Folse & Roughgarden 2010). As previous studies showed individual vs group level of selection can partly be solved by changing the definition of individual and by considering multi-level selection theory (explained in Chapter 4 - Introduction) (Damuth & Heisler 1988). Other levels can be solved by understanding the difference between unit of section and level of selection. The two terms are sometimes used to define the same thing (level of selection), but are much more useful if thinking as two separate definitions (Okasha 2010). Unit of selection is the entity that gets replicated – the replicator (Dawkins 1976), that gets transmitted (Rinkevich 2000), gene being the most well known unit of selection. Level of selection is the phenotype that transmits the information (Rinkevich 2000), the vehicle (Dawkins 1976). It all comes down to that more familiar expression: Selection acts on phenotypes but it is the genotypes that get selected.

Only considering all these arguments together can we fully understand how selection operates. Such multiple level selection ensures that one level does not exploit on other levels (gene replication having fitness costs for individual). Taking the new vision of individuality, individual stays the main, but not only, level of selection. Since selection acts on phenotypes, genes are in most cases units, rather than the level, of selection.

Chapter 5 Dynamics of Aggregation in *P. pallidum*

Work in progress….

ABSTRACT

We report a new aggregation dynamics in *P. pallidum* species. During standard aggregation cells aggregate and aggregates develop into slugs. We discovered that in *P. pallidum* aggregation occurs through two or more steps of population aggregation and disaggregation. Finally, stable aggregates emerge and develop into slugs. This was documented using time-laps microscopy with image acquisition every 5-30 min. Overall 4 different aggregation dynamics were observed; one-step, two-step, two-step: aggregation-disaggregation-aggregation and multiple-step aggregation. Which dynamic will occur depended on environmental conditions. We suggest that within population heterogeneities in starvation rate provide conditions for more complex aggregation dynamics. Our movies show that all aggregation steps before the final involved only a part of the population, the rest of the cell acted as non-aggregating cells. The final step of aggregation resembles the most to standard aggregation dynamics, with streams of aggregating cells and aggregation of the whole population. Preliminary quantitative analysis of two-step aggregation showed that: i) aggregate size distribution in skewed towards aggregates of small size, ii) aggregates are randomly situated in space and iii) there are no differences in size distribution and spatial organization between first and second aggregation step.

INTRODUCTION

I got interested by the phenomenon of aggregation in P. pallidum because it was completely different from one thousand D.discoideum examples I have previously seen. Aggregates appear, they disappear and they reappear in the form of new or old aggregates. First movies were quite magical even though I know magic is not the word we use to explain things in science. I guess pure curiosity for something new that nobody has noticed before was the main motivation to continue exploring the subject. In addition it gave me the opportunity to think about concepts of population level organization and population level optimization.

Aggregation in social amoebae

When there is no more food amoebae start aggregating. Aggregation is based on cell chemotaxis; cells move in the direction of a given chemical signal. Different social amoebae species differ in the chemo-attracting signal (often called acrasins) they use. Phylogeneticly social amoebae can be divided into 4 groups by the order of their genetic distance, group 4 being the most phylogenetic recent and group 1 being the most ancient one (Schaap et al. 2006). Species belonging to group 4, *D.discoideum*, *D. mucoroides*, *D. rosarium* and *D. purpureum* being some of them, almost all use cAMP as chemo-attractant. Other groups use 3 other know attractants; glorin, pterin and folate, or a yet unknown molecule (Schaap 2007). Recently it has been shown that glorin is a cell chemo-attractant used by many ancient group species (group 1 and 2) (Asghar et al. 2012). Although, it's direct role in aggregation was only shown for species *P. pallidum, D. caveatum* and *P. violaceum*. For many species attractant is still unknown.

Evolution of signalling system

Differences, between species and between phylogenetic groups, in the use of chemo-attractants give insights on how the signaling during aggregation and development evolved. Pauline Schaap's group did most of the work on this subject and phylogeny of social amoebae in general. She gives a very nice overview of the field that is schematically represented in Figure 5-1 (Schaap 2011). Microcystis is thought to be one of the most primitive strategies for cell survival in social amoebae (more details in Chapter 1: General Introduction – Social amoebae). By looking at the microcyste formation, in different social amoebae species, it was discovered that in all species cAMP plays a regulatory role in formation and germination of microcystis (Ritchie et al. 2008). Thus, the most primitive survival strategy was regulated by cAMP. Duplication of cAMP receptor gene cAR in groups 1-4 opened new possibilities for the use of cAMP. Using cAMP analogs, that block cAMP binding, and cAMP receptor mutants it was shown that species in groups 1-3 use cAMP signaling for cell differentiation and fruiting body morphogenesis (Alvarez-Curto et al. 2005). During aggregation phase these species use molecules such as glorin, pterin and folte to direct cell movement. In the most recently

evolved group 4, cAR1 cAMP receptor and other developmental genes came under expression control of early aggregation genes. In this way cAMP became both chemo-attractant during aggregation and regulator of development, as in group 1-3 (Schaap 2011). This gives a very nice insight on the evolution of aggregation and development in social amoebae.
All the research on aggregation was done on *D. discoideum*, group 4, species and thus the role of cAMP in aggregation. We will therefore use this system to explain the regulation of aggregation in social amoebae.

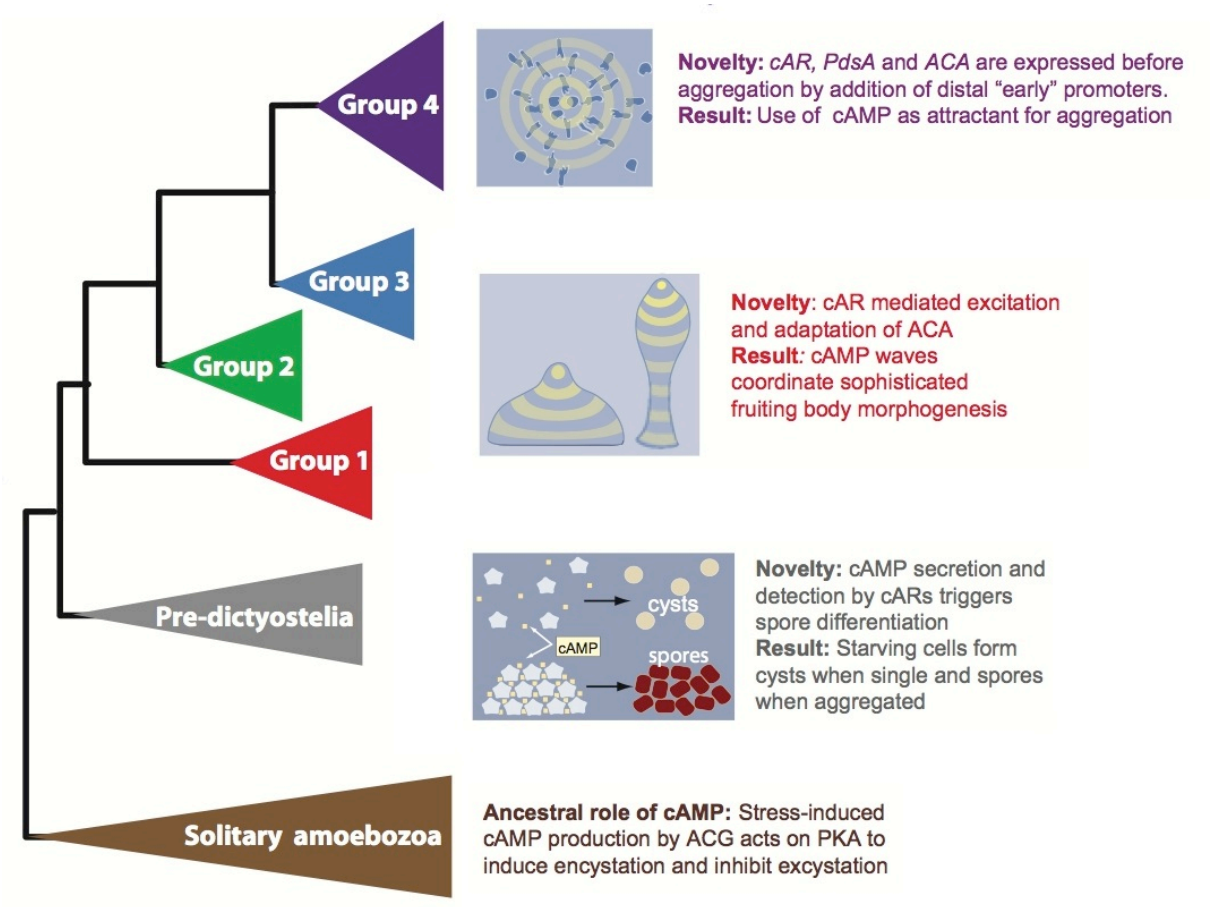


Figure 5 - 1 Evolution of social amoebae cAMP signaling system. In it's most primitive form cAMP served as a regulator of microcyste (endocyste) formation. Extra-cellulary secreted cAMP may have been used in cell decision-making between single cell microcystis and group spore formation. In today species cAMP controls cell differentiation and fruiting body morphogenesis, while molecules such as glorin, pterin and folate direct cell movement during aggregation. Finally, in most recently evolved group cAMP genes came under control of early aggregation genes. This made cAMP a chemo-attractant for aggregation. ACG is highly conserved protein that induces cell differentiation into spores and regulates its germination. PKA is cAMP dependent protein kinase. cAR is a cell surface receptor for cAMP. ACA is an adenylatecyclase that catalyzes conversion of ATP to cAMP. PdsA is a gene producing cAMP phosphodiesterase that degrades cAMP. Figure was adapted with some modifications from (Schaap 2011)

Aggregation in *D. discoideum* – the best studied example

Aggregation with cAMP as chemo-attractant

One of the first signals after starvation is the production and secretion of cAMP. For aggregation to happen threshold level of cAMP is needed (1nM extracellular cAMP). This can only be reached by a population of cells secreting the signal together. This makes aggregation a quorum sensing population level response. Live cell imaging of cells and precise control of cAMP concentrations revelled how exactly aggregation happens (Gregor et al. 2010). Within a population cells start randomly emitting cAMP signal. As they do so extracellular concentration of cAMP rises. After critical extracellular concentration of cAMP is reached, cells start emitting synchronous pulses of cAMP (Figure 5-2A). This leads to even greater increase in extracellular cAMP concentration due to synchronous burst of secretion. The area with more cAMP fires at higher frequencies. This makes it attract even more cells from areas that fire at lower frequencies. The saturating pulse interval that causes cell aggregation is 1 pulse every 6 min. The area that first reaches this pulse frequency is the area that will become an aggregation centre (Figure 5-2C). For aggregation to happen cAMP needs to be secreted in pulses. This is achieved by periods of cAMP production-secretion-removal-production. cAMP removal is performed by a special protein, cAMP-phosphodiesterase (PDE) that degrades cAMP bound to the cell receptors (Kessin 2001). Degrading cAMP has two roles: 1) cAMP receptors become de novo sensible to cAMP and 2) it maintains the cAMP gradient. Without degradation, cell environment would become saturated with cAMP and concentration gradient that directs cell movement would be lost. This chain reaction of synthesis and degradation results in waves of cAMP that guide streams of hundreds to millions of individual cells to aggregation centre (Figure 5-2).

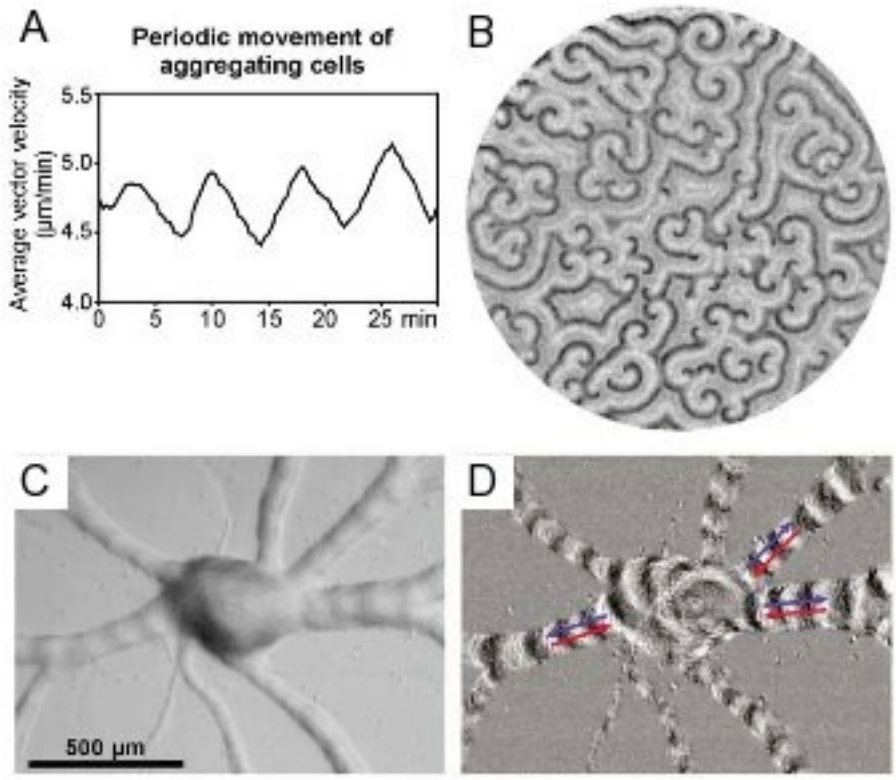


Figure 5 - 2 Aggregation in *D. discoideum*. A) Periodic movement of aggregating cells correspond to periodic cAMP waves, B) Cells plated on agar show dark field waves that correspond to transmitted cAMP waves C) Streams of cells move towards an aggregation center D) Image subtraction shows propagation of cAMP waves that move away from the center (blue arrow) and cells that move towards the center (red arrow). Image from (Dormann & Weijer 2006).

Cell density sensing factor CMF

In addition to cAMP concentration, there is another quorum sensing molecule that controls onset of aggregation. Starved cells secrete glycoprotein called conditioned medium factor (CMF). Threshold concentrations of extracellular CMF need to be reached for cAMP induced aggregation to start. This is reached by increasing the number of cells that secrete CMF. Therefore, CMF acts as a signal that indicates the density of starved cells. For this reasons amoebae cannot aggregate at low densities (Sup. Figure 3-3). This cell density regulated system was proposed to assist synchronous onset of cAMP emission (Jain et al. 1992). Cells starve asynchronously and secrete CMF, but cannot aggregate until sufficient number of starved cells has been reached. When threshold level of cells, and therefore CMF has been reached, all cells become sensitive to cAMP at the same time. This synchronous population level switch increases the synchrony of cAMP emission, threshold levels of cAMP are reached faster and aggregation can begin. CMF controls cAMP mediated aggregation by being involved in cAMP induced signaling

pathway. In the absence of CMF cAMP binds to its receptor carA1 but there is no signal transduction and therefore no aggregation (Haastert et al. 1996). Increasing the concentration of CMF increases the effectiveness of cAMP mediated signal transduction. By this way amoebae cells can sense the density of starved cells in a population and respond to it.

Regulation of aggregate size

Cell counting factor CF

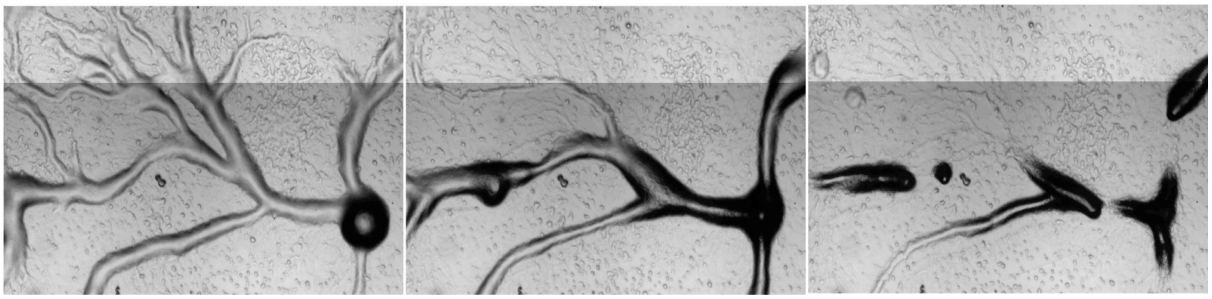


Figure 5 - 3 Regulation of aggregate size in *D. discoideum*. At high cell densities aggregate streams break up due to accumulation of cell counting factor CF. By sensing CF concentration and modifying their behavior according to it, amoebae regulate size of an aggregate.

When working with *D. discoideum* it is easy to observe that from time to time when aggregation streams are very big they break down into smaller ones (Figure 5-3). This happens because a population has a way of counting number of cells in an aggregate. Once there are too many cells, as in the case of big aggregation streams, the population readjusts its size by breaking into smaller aggregates. Regulation of cell number is achieved through a cell secretion of a protein complex called Counting Factor (CF). This protein complex was discovered with an isolation of a mutant strain, called *smlA-,* that forms abnormally small aggregates (Brock & Gomer 1999). When *smlA-* cell conditioned medium was added to wild-type population it caused wild-type cells to make small aggregates as well. The cell extract that caused this phenomenon was purified and called Counting Factor CF. Exact composition of this protein complex is unknown but several studies showed that some of its components are coutin, CF45-1, CF50 and CF60 proteins (Gomer et al. 2011). Concentration of CF regulates aggregate size; high concentrations of CF cause small aggregates and low concentrations causing abnormally big aggregates (Gomer et al. 2011).

Mechanisms of CF action

Over the past decade a number of mathematical and experimental studies revealed in more details how CF regulates aggregate size. Each cell secretes CF, this means that the more there is cells, the higher concentrations of CF. By sensing the concentration of CF a cell can sense the population size and modify its behavior. They do so by changes in cell-

cell adhesion and cell movement. Mathematical models and experimental measurements of CF concentrations, cell motility and cell-cell adhesion showed that high concentrations of CF decrease cell-cell adhesion and increase the randomness of cell movement (Roisin-Bouffay et al. 2000). If there are too many cells in the aggregate stream, concentration of CF will be high which will cause cells to be less adherent to surrounding cells and to start moving around more randomly. This will eventually led to breaking up of the aggregate (Dallon et al. 2006). Experimental measurements have shown that CF affects cell movement and cell-cell adhesion by changing cytoskeleton organization (Tang et al. 2002) and interfering in cAMP transduction pathways (Jang et al. 2002).

Sum up of aggregate size regulation

Regulation of aggregate size is thought to be important for optimal spore dispersion. Small aggregates will not lift the spores high above the ground and therefore spore dispersion will be low. Too big aggregates risk of collapsing due to too long stalks and too heavy spore masses. By regulating group size cells can optimize their dispersal (Gomer et al. 2011). CMF factor insures that aggregation occurs only when certain cell density is reached, ensuring minimal aggregate size. Cell counting factor CF further adjusts aggregate size to ensure optimal spore dispersal.

Aggregation in *P. pallidum*

Glorin

In this study we focus on aggregation in less known species *Polysphondilium pallidum.* As mentioned, this species uses glorin and not cAMP as chemo-attractant. Using biochemical methods and mass spectrometry it was shown that glorin is a small peptide made up of two amino acids, glutamic acid and ornithine (Shimomura et al. 1982). When purified and chemically synthesized it attracts single *P. pallidum* cells. (De Wit et al. 1988) reported the presence of glorin degrading enzyme glorinase that is thought to perform similar role to cAMP-phosphodiestease: it degrades the chemo-attractant making cell receptor de novo sensible to aggregation signal and maintaining the chemo-attractant gradient.

D factor and cAMP

In *P.violaceum* species, which also uses glorin, two additional molecules are important in aggregation: D factor and cAMP. D factor induces aggregation (Newtha & Hannaa 1984), this leads to production of cAMP that inhibits aggregation in surrounding territory (Hanna et al. 1984). It has been suggested that this "ying-yang role" of D factor and cAMP is important in regulating aggregation territories and inhibition of creation of new aggregates in surrounding space (Hanna et al. 1984). The presence of D factor and cAMP in aggregation phase was not reported for *P. pallidum* species. Given that both *P. violaceum* and *P. pallidum* use the same chemo-attracting molecule, glorin, it is possible

that they also share other aggregation important mechanisms. It is sure that cAMP is not involved in *P. palidum* aggregation; null mutants for cAMP receptors cAR, and treatment with cAMP analog, SpcAMPS, which block cAMP receptor activity, showed no effect on *P. pallidum* aggregation. Expression of cAR receptors in *P. pallidum* starts at the end of aggregation which does not exclude the possibility that as in *P. violaceum* it is involved in regulation of late aggregation (Alvarez-Curto et al. 2005). Most mechanism of aggregation in *P. pallidum* remain unexplored.

Our focus: dynamical aggregation in *P. pallidum*

The focus of our interest was aggregation in *P. pallidum* species. When filmed with a time-laps microscope set-up we observed an up to now not described aggregation phenomenon (Figure 5-4, Sup. Movie S5-1). As in *D. discoideum*, starved cells aggregate. This first aggregates are not stable, they tend to disaggregate and cells seem to get "absorbed" by neighboring aggregates. Similar aggregation dydnamics was observed in *D. minutum* species (personal communication V. Nanjundiah) Until know, based on *D.discoideum* aggregation, it was thought that aggregation is a one step process of aggregate formation and maturation. In this preliminary work, we present the first attempts in understanding dynamics of observed *P. pallidum* aggregation. We qualitatively describe the system using movies acquired by time-laps microscopy. We test different quantitative measurements of aggregation and discuss their limits. We are mainly interested in how this dynamical/unstable aggregation affects population level organization. More precisely, how does aggregate size and spatial organization changes from one aggregation to the next one.

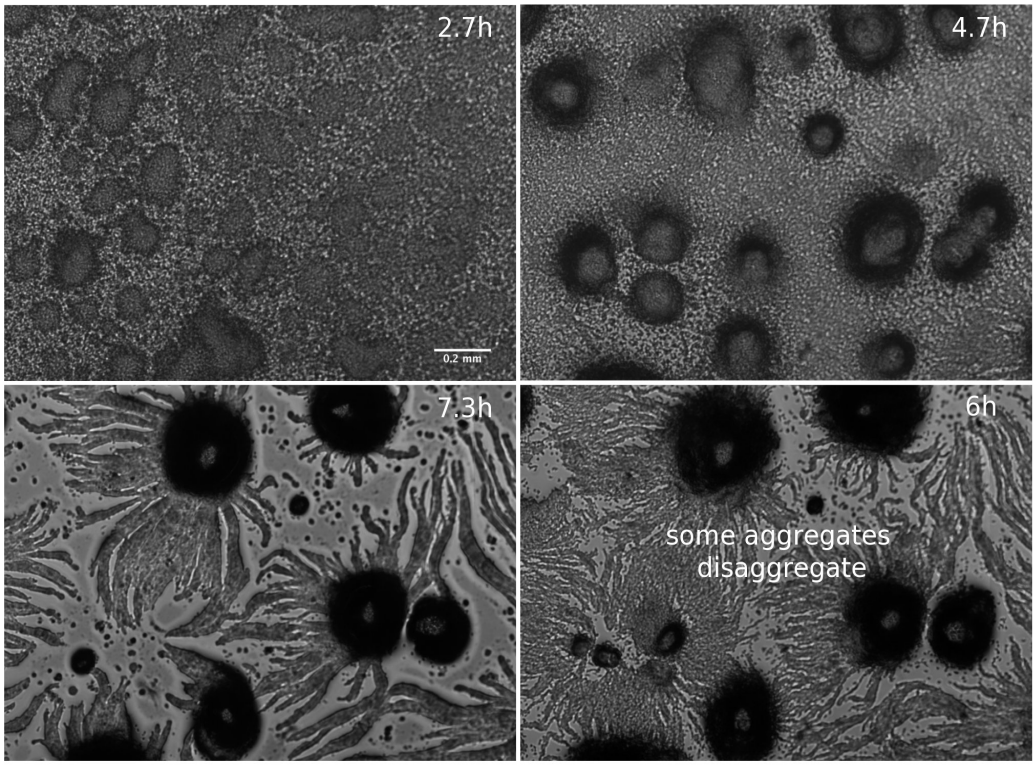


Figure 5 - 4 Dynamical aggregation in *P. pallidum*. When starved, *P. pallidum* cells aggregate. This first aggregates are not stable, they tend to disaggregate and cells seem to get "absorbed" by neighboring aggregates together with stream of other aggregating cells.

RESULTS

Qualitative description

4 different aggregation types

We first observed aggregation in *P. pallidum* as a two-step aggregation described in Figure 5-4 and Sup. Movie S5-1. Cells create first aggregation centers; some of these aggregates will disaggregate and their cells will go to remaining aggregates, together with other surrounding cells. We call this type of aggregation a two-step aggregation. Over the range of experiments preformed we discovered that this is not the only type of aggregation in *P. pallidum*. Figure 5-5 presents a sum-up of 4 different aggregation types we observed: one-step, two-step, two-step: aggregate-disaggregate-aggregate and multi-step aggregation. One-step aggregation is a typical *D. discoideum* aggregation where aggregates do not disaggregate. Aggregates appear and maturate into fruiting bodies (Figure 5-5A). Aggregate-disaggregate-aggregate is a form of two-step process described at the beginning (Figure 5-5C). The difference is that in this case all aggregates disaggregate followed by emergence of new aggregates. The new aggregates may or may not be at the same place as the previous aggregation centers. The fourth aggregation consists of multiple aggregation – disaggregation steps (Figure 5-5D and Sup. Movie S5-2). It can be described as repetitive two-step aggregation in which aggregates appear, disaggregate, appear, disaggregate, appear and disaggregate until the final aggregation step is reached when a few aggregates will "consume" all the cells and develop in to fruiting bodies. By overlapping images at different time points we can see this better (Figure 5-6).

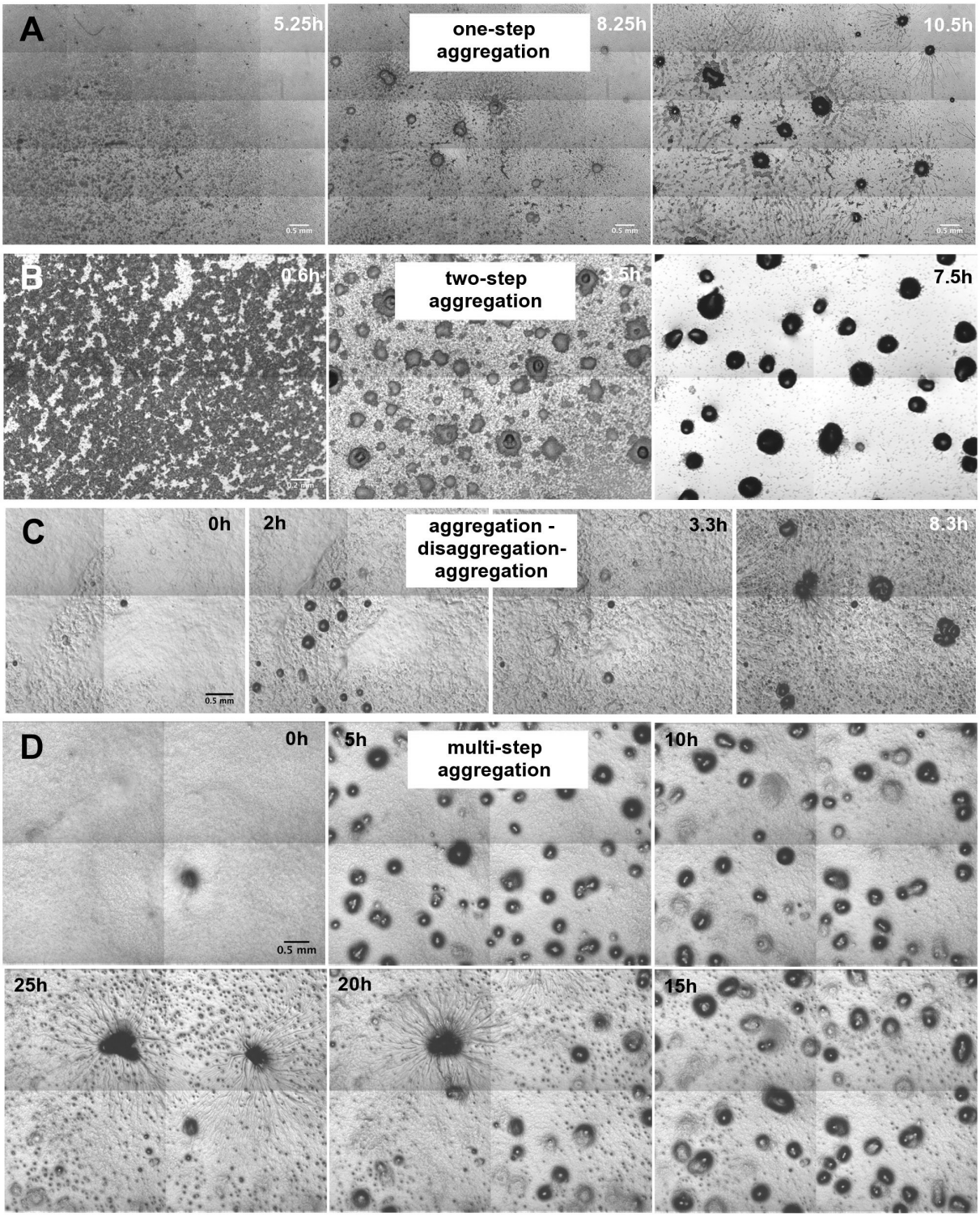


Figure 5 - 5 Different aggregation dynamics in *P. pallidum*. A) Standard social amoebae aggregation is a one-step aggregation where all aggregation centers appear almost simultaneously and proceed to development. B), C) and D) are more complex aggregation types that include aggregate - disaggregate dynamics.

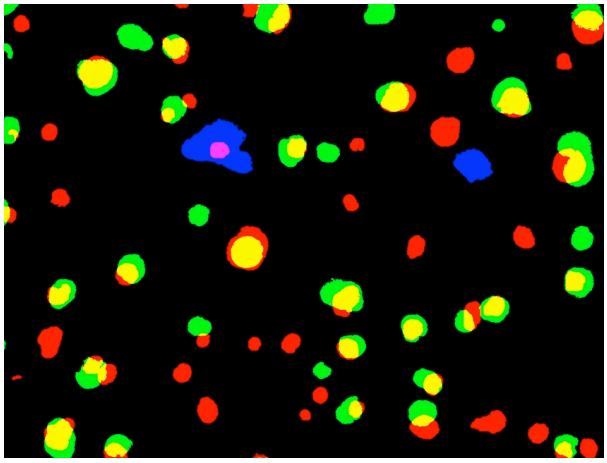


Figure 5 - 6 Changes in aggregate distribution during multiple aggregation. Images from different time points were superimposed to show how aggregates appear disappear/disaggregate and new ones appear. Each color represents aggregate distribution at one time point, red t= 5h, green t=10h and blue, final aggregation, t=25h from the beginning of starvation. Yellow is the result of the overlap between red and green aggregates, these are the aggregates that did not disaggregate in-between t=5h and t=10h. We see that aggregates in the final aggregation appear late in the aggregation and are not always the result of growth of early red or green aggregates.

Environment affects which aggregation type will occur

The 4 different aggregation types were mainly associated with different environmental conditions – different starvation protocols. Two starvation protocols were used: 1) sudden starvation of exponentially growing cells and 2) gradual starvation on bacteria (see Chapter 2: Materials and Methods). Sudden starvation results in sudden transition from exponential growth, in nutrient rich medium, to starvation. Gradual starvation is a more natural starvation during which cells gradually consume bacteria, reach stationary phase and trigger starvation. These tests were inspired from our results on non-aggregating cells in Chapter 3 where different starvation protocols effected non-aggregating cells fraction and therefore aggregation dynamics. In the case of *P. pallidum* different starvation protocols had a clear effect on the type of aggregation. Sudden starvation experiment yielded mainly one-step aggregation and occasionally two-step aggregation dynamics. On the other hand plating cells on bacteria consistently gave one of the other 3 more dynamical types of aggregation. Often different aggregation types would appear on spatially distant areas of the same plate. This suggests that differences in starvation protocol have a strong effect on aggregation dynamics in *P. pallidum*. The two starvation protocols mainly differ in cell starvation state, which was rather synchronous is sudden starvation and more gradual and asynchronous in gradual starvation on bacteria. Therefore, homogenous population with synchronous starvation

may favor one-step aggregation, while heterogeneous population with asynchronous starvation may cause more complex aggregation dynamics.

Population dynamics

By carefully looking at movies reconstructed from our time-laps microscopy we can extract some information on population dynamics during aggregation in *P. pallidum* (Figure 5-7). During first aggregation only a part of the population aggregates, the rest of the cells stay outside of the aggregates and form a dark lawn of non-aggregating cells. This step is followed by disaggregation of certain aggregates, while there is still lots of non-aggregating cells on the agar. In the final aggregation stage, streams of cells appear (Figure 5-4), most of the cells aggregate and the last aggregates disaggregate. Some cells never aggregate as in the case of non-aggregating cells in *D. discoideum* from Chapter 3.

Cells do not always go to the closest aggregate

Low cell density conditions also allowed us to see how cells from disaggregated aggregates get distributed between surrounding aggregates. In Figure 5-7C we indicate a common case where cells from single aggregate go to the closest aggregate but also to more distant aggregates. If all aggregation centers emitted the same aggregation signal and if strength of aggregation signal decreases with distance we would expect that most of the cells would go to the closest aggregate. Example from Figure 5-7 indicates that the situation is more complex and that cells get distributed among differently distant aggregates. This suggests that aggregates emit signals of different strength and that there is a competition between aggregates for the size of aggregation territory. One possible parameter affecting aggregation signal is aggregate size. There is no literature, but we can imagine that: the bigger the aggregate the more cells are producing the aggregation single, and overall aggregate is emitting a stronger signal. Although this may be the explanation in some cases, in Figure 5-7 all aggregates are almost the same size. This indicates that additional parameters may affect aggregation strength. We can speculate that the combination of aggregate size, aggregate distance and yet unknown parameters affect strength of aggregation single. According to the sensed strength of the signal a cell decides in which direction to move.

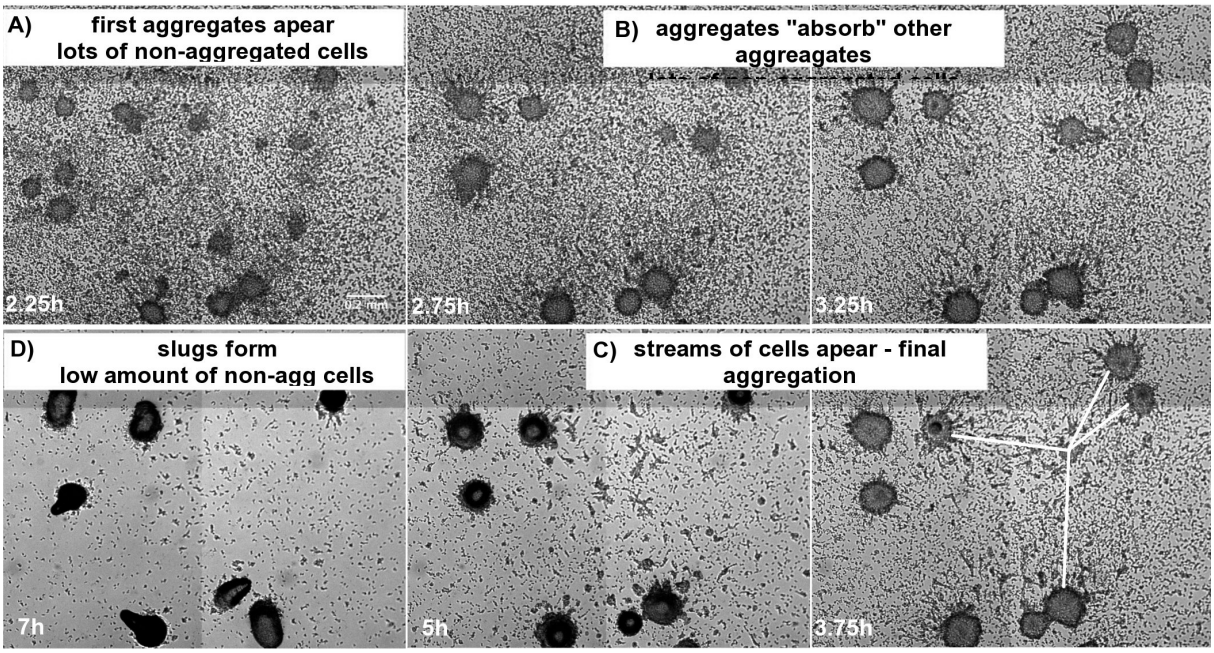


Figure 5 - 7 Population dynamics during two-step aggregation. A) First aggregation step includes only a part of the population. Dark background indicates a tick lawn of non-aggregating cells. B) With time aggregate some aggregates will disaggregate. During this period we still see a tick lawn of non-aggregating cells. C) Suddenly, streams of cells appear and most of the non-aggregating cells go to aggregates. Aggregate rearrangement continues and some aggregates disaggregate. Other aggregates attract their cells. As show with white lines, these cells are attracted to the closest aggregate but also to more distant aggregates, indicating the possibility for competition between aggregates. D) At the end of aggregation slugs form, white background indicates that most of the cells have joined the aggregate. Grey dots are the cells the non-aggregating cells that did not aggregate.

Quantitative analysis

In previous section we gave descriptive analysis of the aggregation in *P. pallidum*. Following these observations we wanted to establish a quantitative way of measuring aggregation dynamic. To do this we looked at changes in: 1) number of aggregates, 2) aggregate size distribution and 3) aggregate spatial organization. In our preliminary results, we propose some ways of measuring these parameters and point to pros and cons of each of these tests. All analysis were performed on processed binary images, like the ones in Figure 5-8 and Chapter 2: Materials and Methods Figure 2-3.

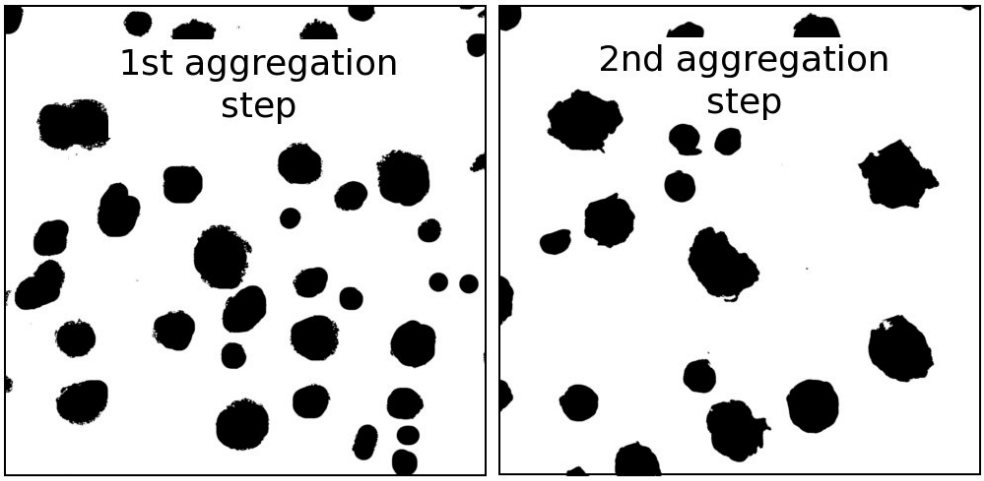


Figure 5 - 8 Processed images used for analysis of two-step aggregation. Aggregate number, size distribution and two-point correlation were performed on these kind of binary images.

Change in the number of aggregates over time

We quantified the number of aggregates at each time step for three different aggregation types: one-step, two-step and multiple-step aggregation (Figure 5-9). This showed that in one-step aggregation aggregates appear almost simultaneously. On the contrary in both two-step and multiple-step aggregation number of aggregates increases and decreases gradually. While in two-step aggregation this happens within 4-5h, during multiple-step aggregation this spans over 25h. Therefore, in multiple-step aggregation there is a long period during which aggregate number gradually decreases while aggregates form, disaggregate and new aggregates form. The fact that in Figure 5-9 both one-step and two-step type reach the same final number of aggregates is rather an experimental chance than a indicator of population regulation of aggregate number. Other one and two-step cases do not show this coincidence in number of aggregates. Tracking number of aggregates over time gives information on the dynamics of aggregate formation over time. It could also be used to quantitatively distinguish different aggregation types. The difficulty of this method is the sometimes not intuitive identification of an aggregate in the background of non-aggregating cells. This makes the

process hand based and sometimes subjective. Still, even with these errors we can get a good approximation on the dynamics of change in aggregate number.

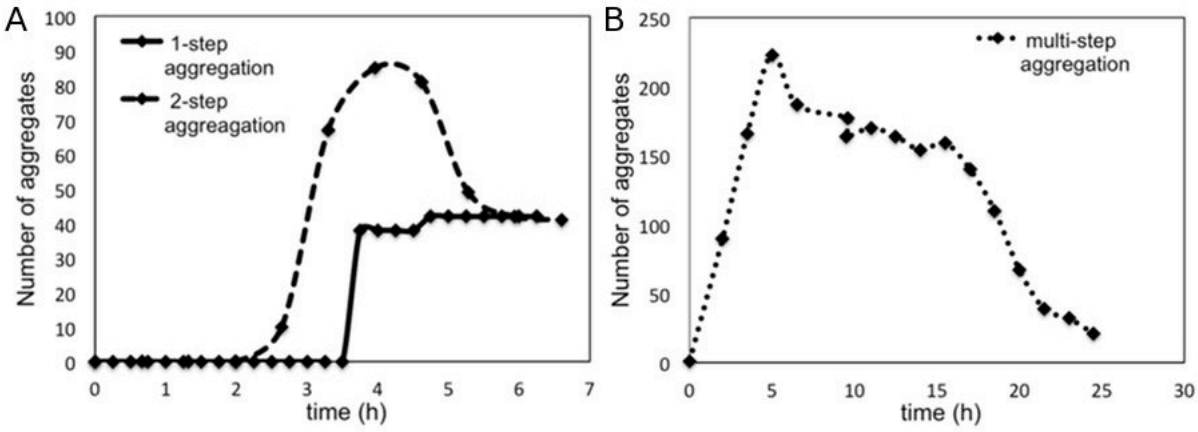


Figure 5 - 9 Change in number of aggregates over time in different aggregation dynamics

Regulation of aggregate size

Presence of cell counting factor like mechanism

In *D.discoideum* protein complex called, CF cell counting factor, regulates size of the aggregate (explained in Chapter 5: Introduction). This mechanism has never been shown in *P. pallidum*. In Figure 5-10 we show that as in *P. pallidum* as in *D. discoideum* big streams break down into smaller ones creating several small aggregates instead of one big. This is a standard *D.discoideum* test for cell-counting phenotype. Thus, we hypothesize that *P. pallidum* could also regulates its aggregate size by a CF cell counting similar mechanism.

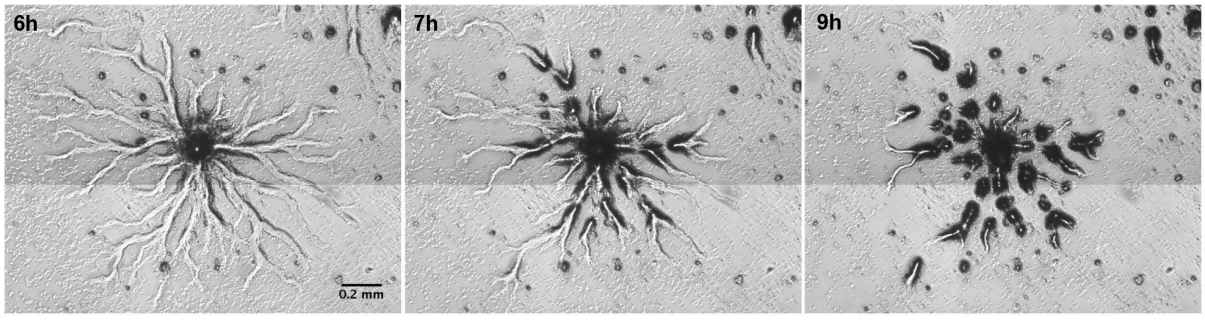


Figure 5 - 10 Regulation of aggregate size in *P. pallidum*. Big aggregates break into multiple smaller ones. This behavior is characteristic for *D. discoideum* population with functional cell counting mechanism and results in aggregate size regulation.

Aggregate size distribution

We were further interested in how the distribution of aggregate size changes during aggregation. If aggregate size is strongly regulated we would expect a biased distribution of aggregate sizes. Variance around the mean could be a way of quantifying the precision of size regulation. A 2D measurement of the aggregate area is taken as a measurement of the aggregate size (see Figure 5-8 for example of images used for analysis). We first look at the aggregate size distribution in a simplest one-step aggregation. In Figure 5-11A we see that based on the 3 experiments it is not clear to which point aggregate size is regulated. It seems to be that within each experiment there is a tendency for certain aggregate size, but with very high size variability. Additional experiments are needed for a conclusion on this issue. Figure 5-11B represents a case of two-step aggregation. Aggregate size was estimated for first and second/final aggregation image. There is a clear tendency for aggregates of certain size, despite the high variability among different experiments. Contrary to our expectations, aggregate size does not change from first to second aggregation. Since in the second/final aggregates are the ones that "absorbed" all the other ones, we would expect them to be bigger and a size distribution to be shifted towards bigger sizes. This is not what we see. One explanation for this may be that at certain size aggregates start growing more in the 3D (height) that in the 2D (diameter). If this is the case, and it seems to be so, measuring aggregate size as aggregate diameter would not be a correct measure. We can therefore only conclude that there does seem to be a regulation of aggregate size in *P. pallidum* and that this regulation is active from the start of aggregation. To understand the change in aggregate size from one aggregation step to another 3D measurement would be needed.

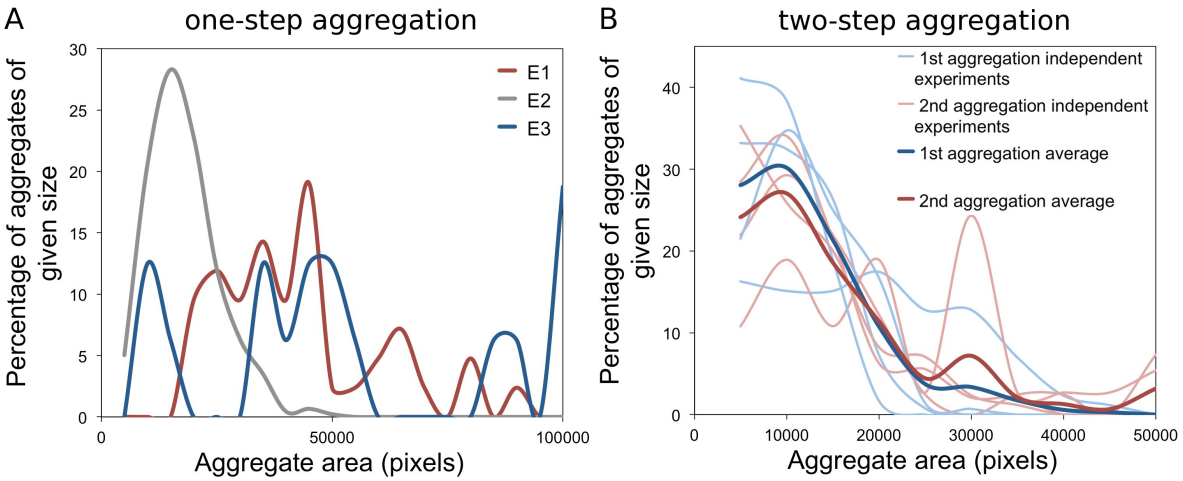


Figure 5 - 11 Aggregate size distribution in one and two-step aggregation types. A) One-step aggregation shows no clear size preference and big between experiment variation in aggregate sizes B) Preference for small aggregate size in 1st and 2nd aggregation step. Average curves in bold are calculated as the mean of all represented experiments.

Spatial structure of aggregation sites

We were particularly interested to see how aggregate spatial organization changes from one aggregation phase to the other. During some aggregation events it seemed that; in the first aggregation step aggregates are randomly (no spatial structure) situated in space, while in the final one aggregates become spatially organized. This impression was supported by some old papers that observed constant size of aggregation territories (Bonner & Dodd 1962; Bonner & Hoffman 1963). We wanted to test these old results with a more quantitative and direct measurement of aggregate size and distance. We decided to measure directly the distance between two aggregates using two-point correlation function.

Two-point correlation function

Two-point correlation function gives the probability of finding simultaneously two points at a given distance r. It is mainly used in the measurements of homogeneity in spacing between galaxies and homogeneity of gels, foams, granular mediums, sand stones etc. (Jiao et al. 2007). When it comes down to image analysis it resembles a lot our aggregate distribution (Figure 5-12).

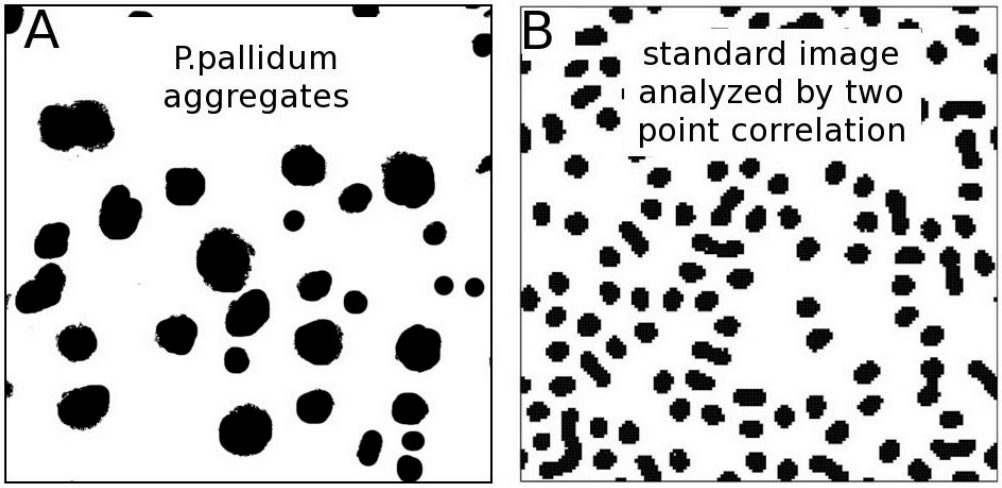


Figure 5 - 12 Processed images used for two point correlation analyses. A) Our *P. palldium* aggregation image and B) computer generated image used as a toy model for two point correlation analysis by (Jiao et al. 2007).

In Figure 5-13 we show two-point correlation function for different spatial organization of aggregates. Let us explain what information we can extract from these representations. Y-axis represent an average probability of finding two points at a given distance x, x-axis. In our case point size is smaller than the size of the aggregate. Aggregate size can thus be considered as distance on x-axis over which there is a high probability of finding particles. The more aggregates have equal sizes the higher the overall probability of finding points within given distance. Distance between aggregates

is empty space, therefore there is 0 probability of finding two points. Depressions and low values in probability therefore represent; distance between the aggregates or very small correlation in aggregate size. In Figure 5-13A cells are equally distant and of equal size. We see that there is a strong correlation for finding aggregates at the distance of around 30 pixels - this is aggregate size, than the probability goes to zero for some time meaning that there are no aggregates in this space – spacing between aggregates. The following oscillations indicate structured presence of neighboring aggregates. In Figure 5-13D aggregates are heterogeneous in size and randomly situated in space, so no clear pattern of aggregate size and distance can be extracted. In between cases of B) heterogeneous aggregate size and homogenous aggregate distance and C) homogenous aggregate size and heterogeneous distance are also presented (Figure 5-13B and C).

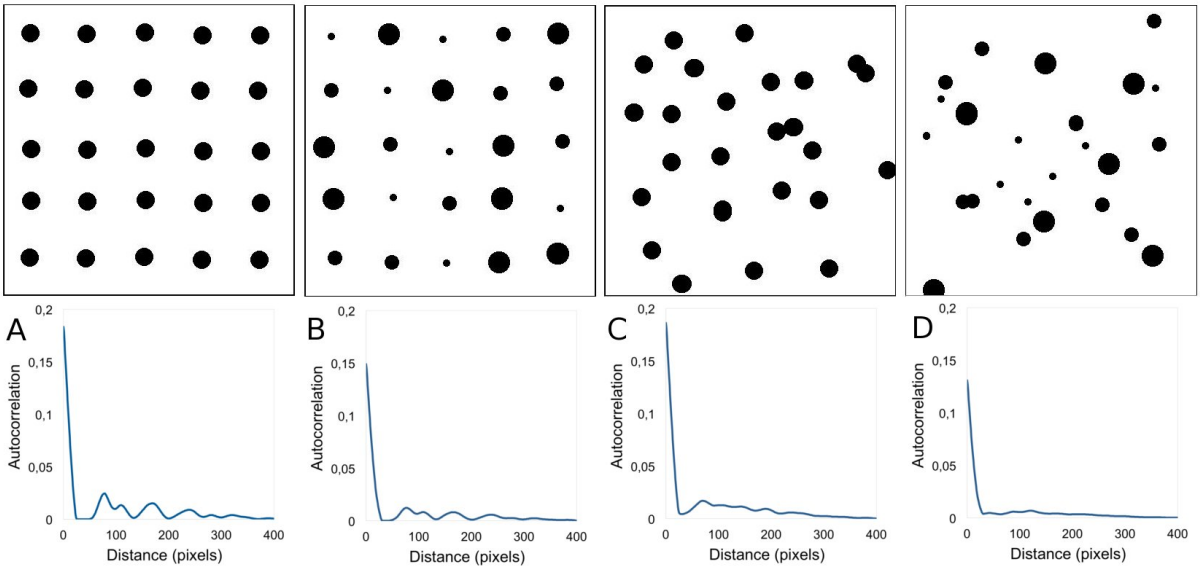


Figure 5 - 13 Two-point correlation analysis of images differing in the aggregate size distribution and spatial structure. A) All aggregates are the same size and are equidistant, B) Aggregates differ in size but are equidistant, C) Aggregates have the same size but are randomly spaced and D) Aggregates differ in size and are randomly spaced.

Problems with two-point correlation analysis

Two-point correlation plugin uses FFT function to speed up the analysis. FFT is a discrete approximation of a real Fourier transform, and would be exact if the number of pixels was infinite and if the objects in our images contained many pixels. In our case, images are around 500x500 pixels and objects area is <500 pixels^2, so there will be discretization artifacts.

Another artifact comes from the effect of number of aggregates on correlation. Since correlation is statistical measure, the more aggregates the higher the correlation value will be (Figure 5-14). This may pose a problem for our measurements because there are sometimes big differences between experiments in number of aggregates. This makes it difficult to compare results from different experiments. In addition, number of

aggregates changes over time; first aggregation has higher number of aggregates than the final one. Thus, we should expect correlation to be lower at final aggregation steps. Therefore, a pattern rather than the values of correlation should be looked at.

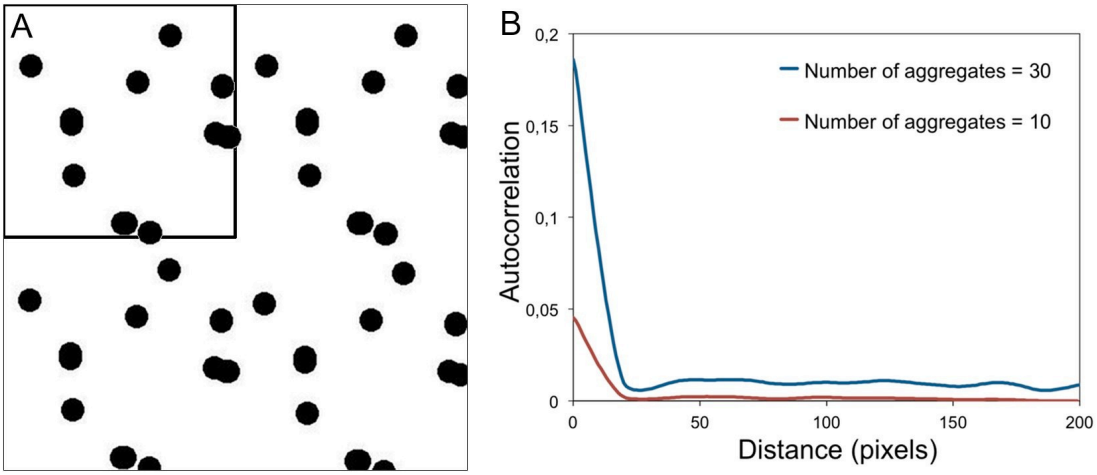


Figure 5 - 14 Number of aggregates effects two-point correlation analysis. A) Hand generated image of randomly situated aggregates of the same size. B) Two-point correlation was performed on the whole image, 30 aggregates, and section of the image, 10 aggregates.

Results

Two-point correlation analyses was performed on aggregation images from one and two-step aggregation cases. Two experiments of one-step aggregation seem to have an oscillatory correlation function in their tails. This indicates that there may be spatial organization of aggregates (Figure 5-15A). The third experiment seems to deviate a lot form this case and makes it difficult to draw any conclusions about spatial organization in one-step aggregation process. Two-step aggregation showed no indications of spatial structure. The fact that correlation function for second aggregation is lower is probably due to smaller number of aggregates, as explained in Figure 5-14.

Aggregate organization in *D.discoideum*

For a comparison, we performed the same tests on *D. discoideum* aggregation. It showed similar distribution of aggregate size to two-step aggregation type in *P. pallidum* (Figure 5-16B). Two-point correlation function was also very similar to *P. pallidum* one with no clear spatial structure (Figure 5-16A). This indicates that both *D. discoideum* and *P. pallidum* show the same pattern in size distribution and spatial structure.

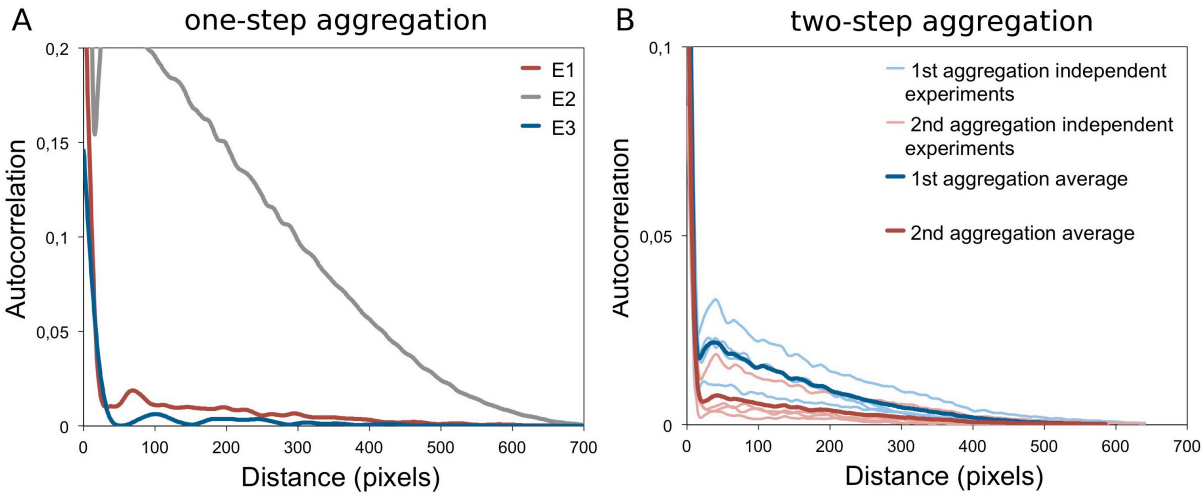


Figure 5 - 15 Two-point correlation of one and two-step aggregation in *P. pallidum*. A) One- step aggregation; experiments E1 and E3 show signs of existence of spatial structure B) two- step aggregation; there are no signs of spatial structure.

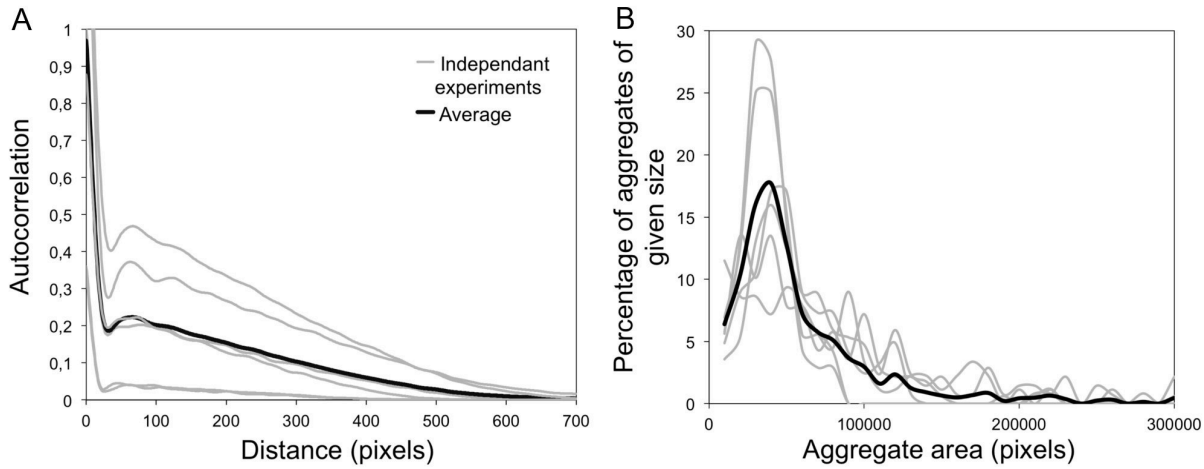


Figure 5 - 16 Aggregation in *D. discoideum*. A) Two-point correlation shows no signs of spatial organization and B) aggregate size distribution is skewed towards small aggregate size.

DISCUSSION

In this study we present a novel dynamics in the aggregate formation in social amoebae species *P. pallidum* (Figure 5-4 and Sup. Movie S5-1 and S5-2). Our aim was to: i) qualitatively describe population level changes during aggregation and ii) quantitatively characterize the dynamics by measuring change in aggregate number, aggregate size and spatial organization. We were particularly interested in population level optimization of aggregate size and structure due to its possible effect of group fitness.

Qualitative description

Standard process of aggregate formation consists of emission of aggregation signal (chemo-attracting chemical), this attracts cells towards aggregation center, aggregates form and develop into a slug or directly fruiting body. Until now it has been thought that all social amoebae undergo the same aggregation dynamics, what we call a one-step aggregation. When observing aggregation in *P. pallidum,* with time-laps microscopy and image acquisition every 5-30 minutes, we have observed a new dynamics in formation of aggregates (Figure 5-4). As in standard aggregation, aggregates form, but they do not directly develop into a fruiting body. Some of the forming aggregates disaggregate and its cells get "absorbed" by surrounding aggregates. "Surviving" aggregates further develop into a slug or a fruiting body. This is what we call a two-step aggregation because it takes two steps of aggregate formation until development into a slug can proceed. In addition, we have observed 3 other types of aggregate: standard one-step aggregation, aggregate-disaggregate-reaggregate and multiple aggregation steps (Figure 5-5). This means that aggregate formation in *P. pallidum* species has a multiple complex dynamics.

Environment affects aggregation dynamic

Which aggregation type will occur depends on the environmental conditions. Sudden starvation conditions gave mainly one-step aggregation and on occasionaly two-step aggregation dynamics, while gradual starvation on bacteria gave consistently two-step, aggregate-disaggregate-reaggregate and multiple aggregation dynamics. The two starvation protocols differed in the cell state, population homogeneity and spatial structure of environment. In sudden starvation experiment cells go suddenly from exponential to starved state. We can assume that due to experimental protocol all cells experience this transition simultaneously. In addition, cells are plated in a very homogeneous lawn with equal cell densities, which means that every cell experiences the same environmental conditions. This high population phenotypic and spatial homogeneity can give more synchronous population onset of starvation, which results in robust one step burst of aggregation. Since this is the standard starvation protocol most studies had probably missed the newly observed aggregation dynamics. On the other hand gradual starvation results in cell-to-cell differences in timing of starvation.

Due to plating technique, spatial structure is rather heterogeneous with areas with higher and lower cell and food densities (Chapter 3, Sup.Figure S3-2). This highly phenotypic and spatially heterogeneous environment gives a population of cells with different starvation levels and aggregation sensitivities which can lead to more dynamic aggregation. We speculate that the more homogenous the environment the more aggregation tends to single step aggregation and the more heterogeneous the environment, the more heterogeneous are the cell states and more dynamic is the aggregation.

Population dynamics during aggregation

Our time-laps microscopy acquired movies give insights on population dynamics during aggregation. We observed that: 1) all the aggregation steps before the final one affect only a part of population, 2) final step seems to be the one that resembles the most to the standard *D.discoideum* aggregation, 3) cells do not always go to the closest aggregate indicating the possibility of competition between aggregates in signal strength.

Dynamic aggregation in other species

Two-step aggregation was also observed, although very rarely, in *D.discoideum* (our observation and personal communication with K.Inouye) and recently in social bacteria *Myxococcus xanthus* by (Zhang et al. 2011a). This indicates that dynamical aggregation may not be a characteristic of *P. pallidum* but a more general property of aggregation with attraction.

Possible mechanism causing observed dynamics

1) Concentration-sensitivity model

It is interesting to speculate on mechanisms producing this aggregation dynamics. One possible scenario could be that dynamics is regulated by differential cell sensitivity to aggregation signal. This could be the consequence of within population differences in starvation rates, as hypothesized in gradual starvation experiment. At the beginning either all cells are not yet sensitive to the signal or the signal is not sufficiently strong to attract all cells. This causes only a population of cells to aggregate. Some aggregates possibly disaggregate because stronger aggregation signals in surrounding aggregates attract their cells. This may also be the reason why disaggregated cells do not always go to the closes aggregate (Figure 5-7C). With time aggregation signal gets higher in concentration, this makes the whole population sensitive to the aggregation. This interplay between signal concentration and cell sensitivity can be a possible explanation for two-step aggregation process. However, additional parameters would be needed to explain why in some cases all aggregates disaggregate and the new aggregates appear as in aggregate-disaggregate-reaggregate model.

2) Traffic jam-attraction model

Second possible scenario is the interplay between chemo-attraction and the traffic jam effect. It has been shown that aggregation can emerge out of random cell movement when cell velocity is dependent on cell density. Aggregates emerge because cells move slower, and therefore stay longer, in areas with higher cell densities, creating a traffic jam (Cates & Tailleur 2013). With time, aggregates appear and disappear, due to the randomness of cell movement. But the end point of pure traffic jam model is infinite increase in aggregate size that leads to one big final aggregate. The study on *M. xanthus* explored to what extent traffic jam effect together with cell-cell attraction can explain two-step dynamics (Zhang et al. 2011a). This gave a monotonical one step aggregation but completely failed at reproducing aggregate disappearance and merging of aggregates. It would be interesting to see if temporally dissociating traffic jam to first aggregation phase and chemo-attraction to second aggregation phase could help in reproducing the observed two-phase aggregation.

Quantitative analysis

Aggregate size distribution and evolutionary consequences

We were mainly interested in how the two-step aggregation dynamic affects population level organization of aggregates. This is interesting because aggregate organization can affect population fitness. It has been suggested that the size of the aggregate affects group fitness. Small aggregates have short stalks that will not lift the spores high above the ground and therefore spore dispersion will be low. Too big aggregates risk of collapsing due to too long stalks and too heavy spore masses (Gomer et al. 2011). By regulating group size, a population optimizes its fitness. In addition, population partitioning into subpopulations of aggregates has consequences on cooperation. Simpson's paradox is one of the cases sole phenomenon of partitioning population into subpopulations of cooperators and defectors enables overall increase of cooperators (Chuang et al. 2009). Cooperation can in addition be stabilized by small group sizes (Hauert, Monte, et al. 2002; Powers et al. 2011) and certain group size distributions (Pena 2012). Overall these studies show that it is reasonable to imagine that cooperative systems have evolved mechanism for regulation and optimization of group size. Social amoebae provide a very nice system to study these mathematical predictions. When aggregating population is repartitioned into distinct subpopulation/aggregates that independently undergo selection. It is known that simple aggregate properties such as size affect group fitness and therefore can be a selective trait. Based on these hypotheses we were interested in effect of aggregation dynamics on aggregate size distribution.

In *D. discoideum* it has been shown that mechanism regulating aggregate size exist (CF protein complex explained in Chapter 5: Introduction). We have observed similar size regulation mechanism in *P. pallidum* (Figure 5-10), supporting the possibility for size regulation. Our aggregate size distribution measurements indicate that in both *D. discoideum* and *P. pallidum* size distribution is positively skewed towards small

aggregates (Figure 5-11B and Figure 5-16B). This indicates the possibility of selection for certain aggregate size in social amoebae. Distribution did not change from first to second aggregation indicating that there is no change in organization over time. The fact that second aggregation did not show increase in mean aggregate size is unexpected. This is probably an artifact of our measurement method. With time aggregates start expanding more in height than in width leaving the impression that their size does not change. A new way of measuring would be needed to correct for this effect. Unfortunately, there is no studies on aggregate size distribution measurement is social amoebae. Any discussion and comparison is therefore limited.

Aggregate spatial structure

In addition to size distribution, we have looked at spatial structure of aggregates. In the 60s a few studies reported a strong tendency of aggregates to be the same size and equally spaced (Bonner & Dodd 1962; Bonner & Hoffman 1963). This was suggested to be the consequence of size of the aggregation territory (all the cells within certain diameter range will finish in the same aggregate). In these studies a size of the stalk was used to calculate number of cells in the fruiting body and from this radius of aggregation was determined. This radius was taken as the distance between aggregates. This is a very indirect measurement of aggregate territory. We wanted to test these old results with a more quantitative and direct measurement of aggregate distance. Our two-point correlation analysis showed no correlation in aggregate distances – meaning that aggregates tend to be situated randomly in space. This was the case for both one-step and two-step aggregation in *P. pallidum* and *D. discoidem* (Figure 5-15 and 5-16A). We have therefore not identified an existence of spatial structure in social amoebae populations. It is important to repeat that two-point correlation analysis is not the most appropriate way of measuring spatial structure in our case. This mainly comes from sensitivity of the method to number of aggregates used and artifacts due to the use of FFT. It still gave some preliminary results and other analysis need to be preformed in the future. Study on *M. xanthus* aggregation used cumulative radial distribution function (CRDF) and found a small increase in spatial organization of aggregates at second aggregation step (Zhang et al. 2011a). Unfortunately, they did not look at how this is coupled with aggregate size distribution. It would be interesting to apply the same CRDF analysis on our data and see what results it would give us on our one step and two step aggregations. Another possible way of analyzing spatial structure would be to look at nearest neighbor distance between aggregation centers. Already this simple analysis would give us the idea on aggregate organization.

CONCLUSION

Overall we report a new aggregation dynamics in social amoebae *P. pallidum*. This new dynamics consists of two or more dynamical cycles of population aggregation and disaggregation, before the further development into a fruiting body proceeds. We present the first attempts to describe this behavior both qualitatively and quantitatively. Our preliminary results give some insights on population dynamic and changes in aggregate number, size and spatial organization. More detailed analysis, replicates and strictly controlled experimental conditions are needed for any solid conclusions to be made.

PERSPECTIVES

Aggregation dynamics observed is a very interesting from both biology and physics perspective. We are very interested in explaining mechanistically how such behavior emerges. To do this we propose two possible models: 1) interplay between strength of aggregation single and cell sensitivity, and 2) interplay between traffic jam effect and attraction directed aggregation. For example, we can imagine testing traffic jam hypothesis by looking if aggregation emerges in liquid nutrient rich cultures with high cell densities. Overall, these are only conceptual frameworks and we need to develop mathematical models and experiments to test them. Developing such models and experiments is a long-term project by itself that we hope to continue exploring in collaborations with specialists in mathematics and physics. We have already discussed the subject with Jean-Paul Rieu's group at Laboratoire Physique de la Matière Condensée et Nanostructures in Lyon and Julien Tailleur at Laboratoire Matière et Systèmes complexes in Paris.

From an evolutionary perspective, population partitioning into distinct subpopulations (aggregates) that face selection independently has evolutionary consequences that have rarely been looked at in experimental system. Here we propose the use of aggregation in social amoebae as a very nice experimental system to test these mathematical predictions. It is easily reproducible in the lab and simple properties such as aggregate size are known to affect group fitness.
In this Chapter we explore different ways of quantifying this population partitioning and point to some weaknesses (such as importance of 3D measurements as aggregate size estimator). Our results are very preliminary and variation between experiments is high. More experiments with strictly controlled cell and bacterial densities are needed to decrease the experimental variation. Our quantitative analysis measurements also need to be improved. We need to develop a plug-in for automatic image processing – most important is the automatic detection of aggregates in the dense background of non-aggregating cells. Second, spatial organization of aggregates needs to be tested using other approaches. First possibility is to look at nearest neighbor distance and the use of

cumulative radial distribution function (CRDF) as was done in (Zhang et al. 2011b). We hope in developing further some aspects of this work with Silvia de Monte's group at Laboratory of Ecology and Evolution in Paris.

SUPPLEMENTARY MOVIES

Sup. Movie S5-1. Two-step aggregation dynamics during *P. pallidum* aggregation

Sup. Movie S5-2. Multiple-step aggregation dynamics during *P. pallidum* aggregation

CONCLUSIONS AND PERSPECTIVES

This thesis represents our work on different aspects of multicellular/cooperative behavior in social amoebae under the evolutionary framework. Evolutionary studies on multicellularity in social amoebae mainly focus on reproductive spore to sterile stalk cell fate. In this thesis we emphasize the importance of looking at other stages of the life cycle. This broader perspective gave us new insights on cooperation and opened new research possibilities. In our work we focused on population-level phenomenon during aggregation phase (Chapter 3 and 5) and interactions over the entire life cycle (Chapter 4).

In Chapter 3 we explore the neglected population partitioning into multicellular/aggregating and unicellular/non-aggregating cells. We describe phenotypically and genetically non-aggregating cell proportion in *D. discoideum* species. We further propose it as a population-level adaptation to fluctuating duration of starvation periods. Population partitioning into multicellular and unicellular state could be an unique example of intersection of microbial cooperation and bet-hedging, two evolutionary concepts whose interactions yet need to be explored. For example, it provides an experimental framework for studying mathematically proposed effects of volunteering and optional participation in social games. In addition, the behavior itself is still largely unexplored and many genetic studies are to be done to understand mechanisms behind population partitioning into two cell fates.

In Chapter 4 we look at the whole life cycle, with multiple competition points, as a common framework for addressing issues of genetic diversity and cooperation in social amoebae. Our computational results showed that multiple competition points could eliminate "social winners". Though we failed to explain strain coexistence. Long-term experimental studies of strain competition are needed to truly confirm these results. Although preliminary, our results emphasize the importance of integrating species ecology in cooperative studies. A more eco-evolutionary view is needed for full understanding of mechanisms driving evolution and maintenance of cooperation.

In Chapter 5 we focus again on aggregation. We have discovered a new aggregation dynamics in *P. pallidum* species. During aggregation population goes through several cycles of aggregation and disaggregation before proceeding with development. Our preliminary results give first insight into population dynamics during aggregation, aggregate size and spatial distribution. More controlled experiments need to be repeated and new analysis methods need to be developed for further analysis. However, this opens new research questions on complex mechanisms directing cell aggregation from both biological and physics perspective. From evolutionary point, aggregation presents a case of population division into subpopulations that experience evolution independently. Mathematically many aspects of such population partitioning has been shown to be important for cooperation. We propose aggregation in the social amoebae as a very good model system for experimentally testing these predictions.

Overall, we show that the social amoebae are an excellent experimental system for looking at population-level phenomenon such as aggregation, cooperation and bet-hedging. They are easy to cultivate in the lab, have short generation time, short life cycle and simple properties such as aggregate size are known to affect group fitness. Our time-laps microscopy set up gives strong technical support for such observations. In addition, our low ratio of fluorescence cell imaging allows simultaneous tracking of individual cell behaviors and population-level phenomenon.

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