

## Group Maintenance in Aggregative Multicellularity

Israt Jahan

Washington University in St. Louis

ORCID: 0000-0002-2950-1664

isratjahan505@gmail.com

Tyler Larsen

Washington University in St. Louis

ORCID: 0000-0002-1566-4788

tjlarsen@wustl.edu

Joan Strassmann

Washington University in St. Louis

ORCID: 0000-0003-0638-8440

strassmann@wustl.edu

David Queller

Washington University in St. Louis

ORCID: 0000-0002-5464-1984

queller@wustl.edu

### Abstract

Aggregative multicellularity occurs when dispersed cells join together to form a highly cooperative unit, in contrast to clonal multicellular organisms formed by cells that remain in contact after descent from a single cell. Because aggregative groups may include non-relatives, aggregative multicellular organisms should be particularly vulnerable to the rise of cheater cells that take advantage of social goods without paying the costs, reducing cooperation, and even threatening extinction. We review the key mechanisms by which aggregative multicellular organisms control cheaters with a focus on the best studied aggregative organisms, *Myxococcus xanthus* and *Dictyostelium discoideum*. These include various passive and active mechanisms to maintain high relatedness within aggregates, to enforce cooperation on aggregate members, and the costs of cheating on other key functions. Ultimately, aggregative multicellular organisms are not that different from clonal organisms descended from a single cell.

### 7.1 Multicellularity and the problem of altruism

Life on earth has changed over its long history. The earliest organisms would have been too small to see, but over time organisms have diversified into endless forms, microscopic and macroscopic. The evolution of multicellularity – which happened not just once but more than twenty times – entailed individual cells banding together to produce the large, often complex bodies of the organisms we see around us (Grosberg and Strathmann 2007; Medina et al. 2003; Buss 1988; Ruiz-Trillo et al. 2007; Bonner 1998; Kaiser 2001). The transition to multicellularity is regarded as one of the major evolutionary transitions (Szathmáry and Maynard Smith 1995), in which formerly independent units (cells) became so dependent upon one another that they thereafter replicated as a combined unit. Like the other major transitions, multicellularity represents a change in the level of organization upon which natural selection acts.

A multicellular organism can be thought of as a group of cells that cooperate, with little conflict, to perform functions that would be impossible for single cells (Queller and Strassmann 2009). Sometimes the benefits cells enjoy by working together can be as simple as advantages of being larger in size. Larger organisms can be better at both avoiding predators and being predators (Kirk 2003; Kessin et al. 1996; Müller et al. 2015; Herron et al. 2019; Pentz et al. 2015) even when their cells are not necessarily contiguous – for example, in the bacterium *Myxococcus xanthus*, which hunts other bacteria in large groups that cooperate to release high concentrations of bactericidal compounds to better lyse and digest their prey (Dworkin and Kaiser 1985; Berleman and Kirby 2009; Daft, Burnham, and Yamamoto 1985; Fraleigh and Burnham 1988; Rosenberg, Keller, and Dworkin 1977). Larger size can provide protective benefits as well - the cells towards the center of a multicellular group can be shielded by the cells on the periphery from chemical and environmental stress, as seen in flocculating yeasts (Smukalla et al. 2008).

A more complicated benefit of multicellularity is that it facilitates the division of labor (Grosberg and Strathmann 2007; Cooper et al. 2020). While single-celled organisms can only divide labor temporally or into different organellar compartments, multicellular organisms can use entire cells to specialize on different tasks. Thus, division of labor can also protect functions of an organism from other functions that would otherwise interfere. For example, in some cyanobacteria, like *Nostoc*, oxygen produced by photosynthesis interferes with the enzymes involved in nitrogen fixation, and so multicellular cyanobacteria split the tasks of photosynthesis and nitrogen fixation into separate cell types (Kumar, Mella-Herrera, and Golden 2010). Non-photosynthetic heterocyst cells specialize in fixing nitrogen by remaining anaerobic behind thick cell walls, and export nitrogen to photosynthetic neighbors. Division of labor is crucial for the function of very complex multicellular organisms like animals, whose bodies can require hundreds of separate cell types working in concert.

Cooperating within a multicellular group can also come with costs, ranging from the energetic costs of producing a public good that all the cells in the group use, like the bactericidal enzymes produced by *M. xanthus* cells in a swarm, to an extreme where some cells sacrifice their lives entirely to benefit other cells. The clearest example of the latter in the context of multicellularity is the cooperation between somatic and germline cells. Somatic cells – like the heterocysts in cyanobacteria – forgo reproduction entirely and thus have no individual fitness. In large multicellular organisms like animals, the vast majority of cells sacrifice themselves and only a miniscule minority are passed on to the next generation.

Costly acts invite exploitation. In the case of a costly public good, for example, it may be in the best interest of an individual cell to stop producing the public good, thus avoiding the cost but continuing to benefit from the public good's production by the rest of the group. An individual that does not cooperate or pay a full share towards the cost of cooperation but receives the benefits from a cooperative group is called a cheater (West, Griffin, and Gardner 2007). If allowed to proliferate, cheaters can result in the destruction of a cooperative trait and the subsequent failure of those dependent on it (Hardin 1968). At high frequency, cheaters can even drive entire populations to extinction (Fiegna and Velicer 2003). Cheaters have been observed in many cooperative systems in nature, and how such groups contend with the threat they pose has been a major question in evolutionary biology (West et al. 2006; West et al. 2007; Riehl and Frederickson 2016). Why should living things cooperate at all if it requires sacrificing their own fitness for others? Why does selection for cheating not preclude the evolution of costly cooperation? How do multicellular organisms persist?

Hamilton's theory of inclusive fitness gives us part of the answer – the altruistic act of sacrificing one's own fitness for others can be favored by selection when it benefits the altruist's relatives (Hamilton 1964a; Hamilton 1964b). According to Hamilton's rule, costly cooperation should be selected for when  $rb > c$ , where  $b$  is the fitness benefit gained by the recipient of a cooperative trait,  $c$  is the cost incurred by the actor, and  $r$  is genetic relatedness between the recipient and the actor. Under Hamilton's rule, alleles underlying costly cooperation can be selected for because, while the costs may reduce a cooperator's own fitness (direct fitness), this cost can be compensated for if cooperation sufficiently benefits the fitness of other individuals likely to carry the same alleles (indirect fitness).

Hamilton's rule, with its emphasis on relatedness, goes a long way towards explaining the evolution and persistence of many multicellular organisms. Most familiar multicellular organisms like plants, animals, fungi, and red and brown algae are composed of cells that descend from a single cell and are therefore genetically identical, with relatedness between cells maximal at  $r = 1$ . Under Hamilton's Rule, altruism by somatic cells in a clonal organism can be favored by selection on the fitness of genetically identical germline cells so long as the total benefits of cooperation outweigh the total costs. Cells within a clonal organism have little to gain by conflict and so the single-celled bottlenecks they undergo can largely sidestep the risks of cheaters. Further, though new cheater mutations can arise within a clonal multicellular organism and reduce relatedness between cells, the single-celled bottleneck ensures that they would enjoy only a single generation of the benefits of cheating before producing progeny that are disadvantaged by consisting entirely of cheating cells (Queller 2000; Buss 2014).

## 7.2 Aggregative multicellularity

Many lesser-known multicellular organisms, however, do not undergo an obligatory single cell bottleneck and instead form by the aggregation of individual cells that may or may not be related. This path to multicellularity, called aggregative multicellularity, does not automatically ensure high relatedness among cells (**Figure 7.1**). Unrelated cells can join the same group, giving cheater genotypes opportunities to increase in frequency at cooperating genotypes' expense, even if doing so results in reduced fitness for the group as a whole. Such fitness reduction is particularly common with an increase of obligate cheaters, which cannot

successfully cooperate on their own. Obligate cheaters readily appear and increase in laboratory populations of both *Myxococcus xanthus* and *Dictyostelium discoideum* (Velicer, Kroos, and Lenski 2000; Velicer and Vos 2009; Kuzdzal-Fick et al. 2011; Gilbert et al. 2007) and can even lead to the extinction of the population, as has been observed in *M. xanthus* (Fiegna and Velicer 2003). Aggregative multicellularity is nonetheless very common, with independently-evolved examples in archaea, bacteria, and eukaryotes (Brown et al. 2012; Chimileski, Franklin, and Papke 2014; Chapter 5).

### Clonal and aggregative multicellularity

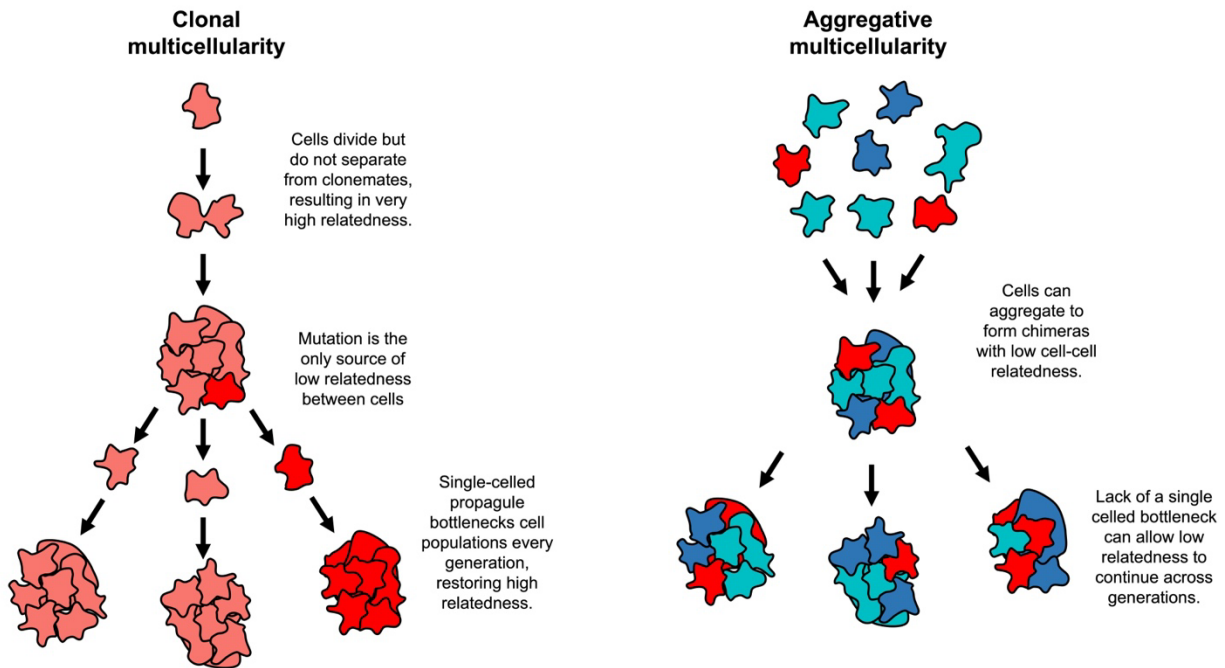


Figure 7.1. Clonal and aggregative multicellularity.

Many bacteria aggregate into cooperative single-species groups. One common form of aggregative multicellularity in bacteria is swarming, in which cells coordinate to move rapidly on solid or semi-solid surfaces via the production of public good surfactants (Harshey 2003; Butler, Wang, and Harshey 2010). Swarming motility has been described in *Bacillus subtilis* (Aguilar et al. 2007; Shapiro 1998), *Proteus mirabilis* (Shapiro 1998), *Pseudomonas aeruginosa* (Lai, Tremblay, and Déziel 2009), and Myxobacteria (Dale Kaiser, Robinson, and Kroos 2010; Velicer and Vos 2009). Cells within the swarm interact with each other and undergo morphological differentiation so that they can glide on surfaces (Julkowska et al. 2004).

Many bacteria – particularly Myxobacteria – also aggregate to form multicellular structures that facilitate survival in harsh conditions and during dispersal. Various Myxobacteria species produce aggregative fruiting bodies consisting of spores and sometimes a stalk that lifts the spores (Velicer and Vos 2009). Fruiting bodies can elevate spores, enhancing dispersal to new environments (Huss 1989; White, Shropshire, and Stephens 1980; Kessin et al. 1996; Kaiser 2001). The process of sporulation often entails death of most of the cells in the aggregate

and may thus represent a form of germ/soma division of labor like that seen in conventional multicellular organisms.

Biofilm formation is another extremely common behavior in microbes that resembles aggregative multicellularity. In a biofilm, cells of one or more species adhere together on a surface, often producing a structure of secreted extracellular matrix that can protect the cells from antimicrobials, shear forces, and host immune systems. The close proximity of microbes within a biofilm can also facilitate the exchange of chemicals. Research over the past decade has revealed that biofilms can be quite complex and sometimes contain many interacting species (Webb, Givskov, and Kjelleberg 2003; Nadell, Xavier, and Foster 2009; Foster 2010; Xavier and Foster 2007; Claessen et al. 2014). When biofilms involve interspecific interactions where relatedness is necessarily zero, they may experience high levels of conflict and unresolved selfishness of members and would not usually qualify as organisms, and (Xavier and Foster 2007; Queller and Strassmann 2009).

Among eukaryotes, aggregative multicellularity has independent origins in Discicristata (Brown, Silberman, and Spiegel 2012), Stramenopiles (Tice et al. 2016), Alveolata (Sugimoto and Endoh 2006), Rhizaria (Brown et al. 2012), Holozoa (Brown, Spiegel, and Silberman 2009) and twice in Amoebozoa (Brown, Silberman, and Spiegel 2011). Most of these eukaryotes share similar lifestyles involving a unicellular stage wherein amoebas feed and divide independently by mitotic division. Upon starvation, amoebas aggregate and enter a multicellular stage that is concerned solely with development, and eventually morph into a fruiting body. There is considerable variation in fruiting body morphology and development among species (Kawabe et al. 2019).

The best-studied organism among the aggregating eukaryotes is the cellular slime mold *Dictyostelium discoideum* and its relatives. *D. discoideum* spends most of its time as a unicellular vegetative cell, hunting bacterial prey in temperate forest soils. Upon starvation, *D. discoideum* amoebas aggregate and undergo a series of developmental steps to form a multicellular motile slug and then a stalked fruiting body consisting of up to four different somatic cell types to support spore production and dispersal (Kessin 2001).

Despite its increased vulnerability to the risks posed by cheaters, aggregative multicellularity has nonetheless persisted in widely disparate taxa, which maintain their multicellularity with a series of mechanisms that limit cheaters from overtaking the cooperative group. In this chapter, we review some of the most important mechanisms, with a special focus on the two best studied aggregative multicellular taxa – the bacterium *Myxococcus xanthus* (Cao et al. 2015; Muñoz-Dorado et al. 2016; Velicer and Vos 2009) and the eukaryote *Dictyostelium discoideum* (Kessin 2001; Medina et al. 2019; Ostrowski 2019; Shaulsky and Kessin 2007; Li and Purugganan 2011). The social stages in the life cycles of *M. xanthus* and *D. discoideum* both involve thousands of individual cells aggregating to form multicellular structures in response to starvation and require the death of a large fraction of the aggregate (more than 90% in *M. xanthus* (Muñoz-Dorado et al. 2016) and approximately 20% in *D. discoideum* (Votaw and Ostrowski 2017)).

These two species thus face similar potential conflicts between cooperators that make the necessary sacrifices and cheaters that do not. In both species, cheater mutations are easy to find

and cheaters can readily increase under experimental evolution (Santorelli et al. 2008; Ennis et al. 2000; Kuzdzal-Fick et al. 2011; Velicer, Kroos, and Lenski 2000). In both, cheating appears to be common between different clones collected from nature (Strassmann, Zhu, and Queller 2000; Buttery et al. 2009; Vos and Velicer 2009; Fiegna and Velicer 2005) though alternative explanations might sometimes apply (Smith, Van Dyken, and Velicer 2014; Wolf et al. 2015; Tarnita et al. 2015). Despite their many differences, *M. xanthus* and *D. discoideum* employ some of the same kinds of mechanisms to prevent these conflicts from destabilizing cooperation (Figure 7.2).

### Aggregative multicellularity in *D. discoideum* and *M. xanthus*

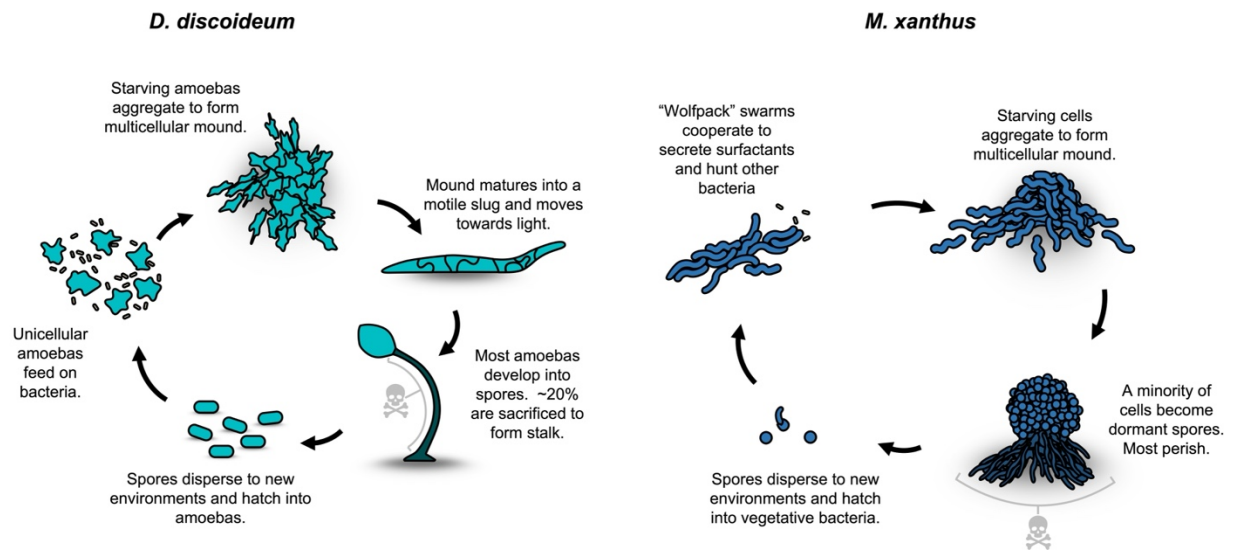


Figure 7.2. Aggregative multicellularity in *Dictyostelium discoideum* and *Myxococcus xanthus*.

### 7.3 Maintaining high relatedness

Inclusive fitness theory's key insight is that even a very costly altruistic trait – like cells sacrificing themselves to help produce a *Dictyostelium* or *Myxococcus* fruiting body – can be selected for if the beneficiaries of that sacrifice are sufficiently related. Similarly, cheaters benefit only when they have someone to cheat, so when relatedness is high and cheaters interact only with other cheaters, there is no incentive to cheat (Bourke 2013; Hamilton 1963; Frank 1995; Frank 2003). As already discussed, relatedness is very high among cells in many conventional multicellular organisms because they undergo a single cell bottleneck during development, while aggregative multicellular organisms without such a bottleneck can potentially include unrelated cells. Even without a strict single cell bottleneck, however, relatedness between cells of these species may be kept high via other mechanisms. In *D. discoideum*, microsatellite markers showed relatedness between cells within the same fruiting bodies collected from nature to be very high – between 0.86 and 0.97 (Gilbert et al. 2007). It is also very high in fruiting bodies of *M. xanthus* isolated from the wild, which were composed of different clonal lineages even when the fruiting bodies were collected just micrometers apart (Kraemer et al. 2016). In a follow-up study on *M. xanthus*, the authors sequenced 120 clones

from 6 naturally isolated fruiting bodies (Wielgoss et al. 2019). While each of the fruiting bodies included genetic variants at a few sites, these were distinguished by just a few mutational changes, which could be traced back to a recent ancestor. The accumulation of variation was due to recent *de novo* mutation in related cells rather than mixing of unrelated cells (Wielgoss et al. 2019).

### 7.3.1 Population structure

One very simple mechanism for maintaining high relatedness is via population structure, wherein cells tend to interact with close relatives as a passive consequence of how they grow and disperse. Though aggregative multicellular organisms like *Dictyostelium* and *Myxococcus* do not undergo an obligatory single celled bottleneck, they do primarily reproduce via clonal division of a mother cell into two identical daughter cells. If movement is limited, microbial populations can grow up as patches of closely related cells radiating out from a single founder. When conditions arise that favor aggregation, cells interact with their nearest neighbors, which tend to be clonemates. In *D. discoideum* laboratory populations, only a couple of millimeters separation between genetically distinct founding spores is required to generate patches of high relatedness, despite the motility of amoebas (Smith, Strassmann, and Queller 2016). When genetically different clones are plated in closer proximity to one another, there is increased mixing and relatedness in the resultant fruiting bodies is lower. Populations of *Dictyostelium* and *Myxococcus* have been observed to be structured the scale of millimeters to centimeters, which is consistent with the high relatedness observed between cells within fruiting bodies (Fortunato et al. 2003; Vos and Velicer 2008; Kraemer et al. 2016).

Further, even when multiple clones are mixed and relatedness is initially low, space constraints can result in a phenomenon called genetic demixing, wherein populations of cells separate into clonal sectors as they grow outwards (**see Figure 7.3**). When space is limited, only a small fraction of cells along the edge of the group can divide. As these cells divide, their progeny push out radially to fill the available space, mostly crowding out the progeny of other cells and thus raising relatedness. This demixing was first shown to occur in bacterial colonies (Nadell, Foster, and Xavier 2010; Hallatschek et al. 2007). Buttery et al. (2012) showed the same phenomenon with *D. discoideum* grown on agar plates.

## Structured growth and genetic demixing

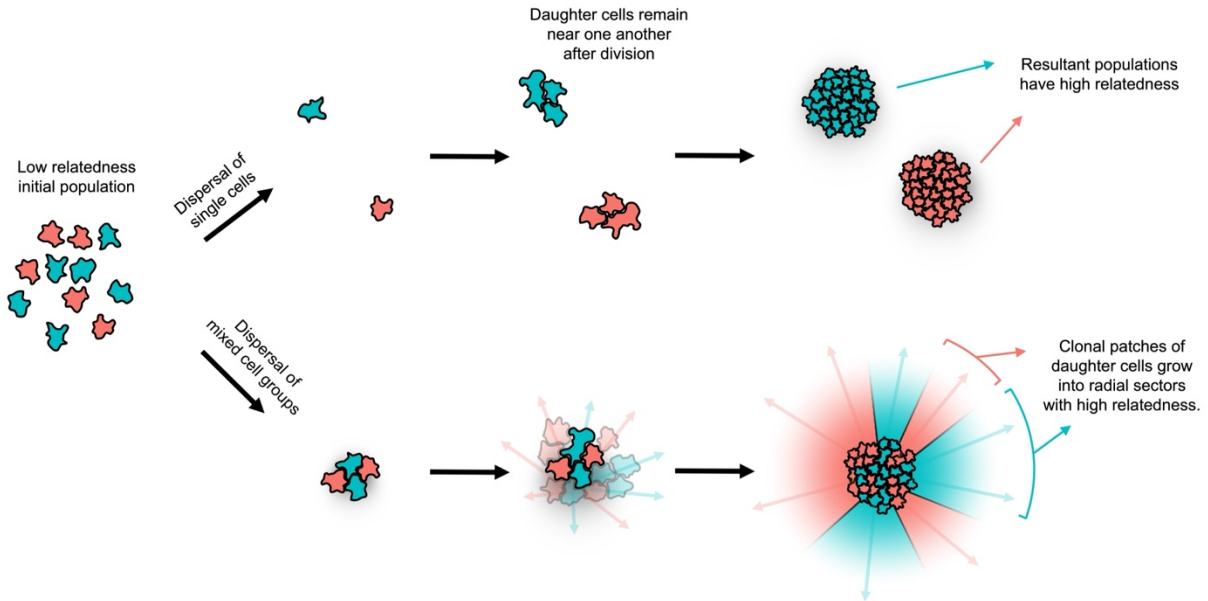


Figure 7.3. Structured growth and genetic demixing.

### 7.3.2 Kin discrimination

While population structure can passively reduce opportunities for cheaters and cooperators to interact with one another, kin discrimination allows cooperators that do risk interacting with cheaters to selectively direct their cooperation towards those individuals most likely to share genes and avoid exploitation by those who do not (Tsutsui 2004). Kin discrimination is widespread in microbes (Medina et al. 2019; Wall 2016; Strassmann 2016; Strassmann, Gilbert, and Queller 2011). In *Dictyostelium* and *Myxococcus*, kin discrimination can help aggregating strains segregate from unrelated strains and preferentially develop in groups with high relatedness between cells.

In the social amoeba species *D. violaceum*, *D. purpureum*, and *D. giganteum*, clonemates sort into mostly separate aggregates in the laboratory (Sathe et al. 2010; Kalla et al. 2011; Mehdiabadi et al. 2006; Mehdiabadi et al. 2009; Sathe, Khetan, and Nanjundiah 2014; Medina et al. 2019). Curiously, in the best studied *D. discoideum*, the degree of kin sorting in fruiting bodies is low (Ostrowski et al. 2008; Gruenheit et al. 2017; Gilbert, Strassmann, and Queller 2012). However, there are genes that seem to be involved in recognition in this species, whether or not they are used for sorting in nature.

Cell adhesion genes were suspected to be important in recognition from the earliest investigations in *Dictyostelium* (Bonner and Adams 1958; Sternfeld 1979). In *D. discoideum*, allrecognition is attributed to transmembrane proteins encoded by two sets of highly polymorphic essential genes, *tgrB1* and *tgrC1* (Hirose et al. 2011; Hirose et al. 2015; Benabentos et al. 2009). Amoebas with compatible pairs of Tgr proteins bind and adhere to one another during aggregation, with a binding affinity that negatively correlates with the degree of



segregation between *D. discoideum* genotypes (Gruenheit et al. 2017). Amoebas with sufficiently different *tgr* alleles segregate within a genetically mixed aggregate and form separate slugs (Gruenheit et al. 2017; Hirose et al. 2011), although these can later fuse and form chimeric fruiting bodies (Ho and Shaulsky 2015). Knocking out either *tgrB1* or *tgrC1* prevents development past the aggregate stage (Benabentos et al. 2009), but the presence of additional *tgrB1* and *tgrC1* alleles does not affect recognition as long as there is at least one compatible allele pair between the cells (Hirose et al. 2011).

The *tgr* recognition system appears to play some role in prevention of obligate cheaters even though it does not lead to complete sorting in *D. discoideum* fruiting bodies in the laboratory. Ho and colleagues found that an obligate social cheater lacking compatible Tgr proteins was excluded from the final fruiting body when *tgr* alleles were incompatible (Ho et al. 2013). They suggest that if obligate cheating occurs due to mutants that have effects early in development, the early segregation of cells within the aggregate and in the initial slugs can largely prevent cheating, even though the slugs later fuse (Ho and Shaulsky 2015). In addition to excluding cheaters, recognition may stimulate competition when chimeras do form. In one study (Noh et al. 2018), but not another (de Oliveira et al. 2019), genes that significantly change expression levels in chimeric mixtures showed rapid evolution, which is consistent with a history of evolutionary conflict between cell lineages.

Kin discrimination in *M. xanthus* is most readily observed in the formation of clear demarcation lines (zones of no or low-density cells) where two unrelated swarms meet on an agar plate. Swarms that are identical or closely related to each other readily merge and so produce no such demarcation zones (Patra et al. 2017; Gibbs, Urbanowski, and Greenberg 2008; Senior 1977).

One should be cautious in classifying a process that segregates clones as having necessarily evolved to discriminate kin from non-kin. In an experimental evolution study of natural isolates of *M. xanthus*, isolated clones rapidly evolved incompatibilities with one another or with their common ancestors (Rendueles et al. 2015), but because there were no foreign clones to interact with during this evolution, incompatibility seems unlikely to have evolved to recognize self or to exclude others. Instead, the result may simply reflect that genetically different clones may not function well together (Mendes-Soares et al. 2014; Foster et al. 2002) which can be due to social incompatibilities evolved in isolation (Rendueles et al. 2015; Ostrowski 2019). For example, if one clone changes its communication system for swarming, those changes might no longer work well with the systems in other clones. This is analogous to hybrid incompatibilities that evolve during allopatric speciation and is expected to occur in any context where complex interactions adapt in isolation from each other (Queller 2018). Even absent the threat of cheaters, these incompatibilities could result in a ‘chimeric load’, such that chimeric aggregates function less well than clonal aggregates, and selection to reduce the likelihood of chimerism via kin-recognition-like mechanisms (Ostrowski 2019).

A less visible form of kin recognition in *M. xanthus* involves exchange of membrane components between adjacent cells (Dey et al. 2016; Pathak et al. 2013; Patra et al. 2017). This can serve both to allow related cells to coordinate swarming, development, and sharing of private goods like lipids (Pathak et al. 2012), lipopolysaccharides (Vassallo et al. 2015), and lipoproteins

(Wall 2014) to assist in the repair of damaged cells, but also allow unrelated cells to harm one another by transferring toxic bacteriocins (Vassallo et al. 2017). Thus, outer membrane exchange acts as a kin discrimination system with dual purposes – its effects can be beneficial or harmful depending on relatedness between the cells (Wall 2016). Outer membrane exchange is facilitated by the two required genes *traA* and *traB* (Pathak et al. 2012). TraA is a highly polymorphic cell surface receptor that, similar to the *tgr* system in *D. discoideum*, facilitates adhesion to and interactions with cells that bear the same allele while TraB may involve the transport of TraA to the cell surface (Cao et al. 2019; Cao et al. 2015).

Recognition systems are not a certain defense against exploitation by cheaters. Cells do not always sort into perfectly related clonal patches; mixed strains of both *M. xanthus* and *D. discoideum* can readily form chimeric groups in the laboratory. In nature, the very high relatedness among cells in *M. xanthus* and *D. discoideum* fruiting bodies is likely to be achieved via a combination of passive population structure and active kin discrimination.

This high relatedness exerts stronger control over obligate cheaters than facultative ones. Facultative cheaters cheat when in mixtures but perform normally when they are alone. High relatedness simply reduces their opportunities to cheat. But for obligate cheaters high relatedness adds an additional big cost by isolating them in groups of obligate cheaters that cannot perform well. Consistent with this difference, facultative cheating appears to be common among natural clones of *D. discoideum* (Strassmann, Zhu, and Queller 2000; Buttery et al. 2009) and *M. xanthus* (Fiegna and Velicer 2005; Vos and Velicer 2009). Yet, despite obligate cheaters readily evolving in the laboratory in both *D. discoideum* (Ennis et al. 2000; Gilbert et al. 2007; Kuzdzal-Fick et al. 2011) and *M. xanthus* (Velicer, Kroos, and Lenski 2000), they have never been isolated from nature in either species. Thus, high natural relatedness may be controlling the obligate mutations, which are the greatest threat to cooperation if they were to spread (Gilbert et al. 2007).

#### 7.4 Enforcement of cooperation

Mechanisms that increase relatedness between group members work by minimizing the opportunities for cheaters and cooperators to interact, but may not eliminate such opportunities entirely. A need thus remains to enforce cooperation in potentially exploitative group members that either could not be excluded by the mechanisms already discussed or that arose *de novo* via the accumulation of random mutations that lower relatedness and convert previously cooperative cells into cheaters. A particularly familiar example of the latter is the spontaneous appearance of cancerous cells in many multicellular organisms, which are analogous to cheaters selfishly exploiting the cooperation of the rest of the cells in the organism (Nunney 2013; Aktipis et al. 2015). As persistent a problem as cancer is for animals, however, cheaters may be even more destructive in aggregative multicellular organisms, because without the opportunity to reset each generation to maximal relatedness through a single cell bottleneck, there is the potential for cheating genotypes to persist over multiple generations. Accordingly, just as animals have evolved genetic mechanisms to actively suppress cancerous cells, for instance by inducing apoptosis (Foster 2011; Evan and Vousden 2001; Singh and Boomsma 2015), aggregative multicellular organisms have evolved enforcement mechanisms to coerce cheaters within the social group to cooperate (Ågren, Davies, and Foster 2019).

The evolution of cheater resistance has been well documented in studies of both *M. xanthus* and *D. discoideum*. In two experimental evolution studies with *M. xanthus*, (Fiegna et al. 2006; Manhes and Velicer 2011), Velicer and colleagues evolved cheater suppression by repeated interactions between populations of a developmentally proficient strain and a developmentally defective cheater that had high fitness in chimeric mixtures with its ancestor, but could not sporulate on its own. In the first study, the cheater rapidly increased in frequency until it became so common that it no longer had enough developmentally competent cooperators to exploit, resulting in the entire population crashing and producing extremely few spores (Fiegna et al. 2006). Interestingly, at the fourth cycle of development, a new mutation occurred in the cheater that allowed for the rescue of the population. The resultant mutant strain (called phoenix because it “rose from the ashes” of the cheater) had a fitness advantage over both wild type and cheater. The phoenix strain can sporulate when it is common, an ability lost by the cheater strain, and rose to apparent fixation in the mixed population. In mixed culture, the phoenix mutant had an advantage over cheaters and wild type at all tested frequencies. The restoration of developmental proficiency by phoenix is not fully understood, but here it was not the wildtype that evolved cheater resistance, but a lineage of the cheater strain itself.

In a similar study (Manhes and Velicer 2011), a developmentally proficient strain was evolved in the presence of a *csgA*– cheater mutant (Velicer, Kroos, and Lenski 2000). By repeatedly replacing the cheater each generation, it was held evolutionarily static, and the non-cheater thus could evolve resistance without evolutionary retaliation. After 20 cycles of repeated development, the wild type cooperators evolved to have higher fitness than cheaters in mixed populations. Interestingly, the evolved populations not only suppressed cheating by cheaters, but increased the absolute fitness of ancestral wild type in a three-party mixture. This benefit to wild type was seen only in the presence of cheater. This benefit of cheater suppression to a third party was likened to policing behavior in some social animals (Zanette et al. 2012; Manhes and Velicer 2011).

Similar studies of cheater resistance have been performed in *D. discoideum*. In a study by Khare et al., the authors evolved the wild type AX4 in the presence of *chtC* mutants which were prevented from evolving. They selected over four rounds of development and isolated a ‘noble resister’ mutant (*rccA*) that was resistant to being cheated by *chtC* but did not cheat AX4 or the original cheater *chtC* (Khare et al. 2009). Hollis performed an evolution experiment using the strains NC4 and AX2, the former of which is a strong cheater of the latter (Hollis 2012). NC4 and AX2-GFP were allowed to grow and develop together but the cheating NC4 was prevented from coevolving. After only 10 generations of social development, AX2-GFP developed defenses against cheating in the social stage along with differences in competitive growth in the vegetative stage. Levin and colleagues employed an experimental design to look for the evolution of cheater resistance in a situation where the cheater was free to evolve in response, and found that non-cheaters evolved to resist the obligate social cheaters without themselves cheating (Levin et al. 2015). This study showed that resistance can evolve on a rapid timescale before the cheater becomes fixed.

### 7.5 Pleiotropic costs of cheating

Cheating can be costly. If the costs are large enough, the benefits gained by being able to exploit cooperators may be too small to compensate and cheating does not spread. Such “intrinsic defector inferiority” (Travisano and Velicer 2004) becomes more interesting when the cheating phenotype itself is beneficial but the allele causing this phenotype also has pleiotropic costs (Foster et al. 2004). To put it the other way around, if the cooperator allele has pleiotropic benefits, it may be protected against cheating. Alleles, or sets of tightly linked alleles, that encode for cooperative traits coupled to other essential functions may thus be naturally resistant to cheaters. Those cooperator alleles that have persisted in aggregative multicellular organisms may therefore be a biased set with pleiotropic advantages (Foster et al. 2004).

There are plausible examples in both *D. discoideum* (Medina et al. 2019), and *M. xanthus* (Velicer and Vos 2009). For example, the *D. discoideum* gene *dimA* is required to receive the signaling molecule DIF-1 that causes cells to differentiate into sterile prestalk cells during development. Cells with *dimA* knocked out are therefore expected to cheat by becoming spores instead of stalk. They do move to the prespore region of the slug, and yet these cells are largely excluded from maturing into spores by an unknown pleiotropic effect (Foster et al. 2004). For this reason, cheating on prestalk cell production leads to a reduction in spores and thus is unlikely to be favored by natural selection (Foster et al. 2004). A similar example can be seen in the *csA*- mutants. These mutants lack functional gp80 adhesion proteins and, on agar substrate, cheat their ancestor with functional gp80, perhaps because their impaired cell adhesion causes them to slide to the prespore region at the back of the slug (Queller et al. 2003). Interestingly, these mutants do not succeed when grown on the more realistic substrate of soil because their impaired adhesion prevents them from getting into an aggregation in the first place (Queller et al. 2003).

Pleiotropic effects can sometimes occur in different individuals, as in sexually antagonistic pleiotropy or pleiotropy underlying local adaptation (Paaby and Rockman 2013). In this light, obligate cheaters can be viewed as carrying a pleiotropic cost (Medina et al. 2019). Though cheaters in mixtures gain a cheating benefit, cheaters that occur in all-cheater groups experience a large cost and this can prevent cheaters from increasing.

It has been recently argued (dos Santos, Ghoul, and West 2018) that pleiotropy cannot explain the maintenance of cooperation because mutations will eventually occur that separate the pleiotropic traits, freeing cheaters from their pleiotropic handicap and enabling their spread. This is a good point for many pleiotropic effects. For example, many bacterial genes are upregulated by quorum sensing and can thus be regarded as pleiotropic effects of the alleles underlying quorum sensing. Cheater strains that did not participate in quorum sensing would suffer costs due to misregulating so many other genes, but it seems likely that these effects can be easily separated by mutations in regulatory regions that remove individual genes from quorum sensing control (dos Santos, Ghoul, and West 2018). The effects of *D. discoideum*'s *csA* knockout allele might also be separable provided a mutant could arise that allowed the gene to be fully expressed during aggregation but suppressed in the slug stage. Some of the obligate cheaters seem less clear. Can one always repair a gene that is developmentally defective on its own? But at least one case is known from *M. xanthus* of an obligate cheater recovering developmental function (Fiegna et al. 2006).

Yet there still appears to be some role for pleiotropy. In general, whether pleiotropy can suppress cheaters relates to the larger unanswered evolutionary question of how much pleiotropy constrains evolution. An example that weighs against the argument that pleiotropic effects can always be separated is senescence. Senescence is thought to result, at least in part, from selection for alleles that pleiotropically cause fitness gains early in life but fitness losses later in life (Williams 1957). If mutation could effectively separate these effects, the later deleterious effects would not have accumulated. Returning to social traits, models suggest the importance of pleiotropy in limiting cancer in large organisms, where cell lineages persist for many generations (Ågren, Davies, and Foster 2019). Finally, pleiotropy may be crucial for certain kinds of frequency dependent cooperation. Some cooperative genes act synergistically such that cooperation is favored only if it can become sufficiently common. Here, pleiotropic effects could allow cooperation to initially increase and if this happens before a mutation separates the pleiotropic effects, cooperation may have reached a frequency where it can be favored on its own (Queller 2019). Understanding the role of pleiotropy in the evolution and maintenance of cooperation will require additional theoretical and empirical work.

## 7.6 Conclusions

In this chapter we discussed how cheaters are controlled in aggregative multicellular organisms, in particular in the best studied systems – *D. discoideum* and *M. xanthus*. We categorized the mechanisms of group maintenance into three main categories – maintaining high relatedness, enforcement of cooperation, and pleiotropic costs of cheating.

High relatedness among group members makes cooperation easier to evolve and maintain because cooperators tend to benefit cooperators and cheaters tend to harm cheaters. At sufficiently high relatedness, cheaters can only hurt themselves. *D. discoideum* and *M. xanthus* can maintain high relatedness passively through structured growth and dispersal that results in structured populations with patches of closely related cells. Relatedness over short spatial scales thus remains high, and it is only at the interfaces between patches that there is opportunity for exploitation. Over longer spatial scales, relatedness will be lower, but the crucial measure is relatedness at the scale of aggregations in which interactions are occurring. Questions remain about the mechanisms of structured growth and dispersal in *Dictyostelium* and *Myxococcus*. What are the vectors for transportation of spores? How well do spores disperse together? What are the distances over which spores can be dispersed? How large a role does demixing play in maintaining relatedness in nature?

A more active way to increase relatedness is kin discrimination – preferentially cooperating with relatives and avoiding harm from non-kin. Kin discrimination has been observed in diverse organisms and may be especially important to aggregative multicellular organisms, in which it can be observed playing a role in clonal segregation (again driving high relatedness within groups) and, for *M. xanthus*, transfer of private goods or toxins via membrane exchange. The potential importance of kin discrimination in *M. xanthus* is underscored by the finding that *M. xanthus* readily evolves social incompatibilities in the laboratory.

The apparent importance of high relatedness in aggregative multicellular organisms suggests that they are not really so different from the more familiar organisms that develop from a single cell. In both cases, high relatedness is maintained. In both cases, this can be due to daughter cells remaining in proximity to each other, although they are detached in one and attached in the other. Kin-recognition or self-recognition systems reinforce high relatedness. High relatedness may be easier to maintain with an obligatory single-cell bottleneck, but aggregative organisms can also achieve it.

When relatedness is imperfect and cheaters and cooperators can interact, a second mechanism – enforcement – comes into play. In *D. discoideum* and *M. xanthus*, cooperators can evolve to resist cheaters in the laboratory, increasing group productivity in the presence of cheaters. These policing mechanisms are somewhat similar to the suppression of cheaters in eusocial insect groups via aggression. Studies of enforcement are relatively new in the field of microbial social evolution and there are multiple unanswered questions. What genes underlie cheater resistance? Do power hierarchies exist between wild cheaters and cooperators, such that strains differ in which other strains they can exploit? Are interactions between cheaters and cheater-resistant cooperators frequency dependent? Do they result in evolutionary arms races?

Finally, cheating may sometimes be inherently costly due to pleiotropic costs of cheating on other important traits. Examples are known from both *D. discoideum* and *M. xanthus*, arguably including obligate cheaters. Sometimes the costly pleiotropic trait may be evolutionarily separable from the cheating trait, allowing cheating to increase. But sometimes, it may not be separable, or it may separate too late, allowing the frequency dependent cooperative trait to reach a sustainable level. Separability is thus an important topic for future studies.

Multicellularity is an ancient, diverse, and highly successful strategy, partly because multicellular organisms have evolved mechanisms by which they control the threat of cheating. In the most familiar multicellular organisms, this is accomplished via the single-cell bottleneck, but there are many examples of aggregative multicellular organisms without single-cell bottlenecks, for which cheating is predicted to be an especially key threat. Though they may seem primitive, aggregative multicellular organisms, too, have ways of maintaining their groups against cheaters, as exemplified by the diverse mechanisms seen in *Dictyostelium* and *Myxococcus*.

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