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Social dynamics in natural populations of *Dictyostelium discoideum*

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Abstract

Evolutionary theory predicts that selection should favour individuals who act in their self-interest, resulting in widespread selfish behaviour. Nonetheless, cooperative behaviours that provide benefits to others are common in nature. Over the past 50 years, evolutionary biologists have established a framework to explain how cooperation can be maintained despite the threat of selfish individuals who fail to cooperate but benefit from the cooperation of others. This framework has mostly been built around social behaviours in animal societies such as social insects. However, the more recent discoveries of social behaviours in microbes, analogous to those observed in animals, allow us to examine the generality and importance of proposed mechanisms that limit selfishness in non-animal societies. The social amoeba *Dictyostelium discoideum* has become an established model system in social evolution studies. Upon starvation, genetically unrelated cells can co-aggregate to form a 'chimeric' fruiting body in which some cells altruistically die to form a supportive stalk while others become viable spores. Theory and empirical work have shown that altruistic stalk formation can select for cheaters: genotypes that preferentially allocate cells to the spores and exploit stalk formation by their social partner. This finding has led to a considerable number of studies that demonstrated proof-of-principle for mechanisms that both promote and limit cheating in this organism. In contrast, few studies have examined whether cheating is widespread in nature, nor assessed the importance of the previously identified mechanisms that may limit this behaviour. In this thesis, I aimed to bridge the gap between the laboratory and a more natural situation. Using a diverse collection of natural strains of *D. discoideum*, I investigated the frequency and intensity of cheating among natural strains, the mechanisms that limit this behaviour, and the potential evolutionary consequences of interactions between cheaters and cooperators. Overall, this thesis will contribute to the growing body of work that examines how cooperative behaviours can be maintained in non-animal societies.

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Chapter 1.

General Introduction

Cooperation is widespread in nature

Cooperative behaviours, those that confer a benefit to another individual and are favoured by natural selection for this reason, are common in nature (Sachs *et al.*, 2004; West *et al.*, 2006; West, Griffin and Gardner, 2007a; Clutton-Brock, 2009; Hatchwell, 2009; Silk, 2009). Prairie dogs and meerkats use alarm calls to alert their fellow group members about approaching predators, despite endangering themselves in the process. Similarly, many animals engage in cooperative breeding, wherein some individuals forgo breeding themselves and instead raise the offspring of others (Griffin *et al.*, 2003). These cooperative behaviours however pose a conceptual problem: why does natural selection promote behaviours that reduce an individual's fitness relative to that of others in the group?

Even more extreme examples of cooperation are observed in the eusocial animals, which include all termite species (infraorder Blattodea), numerous Hymenoptera species (ants, wasps, and bees), some other insects, and the naked mole rat (*Heterocephalus glaber*) (reviewed in Wilson 1975; Hölldobler and Wilson 2009). Eusociality is characterized by *i.* reproductive division of labour where only a subset of individuals within a colony reproduces, *ii.* cooperative brood care, and *iii.* multiple generations living together (Crespi and Yanega, 1995). In the case of the eusocial Hymenoptera, the queen reproduces, and workers specialize in non-reproductive tasks, such as caring for the young, protecting the nest, and foraging for food (Wilson, 1975; Robinson, 1992; Smith *et al.*, 2008). Under obligate eusociality, the workers typically do not reproduce and are generally sterile (though they can lay unfertilized eggs under some conditions when the queen is absent) (Crespi and Yanega, 1995; Boomsma, 2007, 2009). Obligate sterility is an extreme example of cooperative behaviour, called altruism, where the workers have no (with exceptions) opportunity for direct fitness. Not surprisingly, the evolutionary origins and maintenance of eusociality have interested many evolutionary biologists (Hamilton, 1964a, 1964b; Maynard Smith, 1964; Trivers, 1971; Szathmáry and Smith, 1995; Sachs *et al.*, 2004; Lehmann and Keller, 2006; Nowak, 2006; Sachs, 2006; West *et al.*, 2006).

In this Chapter, I begin by examining the two primary theories that explain how cooperation can evolve. I then explore the incentives to cheat that can threaten cooperation, defined as behaviours that allow individuals to gain benefits from cooperation by others without contributing their fair share of the costs. Using examples from different social systems, I explain the problem of cheating and the mechanisms that might limit it and promote cooperation. Finally, I introduce the focal organism of this thesis: the social amoeba *Dictyostelium discoideum*. I explain why this microbe has emerged as an important model organism for the study of cooperation and conflict.

Major theories for how cooperation can evolve

In this section, I outline the two main theories for the evolution of cooperation. These theories illustrate that cooperation hinges on the benefits the actor derives, either directly through reciprocity or indirectly through kin selection (Hamilton, 1964a, 1964b; Trivers, 1971; Grafen, 1984).

Reciprocity—you scratch my back, and I'll scratch yours

The first primary theory on how cooperation can evolve posits that an individual can benefit from a costly act if they are likely to receive aid in the future (Trivers, 1971). This concept is called indirect or reputation-based reciprocity (Boyd and Richerson, 1989). Cooperation via indirect reciprocity requires several key factors, including repeated interactions between individuals, the capacity for individuals to recognize and remember one another, and, ultimately, that the costs of offering aid in the present must be outweighed by the benefits of receiving aid in the future (Lehmann and Keller, 2006).

An example of indirect reciprocity occurs in vampire bats (*Desmodus rotundus*) (Wilkinson, 1984). These bats live in social groups and sustain themselves by feeding on the blood of mammals. Individuals who are unable to feed for a short period are at high risk of starvation. Wilkinson (1984) observed that individuals who obtained food shared it with those who had not, and therefore suggested that this food sharing was altruistic. Further research revealed that individuals who previously received aid would reciprocate later, indicating reciprocity (Wilkinson 1988).

It is important to note that indirect reciprocity requires the processing of complex information. Consequently, some researchers argue that this type of reciprocity may be limited to a select group of organisms, with less complicated forms of reciprocity likely prevailing in most social groups (Clutton-Brock, 2009; Davies, Krebs and West, 2012).

Group selection

Wynne-Edwards (1962) proposed that cooperation could be favoured by natural selection if individuals benefit the collective, referred to as group selection (West, Griffin and Gardner, 2008). An illustrative example of this idea involves two groups: one group harbours individuals that fail to limit their birth rates, leading to over-exploitation of resources, and another group harbours individuals that regulate their birth rates and do not deplete their resources. Since the group that over-exploits their resources would experience reduced reproductive success compared to the group that restrained exploitation of resources, cooperative behaviours favouring resource conservation would be inherited by the next generation. However, group selection was rapidly rebutted by both

theoretical and empirical research that amongst others demonstrated that the idea had too many unrealistic assumptions, such as the absence of migration (Hamilton, 1964a, 1964b; Maynard Smith, 1964; Williams, 1966; West, Griffin and Gardner, 2007b).

Group selection nonetheless regained attention in later years through modifications that incorporated both group-level and individual-level selection, a concept known as multilevel selection (MLS) (Wilson and Wilson, 2007). In the MLS framework, a population is subdivided into subpopulations, each comprising either all cooperators, all non-cooperators, or a mix of both. Within groups, non-cooperators outcompete cooperators. However, groups containing more cooperators contribute more offspring to the next generation than those with fewer cooperators. In this scenario, a gene for cooperation can increase in frequency in the overall population, even as it decreases within each subpopulation. Consequently, MLS shows how cooperation can evolve, despite selection favouring non-cooperators within each subpopulation.

Similar to the old idea, researchers criticised the new idea of group selection (Grafen, 1984; Queller, 1992; West, Griffin and Gardner, 2007b, 2008). First, group-level selection is predicted to be relatively weaker compared to individual-level selection and thus may not be a significant evolutionary force in nature (reviewed in West, Griffin and Gardner (2008)). Moreover, as subpopulations often comprise close kin, this leads to conceptual overlap between MLS and kin selection (discussed below) (Queller, 1992; Gardner, West and Barton, 2007).

Kin selection

The prevailing theory that explains the evolution of cooperation is kin selection theory, also known as inclusive fitness theory (Hamilton, 1963, 1964a, 1964b; West-Eberhard, 1975; Lehmann and Keller, 2006). Inclusive fitness theory divides an individual's fitness into two components: direct and indirect fitness, both measured in terms of lifetime reproductive success. Direct fitness refers to the lifetime reproduction of a given individual, whereas indirect fitness is the fitness an individual gains from the reproduction of relatives, who also carry and pass on the individual's genes. Inclusive theory is encapsulated in Hamilton's rule, which states that an allele for cooperation will be favoured by natural selection when $rB–C>0$, where C is the fitness cost incurred by the actor, B is the fitness benefit received by the recipient, and r represents the relatedness between the actor and the recipient (Hamilton, 1963, 1964a).

Relatedness: what it is, why it matters, and how it is measured

The relatedness term of Hamilton's Rule describes "the statistical measure of the genetic similarity between interacting individuals (potential beneficiaries of the altruistic behaviour) relative to the average population-wide genetic similarity (competing individuals)'' (Grafen, 1985). Genetic similarity is typically estimated by co-ancestry, i.e., identity by descent (IBD) from a common ancestor, but this is not essential (Hamilton, 1964a, 1964b, 1970; Grafen, 1991; Lehmann and Keller, 2006; Gardner and West, 2010). As discussed below, relatedness is distinct from a genealogical relationship. In the following sections, I illustrate this point by briefly discussing several aspects of relatedness: how relatedness is defined and quantified, that the relevant estimate of relatedness should be at the level of a single gene, causes of relatedness, and how relatedness is used in empirical studies.

The definition of relatedness

Coancestry, or kinship, quantifies the genetic similarity between individuals by estimating the probability that they share genes identical by descent from a common ancestor (Hamilton, 1970; Grafen, 1985; Queller, 1994). Importantly, coancestry is measured relative to a reference population. This requires knowing the frequency of the allele at the locus of interest in the population, as well as the allele frequency in both in the actor and the recipient (Hamilton, 1964a; Grafen, 1991). Specifically, if the population average gene frequency matches the recipient's gene frequency, the relatedness between actor and recipient is zero; in this case, any help provided by the actor to the recipient will not change the allele's frequency in the population. In contrast, when the allele frequency in the recipient is greater than the population average, relatedness is positive; help provided by the actor will increase the allele's frequency in the population.

From these examples, it follows that under diploidy and random mating, two randomly chosen individuals are by definition unrelated on average, meaning their genetic similarity aligns with the population average gene frequency (Hamilton, 1964a; Grafen, 1991). Consequently, the relatedness of offspring to their parents is $^1/_2$, assuming the parents have zero relatedness to each other. With each generation of random mating, relatedness declines by half, rapidly approaching zero across multiple generations. This implies that continuous random mating diminishes relatedness, converging it toward the population average gene frequency and effectively eliminating close genetic ties. In contrast, mechanisms of inbreeding (preferential mating between relatives) and asexual reproduction (mating with yourself) can maintain higher-than-average relatedness among relatives compared to random mating (Hamilton, 1964b; Michod, 1979; Breden and Wade, 1981; Weir, Anderson and Hepler, 2006).

Quantification of relatedness

Coancestry is often calculated as the relatedness (or coancestry) coefficient, which is the probability that alleles at the same locus in two individuals are copies of the same ancestral allele (Rousset, 2013). These calculations can be based on either path analysis of a pedigree (family tree) or estimated using genetic marker data. Path analysis has traditionally been the method of choice for estimating relatedness based on genealogical relationships, as it accounts for multiple paths of allele transmission through multiple common ancestors and can model the effects of inbreeding on relatedness estimates. However, this method introduces several challenges. Specifically, it requires detailed observations of social behaviours within populations, such as multiple mating events in females, paternity assessments, and clear definitions of generational boundaries, which can be especially difficult to determine in long-lived organisms. Additionally, path analysis assumes no recombination during meiosis—an assumption that can strongly deviate from actual genome sharing (i.e., instead of an average of 50% genome sharing between parent and offspring, this can range between 37% and 63%) (Speed and Balding, 2015). This can make the calculation of relatedness based on path analysis challenging.

More recently, genetic marker data-based methods have been developed to calculate average relatedness based on genome similarity between individuals (Pamilo, 1982; Queller and Goodnight, 1989; Lynch and Ritland, 1999; Weir, Anderson and Hepler, 2006; Speed and Balding, 2015). In contrast to path analysis, these methods are based on realized/actual genome sharing rather than expected sharing. Similarity can be assessed at various sites in the genome: across the entire genome, at sequences of housekeeping genes, at different microsatellite loci, or at specific loci that confer the social traits of interest—if these loci are known. Regardless of choice of marker, these methods compare allelic similarity between individuals while accounting for allele frequencies within the population (Weir, Anderson and Hepler, 2006).

Queller and Goodnight (1989) developed marker-based estimator based on allozymes, microsatellites, and single nucleotide polymorphisms (SNPs) to infer relatedness. They emphasized that genetic similarity at rare alleles should be given greater importance, as this allele sharing is less likely to occur by chance, thereby improving the reliability of relatedness estimates. This approach requires selecting markers with a higher allelic diversity—ideally, the actual gene controlling the altruistic trait under study. Advantages of Queller and Goodnight's method include its suitability for small sample sizes, its applicability in estimating relatedness between pairs of individuals, and its accuracy in populations with variable genetic diversity.

Applying relatedness to examine the evolution and maintenance of cooperation

Relatedness calculations have been used to explore the potential for cheaters mutants to invade a population (Velicer, Kroos and Lenski, 1998; Griffin, West and Buckling, 2004; Gilbert *et al.*, 2007; J. J. Kuzdzal-Fick *et al.*, 2011; Bastiaans, Debets and Aanen, 2016). For example, Gilbert *et al.* (2007) determined the level of relatedness required in populations of the social amoeba *Dictyostelium discoideum* to prevent invasion by a cheater mutant *fbxA*$^{-}$. This mutant has a mutation in protein involved in cell-fate decisions, which causes its carriers to behave selfishly by avoiding the altruistic act of stalk formation and preferentially becoming viable spores (discussed in more detail later). As a result, the cheater can rapidly spread through a population up until it would limit its own spread by not being able to rely on stalk formation by others. Gilbert *et al.* showed that this threshold of relatedness was at r=0.75. Comparing this to the level of relatedness they observed in natural populations of r=0.86 based on polymorphic microsatellite loci, they concluded that relatedness in nature is sufficiently high to limit the spread of this cheater.

Griffin *et al*. (2004) examined if the cooperative trait of pyoverdine production in the bacterium *Pseudomonas fluorescence* can be exploited by nonproducing cheats under high and low levels of relatedness. In addition, they manipulated the scale of competition (local versus global), to test the effect of limited dispersal, or population viscosity, on the evolution of cooperation. Specifically, social evolution theory predicts that limited dispersal leads to a higher relatedness, but also increased competition among kin, potentially cancelling out the benefits of cooperation (Taylor, 1992; Queller, 1994; Frank, 1998). In line with theory, they found that high relatedness favours cooperation, but when competition was local, cooperation was significantly reduced.

Collectively, these studies demonstrated the importance of relatedness on the evolution and maintenance of cooperation. In addition, they underscore that the relevant measure of relatedness is with respect to the gene for the altruistic trait and that population structure must be taken into account for accurate predictions about when cooperation is favoured.

Greenbeard genes

Dawkins first introduced the concept of a greenbeard gene to illustrate that the relevant relatedness for altruism is at the level of a single gene, and that this does not require genome wide relatedness (Dawkins, 1976). A greenbeard is a hypothetical gene that can do two things: (1) recognize copies of itself in other individuals, and (2) direct benefits to those carrying the same gene (Hamilton, 1964a; Dawkins, 1976; Gardner and West, 2010; McGlothlin *et al.*, 2010). Dawkin's greenbeard refers to any

visible phenotypic trait (such as a green beard) that reliably signals the presence of a specific gene. If the altruistic trait produces a readily observed phenotype, individuals who carry the relevant allele can recognise and then direct altruism to others who carry that same allele (Gardner and West, 2010). Alternatively, a greenbeard can act by harming individuals that do not carry the gene.

The greenbeard gp-9 locus in red fire ants

Dawkins' greenbeard was a thought experiment only. The existence of greenbeard genes was long been considered biologically unrealistic, as it was unlikely that a single locus can encode both the ability to recognise kin and behave preferentially to them. However, several empirical studies support their existence (Keller and Ross, 1993; Queller *et al.*, 2003; Sinervo and Clobert, 2003; Smukalla *et al.*, 2008). The best known example is the *gp-9* locus in red fire ants (*Solenopsis invicta*) (Keller and Ross, 1993). Red fire ants are eusocial insects that establish colonies consisting of either one ('monogynous') or multiple queens ('polygynous'). The *gp-9* locus, which has two primary alleles, *B* and *b*, influences an individual's ability to recognize and interact differentially to individuals with distinct sets of pheromones. In polygyne colonies, *bb* queens do not survive, so all queens are either *BB* or *Bb*. However, when queens initiate reproduction, workers that carry the *b* allele attack and kill the *BB* queens—i.e., attack those not carrying the *b* allele. Effectively, the workers' *b* alleles recognise whether the queen harbours the same allele, and they accept (i.e., work for) the queen only if she does. The *gp-9* locus is considered an example of a harming greenbeard, since individuals lacking the greenbeard gene are attacked, rather than individuals harbouring the correct allele benefiting.

Complexities of greenbeards

While theoretical models and a few empirical examples of greenbeards exist, their role in the evolution of social traits remains uncertain. Theory suggests that greenbeards may be genomic outlaws (Madgwick, Belcher and Wolf, 2019). Specifically, because greenbeard genes enhance their own fitness—acting as a type of selfish element—at the expense of that of other genes in the genome, they are expected to promote genetic conflict with other loci, leading to their eventual suppression (discussed in more detail later) (Grafen, 2006; Helanterä and Bargum, 2007). Additionally, the expected strong selection on greenbeards should drive the rapid spread and fixation of greenbeard alleles (Crozier, 1986). Once fixed, greenbeards can no longer exhibit conditional social behaviour, rendering them evolutionary unimportant. Finally, through mutation and recombination, "falsebeards" can emerge—genes that possess the greenbeard signal but do not engage in social behaviour (Dawkins, 1976; West *et al.*, 2006; Gardner and West, 2010; Biernaskie, West and Gardner, 2011). Because falsebeards avoid the costs associated with social behaviour, they can cheat on

greenbeards and are likely to drive greenbeards to extinction. Ultimately, these dynamics suggest that greenbeards might not play a significant long-term role in the evolution of social traits.

Causes of relatedness

As discussed above, because the relevant relatedness to selection on altruistic behaviours is at the level of a gene, relatedness can potentially occur in the absence of a genealogical relationship, i.e., it does not require alleles to be identical-by-descent (IBD) (Hamilton, 1964a, 1964b, 1970; Frank, 1998). Instead, relatedness requires alleles in a gene of interest in two individuals to be identical-by-state (IBS), which simply means that alleles are the same, irrespective of whether they are from the same or different recent ancestor.

One potential mechanism by relatedness can potentially occur in the absence of a genealogical relationship is through horizontal gene transfer (HGT) (Frost *et al.*, 2005; Sørensen *et al.*, 2005; Thomas and Nielsen, 2005; Mc Ginty *et al.*, 2013). HGT describes the transfer of genetic material between individuals other than by vertical (parents-offspring) transfer, e.g., bypassing traditional inheritance. HGT is ubiquitous in microbes, occurring via transformation, transduction, or conjugation, and it has been suggested as a mechanism to stabilize cooperation in these organisms (Smith, 2001; Mc Ginty *et al.*, 2013; Dimitriu *et al.*, 2014). For example, Dimitru *et al.* (2014) used a synthetic system of *Escherichia coli* to demonstrate that the HGT of cooperative genes to non-cooperative individuals in a structured population could favour public good production. However, similar to greenbeards, it remains unclear whether HGT promotes social traits in the long-run (Mc Ginty, Rankin and Brown, 2011; Dewar *et al.*, 2021, 2024).

Applications of Hamilton's rule

The significance of Hamilton's rule lies in its demonstration that genes promoting behaviours deleterious to an individual's direct fitness, termed altruistic traits, can nevertheless evolve by natural selection if they sufficiently increase the individual's indirect fitness. In other words, alleles for altruistic behaviours can increase in a population if the benefits to relatives, adjusted for their degree of relatedness to the actor (rB), outweigh the direct fitness cost incurred by the actor (C). Kin selection is therefore a crucial explanation for the evolution of altruism and predicts that costly forms of cooperation should primarily occur within groups of related organisms.

Over the last 50 years, Hamilton's rule has proven instrumental in explaining altruistic behaviours across social organisms, including mammals, birds, and social insects, underscoring the crucial role of

relatedness in the evolution of cooperation (Hamilton, 1964a; West-Eberhard, 1975; Queller and Strassmann, 1998; Sachs *et al.*, 2004; West, Griffin and Gardner, 2007a; Bourke, 2011; Kay, Lehmann and Keller, 2019). Notably, Hamilton's rule can explain highly cooperative behaviours observed in eusocial Hymenoptera. Eusocial Hymenoptera exhibit haplodiploidy, a unique reproductive system where the sex of an individual is determined by fertilization. Males arise from unfertilized, haploid eggs, and females arise from fertilized, diploid eggs (Gardner and Ross, 2013). Due to this system, full sisters in haplodiploid species have a relatedness of r=0.75 to each other but only have a relatedness of r=0.5 to their own offspring. Consequently, haplodiploidy promotes altruistic behaviour, such as cooperative rearing of sisters among female workers, as they show greater relatedness to their sisters than their own offspring. Eusocial insects, including haplodiploid species, have played an important role in validating inclusive fitness theory for the evolution of cooperation. Studies on these insects have consistently confirmed the central importance of relatedness in driving the evolution of highly cooperative behaviours, including altruism (Wenseleers and Ratnieks, 2006a; Bourke, 2011; Strassmann *et al.*, 2011; Gardner and Ross, 2013). Do note that while early studies on haplodiploidy were instrumental in developing theoretical frameworks, subsequent research suggests that its influence on the evolution of eusociality may be negligible (Alpedrinha, Gardner and West, 2014).

Critiques of kin selection theory

Kin selection theory has long been the dominant framework for predicting the evolution of eusociality and other social behaviours, but it has faced criticism from some (Traulsen and Nowak, 2006; Wilson and Wilson, 2007; Nowak, Tarnita and Wilson, 2010). Critics argue that the theory is overly restrictive, overemphasises the importance of kinship relative to other means that confer relatedness, has limited applicability, and lacks sufficient empirical support (Ferriere and Michod, 2011; Herre and Wcislo, 2011; Rousset and Lion, 2011; Strassmann *et al.*, 2011). They instead propose that natural selection, with an emphasis on multilevel selection and population structure, offers a more robust and 'easier' explanation for these phenomena. However, these critiques have been met with significant opposition, with many arguing that they misinterpret the existing theory and greatly underestimate the extensive empirical evidence supporting kin selection (Ferriere and Michod, 2011; Herre and Wcislo, 2011; Rousset and Lion, 2011; Strassmann *et al.*, 2011; West and Gardner, 2013).

In conclusion, both reciprocity and kin selection offer explanations for why individuals engage in costly behaviours that confer benefits to others. In both cases, the benefits of cooperating must outweigh its associated costs. Reciprocity operates solely through direct fitness benefits, where the actor ultimately receives a direct fitness benefit that mitigates the costs. In contrast, kin selection involves

indirect fitness benefits, where the actor's direct fitness costs of cooperation are mitigated by benefits accrued through the reproduction of related individuals.

Cooperation is susceptible to selfish behaviours

Evolutionary theory predicts that selection should favour individuals who act in their self-interest, ignoring the interests of others (Trivers, 1971; Axelrod and Hamilton, 1981; Sachs *et al.*, 2004; Burt and Trivers, 2006; Foster and Wenseleers, 2006; Nowak, 2006). In scenarios where cooperation is based on a common resource or public good that benefits all group members, selfish individuals are commonly termed cheaters. These individuals reap the benefits of the cooperation by others without contributing to the collective effort – in other words, they gain the benefits of cooperation without paying its associated costs (Hamilton, 1964a; Axelrod and Hamilton, 1981; Szathmáry and Smith, 1995; Frank, 2003; Bourke, 2011; Ghoul, Griffin and West, 2013). This concept was illustrated by Hardin (1968) in "The Tragedy of the Commons", which describes that individual self-interest leads to the depletion of a common resource upon which everyone relies. Hardin originally explained this concept using a group of herders who all had access to a common grazing area. Each herder aimed to maximize their profits by grazing as many animals as possible on the shared land., i.e., acting in their self-interest. However, by doing so, the high number of animals prevented the grass from regenerating in time, ultimately depleting the food due to overgrazing and causing financial losses for all herders. The "Tragedy of the Commons" therefore demonstrates how selection in cooperative systems may drive individuals to act in their self-interest, disregarding potential detrimental consequences to the group.

Game theory is another fundamental framework that researchers use to analyse and understand how individuals decide to contribute to collective or public goods (i.e., cooperate) that affect the welfare of the entire group (Trivers, 1971; Axelrod and Hamilton, 1981). The framework is used to examine interactions between individuals (players) that can employ different strategies associated with different outcomes, which also depend on other player's strategies. The example of Prisoner's Dilemma is commonly used to illustrate game theory (Axelrod, 1984). Here two suspects are arrested and given the choice to confess or remain silent. The length of the sentence depends on their mutual choice. If both individuals confess (defect), they both receive a higher sentence. If both individuals remain silent (cooperate), they receive a minimal sentence. And, if one individual confesses and the other remains silent, the defector goes free, and the cooperator gets a severe sentence. This example illustrates that individuals are incentivised to not cooperate despite potential benefits from collective cooperation.

Both the Tragedy of the Commons and the Prisoner's Dilemma are classic examples that illustrate scenarios where individuals acting in their self-interest can lead to suboptimal outcomes for the group. These ideas are complementary, fundamental concepts that explain why cooperation can be challenging to achieve without mechanisms that enforce or encourage it (described later).

Consistent with the expectation that cooperation should select for selfish behaviours, examples of selfishness and cheating are observed in nature (Dawkins, 1976; Werren, Nur and Wu, 1988; Szathmáry and Smith, 1995; Hurst, Atlan and Bengtsson, 1996; Michod and Roze, 1997; Nunney, 1999; Strassmann, Zhu and Queller, 2000; Dobata and Tsuji, 2009; Sachs, Ehinger and Simms, 2010; West *et al.*, 2015; Leeks, West and Ghoul, 2021). For example, in honeybees (*Apis mellifera*), researchers observed workers laying their own eggs instead of tending to those of the queen—where the eggs of the workers are all males as a result of haplodiploidy (Ratnieks, 1993; Foster and Ratnieks, 2000; Hammond and Keller, 2004; Ratnieks, Foster and Wenseleers, 2006). This behaviour negatively impacts colony productivity, as the workers divert energy away from their usual tasks (Dobata and Tsuji, 2013). The evolutionary explanation for such behaviours lies in the higher relatedness of workers to their own offspring (r=0.5 to their sons) compared to the queen's offspring (r=0.25 to their brothers) (Hamilton, 1964a; Woyciechowski and Łomnicki, 1987; Ratnieks, 1988). Therefore, kin selection theory not only explains altruism but also when social behaviours that undermine cooperation can evolve—specifically, when genetic relatedness (r) between individuals decreases. As discussed later, the occurrence of worker-laying often rapidly selects for 'worker-policing', where other workers remove and consume the eggs, limiting the success of these selfish individuals.

Selfishness is not limited to social groups; it also occurs at the level of the gene, genome, or cell within individuals (Werren, Nur and Wu, 1988; Hurst, Atlan and Bengtsson, 1996; Jaenike, 2001; Burt and Trivers, 2006; Werren, 2011). For example, during fair meiosis, both homologs in each paired chromosome should have an equal chance of being transmitted to the gamete, resulting in a 50% probability of receiving any given homolog (Mendel 1985). However, selfish genetic elements (SGEs) can manipulate the meiotic process for their benefit, a phenomenon known as meiotic drive (Hurst, Atlan and Bengtsson, 1996; Jaenike, 2001; Burt and Trivers, 2006; Werren, 2011; Hurst, 2019). This manipulation enables SGEs to rapidly spread through a population, irrespective of potential detrimental effects on their host (Burt and Trivers, 2006; Lindholm *et al.*, 2016). In *Drosophila* species, for example, sex ratio distorters on the X chromosome can bias the segregation of the X and Y chromosomes during meiosis, which may lead to a higher proportion of the offspring that carries the X chromosome. If widespread, this process can rapidly disrupt population balance, leading to

population decline, or in extreme cases, extinction (Jaenike, 2001; Phadnis and Orr, 2009). In summary, examples such as worker-laying in honeybees and sex ratio distorters in *Drosophila* illustrate that selfish behaviours, if not unchecked, can emerge, and rapidly spread within cooperative societies.

Evolutionary conflict

Conflict describes when the evolutionary interests of different individuals (or generally, parties) are in opposite directions (Queller and Strassmann, 2018). Conflict can again be split up into two types: potential and actual conflict. As the names suggest, potential conflict describes when the interest of two parties *should* be in opposite directions and actual conflict describes what we actually observe. In a later section I discuss the mechanisms that often prevent potential conflicts from escalating into actual conflicts. In this section, I provide two examples where potential conflict has turned into actual conflict and describe how this manifested in the phenotype of the group or higher-level unit.

Sex allocation conflict in insect societies

Inclusive fitness theory explains that in hymenopteran societies potential conflict should arise between the queen and workers over the rearing of sons versus queens (Trivers and Hare, 1976). As previously mentioned, as a result of haplodiploidy, in colonies of singly-mated queens, workers are related to males (brothers) at r=0.25 and to their full-sisters at r=0.75. Hence, from a worker's perspective, this leads to an optimal sex ratio of 3:1 in favour of females. In contrast, from the queen's perspective, the optimal sex ratio is 1:1 resulting from equal relatedness to all her offspring (r=0.5) (Fisher, 1930). Consequently, the different reproductive optima of the queen and her workers predict that potential conflict may emerge over sex allocation (Ratnieks, Foster and Wenseleers, 2006). Further complicating this idea, conflict over sex allocation should be absent (or limited) in colonies of multiply-mated queens, where relatedness between workers and young queens (half-sisters) is at r=0.25, while the relatedness between a sister and her brother remains at r=0.25 (Boomsma and Grafen, 1991).

Both the queen and the workers have evolved adaptations that can manipulate the sex ratio in their favour (summarized in Trivers and Hare (1976), Helanterä and Ranieks (2009)). The queen can manipulate the sex ratio by laying more male eggs or laying female eggs that can only develop into workers (and not queens) (de Menten *et al.*, 2005). Workers, in turn, can manipulate the sex ratio by eliminating male eggs, allocating resources preferentially to female eggs, and altering the frequency of queens reared from female eggs (Sundstrom, Chapuisat and Keller, 1996; Hammond and Keller,

2004; Reuter *et al.*, 2004). Who ultimately wins this struggle? All possible outcomes of colony sex ratio conflicts are found, including outcomes in favour of the workers, the queen, and, in some cases, a compromise between the optimal ratios by workers and the queen, with a slight advantage for the workers (Trivers and Hare, 1976; Mehdiabadi, Reeve and Mueller, 2003; Ratnieks, Foster and Wenseleers, 2006; Helanterä and Ratnieks, 2009). These studies collectively demonstrate that theory can predict conflict based on different reproductive optima within a social group. In the case of certain eusocial insects, the presence of various mechanisms used by both parties (workers and queen) to shift the colony sex ratio in their favour highlights the ongoing nature of this conflict.

Intragenomic conflict

Similar to conflict between individuals, conflicts can arise between the different genomic elements within an individual, termed intragenomic conflict (Hurst, Atlan and Bengtsson, 1996; Gardner and Úbeda, 2017). For example, in many eukaryotes, nuclear genes are inherited from both parents, while cytoplasmic genes (mitochondrial and chloroplast) are uniparentally inherited, often maternally (Correns, 1908; Kuroiwa, 2010). Given the distinct inheritance patterns of nuclear and cytoplasmic genes, inclusive fitness theory predicts potential conflict over the sex determination of the offspring (Hurst, Atlan and Bengtsson, 1996; Frank, 2000; Gardner and Úbeda, 2017).

An example of intragenomic conflict is predicted and observed in hermaphroditic plant species. Hermaphroditic plants possess both female (ovule) and male (pollen and anther) reproductive organs on the same individual (Renner and Ricklefs, 1995). Cytoplasmic genes are only transmitted through ovules, while nuclear genes are transmitted through both ovules and pollen—meaning that cytoplasmic inherited genes may promote their spread through the population if resource allocation to the male reproductive organ can be stopped.

Field studies on 140 hermaphroditic plant species have revealed that many populations harbour individual plants that lack anthers, meaning they are ‘male-sterile’ (=female) (Rivkin, Case and Caruso, 2016). This phenomenon is called cytoplasmic male sterility (CMS). These findings suggest that indeed selfish cytoplasmic genes can promote their own spread in the population by increasing resources directed to the ovules and inhibiting resources directed to the pollen and anthers (Lewis, 1941). As I discuss later, selfish cytoplasmic elements can rapidly select for nuclear genes that restore male fertility, thereby limiting their success (Frank, 1989). In fact, entire populations of Thyme (*Thymus vulgaris*) were found in which all individuals carry both a CMS gene and a restorer gene (Thompson,

Manicacci and Tarayre, 1998). Nonetheless, the common occurrence of gynodioecy (=coexistence of females and hermaphroditic plants) in nature suggests that these conflicts are ongoing.

Hypotheses for the maintenance of cooperation

"Genes may cooperate for two reasons: either it pays for them to cooperate, or they are forced to do so" Hurst 1996

While theory predicts that conflict could arise in any cooperative group where relatedness is less than <1 (i.e., individuals are non-clonal), instances of actual conflict are rarely observed in nature. This discrepancy between prediction and observation hints at the existence of mechanisms that limit or prevent selfish behaviour and promote cooperation. (Note that, the absence of conflict can also result from the absence of genetic or phenotypic variation that is necessary for natural selection to act upon, including selfish behaviour and mechanisms that counter them). In the following section, I outline two mechanisms that might limit selfishness within cooperative systems: (*i*) kin selection, whereby the benefits of cooperation are restricted to relatives, who share and therefore pass on the alleles for these cooperative behaviours, and *(ii)* enforcement, whereby mechanisms of partner choice, partner-fidelity feedback, and partner manipulation shift the cost/benefit ratio in favour of cooperation. An overview of these concepts is illustrated in Figure 1.1.

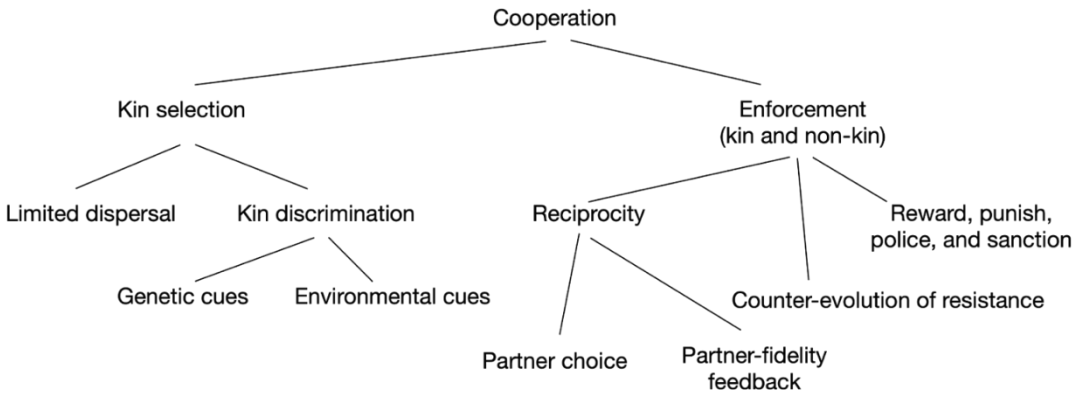

Figure 1.1. Overview of concepts for how cooperation can be maintained. Figure modified from Davies *et al.* (2012).

Kin selection

Previously, I discussed how altruistic behaviours among relatives can be explained by kin selection. However, ensuring that the benefits of cooperation are directed primarily towards kin rather than non-kin requires a mechanism of kin discrimination (Boehm, 2006). This discrimination can be

established through direct genetic cues of relatedness, indirect environmental cues, or a combination of both (Gamboa, Reeve and Holmes, 1991; Mateo, 2002; Holmes, 2004; Penn and Frommen, 2010).

An example of kin discrimination based on an environmental cue is observed in the long-tailed tit (*Aegithalos caudatus*), a small bird that exhibits cooperative breeding (Russell and Hatchwell, 2001). At the start of the breeding season, all pairs initially breed independently. However, many nests fail due to predation. Some failed breeding pairs initiate new nests and some pairs choose to help rear the offspring of other pairs instead (Hatchwell *et al.*, 2004). Helping is considered altruistic because these individuals forgo their own reproduction to benefit that of others (Hatchwell *et al.*, 2001; Russell and Hatchwell, 2001).

Consistent with kin selection theory, Russel & Hatchwell (2001) demonstrated in a choice experiment that helpers consistently chose to provide aid to relatives over non-relatives, suggesting a mechanism of kin discrimination exists. Subsequent research demonstrated that this kin discrimination is based on 'churr calls' used for short-range communication (Sharp *et al.*, 2005). Playback experiments using recorded churr calls from relatives and non-relatives showed that individuals were more attracted to nests playing calls from relatives. To examine whether relatedness was inferred from genetic or environmental cues, the researchers performed cross-fostering experiments, where eggs were swapped between nests. First, they found that the churr calls of foster siblings (non-related) raised in the same nest were more similar than those of true siblings (related) raised in different nests, suggesting that the call differences are acquired rather than hereditary. Second, they found that helping was directed toward individuals who were reared together, rather than genetically related individuals from different nests. These findings provided support for the idea that kin discrimination, in this case using an environmental cue, is one mechanism that allows the maintenance of cooperation.

Kin discrimination based on genetic cues is observed in Hymenoptera (Sundström, 1994). As discussed previously, the optimal sex ratio from the worker's perspective is influenced by whether the queen mates once or multiple times. However, this does require the workers' ability to assess whether the queen has engaged in multiple matings. Boomsma *et al.* (2003) demonstrated that workers could discriminate between full-sisters and half-sisters based on a chemical cue in the hydrocarbons of their cuticles. Workers from a singly mated queen share the same hydrocarbon profile, while workers from multiply mated queens showed variation in their profiles. In a comparative study, Boomsma *et al.* (2003) found that as the variation in hydrocarbon profiles increased in the population, the sex ratio

shifted in favour of more males. This finding suggests that workers can assess the degree of relatedness to other workers based on olfactory cues and adjust the sex ratio accordingly (Sundström, 1994; Boomsma *et al.*, 2003). In short, experiments with long-tailed tits and Hymenoptera highlight that mechanisms of kin discrimination, which allow altruistic behaviours to be directed towards closely related individuals, are crucial components of kin selection.

Enforcement

In addition to kin selection, enforcement is the second major mechanism crucial for the maintenance of cooperation (Singh and Boomsma, 2015; Ågren, Davies and Foster, 2019). Enforcement can be defined as any act that evolves, at least in part, to limit selfish behaviours (Ågren, Davies and Foster, 2019). Mechanisms of enforcement include *(i)* partner choice and associated mechanisms that increase interactions with cooperators, *(ii)* partner-fidelity feedback mechanisms that align fitness interests through long-term stable associations, and *(iii)* partner manipulation mechanisms that modify individual behaviour. Below, I discuss five examples of enforcement: partner choice in a plant-pollinator mutualism, partner-fidelity feedback in a host-symbiont system, sanctioning in a host-legume mutualism, worker policing in eusocial insects, and repression of selfish genetic elements within a genome.

Partner choice through punishment in the yucca plant-yucca moth mutualism

Enforcement through partner choice involves mechanisms that enable individuals to select cooperative partners and control the extent of cooperation with them (Axelrod and Hamilton, 1981; Sachs *et al.*, 2004; Foster and Wenseleers, 2006; Ågren, Davies and Foster, 2019). An example of partner choice occurs in the yucca moth-yucca plant mutualism (Riley, 1892) in which reciprocal specialisation has led to mutual dependence. The yucca plant relies on the yucca moth for pollination, while the moth's larvae rely on the plant's developing seeds for their development. Specifically, the moth first collects pollen from yucca flowers and stores it underneath her head. She then selects flowers suitable for oviposition in which she cuts into the ovary wall and lays her egg inside. The moth can lay multiple eggs in a single flower, and by doing so makes multiple cuts, before moving on to the next flower. After oviposition, the moth walks to the stigma and places the pollen on it. When the larvae hatch, they feed on the developing seeds.

Whereas the moth can obtain a higher fitness from laying more eggs in a single flower, the increasing number of larvae within a flower is predicted to reduce the fitness of the host plant by damaging a large fraction of the developing seeds (Powell, 1992). A study by Pellmyr and Huth (1994)

demonstrated that plants can prevent overexploitation by their social partners by selectively aborting flowers with heavy egg loads, killing the eggs and larvae, and retaining flowers with lower egg loads and relatively higher pollination loads. This result suggests that the plant can regulate the number of eggs placed within flowers by choosing not to cooperate with 'less cooperative' moths that lay too many eggs.

Partner-fidelity feedback in the Vibrio fischeri-squid mutualism

Mutualism can be evolutionary stable when species engage in a long-term stable partnership with repeated interactions, known as "partner-fidelity feedback" (Bull and Rice, 1991; Sachs *et al.*, 2004; Foster and Wenseleers, 2006). In such cases, when one party cooperates, it enhances the fitness of the other party, and vice versa, creating a mutual incentive for both to cooperate. An example of partner-fidelity feedback is observed in the highly specific interaction between the bioluminescent bacterium *Vibrio fischeri* and the Hawaiian bobtail squid (*Euprymna scolopes*) (McFall-Ngai and Montgomery, 1990; Montgomery and McFall-Ngai, 1993; Jones and Nishiguchi, 2004; Nyholm and McFall-Ngai, 2021). In each generation of the squid, a juvenile squid recruits exclusively *V. fischeri* from the surrounding environment within a specialized cavity known as the "light organ". This association benefits both partners: the bacteria receive nutrients and protection, while the squid benefits from bacterial bioluminescence at night. Bioluminescence of the bacteria mimics moon- and starlight which allows the squid to hunt prey and avoid being predated on itself. Hence, the positive phenotypic effects both parties provide to each other create a positive feedback loop that stabilises cooperation (Foster and Wenseleers, 2006).

Partner choice and partner manipulation in the host-legume mutualism

Enforcement through sanctioning is observed in the legume-rhizobia mutualism (Kiers et al. 2003). Legumes provide carbon and oxygen to rhizobia that inhabit its root nodules, and the rhizobia fixate atmospheric nitrogen and provide this to their host (Fred, Baldwin and McCoy, 1932). However, nitrogen fixation by the bacteria is energetically costly and diverts energy away from the growth of the bacteria themselves (Burdon *et al.*, 1999). In many cases, multiple bacterial species inhabit the root nodules of a legume plant, and these species or strains can vary in the extent to which they supply nitrogen to their host—essentially their degree of cooperation (Burdon *et al.*, 1999). Consequently, selection might favour cheater bacteria that invest less in nitrogen fixation themselves, while reaping the benefits of fixation by more cooperative rhizobia (Kiers *et al.*, 2003; Oono, Anderson and Denison, 2011). Kiers *et al.* (2003) tested whether host plants can differentiate between cooperative and non-cooperative bacteria and exclude those nodules that house non-cooperative individuals. They

mimicked bacterial selfishness by replacing the airflow from some nodules with a gas mixture containing very low levels of nitrogen. Indeed, the host plant could reduce its resource allocation to the non-cooperative root nodules, resulting in a relatively lower nodule mass. This finding supports the idea that cooperation can be enforced through either partner choice (i.e., by preferentially interacting with cooperative bacteria) or partner manipulation (by sanctioning non-cooperative bacteria).

Worker policing in eusocial insects

Earlier I described how workers in eusocial insects sometimes lay unfertilized eggs that develop into males (r=0.50) rather than rearing the queen's sons (r=0.25). However, in colonies where the queen is multiply mated, the relatedness of non-laying workers to their nephews is reduced (offspring of laying workers, r=0.125). This reduced relatedness occurs because the female shares only 0.25 of her genes with her half-sisters, and therefore relatedness to these workers' sons is only r =0.125, compared to male offspring of the queen, r=0.25. Consequently, while selection is predicted to promote workers to produce sons, it also promotes other workers to suppress those that perform this behaviour (Woyciechowski and Łomnicki, 1987). To test this idea, Ratnieks and Visscher (1989) conducted an experiment in which they introduced male eggs into colonies that were either laid by the queen or a worker. While most queen-laid eggs were not removed by the workers, almost all worker-laid eggs were quickly eliminated and consumed by the workers within seven hours after introduction. This finding supports the prediction that workers can distinguish queen-laid from worker-laid eggs and that worker-laying is suppressed or 'policed' by other workers. Subsequent research also found that worker policing prevails more in colonies with multiply-mated queens, as average worker relatedness decreases as the queen's number of matings increases (Foster and Ratnieks, 2000; Hammond and Keller, 2004; Wenseleers and Ratnieks, 2006b). These findings demonstrate that mechanisms of enforcement, such as policing, can contribute to the maintenance of cooperation, and that these mechanisms also prevail in social groups that exhibit already a high relatedness.

Repression of selfish genetic elements

Leigh proposed that selfish genetic elements (SGEs), owing to their fitness costs on the transmission of other genes, should select for mechanisms that rapidly suppress them and restore cooperation (Leigh, 1977). This counter-evolutionary process is also called "the parliament of genes", drawing an analogy to decision-making in a parliamentary system. Specifically, other genes within the genome have a unified interest in the suppression of SGEs, and conflicts over transmission are often settled in

favour of the majority, resulting in random transmission (Dawkins, 1976). Consistent with this idea, various empirical studies have provided evidence for the suppression of SGEs (reviewed in Rice and Holland, 1997). For example, as mentioned earlier, cytoplasmic male sterility (CMS) in hermaphroditic plants arises because it provides a transmission advantage to maternally inherited cytoplasmic genes, as reproductive effort is not 'wasted' on the production of pollen (where 'wasted' refers to the perspective of a cytoplasmic gene). These variants can then spread in populations, so long as there is no pollen limitation. These SGEs, however, are countered by the rapid evolution of nuclear restorer alleles that suppress mitochondrial dysfunction and restore pollen fertility (Schnable and Wise, 1998). In other words, the faster rate at which the CMS genes spread creates a context in which there is selection for restorer genes to suppress their effects (Hurst, Atlan and Bengtsson, 1996).

In summary, empirical studies provide strong evidence that enforcement mechanisms play a significant role in the maintenance of cooperation within non-clonal social systems. Enforcement can promote cooperation in interactions that occur either once or persist throughout a lifetime. In the absence of kin selection, enforcement is predicted to play a crucial role in the maintenance of cooperation between non-relatives and different species (Frank, 1995, 2003; El Mouden, West and Gardner, 2010; Ågren, Davies and Foster, 2019). Nonetheless, enforcement can also play an important role in social systems with high relatedness, as observed in eusocial insects (Ratnieks and Visscher, 1989; Singh and Boomsma, 2015).

Social evolution in microbes

So far, my focus has been on describing cooperation and conflict in macro-organisms. However, microorganisms also exhibit complex and coordinated behaviours that involve cooperation (Crespi, 2001; West *et al.*, 2007; Gonçalves *et al.*, 2020). Examples of microbial cooperation include biofilm formation (Kolter and Peter, 2006), quorum sensing (Diggle *et al.*, 2007; Williams *et al.*, 2007), the production of molecules for nutrient acquisition (West and Buckling, 2003), collective swarming and hunting (Velicer and Vos, 2009), and fruiting body formation (Strassmann, Zhu and Queller, 2000; Kessin, 2001). This prompts the question of whether social evolution theory, initially developed to explain cooperation in social animals, can be extended to explain cooperation in microbes.

An example of social evolution in the bacteria *Pseudomonas*

Microbial cooperation has been extensively studied in the bacteria *Pseudomonas fluorescens* and *P. aeruginosa* (West and Buckling, 2003; Griffin, West and Buckling, 2004; Rolf Kümmerli *et al.*, 2009; Butaitė *et al.*, 2018; Butaitė, Kramer and Kümmerli, 2021; Figueiredo, Wagner and Kümmerli, 2021).

In certain *Pseudomonas* species, cooperation involves the production of a public good: a resource that is metabolically costly for the actor to produce but benefits other cells nearby—in this case, iron scavenging molecules (Varma and Chincholkar, 2007; West *et al.*, 2007).

Iron is an essential nutrient for bacteria. It is crucial for various cellular processes, including energy production, DNA synthesis, and gene expression regulation (Andrews, Robinson and Rodríguez-Quiñones, 2003). However, iron is often limited in nature and exists predominantly in the ferric (Fe^{3+}) form, which microbes cannot readily solubilize (Lindsay and Schwab, 1982). To take up iron, bacteria produce siderophores—secondary metabolites that bind to and solubilize iron from the environment, making it available for uptake by the cell (Ratledge and Dover, 2000; Greenwald *et al.*, 2007). Importantly, the siderophore-iron complex can also be taken up by surrounding cells with compatible receptors, leading to potential for exploitation of siderophore production (West and Buckling, 2003; Griffin, West and Buckling, 2004; Jiricny *et al.*, 2010; Ghoul *et al.*, 2014; Kramer, Özkaya and Kümmerli, 2020).

In natural populations of *Pseudomonas fluorescens*, considerable variation exists in the production of the siderophore pyoverdine (Bruce *et al.*, 2017; Butaitė *et al.*, 2018). Some strains produce minimal pyoverdine, referred to as non-producers. Laboratory experiments in which non-producers and producers from the same site were mixed under conditions that favour cheating (low iron availability), demonstrated that some non-producers could exploit the production by producers, consistent with theoretical predictions (Bruce *et al.*, 2017).

The coexistence of pyoverdine producers and non-producers in natural populations led to studies that examined the mechanisms that maintain cooperation (Griffin, West and Buckling, 2004; Harrison *et al.*, 2008; Jiricny *et al.*, 2010; Kümmerli *et al.*, 2015; Bruce *et al.*, 2017; Butaitė, Kramer and Kümmerli, 2021). One tested hypothesis is that cooperation may be maintained through the rapid evolution of resistance against non-producers (Butaitė, Kramer and Kümmerli, 2021). To test this idea, researchers isolated *Pseudomonas* strains from natural communities and determined their pyoverdine variant and quantity during clonal growth. In addition, they performed cross-inoculation experiments where non-producers were tested in the presence of producers from the same site and from different sites. These experiments aimed to test for evidence of local or co-adaptation, predicting that cheating should rapidly select for new adaptations to resist cheating, resulting in lower levels of cheating in same-site compared to different-site pairings. The results showed variation in the ability of non-producers to exploit pyoverdine production by producers, both in same-site and different-site pairings. However,

there was no evidence of reduced exploitation in same-site versus different-site pairings that would indicate local adaptation of producers to non-producers. Instead, the ability of non-producers to exploit siderophore production by producers increased when strains were more closely related, likely resulting from compatible receptors derived from common ancestry.

In summary, while this study did not confirm evolved resistance against cheaters as a mechanism to maintain cooperation, it underscores the parallels in social behaviours between microbes and macro-organisms. The ease of manipulating microbes offers a unique opportunity to examine the prevalence and relevance of mechanisms proposed to maintain cooperation in nature.

In this thesis, I focus on another microbe—the soil amoeba *Dictyostelium discoideum*. In the following sections, I describe the unique life cycle of *D. discoideum*, which has made it a useful model organism for studying cooperation and conflict (Strassmann and Queller, 2011). I also discuss various hypotheses that seek to elucidate how cooperation is maintained, placing a particular emphasis on the evolution of mechanisms to repress cheaters.

The model organism *Dictyostelium discoideum*

The social amoeba *D. discoideum* is primarily found in the upper layer of the soil in deciduous forests. So far it has been found primarily in eastern North America, particularly along the Appalachian mountains, as well as in East Asia (Swanson, Vadell and Cavender, 1999). *D. discoideum* belongs to the class of Dictyostelids in the major taxonomic group of the Amoebozoa (Baldauf *et al.*, 2000). A defining characteristic of the Dictyostelids is their remarkable transition from unicellular amoebae to a multicellular stalked fruiting body structure (Schaap *et al.*, 2006).

As individual cells, the haploid amoebae of *D. discoideum* primarily feed on other microorganisms and reproduce through mitosis (Kessin, 2001). However, when food becomes scarce, the cells transition from a vegetative stage of feeding and growth to a developmental stage. Depending on environmental conditions, this transition can lead to the formation of either a macrocyst in the sexual cycle or a fruiting body in the asexual cycle (Fig. 1.2) (Raper, 1935; Kessin, 2001; Bonner, 2003; Chisholm and Firtel, 2004).

Macrocyst formation in the sexual life cycle

In the sexual life cycle of *D. discoideum*, which occurs in a wet and dark environment, two haploid cells (amoebae) with compatible mating types can fuse to create a diploid zygote (Fig. 1.2) (Erdos, Raper

and Vogen, 1973; Wallace and Raper, 1979; Saga and Yanagisawa, 1982; Bloomfield *et al.*, 2010a). The zygote attracts the surrounding haploid cells, which are cannibalized, and their cells materials used to form three layers of cellulose. Within the zygote, meiosis takes place, followed by multiple rounds of mitosis, resulting in the production of many recombinant haploid amoebae. The final product of this process is called a macrocyst, which is a hardened structure capable of surviving harsh environments for longer periods. When conditions are favourable again, the macrocyst can germinate to release hundreds of amoebae. While macrocyst formation is also interesting from a social evolution perspective (Douglas, Queller and Strassmann, 2017), this thesis will focus on the asexual life cycle of *D. discoideum*.

Cooperative fruiting body formation in the asexual life cycle

Under conditions of dryness, food deprivation, and high cell numbers, *D. discoideum* enters its asexual life cycle in which a fruiting body is formed (Kessin, 2001). Initially, up to hundreds of thousands of cells aggregate into a tightly packed mound structure (Fig. 1.2). This structure gradually morphs into a slug, which exhibits a strong attraction to heat and light, guiding it to the soil surface (Bonner *et al.*, 1950; Kessin, 2001). During the slug stage, cells start to differentiate into distinct cell types. Approximately 10-30% of the cells adopt prestalk fate and redistribute themselves towards the anterior of the slug. The remaining 70-90% of the cells adopt prespore fate and redistribute themselves towards the posterior of the slug (K. B. Raper, 1940; Bonner and Dodd, 1962). As the slug morphs into a fruiting body structure, the prestalk cells differentiate into stalk cells, undergoing vacuolization to form a rigid stalk composed of dead cells. The prespore cells migrate up the stalk and differentiate into viable spores. These spores can be dispersed by small invertebrates or other organisms (J. Smith, Queller and Strassmann, 2014). When environmental conditions are favourable a single spore can germinate to release a single amoeba, completing the life cycle.

Importantly, the self-sacrifice of the stalk cells is considered altruistic, as this behaviour likely benefits the spore cells by aiding their survival and dispersal via elevation in the air (Kessin, 2001; Kuzdzal-Fick *et al.*, 2007; Strassmann and Queller, 2011; J. Smith, Queller and Strassmann, 2014). The self-sacrifice of stalk cells in *D. discoideum* is therefore akin to the altruistic behaviour observed in eusocial insects, where workers forgo their own reproduction to care for the offspring of the queen (Davies, Krebs and West, 2012).

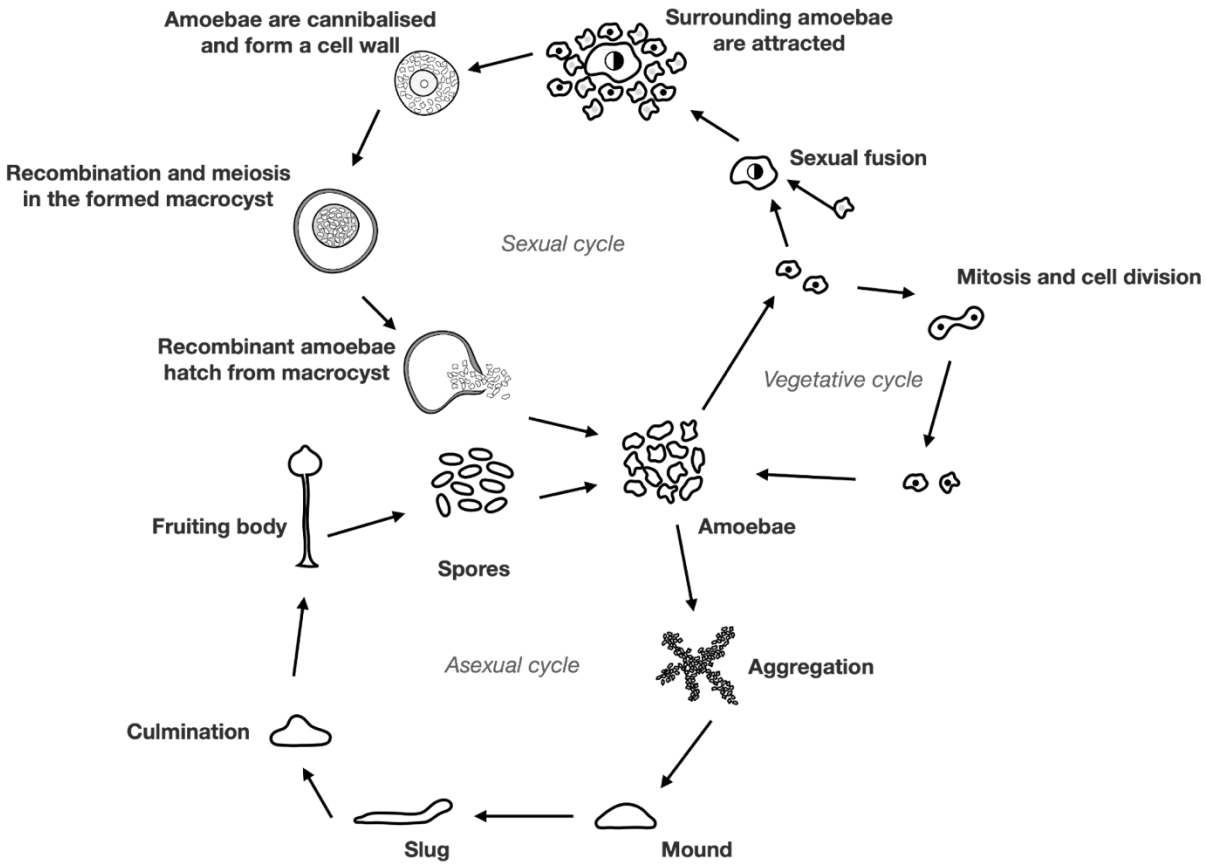

Figure 1.2. The life cycles of the social amoeba *Dictyostelium discoideum.* *D. discoideum* can undergo a sexual and asexual life cycle in which a macrocyst and fruiting body are formed respectively. Not drawn to scale. Figure modified from Flowers *et al.* (2010).

Kin selection theory explains that the altruistic act of becoming a stalk cell can be favoured by selection if it provides sufficient benefits to genetically related individuals (kin) (Hamilton, 1964a). In simpler terms, when cells within a fruiting body are clonal or sufficiently related, the costs of the self-sacrifice incurred by the stalk cells can be compensated by the benefits gained by the spores. However, unlike most multicellular organisms, *D. discoideum* becomes multicellular through aggregation. Unlike purely clonal structures, a 'chimeric' fruiting body can be formed, where potentially genetically different individuals can coexist (Filosa, 1962; Buss, 1982; Ennis *et al.*, 2000; Strassmann, Zhu and Queller, 2000; Foster *et al.*, 2002).

In cases of chimerism (i.e., $r<1$), evolutionary theory predicts that conflict over reproductive fate may arise. Selfish genotypes, or cheaters, can increase their relative fitness by reducing their investment in the stalk while exploiting the investment made by others in the group (Fig. 1.3) (Buss, 1982; Strassmann, Zhu and Queller, 2000; Hudson *et al.*, 2002). If not limited or prevented, cheaters could rapidly spread through a population, potentially leading to the breakdown of cooperation within the

population (discussed further below). An important aspect of fruiting body formation in *D. discoideum* is that stalk formation is a type of public good, implying that a genotype that does not contribute to the formation itself is not a better competitor but instead *exploits* the investment by other strains—which makes this an act of cheating (Ghoul, Griffin and West, 2013). Additionally important, cheating does not imply a conscious decision nor does it imply evolutionary success per se (Tarnita *et al.*, 2015; Wolf *et al.*, 2015; Martínez-García and Tarnita, 2016; Ostrowski, 2019).

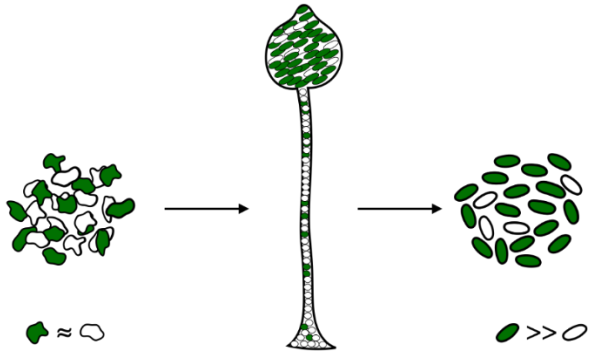

Figure 1.3. The formation of a genetically mixed, chimeric, fruiting body formation can select for cheaters that avoid contributing their fair share to stalk formation and consequently become overrepresented in the spores. In the illustration, the cheater is depicted in green. The fraction of the cheater in the spores exceeds what it was in the cells before development. The figure has been adapted from Ostrowski (2019).

The apparent persistence of altruistic fruiting body formation in *D. discoideum*, despite the potential for conflicts to arise over reproductive outcomes, has raised several questions related to the evolution and maintenance of this behaviour. In the research chapters of this thesis, I will focus on three key questions: *(i)* How widespread is cheating in nature, *(ii)* What adaptations exist in nature that limit or prevent cheating, and (*iii*) Have these adaptations led to divergence in social behaviours across and within populations? To provide a foundation for these questions, in this chapter, I review prior work that examined cheating between natural strains of *D. discoideum*. I then discuss the experiments that identified potential cheating mechanisms and mechanisms that limit or prevent this behaviour.

Evidence for cheating in nature

Several studies have examined the social interactions among natural strains of *D. discoideum* and provided evidence of cheating (Strassmann, Zhu and Queller, 2000; Fortunato *et al.*, 2003a; Gilbert *et al.*, 2007; Buttery *et al.*, 2009; Buttery, Thompson and Wolf, 2010; Flowers *et al.*, 2010; Parkinson *et al.*, 2011). Strassmann *et al.* (2000) were the first to quantify the magnitude of cheating in twelve pairwise mixes between strains sampled from a 1.5 km^2 area in North Carolina. To do this, they mixed the spores of five fruiting bodies from two strains with *Klebsiella aerogenes* as a food source and plated the entire mixture on semi-food-rich plates. In the slugs that formed in these mixes, they

sampled the posterior (prespore) and anterior (prestalk) regions. They isolated DNA from these regions and performed PCR amplification of the DNA at three microsatellite loci. They quantified the brightness of the PCR bands and used these estimates to quantify the fractions of the two strains in the prestalk and prespore regions of the slugs. They found that in approximately half of the mixtures, one strain was overrepresented in the prespore region compared to its social partner, suggesting that natural strains may regularly cheat on each other. This pioneering work motivated subsequent studies to investigate the prevalence and mechanisms of cheating in more detail.

Following the influential work by Strassmann and colleagues, additional studies have also reported instances of cheating among natural strains of *D. discoideum* (Fortunato, Queller and Strassmann, 2003; Buttery *et al.*, 2009; Buttery, Thompson and Wolf, 2010; Flowers *et al.*, 2010; Wolf *et al.*, 2015). Notably, except for Flowers *et al.* (2010), there has been considerable overlap in the set of strains used across these studies, limiting the diversity of strains tested. Therefore, to examine if cheating is common in nature, in Chapter 3, I quantify the frequency and intensity of this behaviour across a much broader range of strains from many more populations. Nonetheless, despite the limited number of strains tested, the evidence to date suggests that cheating occurs in natural populations, prompting further exploration.

Evidence for cheating in the laboratory

Finding the genes and mechanisms associated with cheating in *D. discoideum* has primarily been done using genetically modified lab strains. To investigate the genes involved in cheating, Kuspa and Loomis (1992) used restriction enzyme-mediated insertional (REMI) mutagenesis on the lab strain, AX4. REMI mutagenesis generates random insertions into genomic restriction sites, with some insertions potentially leading to mutations in genes associated with cheating (Schiestl and Petes 1991). To enrich for cheaters, the REMI generated mutant pool was put through repeated rounds of development favouring strains that preferentially form spores rather than stalk cells. As a result of this effort, some of the enriched mutants had completely lost their ability to fruit by themselves and instead relied on their social partners to form the stalk, therefore called “obligate cheaters” (Ennis *et al.*, 2000; Nelson *et al.*, 2000).

In examining specific cases of obligate cheaters, one notable example is mutant *fbxA*$^{-}$ (formerly known as *chtA*). *fbxA*$^{-}$ harbours a mutation in an F-box A protein involved in cell-fate decisions that prevents the differentiation of vegetative cells into stalk cells. This mutation results in aberrant clonal development, typically not progressing beyond the mound stage (Ennis *et al.*, 2003). Consequently,

obligate cheaters may spread through a population until the point where they limit their own spread by drastically lowering spore production. Whereas obligate cheaters rapidly emerge in laboratory settings (J. J. Kuzdzal-Fick *et al.*, 2011), their existence in nature remains an open question. Some attempts have been made to isolate obligate cheaters or 'stalkless' strains from nature, but these efforts have proven unsuccessful, suggesting that obligate cheaters may be rare in nature (further discussed below) (Gilbert *et al.*, 2007; Medina *et al.*, 2019; Ostrowski, 2019).

Santorelli *et al.* (2008) similarly used REMI mutagenesis to select for cheaters. In contrast to the previous study, they introduced a round of clonal development after ten rounds of development. By doing so, they excluded obligate cheaters and instead isolated 'facultative' cheaters—strains that cheat when developed with non-self, but retain their ability to produce functional fruiting bodies when developed clonally (Buttery *et al.*, 2009; Strassmann and Queller, 2011). The sequencing of the facultative cheater mutants they obtained revealed over 150 genes involved in different pathways that, if mutated, led to cheating (Santorelli *et al.*, 2008).

Some of the mutants isolated by Santorelli and colleagues have been further analysed. For example, cheater mutant C (*chtC*$^{-}$) increases its representation in the spores in a chimera by altering its cell fate during development (Khare and Shaulsky, 2010). During the slug stage, *chtC*$^{-}$ initially generates a prestalk cell fraction that is similar to that of its social partner. However, as development proceeds, some of these prestalk cells transdifferentiate into prespore cells, which eventually results in the overrepresentation of *chtC*$^{-}$ in the spores (Khare and Shaulsky, 2010). The mechanism by which *chtC*$^{-}$ acts can therefore be considered a type of self-promotion.

Another cheater, cheater mutant B (*chtB*), becomes overrepresented in the spores in chimera by reducing the expression of the prespore gene *cotB* in its social partner, which in turn results in its reduced spore production (Santorelli *et al.*, 2013). Importantly, unlike the clonal costs that limit the spread of obligate cheaters, facultative cheaters that retain their ability to cooperate when by themselves may spread in nature (if not limited by mechanisms I discuss below) (Santorelli *et al.*, 2008).

In addition to obligate and facultative cheaters, the third category of cheaters is called fixed cheaters (Buttery *et al.*, 2009). Fixed cheating can arise if strains differ in their inherent allocation of cells to the spores and stalk (i.e., spore-to-stalk allocation)—even if two strains do not change these allocations in a chimera, the strain with the intrinsically greater spore allocation will become overrepresented in

the spores. In doing so, it may also benefit from the greater stalk allocation of its partner strain, which is why this behaviour can be considered a type of cheating. For example, if strain A typically allocates 90% of cells to spores and 10% to the stalk, while strain B allocates 70% to the spores and 30% to the stalk and the strains do not alter this ratio in a chimera, then strain A will obtain 56% (=90/160) of the spores in the mix.

Fixed allocation cheating minimally requires that strains differ in their spore-stalk ratios. Is this true? Consistent with this requirement, natural strains do appear to vary in their inherent spore-to-stalk allocation, even within sites (Buttery *et al.*, 2009; Votaw and Ostrowski, 2017). Importantly, Buttery et al. (2009) found that when mixed, these inherent differences could predict the outcomes in terms of spore representation. Consequently, as one might predict that this process selects for reduced stalk investment, this finding raises questions about how optimal or sufficient levels of stalk formation are maintained in nature (Hudson *et al.*, 2002). These questions are further explored in Chapters 2, 4 and 5.

How common is chimeric fruiting body formation in nature?

The occurrence and frequency of chimerism in nature play a crucial role in determining whether fixed and facultative cheating might occur and how these behaviours impact the maintenance of cooperative fruiting body in *D. discoideum*. Unfortunately, assessing how common chimerism is in nature poses challenges because fruiting bodies are too small to be sampled from nature directly. Researchers must therefore rely on indirect measurements to estimate the occurrence of chimerism. For example, one study examined the number of genotypes within small (0.2 g) soil samples. Here, a genotype was based on the genetic similarity at five microsatellite loci. They found that 63% of samples contained more than one genotype, with some samples containing up to six genotypes. Based on the differences at five microsatellite loci, this resulted in an average relatedness of approximately 0.52 (Fortunato *et al.*, 2003a). Another study estimated relatedness in fruiting bodies collected from deer scat pellets brought back to the laboratory (Gilbert *et al.*, 2007). Based on three microsatellite loci, they found that 77% of fruiting bodies consisted of a single strain and 23% consisted of multiple strains, resulting in a minimum relatedness of 0.86. The high relatedness within individual fruiting bodies suggests the existence of mechanisms that limit most of the genetic mixing among closely occurring strains. The methods to estimate relatedness in nature and the possible implications of these findings are discussed in more detail in Chapter 2.

In summary, behaviours consistent with cheating have been observed among strains isolated from nature. In addition, prior research mostly using laboratory-generated mutants has identified various mechanisms of cheating, including obligate, fixed, and facultative. The impact of these different mechanisms on the maintenance of cooperative fruiting body formation in *D. discoideum* likely varies depending on the genetic diversity in natural populations and the frequency of chimerism in nature.

What mechanisms may limit cheaters?

The apparent ease with which cheaters can arise in laboratory conditions, coupled with observations of cheating among natural strains and the co-occurrence of genetically distinct strains in small soil samples, prompted questions on how cooperation may be maintained in *D. discoideum*. In the following sections, I describe two mechanisms proposed to restrict cheating and maintain cooperation: high relatedness and the counter-evolution of repression to cheating.

High relatedness prevents cheaters from emerging

Two studies have explored the role of relatedness in preventing the success of cheaters in *D. discoideum* (Gilbert *et al.*, 2007; J. J. Kuzdzal-Fick *et al.*, 2011). Using experimental evolution, Kuzdzal-Fick *et al.* (2011) tested if obligate cheaters (strains unable to fruit independently) could evolve under conditions of low or high relatedness. Under conditions of low relatedness, achieved by random mixing of newly emerging mutants into the population during each transfer, about a third of the populations evolved obligate cheaters. When co-developed with their ancestor, the proportion of cheaters in a mix exhibited a negative correlation with total spore production, indicating that at low relatedness, the spread of obligate cheaters can undermine cooperation. Conversely, under conditions of high relatedness, achieved by maximizing drift and minimizing selection through the introduction of a single-celled bottleneck for each social generation, no obligate cheaters evolved. In short, these findings suggest that high relatedness can protect a population against obligate cheaters.

In another study, they tested if the level of relatedness observed in nature is sufficient to limit the spread of the obligate cheater *fbxA*$^-$ (Gilbert *et al.*, 2007). The researchers determined how high relatedness must be to prevent invasion of *fbxA*$^-$. At a relatedness of >0.75 (from the perspective of the mutant), *fbxA*$^-$ decreased in frequency because it could no longer rely on fruiting body formation by the wild-type strain, resulting in net selection against *fbxA*$^-$. Comparing this threshold to the observed level of relatedness in fruiting bodies sampled from plated deer scat pellets (r=0.86, as discussed earlier), they concluded that the level of relatedness in nature would be sufficiently high to limit the spread of obligate cheaters. This study, together with the work by Kuzdzal-Fick *et al.* (2011), underscore the crucial role of relatedness in maintaining cooperation in *D. discoideum*.

High relatedness can result from mechanisms of allorecognition

Similar to macroscopic organisms, altruism in *D. discoideum* relies on mechanisms that ensure that cooperation is directed towards kin (Hamilton, 1964b; Buss, 1982; Grosberg and Quinn, 1986; West *et al.*, 2007; Bourke, 2011). First, high relatedness can result from passive mechanisms such as spatial structuring, where related individuals cluster together due to limited dispersal. Indeed, empirical work demonstrated that genetically different cells a few millimetres apart on an agar plate could generate a high level of genetic relatedness in the resulting fruiting bodies (Smith, Strassmann and Queller, 2016).

A second way to achieve an assortment of kin is through the active process of kin discrimination: the identification of, and preferential cooperation with, kin (West, Griffin and Gardner, 2007a; Ostrowski *et al.*, 2008; Strassmann, 2016). Kin discrimination has been demonstrated in *D. discoideum* and other Dictyostelids (Kaushik, Katoch and Nanjundiah, 2006; Mehdiabadi, Jack, Tiffany Tally Farnham, *et al.*, 2006; Ostrowski *et al.*, 2008; Flowers *et al.*, 2010; Sathe, Khetan and Nanjundiah, 2014; Gruenheit *et al.*, 2017). In *Dictyostelium*, genetically different strains will initially co-aggregate but then partly segregate to form fruiting bodies with a relatedness higher than expected under random mixing.

The genes responsible for kin discrimination in *D. discoideum* are *tgrB1* and *tgrC1* (Benabentos *et al.*, 2009; Hirose *et al.*, 2011). These genes code for the cell-surface proteins TgrB1 and TgrC1, which together form a ligand-receptor pair. This pair operates during the aggregative stage, functioning as an allorecognition system to distinguish between self and non-self in neighbouring cells (Benabentos *et al.*, 2009; Hirose *et al.*, 2011, 2017). Genomic analyses of *tgrB1* and *tgrC1* alleles in natural strains revealed a remarkably high level of polymorphism compared to the average variation in *D. discoideum*'s genome, aligning with the expectation that genes involved in allorecognition undergo rapid evolution or selection maintains it (West-Eberhard, 1983; Benabentos *et al.*, 2009; Ostrowski *et al.*, 2015). This diversity is predicted to enable cells to accurately assess their relatedness to other cells in a mixed population.

Ho and colleagues proposed that the segregation mediated by the *tgrB1*/*tgrC1* locus may serve as a mechanism to prevent cooperation with potential cheaters (Ho *et al.*, 2013). To test this hypothesis, they engineered strains that differed at a cheating gene and/or at the *tgrB1*/*tgrC1* locus, maintaining isogenic conditions otherwise. They predicted that if the *tgrB1*/*tgrC1* locus is responsible for the segregation of kin from non-kin, a cheater identical to its social partner at the *tgrB1*/*tgrC1* locus could evade kin recognition-mediated segregation. Conversely, a cheater non-identical to its social partner

at the *tgrB1*/*tgrC1* locus should segregate from its social partner, indicating kin recognition. Their findings supported both predictions, indicating that genetic dissimilarity at the *tgrB1*/*tgrC1* locus serves as a defence mechanism against cheaters. However, segregation was not always perfect, and there were associated costs in terms of reduced spore production. Imperfect segregation was likewise observed in experiments involving natural strains of *D. discoideum* (Ostrowski *et al.*, 2008). In short, these studies underscore that genetic dissimilarity at the *tgrB1*/*tgrC1* locus might serve as a defence mechanism against cheaters in *D. discoideum*, despite its imperfect function and associated costs.

In summary, prior work in *D. discoideum* suggests that both kin discrimination and relatedness are crucial for the maintenance of cooperation in natural populations. In addition, the high level of polymorphism in the genes responsible for kin recognition may reflect the need for these mechanisms, i.e., suggesting that the potential threat of being cheated on in nature is high (Ho *et al.*, 2013; Ostrowski, 2019).

Counter-evolution of resistance to cheating

The second major mechanism proposed to prevent cheaters from becoming successful is the counter-evolution of resistance to cheating (Frank, 2003; Travisano and Velicer, 2004; Khare *et al.*, 2009; Manhes and Velicer, 2011; Hollis, 2012; Levin *et al.*, 2015; Ostrowski, 2019). Resistance to cheating can result from different mechanisms. For example, genotypically different cells might enforce cooperation by punishing cheaters, similar to what is observed in social insects (Queller and Strassmann, 1998; Ratnieks, Foster and Wenseleers, 2006; Ratnieks and Wenseleers, 2008). Another possibility is that the emergence of cheating might rapidly select for mutations that suppress cheating and enforce cooperation, similar to the evolution of restorer genes in response to SGEs that cause meiotic drive in *Drosophila* (Atlan *et al.*, 1997; Jaenike, 2001; Larracuente and Presgraves, 2012). Below, I discuss the three studies that have examined the possibility for counter-adaptations to evolve in response to cheating in *D. discoideum* (Khare *et al.*, 2009; Hollis, 2012; Levin *et al.*, 2015).

Evidence for repression to evolve in the laboratory

Khare *et al.* (2009) used a genetic screen to select for mutants able to resist a facultative cheater. To do so, they introduced the facultative cheater mutant *chtC*$^{-}$ into a pool of REMI mutagenesis mutants resistant to the antibiotic blasticidin and allowed the population to develop fruiting bodies. They collected the spores and grew these in the presence of blasticidin to eliminate *chtC*$^{-}$. This process was repeated for a total of six selection cycles. After six cycles, the majority of the experimental lines consisted mainly of a single genotype that had evolved resistance to cheating by the *chtC*$^{-}$ mutant. To

test whether resistance was specific to the mechanism of cheating, one of the evolved resistant strains, mutant *rccA* (resistor of chtC A), was co-developed with another known facultative cheater mutant, *LAS1*. The results showed that the *rccA* mutant was unable to confer resistance against the *LAS1* cheater mutant, indicating that resistance may be specific to the mechanism of cheating (similar results were found in a recent study by Miller *et al.* (2023)). This study thus demonstrated that in a pool of artificially created genetic variants, cheating can rapidly select for resistance to cheating, without adopting the behaviour themselves.

Hollis (2012) investigated whether resistance to cheating could evolve in a population through spontaneous mutation. To test this idea, cooperative strain AX2 was co-developed with a known strong facultative cheater of this strain, NC4. Two types of evolution experiments were performed in which the cheater either was or wasn't allowed to co-evolve (i.e., two- and one-sided evolution respectively). Similar to Khare *et al.* (2009), in the one-sided evolution selection regime, the population of AX2 evolved resistance to the cheater NC4. In the two-sided evolution selection regime, where both AX2 and NC4 were allowed to evolve, varying levels of evolved resistance to cheating were observed across experimental lines. One possible explanation for this result is that in response to evolved resistance, the cheater mutant evolved new counter-adaptations to overcome evolved resistance by the cooperative strain. In short, complementary to the study of Khare *et al.* (2009), this work provided evidence that resistance to cheating can result from evolved mutations rather than from existing genetic diversity. However, the strength of resistance might vary depending on whether the cheater can rapidly evolve new strategies to counter resistance.

The third study that examined if the counter-evolution of resistance to cheating could evolve in *D. discoideum* was conducted by Levin *et al.* (2015). This research built upon the experimental lines evolved under low relatedness from Kuzdzal-Fick *et al.* (2011). As discussed earlier, some of these experimental lines had evolved obligate cheaters, called 'non-fruiters', alongside developmentally proficient genotypes, called 'fruiters'. Levin's study examined whether the evolved fruiters in these populations had conferred resistance to their coevolved non-fruiters. To test this idea, they isolated both the evolved fruiters and evolved non-fruiters by plating the spores of the evolved population at a very low density, resulting in individual plaques of amoebae from single spores. They identified non-fruiters by screening for plaques that did not proceed beyond the aggregation or mound stage, indicating their inability to form fruiting bodies. In the first set of experiments, the collective of non-fruiters was co-developed with its ancestor, and with the collective of non-fruiters. The results showed that while non-fruiters could cheat their ancestor, they were unable to cheat their co-evolved fruiters.

In a second set of experiments, individual clones of non-fruiters were co-developed with their ancestor, and with individual clones of fruiters. Individual clones of non-fruiters consistently cheated on their ancestor but varied in their ability to cheat on individual clones of coevolved fruiters, akin to the results in Hollis (2012). This indicates that populations of *D. discoideum* could partly evolve resistance to obligate cheaters before these cheaters completely dominated the population.

Together, the three discussed studies demonstrated that cheaters can rapidly select for counter-adaptations that resist cheating, thereby contributing to the partial restoration of spore equity. These findings provide support for the hypothesis that cooperation in *D. discoideum* may also be maintained through mechanisms of enforcement, specifically via the evolution of resistance to cheating.

Social adaptations that confer cheating and resistance might drive evolutionary arm-races

So far, I have discussed empirical work demonstrating that the presence of cheaters can rapidly lead to the evolution of resistance and restore cooperation. However, in rare instances, resistance may instead select for stronger cheating or other counter-repression mechanisms, initiating a positive feedback loop of adaptations (Dawkins and Krebs 1979; Haig 1993d; Hurst 1996). These evolutionary arms races are well-established in various biological interactions such as host-pathogen, host-parasite, and plant-pollinator relationships and have been shown to lead to both within-population polymorphism and between-population divergence in associated traits (Dawkins and Krebs, 1979; West-Eberhard, 1983; Rice and Holland, 1997; Dybdahl and Lively, 1998; Frank, 2000; Thompson, 2005; Brockhurst *et al.*, 2014; Queller and Strassmann, 2018). While arms races have been extensively studied in the context of interspecific interactions, whether they arise from social conflict within a single species, as suggested by the empirical work of Khare, Hollis and Levin, remains limited (Ostrowski, 2019). Nonetheless, genomic data from natural strains of *D. discoideum* has also revealed signatures of increased selection on genes associated with cheating and resistance, indicating potential coevolutionary dynamics (Ostrowski et al. 2015; Noh et al. 2018). However, further research is required to directly test whether social conflict drives evolutionary arms races and shapes intraspecific interactions and cooperative behaviours in nature.

Research objectives

In conclusion, the discussed research underscores the current knowledge gaps surrounding altruistic fruiting body formation in *D. discoideum*. These gaps include uncertainties about the frequency and extent of cheating in natural populations, as well as the mechanisms restricting this behaviour. Additionally, the specific drivers of cheating and the evolutionary consequences of its occurrence in a

natural setting remain unknown. This thesis seeks to address these gaps by connecting insights from laboratory studies to more natural scenarios. The following sections outline the specific objectives for each chapter.

In **Chapter 2**, I describe the evolution of aggregative fruiting body formation in *D. discoideum* and other taxa. This review discusses the potential benefits and evolutionary mechanisms that underlie stalk formation in various taxa. In addition, I explore the taxonomic diversity of aggregative fruiting species, emphasizing variations in stalk morphology and potential functional differences among the species and lineages within dictyostelids. This chapter contributes to what we know about the ecological and adaptive significance of stalked fruiting body formation.

In **Chapter 3**, I focus on two questions: *i.* How common are cheaters in natural populations? And *ii.* Does resistance to cheating arise in nature? To answer these questions, I first quantify the intensity of cheating and frequency of cheaters in fifteen natural populations of *D. discoideum*. To test if resistance readily evolves, I compare the frequency of cheaters in pairs of strains from the same population ('local') partners with that observed in pairs of strains from different populations that lack an evolutionary history together ('foreign'). Additionally, I examine two other mechanisms that might limit cheaters from becoming successful: *i.* cheaters limit their own spread because they reduce groups that harbour them, and *ii.* kin discrimination excludes potential cheaters from the social group. These experiments address the prevalence and intensity of cheating in nature and examine potential mechanisms that exist to limit this behaviour.

In **Chapter 4**, I examine the outcomes of social interactions between strains to elucidate one commonly observed aspect of social groups in nature: the tendency to form a linear dominance hierarchy. A linear hierarchy implies that one individual dominates all others, the second dominates all but the most dominant, and so forth. A common explanation for linearity is that social success is predetermined based on certain dominance characteristics, such as increased size or strength that are additive across different social partners. Interestingly, linearity was also found among a set of natural strains of *D. discoideum* (Fortunato, Queller and Strassmann, 2003). In lieu of other fitness equalizing mechanisms, linearity is predicted to lead to a loss of social trait variation over time, as the best genotype outcompetes the others. However, it is unknown whether linearity is common in natural populations of *D. discoideum*, given that its observation has been limited to a single set of seven strains at one site only. In this chapter, I assess the linearity of the dominance hierarchy in four natural populations of *D. discoideum*. I also determine whether linearity results from pre-existing variation in

dominance abilities among strains. Finally, I employ a quantitative genetics analysis to explore underlying factors that influence social dominance. This analysis examines the influence of the social genetic environment on social traits, a factor often overlooked but increasingly shown to be important in their evolution.

In **Chapter 5**, I investigate whether strains and populations have undergone divergence in fruiting body morphology, in particular stalk investment, due to diverse abiotic or biotic environments. This examination involves measuring stalk length, spore number and spore size in twenty strains from four natural populations. The allocation of resources to the spores versus the stalk serves as a metric for the level of altruism investment within a group, and evolutionary theory predicts that this ratio can be influenced by various factors. Moreover, whereas prior work suggests that strains with increased spore-to-stalk ratios can have a competitive advantage, few studies have examined the diversity in these traits within different natural populations. Lastly, assessing the fruiting body morphology across many strains will allow me to examine possible biophysical limitations to the production of a stalk fruiting body structure. The outcomes of this chapter will contribute to current research on phenotypic diversity of social traits in nature. Additionally, it will address the possible impact of cheaters on the maintenance of altruism investment within social groups.

In **Chapter 6** I provide concluding remarks on the research presented in this thesis.

Chapter 2.

Group Transformation: Fruiting Body and Stalk Formation

Cathleen M. E. Broersma and Elizabeth O. Ostrowski

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Abstract

Throughout the eukaryotic tree of life, amoeboid organisms have evolved that aggregate upon starvation and form multicellular fruiting bodies, consisting of a ball of spores atop a stalk. This chapter discusses the remarkable convergent evolution of a stalked fruiting body in these different taxa. It then discusses a well-studied group of aggregative fruiters, the cellular slime moulds, in more detail. These organisms exhibit substantial variation in their stalk formation and composition, which allows a better understanding of the evolution, maintenance, and possible functions of stalked fruiting bodies, but also points to potential costs and benefits of different types of stalks.

Introduction

Dispersal is essential to the life history of many organisms (Bowler and Benton, 2005; Kokko and López-Sepulcre, 2006). It can enhance survival when environmental conditions deteriorate, promote outbreeding, and broaden a species' geographic range. Dispersal often occurs in response to environmental cues, is regulated during ontogeny, and may involve specialized structures or cells (propagules) that promote long-distance travel and survival under adverse conditions.

Dispersal can potentially be accomplished by locomotion, and many single-celled organisms have mechanisms of active locomotion that enable travel over short distances. For example, some prokaryotic and eukaryotic microorganisms can swim using their flagella. Amoebae crawl across surfaces by extending and retracting pseudopods. Some organisms, such as myxobacteria, can glide over surfaces alone or in groups. However, microscopic organisms typically rely on passive mechanisms for dispersal over greater distances. Passive mechanisms include dispersal by wind or water or by hitching a ride on a larger organism. It can be facilitated by a resting state, such as a spore or cyst.

In addition to forming hardy cysts or spores that protect cells in harsh environments, many organisms also produce fruiting bodies that promote dispersal by lifting spores into the air. Fruiting bodies are produced by diverse taxa, ranging from fungi to bacteria to amoebae. Most have a similar morphology, consisting of some sort of stalk that lifts and supports a spore-producing head, resulting in a lollipop or umbrella morphology. Fruiting bodies can consist of only one or a few spores produced by cell division ("sporocarps") or they can be multicellular, usually produced through aggregation of cells ("sorocarps") (Kang *et al.*, 2017; Spiegel *et al.*, 2017). Formation of single-celled versus multicellular fruiting bodies likely entails different costs and benefits, as do the different ways of achieving these

structures, through aggregation or cell division, referred to as “coming together” versus “staying together” (Tarnita, Taubes and Nowak, 2013).

In this chapter, I discuss what is known about the evolution and function of stalked fruiting bodies in taxa that exhibit aggregative multicellularity. I begin by discussing amoeboid organisms throughout the tree of life that form these structures. The convergent evolution of similar morphologies, accomplished through diverse means, suggests that they are adaptations. Nevertheless, while it seems likely that stalked fruiting bodies confer a benefit, exactly what they are an adaptation for, and why they evolved and persist, remains subject to debate. In the later sections, I turn our attention to a large clade of sorocarpic amoebae—the Dictyostelia—that are well-studied for their aggregative multicellularity from both a developmental and an evolutionary perspective.

Aggregative fruiting is found throughout the eukaryotic tree of life

Many taxa that undergo aggregative multicellularity to form fruiting bodies have an amoeboid single-cell state. Thus, these organisms are called “sorocarpic amoebae” or “cellular slime moulds”. The first sorocarpic amoeba to be discovered, *Dictyostelium mucoroides*, was isolated by Brefeld in 1869. The Dictyostelia, which comprises a large clade within the Amoebozoa, are still the most well-studied of the taxa that undergo this morphological transformation (Shadwick *et al.*, 2009). However, sorocarpic amoebae can be found in five of the six eukaryotic supergroups: Amoebozoa, Opisthokonta, Excavata, Stramenopiles and Rhizaria (Fig. 2.1). Molecular reconstruction indicates that sorocarpy evolved independently in most of these taxa, as summarized in Brown and Silberman (2013).

Sorocarpic amoebae have a characteristic life cycle, consisting of separate unicellular and multicellular stages, the latter of which is achieved through aggregation. In all cases, the transition to multicellularity involves a switch from a stage of feeding and cell division to one of development and differentiation. When nutrients are abundant, the single-celled amoebae feed on soil microorganisms and increase in number by cell division. Upon starvation, anywhere from a few to hundreds of thousands of amoebae aggregate through chemotaxis and cooperatively form a fruiting body structure, consisting of dispersal propagules and a stalk that supports them. Despite these commonalities, however, there are numerous differences among different types of sorocarpic amoebae in the formation and morphology of their fruiting bodies. Below, I emphasize some notable differences among the different taxa and how these different structures, achieved through different routes, nevertheless result in a stalked fruiting body structure (Fig. 2.1).

Guttulinopsidae (Rhizaria)

Species from the genus *Guttulinopsis* are the only known aggregative fruiters in the supergroup Rhizaria, which contains mostly unicellular eukaryotes (Brown *et al.*, 2012). In the most common species, *G. vulgaris,* the stalk is composed of multiple different compartments, some of which contain dead cells, which can hold aloft one or several sori (Fig. 2.1A) (Raper, Worley and Kessler, 1977). *G. vulgaris* thus represents an example of reproductive division of labour; only some cells undertake reproduction and others instead provide non-reproductive, structural support.

Acrasidae (Excavata)

The species *Acrasis rosea* was the first non-dictyostelid sorocarpic amoeba to be discovered (Olive and Stoianovitch, 1960). Following aggregation into a mound, a single cell at the base of the mound differentiates into an encysted stalk cell. Cells on top of the stalk cell subsequently encyst as well, resulting in a stalk that extends upward and holds aloft the rest of the population. After the stalk is complete, the remaining cells in the aggregate form chains and encyst to become spore cells. This results in a fruiting body that resembles a tree-like structure, consisting of a main branch and smaller offshoots (Fig. 2.1B). Although all cells in the fruiting body are viable, it is unknown whether there are fitness costs or benefits associated with adopting different positions in the fruiting body (Kaushik and Nanjundiah, 2003).

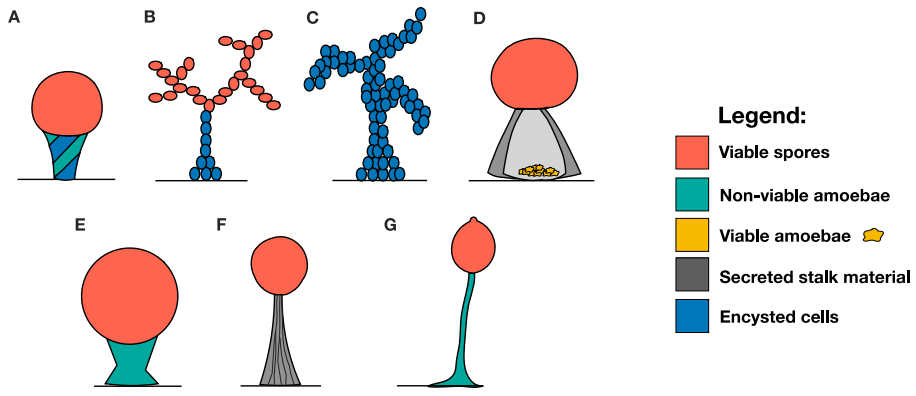


Figure 2.1. Fruiting body morphologies throughout the eukaryotic tree of life. (A) *Guttulinopis* - Rhizaria, (B) *Acrasis rosea* - Excavata, (C) *Copromyxa protea* - Amoebozoa, (D) *Fonticula alba* - Opisthokonta, (E) *Sorodiplophrys stercorea* - Stramenopiles, (F) *Sorogena stoianovitchae* - Alveolata, (G) *Dictyostelium discoideum* - Amoebozoa. Fruiting bodies are not drawn to scale.

Copromyxa (Amoebozoa)

The genus *Copromyxa* consists of only two species, only one of which, *Copromyxa protea,* exhibits aggregative fruiting (Brown, Silberman and Spiegel, 2011). In *Copromyxa*, fruiting body formation involves the aggregation of cells, with those at the apex becoming encysted (Fig. 2.1C). Like *A. rosea*, the fruiting body consists of a column of cells, and the existence of any position-dependent fitness difference is unknown. Kaushik and Nanjundiah (2003) describe this process as "coming together and sticking to each other", a more primitive evolutionary form of multicellularity compared to other species of sorocarpic amoebae that show complex division of labour, where cells adopt distinct and irreversible cell fates.

Fonticula (Opisthokonta)

Multicellularity evolved multiple times within the Opisthokonta, manifested by different forms: metazoan (animals) and fungi, which arise by cell division from a single starting cell, and aggregative fruiting forms (Ruiz-Trillo *et al.*, 2007; Brown, Spiegel and Silberman, 2009; Fisher, Shik and Boomsma, 2020). Among the latter is the taxon *Fonticula,* which currently consists of only a single species, *Fonticula alba*. *F. alba* was isolated only once by Olive and Stoianovitch (1960) and never rediscovered, although the original isolate has been retained. Following aggregation into a mound, the cells secrete a Golgi-derived extracellular matrix, forming a hollow volcano-shaped stalk tube. Except for a small number of cells that continue to produce stalk material, the cells in the stalk are mechanically forced out of the top of the structure. These cells encyst, forming a ball of spores suspended in a thin slime sheet, which collapses a few days after maturation (Fig. 2.1D) (Worley, Raper and Hohl, 1979; Brown, Spiegel and Silberman, 2009).

Sorodiplophrys (Stramenopiles)

Little has been published about *Sorodiplophrys stercorea* (Dykstra and Olive, 1975; Tice *et al.*, 2016). Like other sorocarpic amoebae, however, single-celled amoebae aggregate and form a fruiting body. The final fruiting body structure consists of a small, thick stalk composed of secreted material and dead cells that supports a spherical, golden, mucoid sorus (Fig. 2.1E). The presence of dead stalk cells indicates that this organism shows reproductive division of labour as well.

Finally, while many examples of aggregative multicellular fruiting body formation involve sorocarpic amoebae, it is worth noting that there are additional non-amoeboid taxa that undergo cooperative fruiting.

Myxobacteria are a clade of prokaryotic organisms that undergo aggregative fruiting in response to starvation. Their fruiting bodies can vary dramatically among different species. For example, some species form clear stalks that lift up the spores, whereas in others, the stalk is reduced or absent (Velicer and Vos, 2009). The best-known species is *Myxococcus xanthus*, which has been used as a model system for cooperation and conflict. The life cycle of *M. xanthus* is similar to that of social amoebae: the soil-dwelling bacteria prey upon other microbes, sometimes cooperatively as a swarm (Mauriello *et al.*, 2010). Upon starvation, the bacteria aggregate into mounds and form fruiting bodies, where only a fraction of the cells differentiate into spores, and others either remain as rod-shaped cells or undergo autolysis (Fig. 2.1F) (Varon, Cohen and Rosenberg, 1984; Nariya and Inouye, 2008). The percentage of cells that become viable spores is much lower than in some of the other eukaryotic species discussed so far, with a non-spore percentage of up to 90% in *M. xanthus*, at least under laboratory conditions (Velicer and Vos, 2009). The reason for the variation in fruiting body morphology among species, including in the formation of a stalk, is not well understood (Velicer and Vos, 2009).

Sorogena stoianovitchae (eukaryotic supergroup Alveolata) is unique among ciliates in undergoing aggregative fruiting (Olive and Blanton, 1980; Sugimoto and Endoh, 2006). In its unicellular stage, it feeds on other ciliate species. Upon food shortage, however, it aggregates beneath the water surface and forms an aerial fruiting body. The stalk is produced via collective secretion of a mucous material by the entire population. The stalk lifts the population out of the water, after which each of the cells becomes encysted and together form a sorus (Sugimoto and Endoh, 2006).

Fruiting body formation in the Amoebozoa

Within the Amoebozoa, the group historically known as *Eumycetozoa* (true slime moulds) consisted of three major classes of organisms: *Protostelids*, *Myxogastrids*, and *Dictyostelids.* The latter two groups are monophyletic, whereas molecular analyses indicate that protostelids are not. For this reason, they are now sometimes referred to as “protostelioid amoebae” rather than “protostelids”, to emphasize common elements of their morphology in lieu of a phylogenetic classification (Shadwick *et al.*, 2009). Protostelioid amoebae undergo sporocarpic development (Spiegel *et al.*, 2017). The amoeba secretes an extracellular matrix, which forms a stalk that lifts the amoeba. The amoeba then differentiates into a spore, sometimes following cell division (Furtado and Olive, 1971; Lahr *et al.*, 2011; Spiegel *et al.*, 2017). Thus, sporocarpy in protostelids results in the production of microscopic fruiting bodies that contain only one or a few cells.

The plasmodial slime moulds belong to the monophyletic group *Mxyogastrea*, another taxon of sporocarpic amoeba. Plasmodial slime moulds are named for the slimy structure they produce—called a plasmodium—which forms when amoeboid cells undergo repeated rounds of mitosis without cytokinesis. This process results in a single, massive, multi-nucleated cell with a continuous cytoplasm, which can reach many meters in size. When conditions turn bad, the plasmodium produces masses of stalked fruiting bodies, a process that occurs not through growth, but through rearrangement of the existing biomass (Stephenson and Schnittler, 2017). Thus, they form fruiting bodies, albeit not achieved through aggregation. Spores are mostly wind-dispersed from the fruiting bodies and germinate to form the plasmodium again. Plasmodial slime moulds were the likely inspiration for the 1950s horror film *The Blob*. Like protostelids, the stalks produced by plasmodial slime moulds are acellular (i.e., secreted). Unlike the protostelids, however, their fruiting bodies are macroscopic.

Finally, the dictyostelids consist of more than 160 species (Romeralo *et al.*, 2011). Their phylogenetic tree contains many long, unbroken branches, which suggests that they have been undersampled and that the true diversity of the group is even greater (Romeralo *et al.*, 2011). Although the phylogeny has been revised over the years, recent phylogenies based on SSU rRNA and alpha-tubulin sequences group dictyostelids into two major clades, the Dictyosteliales and the Acytosteliales, each of which is composed of two groups—resulting in groups 1-4, referred to below. These groups are then further subdivided (e.g., into groups 2A and 2B) (Romeralo et al. 2011). The model organism *D. discoideum,* discussed in detail below, belongs to group 4.

Comparative analyses indicate that the formation of a stalked fruiting body is conserved within the dictyostelids (Schaap *et al.*, 2006; Heidel *et al.*, 2011; Romeralo *et al.*, 2011; Sucgang *et al.*, 2011). Romeralo et al. (2013) combined genetic data from 99 species with the phenotypic data of 24 traits in each of these species. This work suggests that their last common ancestor (~0.6-1.0 billion years ago) formed fruiting body structures that lift spores in the air. However, as I emphasize below, the species show substantial variation in the formation and appearance of their fruiting bodies (Fig. 2.1). The evolutionary drivers of such diversity in fruiting structures are still being investigated. Nevertheless, this diversity makes this group suitable to study the function of a stalk, its associated costs and benefits, and the possible functional constraints on this structure.

Morphological variation among dictyostelids, with emphasis on stalk formation

In this section, I discuss fruiting body formation and function from the perspective of the model organism *D. discoideum*. I start by describing the life cycle and stalk formation in *D. discoideum*. I then

describe some of the morphological variation within dictyostelids, particularly in when and how they form their stalked fruiting bodies.

D. discoideum is a soil-dwelling amoeba frequently isolated from the upper layer of the soil of mostly deciduous forests located in the temperate zone (Swanson, Vadell and Cavender, 1999; Landolt, Stephenson and Cavender, 2006). Its life cycle is broadly similar to many of the sorocarpic amoebae described in previous sections. However, its developmental cycle is more complex, as it involves division of labour, extensive cell-cell communication and coordinated cell death. *D. discoideum* initiates aggregation in response to deteriorating environmental conditions. Starving cells cease phagocytosis, secrete extracellular cyclic adenosine monophosphate (cAMP), and respond chemotactically to sources of cAMP, which results in cell streaming and aggregation to form a mound. In the mound, cells initially differentiate into either pre-stalk or pre-spore cells, indicative of their eventual cell fates in the later fruiting body. The pre-spore cells move to the top of the mound, which forms a tip that elongates into a finger-like structure that falls to the surface. The resulting worm-like structure, called a slug, migrates away from the site of aggregation. Following migration, and upon detecting cues such as overhead light, the slug transforms into a multicellular fruiting body. Cells at the anterior of the slug undergo apoptosis, having vacuolized and hardened to form a rigid, dead cellular stalk. The remaining cells move to the top of the stalk and differentiate into viable spores, which disperse and germinate to release single-celled amoebae (Fig. 2.1G). In addition to this multicellular stage, which results in asexually produced spores, *D. discoideum* has a sexual stage. The sexual stage also involves aggregation and cell sacrifice (through cannibalism). It results in the formation of a durable structure, called a macrocyst, that is not stalked (Bloomfield 2010b; Bloomfield, 2011; see also Schapp chapter). Meiosis takes place during the formation of the macrocyst, and the amoebae that later emerge are recombinants.

D. discoideum is a model system for cell biology, developmental biology, chemotaxis, and host-pathogen interactions (Williams, 2010; reviewed in Bozzaro, 2019). It is genetically tractable, has a precise 24-hour development cycle, and terminal differentiation results in a small number of distinct cell types. *D. discoideum* is also notable for its relatively stable cell-type proportions: approximately 80% of cells in the posterior of the slug will form the spore-containing sorus, whereas ~20% of cells in the anterior die to form the stalk. These cell-type proportions can partially re-establish following perturbations, for example, by ablation of either the anterior (prestalk) or posterior (prespore) sections of the slug (Kenneth B. Raper, 1940; Ràfols *et al.*, 2001). The robustness of its spore-stalk cell proportions is of interest to developmental biologists interested in how multicellular organisms

achieve and maintain specific cell-type proportions, as well as evolutionary biologists interested in whether and how an altruistic stalk can be maintained.

While *D. discoideum* is by far the best studied of the social amoebae species, it exhibits a variety of traits that are somewhat uncommon among dictyostelids. Below, I focus on three morphological traits, namely stalk composition, stalked migration and clustering and branching patterns, comparing *D. discoideum* to other species. Although it is difficult to ascertain the adaptive significance of this variation in morphology, I discuss the functional implications of the different structures and some of their potential costs and benefits.

Cellular versus acellular stalks

Aggregative multicellularity—by virtue of allowing unrelated cells to collaborate to form a multicellular individual—presents opportunities for conflict, especially if there are different fitness costs and benefits associated with the adoption of different cell fates. This problem is particularly severe in social amoebae, where cells that form the stalk will die and the remainder will live, providing a large fitness advantage to strains that can avoid the stalk fate. Conflict is thought to emerge over which cells will adopt the dead-cell fate and which will survive into the next generation. The opportunity for different genotypes to co-aggregate means that selection has the opportunity to favour genotypes that behave selfishly (Ostrowski, 2019).

Stalk formation does not necessarily require self-sacrifice. The acytostelids (Group 2A), for example, form acellular stalks, consisting of a hollow tube that is made from secreted cellulose, with all cells subsequently forming viable spores atop the stalk (Mohri *et al.*, 2013). In contrast, cellular stalks are made from an inner layer of hardened vacuolized stalk cells and an outer layer of cellulose (Gezelius, 1959). Why some species evolved to use cellular stalks, whose formation depends on the death of a fraction of the population, while others form stalked fruiting bodies without such a sacrifice, is not known.

At present, I can only speculate about why these differences might have evolved. One possibility is that cellular stalks might provide stronger support, allowing larger aggregate sizes and taller stalks that can support more spores. For example, acytostelids have a smaller aggregate size and form smaller structures compared to species with cellular stalk formation (170-1200 mm versus 1200-8200 mm), which might be consistent with a weaker stalk in the former (Raper 1956b; Schaap et al. 2006). Additionally, Kaushik and Nanjundiah (2003) point out that the production of an acellular stalk might be energetically costly to the cells and therefore detract from their ability to survive for long periods.

The division of labour achieved through the formation of a cellular stalk might entail benefits for the survival of the spores.

One possible benefit of acellular stalk formation is the ability to produce a fruiting body with a smaller population. This potential benefit is apparent in *Dictyostelium lacteum*, the only species known to be capable of producing both cellular and acellular stalks. When food availability is low, the species forms a small, acellular stalk, like that produced by acytostelids; only at higher cell numbers is a larger, cellular stalk formed (Bonner and Dodd 1962; Bonner 2006). While *D. lacteum* is the only species known to be plastic for cellular stalk formation, it is possible that other dictyostelids possess similar plasticity but remain to be discovered, or that their plasticity has simply not been noticed. Finally, although some have speculated that acytostelids could represent an intermediate stage in the evolutionary transition from simple (e.g., single cell type, no division of labour) to complex (multiple cell types with division of labour) multicellularity (Olive 1975; Bonner 2003), a molecular study by Romeralo (2013) concluded that the most recent common ancestor of the dictyostelids likely already displayed cellular stalk formation, suggesting that acellular stalk formation is a derived trait.

Stalked migration

In some species, aggregation of cells is followed by the formation of a slug that migrates away from the point of aggregation (Fig. 2.2). For example, in *D. discoideum*, the slug forms approximately 12 hours after the onset of starvation, and it can travel long distances from the site of aggregation, resulting in movement of up to 6 cm in a week (Jack *et al.*, 2011, 2015). In *D. discoideum*, the slugs are strongly phototactic, moving towards a directional light source. Migration ceases once light is overhead, which triggers the culmination to form a fruiting body. The combination of attractants (light and heat) and repellents (high ammonia levels) is thought to direct slugs upwards through the soil, into an open area suitable for fruiting body formation (Raper 1984; Bonner and Lamont 2005).

Slug migration is thought to have evolved several times in the major groups of the dictyostelids (Romeralo et al. 2013). In *D. discoideum*, the stalk is not formed until after slug migration, during the final stages of development. However, in the majority of dictyostelid species, stalk is continuously produced from the rear of the slug during its migration (Fig. 2B). Ancestral trait reconstruction suggests that the last common ancestor of the dictyostelids was likely a stalked migrator, with only a few species (*D. discoideum, D. polycephalum, D. citrinium, D. intermedium* and *D. dimigraformum*) having evolved stalkless migration (Schaap et al. 2006; Schaap 2007; Romeralo et al. 2013).

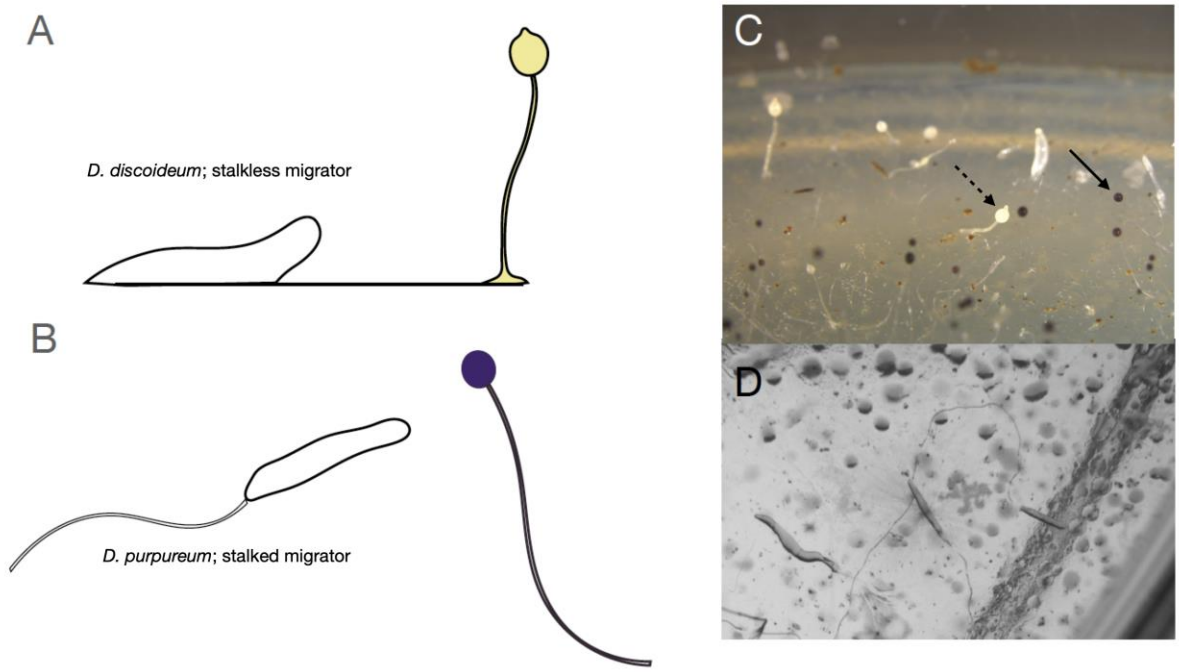


Figure 2.2 Stalked versus stalkless migration among dictyostelid species. (A) *D. discoideum* and (B) *D. purpureum,* both Group 4 species. *D. discoideum* has stalkless migration and an upright, sturdy stalk with a flattened disk of cells at its base. Its slugs lie on the substrate, with the tip (pre-stalk zone) in the air. It produces yellow sori. *D. purpureum* has stalked migration, produces a slender stalk that lacks a basal disc, and its sori are purple. (C) *D. discoideum* and *D. purpureum* co-occurring on the same soil isolation plate. The pale yellow fruiting bodies are *D. discoideum* (dashed arrow), whereas the dark fruiting bodies are *D. purpureum* (solid arrow). (D) A different soil isolation plate, showing an unidentified dictyostelid species with stalked migration. Images taken by E.O. Ostrowski.

Several studies have addressed the potential costs and benefits of stalked versus stalkless migration (Bonner, 1982; Gadagkar and Bonner, 1994; Jack *et al.*, 2011). Jack et al. (2011) quantified the costs of slug migration in *D. purpureum* (a stalked migrating species) and *D. discoideum* (which does not undergo stalked migration). Both species showed a trade-off between migration and sporulation, as they show a similar decrease in sporulation after controlling for differences in migration distance. The authors suggest that the two species could have adapted their behaviour to different stages of the life cycle. As *D. purpureum* produces much taller fruiting bodies than *D. discoideum* (>7 mm versus 3-7 mm), the authors suggest that *D. purpureum* invests less in migration (active dispersal) and more in fruiting body size (passive dispersal). In contrast, *D. discoideum* invests relatively more in active dispersal so it can reach suitable fruiting locations further away, though it produces a shorter fruiting body structure.

That the stalk might serve a different or additional purpose in different dictyostelids was proposed by Bonner (1982) and later tested by Gilbert et al. (2012). They showed that the stalked migrator *D. giganteum* could use its stalk as a bridge to traverse small gaps in the substratum. In contrast, slugs of stalkless migrator *D. discoideum* were not able to traverse these same gaps, suggesting one potential advantage of stalked migration. Regardless of some of the potential benefits of stalked migration, it is surprising that cells might co-aggregate to form a slug with no guarantee as to how large of a stalk—and thus, how big of a cell sacrifice—will be made.

Because the costs of stalk formation are likely to be greater for stalked migrators like *D. purpureum*, one might expect that higher levels of relatedness within cooperative fruiting bodies would likewise need to be greater. Indeed, while both *D. discoideum* and *D. purpureum* possess mechanisms of kin discrimination, and thus imperfectly separate out during chimeric development (Mehdiabadi, Jack, Tiffany Talley Farnham, *et al.*, 2006; Ostrowski *et al.*, 2008), *D. purpureum* seems to segregate more completely than *D. discoideum*. It would be interesting to know whether *D. purpureum* has a stronger history of selection on the genes that underpin its kin discrimination. These genes have been identified in *D. discoideum* (Hirose *et al.*, 2011), but are not yet known in *D. purpureum*.

Clustering and branching patterns

In *D. discoideum,* each aggregate gives rise to a fruiting body, which consists of a relatively thick, non-branched stalk that holds aloft a single sorus. This morphology is common among species in Group 4, but outside of this group there is a large variety of structures that differ in their degree of clustering and branching (Fig. 2.3). In some species, secondary tips form after aggregation, giving rise to multiple, closely spaced fruiting bodies (referred to as “gregarious” development) or fruiting body structures with multiple sori emanating from one stalk (“like flowers in a vase”, Raper 1956). Fruiting bodies can also be branched and/or consist of whorls (Schaap et al. 2006; Baldauf and Strassmann 2017). The extent of the branching and clustering is also plastic, as it can depend on cell density (Bonner and Dodd, 1962; Romeralo *et al.*, 2013). The general pattern was that larger structures—i.e., branched, and clustered—tend to form in response to high cell density, whereas unbranched and solitary structures would emerge at low cell density.

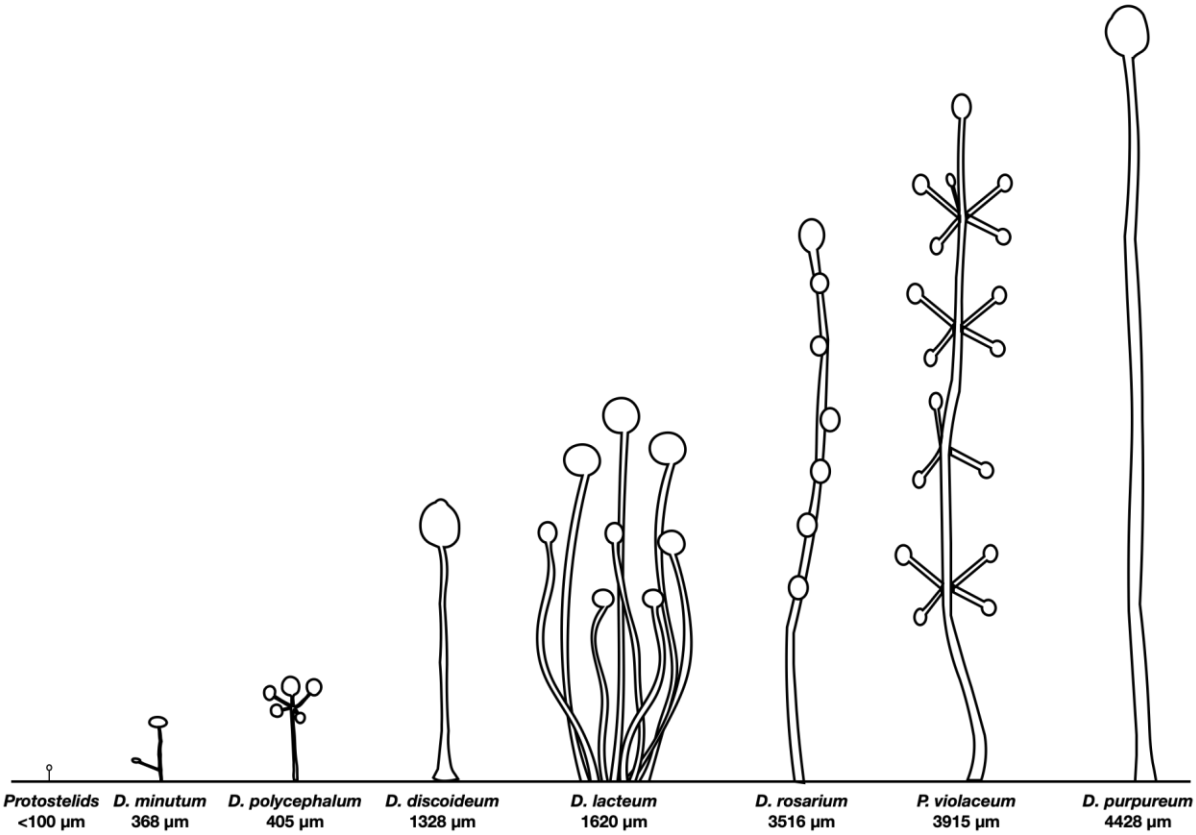


Figure 2.3. Examples of clustering and branching patterns in the fruiting bodies of protostelids and dictyostelids. Approximate fruiting body height is indicated below each species, based on descriptions in Raper (1984), except for *P. violaceum*, where the value listed is the mean stalk length from Romeralo et al. (2013). Note that fruiting body size can vary substantially depending on plating conditions, so only an approximate range is provided.

Altruism, stalk formation, and the maintenance of multicellularity in *D. discoideum*

In previous sections, I discussed numerous examples of aggregative multicellularity that result in the formation of a stalked fruiting body. I emphasized that, in many cases, many or all the cells in the fruiting body remain viable, although there could be fitness costs associated with exactly which role is adopted by a given cell. I also emphasized that some species form fruiting bodies with secreted, non-cellular stalks, and others form extensive cellular stalks throughout migration, resulting in a potentially large and unpredictable cell sacrifice.

The formation of cellular stalks by dictyostelids is of special interest to evolutionary biologists. Stalk formation is likely altruistic, in that some give up opportunities for direct fitness to form a structure that appears to benefit the rest. This differentiation into spore and stalk cells is analogous to the differentiation into soma and germline that is seen in complex multicellularity. Stalk formation is a

clear example of reproductive division of labour, where some cells specialize in reproduction, and others specialize in non-reproductive functions.

Certain features of the *Dictyostelium* life cycle mean that its reproductive division of labour might be evolutionarily fragile. Aggregative multicellularity potentially permits multiple different genotypes to co-aggregate and form chimeric multicellular structures. This genetic diversity in combination with strong fitness consequences for becoming stalk vs spore means that natural selection can operate during this stage of the life cycle. Thus, all else being equal (and it may not be), natural selection should favour genetic variants that can avoid the costly role of the stalk and disproportionately adopt the high-fitness spore fate. The problems posed by chimerism in *Dictyostelium* and its potential consequences for the evolution of multicellularity are discussed more fully in Chapter 7 (Jahan et al. 2022). Given these opportunities for selection to favour stalk-avoiders, one long-standing question is the extent to which opportunities for conflict may have influenced how multicellularity evolves (e.g., whether aggregative multicellularity is successful) and whether it is evolutionarily stable. In *Dictyostelium*, this possibility has led to interest in whether stalkless forms might evolve in nature.

Hunting for stalkless strains in nature

There have been limited attempts to find stalkless strains in nature. Buss (1982) reported the existence of a stalkless *Dictyostelium* strain. While growing and isolating *Dictyostelium* fruiting bodies from soil samples, he observed two distinct fruiting body morphologies. One strain showed a standard stalked morphology, whereas the other produced a ball of spores directly on the substratum, without a stalk. Buss described the latter strain as a "somatic cell parasite", because it was capable of "reproducing itself and spreading infectiously" at the costs of the rest of the population (Buss, 1982). To study whether such a stalkless strain could be maintained in the population over multiple generations, he co-cultured the stalked and stalkless strain over several generations. The stalkless strain increased in frequency from rarity and, at a sufficiently high starting ratio, it could become fixed. However, when the stalkless strain was mixed with a different set of strains isolated from the same soil sample, no chimeric fruiting bodies were observed, indicative of some sort of recognition mechanism present in the other strains able to distinguish self from non-self. Unfortunately, the stalkless strain was subsequently lost (Buss, pers. communication), making it impossible to study it further. This means that it is not possible to carry out molecular analyses to establish which dictyostelid species it was or to identify the genetic changes responsible for its unusual morphology.

More recently, Gilbert *et al.* (2007) attempted to isolate stalkless strains from natural populations. Unfortunately, dictyostelid fruiting bodies are too small and infrequent to find simply by directly

examining soil samples. Moreover, finding stalkless strains—given that they do not form the recognizable macroscopic fruiting body—is even more challenging. However, previous work showed that fruiting bodies can sometimes be observed on animal dung pellets (Raper, 1984; Stephenson and Landolt, 1992). Gilbert and colleagues collected dung pellets, brought them back to the lab, and incubated them with or without additional bacteria for food until fruiting bodies formed (Gilbert et al. 2007). They then collected the spores from 95 of these fruiting bodies and plated them at low density on a lawn of bacteria. Under these conditions, well-spaced spores germinate and divide to produce circular plaques—clearings in the bacterial lawn where the amoebae have devoured the prey. At the centre of each plaque, where the amoeba cell density is high and food has been depleted, fruiting bodies will form if the strain is capable of multicellularity. Thus, dilution plating of spores to see whether they give rise to fruiting bodies is a way to screen for the presence of strains that have lost multicellularity, yet previously managed to join and form a stalked fruiting body with others, or that exhibit other morphological alterations. However, despite screening >3,300 plaques, Gilbert and colleagues observed no stalkless morphologies.

To our knowledge, the works by Buss (1982) and Gilbert *et al.* (2007) are the only studies that have attempted to identify and/or quantify the frequency of stalkless strains in nature. In the future, as new methods for single-cell genomics improve, it might be possible to use culture-independent methods to isolate, sequence, and identify each amoeba cell in a soil sample, enabling the identification of natural isolates that have lost multicellular development or evolved novel morphologies not currently recognized. For now, however, whether *D. discoideum*'s aggregative fruiting can lead to selfishness that threatens the maintenance of the stalk in nature remains unknown.

Laboratory and theoretical studies of stalklessness

The observation by Buss (1982) of a stalkless morphology motivated laboratory studies of stalk-avoiding mutants as well as theory to address the circumstances under which stalklessness might evolve and persist. For example, two studies examined an insertion mutant (*fbxA*$^{-}$ mutant) that contributes less to the stalk in chimera with the wild-type strain (Ennis *et al.*, 2000; Nelson *et al.*, 2000). When developed clonally, the mutant forms aberrant fruiting bodies that contain few to no spores or fail to initiate stalk production altogether (Ennis *et al.*, 2000; Gilbert *et al.*, 2007). However, when co-developed with a wild-type strain, it produces a disproportionate fraction of the spores. Ennis and colleagues (2000) speculated that the *fbxA* gene takes part in the regulation of a complex involved in

the differentiation in spore and stalk cells, where deletion of the gene causes the stalk cell differentiation pathway to be halted.

Gilbert and colleagues (2007) subsequently used the *fbxA*$^{-}$ mutant to examine the extent to which such a strain that does not contribute fairly to the stalk might increase in frequency in a population owing to its advantage in spore production in chimera. However, such a strain may face a disadvantage at high frequency, if it has displaced the very strain it relies on to sporulate. The impacts of these different frequencies can be quantified as relatedness, which encompasses the degree to which the mutant interacts with self (r = 1) or with the stalk-proficient wild-type (r = 0) to build a fruiting body. The authors found that when r > 0.75, the *fbxA*$^{-}$ mutant decreased in frequency, indicating net selection against the mutant. This work demonstrated that sufficiently high relatedness could be essential for preventing the invasion and takeover of populations by non-stalk-forming strains.

The above empirical examples suggest that stalk-avoiding strains, provided that the behaviour is costly in the absence of a cooperating strain, could be selected against when relatedness is high—but is relatedness in natural populations sufficiently high to accomplish this? Relatively little is known about relatedness in nature, especially over the small spatial scales in the soil where different strains might encounter one another and co-develop to form chimeric fruiting bodies. Fortunato and colleagues genotyped natural isolates from minute soil samples collected using a plastic straw with a diameter of 6 mm (Fortunato *et al.*, 2003b). Of 26 soil samples that contained *Dictyostelium*, 63% yielded more than one genotype, with as many as 9 distinct genotypes from a single soil sample. These results yielded an estimate of average genetic relatedness of 0.52. In addition, Gilbert collected and genotyped 88 individual fruiting bodies that emerged from 25 dung piles incubated in the lab (Gilbert *et al.*, 2007). Seventy-seven percent of the fruiting bodies contained only a single genotype, which yielded a minimum relatedness of r = 0.86—high enough to support their hypothesis that relatedness would be high enough to select against the *fbxA*- mutation in nature (i.e., 0.86>0.75).

The hypothesis that high genetic relatedness would be sufficient to stop the spread of non-stalked mutants was further supported by Kuzdzal-Fick et al. (2011). They carried out laboratory evolution experiments that involved multicellular development under either high or low relatedness conditions (i.e., in genetically clonal or diverse groups, respectively). Approximately one-third of populations evolved at low relatedness harboured strains that could not form a stalked fruiting body when developed clonally but were disproportionately represented among the spores when co-developed with their ancestor. Conversely, no losses of multicellularity occurred in the high-relatedness

experiment. Taken together, these experiments collectively support that high relatedness is an important condition for the maintenance of the stalked fruiting body in this organism.

Hudson et al. (2002) developed a mathematical model that sought to address the evolutionary stability of stalk formation. This model made two assumptions about how stalks influence fitness: (1) that the fitness of a stalkless strain would be low and (2) that dispersal success increases with stalk size. They showed that stalk formation could still be maintained in populations founded by genetically unrelated individuals. However, they also showed that the presence of selfish genotypes, which contribute less to the stalk, could drive the evolution of suboptimal stalk sizes—that is, reductions in the allocation to the stalk relative to what would be optimal, assuming that dispersal is an important fitness component. Similarly, a model by Brännström and Dieckmann (2005) supported the potential for the coexistence of multiple genotypes (e.g., stalked and stalkless) within a single population. Taken together, these experiments suggest that one way to identify stalkless strains in nature might be to look for them under natural conditions where relatedness is low (i.e., genetic diversity is high) or the importance of dispersal is low. Moreover, while it remains unknown how essential stalk formation is for dispersal per se (as opposed to simply protecting the spores by lifting them in the air), one study did show that spores from an intact, stalked fruiting body were more likely to be acquired by an insect vector than those that were placed directly on the substrate; the spores also survived passage in the insect gut when ingested (Jeff Smith, Queller and Strassmann, 2014). This finding thus provides some support for the role of the stalk in facilitating spore dispersal.

Naturally occurring variation in stalk size

The model by Hudson *et al.* (2002) suggested that the size of the stalk in *D. discoideum* could reflect a trade-off between its cost to the cells that die and its benefit to the surviving spores, and thus that its size may be sub-optimal, at least for dispersal. Similarly, the study by Brännström and Dieckmann (2005) supported the idea that within-population polymorphisms could arise, such that one might expect to observe natural variation in the degree of stalk investment. To what extent is there evidence that this variation exists?

Within *D. discoideum*, only a few studies have looked at natural variation in overall stalk or fruiting body size, as well as equity of spore-stalk allocation between strains (Buttery *et al.*, 2009; Votaw and Ostrowski, 2017). Buttery *et al.* (2009) estimated spore-stalk allocation alone and when strains were co-developed in pairwise combinations for six strains from a site in North Carolina. Spore allocation was estimated in two ways: from images of individual fruiting bodies, followed by estimation of

relative sorus and stalk volume, and by comparing the spore production of strains clonally and following development in pairwise chimeras, under the assumption that more spores indicate less allocation to stalk. Using the latter approach, they observed variation in spore allocation (up to 2.8-fold difference) among strains (Buttery *et al.*, 2009).

Ostrowski and Votaw (2017) looked at variation among strains, among sites, and in clonal versus pairwise chimerae using strains from two geographically distant sites, one in North Carolina and one in Texas. In addition to imaging and measuring stalk height and spore number, GFP reporter strains were used to estimate spore-stalk allocation. They observed variation among strains within both populations in stalk allocation, but also larger size overall for Texas strains compared to those from North Carolina. These results underscore that increases in stalk size can be accomplished through two routes: either by allocating a higher fraction of cells to the stalk or by forming larger fruiting bodies altogether, presumably through production of fewer, larger aggregates from the same starting cell number. In addition, after controlling for differences in overall fruiting body size, within-population variation in relative stalk size was observed in both populations, similar to the findings by Buttery *et al*. (2009). These studies together indicate that some polymorphism in clonal and chimeric spore-stalk allocation occurs among strains within a given site, but also that the morphology of the fruiting bodies can evolve divergently among locations.

Overall, the studies of altruistic stalk formation by *D. discoideum*, clonally and in chimera, illustrate the potential for conflict in organisms that undergo aggregative multicellularity with division of labour. Aggregative multicellularity can lead to genetic diversity within the multicellular organism, providing the fuel for natural selection, whereas division of labour generates strong competitive advantages to cells that avoid the altruistic fate. Studies in this organism thus help to validate the predictions of evolutionary theory about the problems of aggregative multicellularity.

Conclusions

Aggregative fruiting has independently evolved in five of the six supergroups of the Eukarya, suggesting that the formation of a stalk is a morphological adaptation. Nevertheless, there is substantial diversity in how these structures are formed—whereas some stalks are composed of dead cells (and thus involve cell sacrifice), others form by secretion, such that all cells potentially survive as spores. Differences in how stalks are formed in organisms that undergo aggregative multicellularity have important implications for the evolutionary maintenance of this trait.

Substantial variation in how stalked fruiting bodies form exists within dictyostelids alone, a large clade consisting of more than 150 species within the Amoebozoa. Here I described variation among species in the composition of the stalk (acellular versus cellular), the timing of its production (during or after migration), and its branching morphology (branched or unbranched, whorled, or not). Unfortunately, the explanations for the variation in these features are not known, although several studies provide information about some of the potential functions of the stalk. For example, spores that sat atop stalks were more likely to be picked up by an insect vector (J. Smith, Queller and Strassmann, 2014), and stalks emanating from the rear of the slug can help in traversing gaps in the soil (Gilbert *et al.*, 2012)—yet, whether and how these features are used in nature remains to be seen. In addition, while some stalk features may provide a fitness advantage, there may also be functional constraints imposed by development or physics. For example, acellular stalks have the benefit of not necessitating cell sacrifice, but these structures may not support as many spores, or the spores may be of lower quality (Kaushik and Nanjundiah 2003).

In those species that form cellular stalks, death of a fraction of the cells presents opportunities for conflict, as strains that avoid forming the stalk and disproportionately form spores should have a fitness advantage. In the model organism *D. discoideum*, studies of stalk-avoiding mutants and the behaviours of natural isolates, as well as mathematical models have all contributed to our understanding of the evolutionary maintenance of altruistic stalk formation. These studies confirm an essential role for relatedness, but whether relatedness is high enough in nature to prevent takeover by stalk-avoiding strains remains uncertain. Future studies would benefit from consideration of how the evolution of morphology has been impacted by variation in relatedness across populations, as well as of other factors that might also promote the evolutionary maintenance of cooperative multicellularity.

Chapter 3.

Cheating and mechanisms that limit cheating in natural populations of a social amoeba.

Abstract

Cooperative societies are vulnerable to exploitation by cheaters, who reap the benefits of cooperation without contributing their fair share of the costs. Theory and experimental work, mostly using lab-generated mutants, have shown that cheaters can rapidly emerge and threaten cooperative societies. However, few studies have examined the prevalence and intensity of cheating in nature, nor the importance of mechanisms that may limit cheaters. In this chapter, I used the social amoeba *Dictyostelium discoideum* to examine if cheating occurs in nature. In addition, I tested one hypothesis that explains how cooperation might be maintained: cheating rapidly selects for counter-adaptations to resist cheating. I did this by asking whether cheaters were less common among local (sympatric) strain pairs than foreign (allopatric) pairs. Specifically, if resistance suppresses cheating, then I expected strong local cheating to be rare. The results showed that cheaters were moderately rare and were found in some populations but not others. In addition, there was no strong evidence that populations evolved resistance to their local cheaters. Instead, limited evidence showed that the strongest cheating occurred between local strain pairs, hinting that cheating may be selectively favoured. In response to these findings, I briefly considered other factors that may explain both cheating and the absence of cheating in nature. This study is one of the first that examined the occurrence of cheating across multiple natural populations of a social microbe, and its findings suggest diverse and complex social interactions may prevail in nature.

Introduction

Cooperative societies can be vulnerable to exploitation by non-cooperative 'cheaters': individuals who gain the benefits of cooperation without bearing their fair share of the costs (Trivers, 1971; Axelrod and Hamilton, 1981; Burt and Trivers, 2006; Foster and Wenseleers, 2006; Nowak, 2006; Ghoul, Griffin and West, 2013). If left unchecked, cheaters can lower group productivity or, in extreme cases, cause the collapse of the cooperative group itself (Hardin, 1968; Fiegna and Velicer, 2003; Rainey and Rainey, 2003; Greig and Travisano, 2004; Dobata and Tsuji, 2009). Given that cooperative behaviours are nonetheless widespread in nature, this observation suggests the existence of mechanisms that limit or prevent the spread of these cheaters and promote cooperation.

One way cheating can be prevented is if individuals limit cooperation to relatives. In this case, the benefits of cooperation accrue to individuals who pass on the altruist's genes (Hamilton, 1964a). However, cooperation is also found in groups of unrelated individuals, suggesting there may be alternative mechanisms to limit cheating in these systems. One possibility is that cheating can be

limited through the counter-evolution of mechanisms to resist or suppress it, collectively referred to as enforcement (Frank, 1995, 2003; El Mouden, West and Gardner, 2010; Singh and Boomsma, 2015; Ågren, Davies and Foster, 2019). Increasing evidence across diverse cooperative systems suggests the potential importance of such mechanisms (summarised in Ågren *et al*. (2019)). For example, in eusocial insects, workers who selfishly lay their own eggs instead of raising those of the queen are punished and policed by other workers, who destroy the eggs or attack the worker (Wenseleers and Ratnieks, 2006b). At the genic level, selfish genetic elements that cause meiotic drive can select for genetic changes at other loci to suppress their effects and restore equal transmission (Frank, 1989). Finally, in social groups of microbes, cheaters who exploit a public good can be suppressed through the counter-evolution of resistance by others (Fiegna *et al.*, 2006; Khare *et al.*, 2009; Manhes and Velicer, 2011; Hollis, 2012; Tarnita *et al.*, 2015; Wang *et al.*, 2015; Miller, Sidell and Ostrowski, 2023).

While the above examples suggest that mechanisms to resist or suppress cheating could be important for the maintenance of cooperation, few studies have examined their prevalence and relevance in nature (Bruce *et al.*, 2017; Butaitė, Kramer and Kümmerli, 2021). Here I will test this hypothesis in natural populations of the soil amoeba *Dictyostelium discoideum*, a model system where both cheating and resistance evolve readily, at least in laboratory environments (Khare *et al.*, 2009; Hollis, 2012; Levin *et al.*, 2015; Miller, Sidell and Ostrowski, 2023). In this system, individual amoebae aggregate upon starvation and cooperate to form a multicellular fruiting body. The fruiting body consists of a sorus that contains viable spores that sits atop a stalk of nonviable cells. The self-sacrifice of the stalk cells is altruistic, as their death likely provides a dispersal benefit to the spores (Huss, 1989; Kessin, 2001; Chisholm and Firtel, 2004; Kuzdzal-Fick *et al.*, 2007; J. Smith, Queller and Strassmann, 2014).

Because *D. discoideum* becomes multicellular through aggregation, unrelated cells can end up in a genetically mixed fruiting body structure, called a chimera (Filosa, 1962; Buss, 1982). Under chimerism, selection may favour cheaters: individuals that form proportionally more spores and exploit stalk formation by others in the group (Buss, 1982; Strassmann, Zhu and Queller, 2000). Indeed, various studies have demonstrated that cheating can rapidly emerge in a laboratory environment and cheaters are found in mixes between natural strains (Strassmann, Zhu and Queller, 2000; Fortunato *et al.*, 2003a; Gilbert *et al.*, 2007; Buttery *et al.*, 2009; Buttery, Thompson and Wolf, 2010; Flowers *et al.*, 2010; Parkinson *et al.*, 2011). Likewise, laboratory studies have shown that counter-evolutionary changes can also arise that can suppress cheaters (Khare *et al.*, 2009; Hollis, 2012; Levin *et al.*, 2015; Miller, Sidell and Ostrowski, 2023).

In this chapter, I asked two questions:

1. **How common are cheaters in natural populations?** Are they found in every population or just a few? Do some populations harbour more cheaters than others? Empirical studies that examined cheating between natural strains have mostly been limited to a handful of strains from a single site in North Carolina, and it is currently unknown if cheaters are widespread in nature (Strassmann, Zhu and Queller, 2000; Fortunato, Queller and Strassmann, 2003; Buttery *et al.*, 2009; Flowers *et al.*, 2010).
2. **Does resistance to cheaters arise in nature?** Laboratory evolution experiments have shown that cheaters can select for resistance (Khare *et al.*, 2009; Hollis, 2012; Levin *et al.*, 2015; Miller, Sidell and Ostrowski, 2023). However, despite these proof-of-principle demonstrations of what is possible, we know little about whether cheaters *are* suppressed in natural populations.

To look for evidence for the second question, I compared the frequency and intensity of cheating among groups of co-occurring individuals (in this case, isolates from the same small soil sample; 'local') to those who likely lack any evolutionary history together (in this case, isolates from different soil samples, collected at varying distances, up to >1000 km apart; 'foreign'). Using this information, I tested three predictions: If resistance evolves readily, then cheaters should be suppressed by their local social partners and therefore cheating should be uncommon within populations (prediction 1). Moreover, if resistance is specific to local cheaters, then I may also find that strains can cheat on foreign social partners (prediction 2), since the evolved resistance mechanisms may not be effective against an unfamiliar cheater (Miller, Sidell and Ostrowski, 2023). Finally, although I expected suppression will limit the success of cheating, there may be time lags between when newly selected cheating mutations arise and the counter-evolution of resistance. Therefore, I also predicted that the strongest examples of cheating might be observed in local strain pairs (prediction 3). These predictions are illustrated in Figure 3.1.

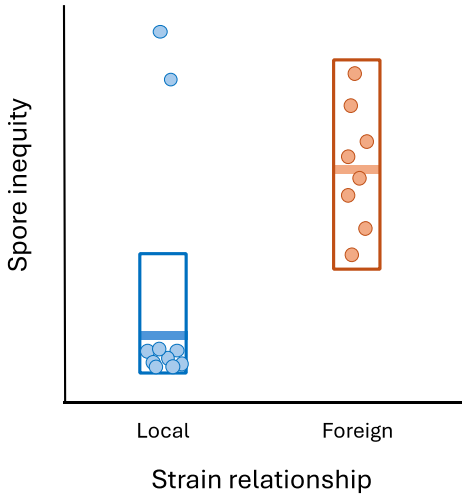


Figure 3.1. Schematic of the patterns of spore inequity in local and foreign strain pairs that I may find if resistance occurs in nature. Details are provided in the text.

In addition to testing the above predictions on resistance, I also examined two other mechanisms that may promote the maintenance of cooperation: *i.* cheaters limit their own spread because they reduce the fitness of groups that harbour them, and *ii.* kin discrimination excludes potential cheaters from the social group. Ultimately, this study aimed to examine the prevalence of mechanisms that prevent or limit social cheating in cooperative societies in nature.

Materials and Methods

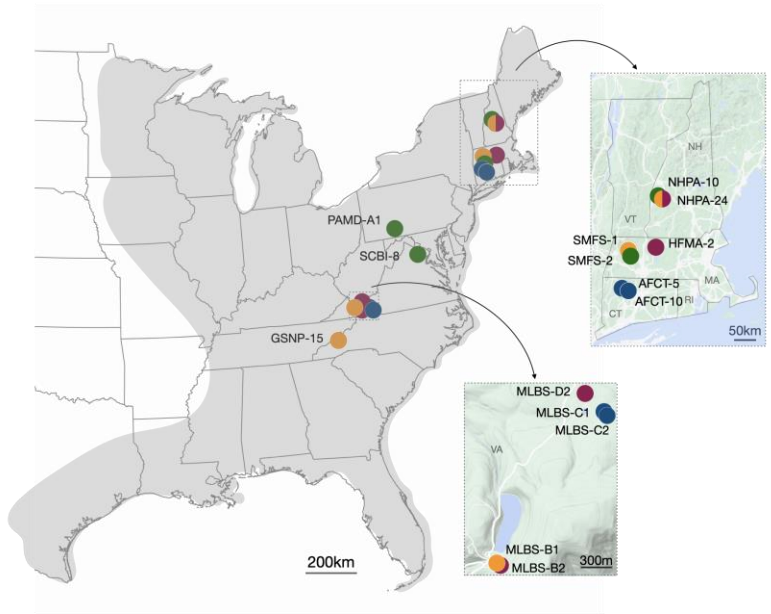
Strains

The strains used in this study were isolated from soil samples collected by members of the Ostrowski laboratory, as described by Kuzdzal-Fick et al. (2023). Care was taken to rapidly isolate strains of *D. discoideum* from the soil samples. This was either done in the US soon after collection, or soil samples were frozen and transported to New Zealand. Note that spores of *D. discoideum* can readily be stored at and revived from temperatures below 0°C. All experiments were performed in New Zealand. A "site" in this study represents a small soil sample originally taken from the surface of a 10-by-10 cm plot. I selected five strains from each of the fifteen sites that vary in distance from metres to >1,000 km apart. For one site (NHPA-24), I used two separate sets of five strains, resulting in a total of 80 isolates (=14 sites x 5 strains + 1 site x 10 strains). These fifteen sites cover a large fraction of *D. discoideum*'s geographical distribution in the US (Fig. 3.2A) (Raper, 1935; Swanson, Vadell and Cavender, 1999). The list of strains and the GPS coordinates of the sampling sites are provided in Table S3.1.

Overview of the experimental design

I performed four sets of experiments between 2019 and 2021. In each set, I tested the interactions between strains from four sites (Fig 3.2A and B). I used site NHPA-24 in two sets but used different strains here. The geographical distances between sites within a set are shown in Figure 3.2B. Within each set, I mixed each strain with two 'local' (same site) and two to three 'foreign' (different site) partner strains, resulting in a total of 27 local and 33 foreign strain pairs tested per set. I performed all mixes within a set on the same day and I replicated each set four times. Each replicate was temporally independent and started from frozen spore stocks.

A



B

Geographic distance (in km) matrices

Set 1	GSNP-15	SMFS-1	NHPA-24	MLBS-B2
GSNP-15	0	1201	1,323	324
SMFS-1		0	131	878
NHPA-24			0	1,001
MLBS-B2				0

Set 2	AFCT-10	MLBS-C2	MLBS-C1	AFCT-5
AFCT-10	0	817	817	0.184
MLBS-C2		0	0.002	817
MLBS-C1			0	817
AFCT-5				0

Set 3	HFMA-2	NHPA-24	MLBS-B1	MLBS-D2
HFMA-2	0	105	917	914
NHPA-24		0	1,001	999
MLBS-B1			0	2.93
MLBS-D2				0

Set 4	NHPA-10	SMFS-2	SCBI-8	PAMD-A1
NHPA-10	0	132	733	735
SMFS-2		0	608	620
SCBI-8			0	133
PAMD-A1				0

Figure 3.2. (A) Map of the eastern United States showing the locations of the fifteen sites. The shaded region indicates the approximate boundary of *Dictyostelium discoideum* species' distribution in the United States. **(B) The geographical distance (in kilometres) between sites.** The experiment consisted of four "sets". In each set, strains from the same site were tested against a selection of strains from different sites.

Cultivation, cell staining, and developmental assays

At the start of each replicate, I inoculated the spores of the relevant strains from freezer stocks on a lawn of *Klebsiella pneumoniae* on SM agar plates (Formedium Ltd, with 2% agar). Once fruiting bodies had formed, I collected the spores and plated $5x10^5$ of them on fresh SM plates with *K. pneumoniae*. I harvested the cells when the cells were at mid-exponential growth, after approximately 40 hours. I washed the cells three times (450 x g, 3 min) in KK2 buffer (14.0 mM K_2HPO_4 and 3.4 mM KH_2PO_4, pH 6.4) to remove the bacteria and resuspended them at a density of $1x10^7$ cells/ml in KK2.

To distinguish the two strains in a mix, I treated one strain of each pair with the fluorescent dye CellHunt Green CMFDA (Molecular Probes), which was diluted in DMSO according to the manufacturer's specifications. I added the fluorescent cell dye to a concentration of 20 µM, incubated the cells on a shaker for 30 minutes in the dark, washed them once in KK2, and incubated them again in KK2 for 30 minutes to allow the efflux of the excess dye. After staining, I washed the cells twice in cold KK2 and resuspended them in cold KK2 at a final density of $1x10^8$ cells/mL. I treated the unlabelled strains with DMSO only, and these samples underwent the same treatment as the labelled strains.

Following labelling, I combined the labelled and unlabelled cells in equal proportions for each mix and deposited a 50 µL aliquot in a 3-by-3 square (an area of 1 cm^2) on a 47 mm gridded 0.45 µm nitrocellulose filter, for a total of $5.0x10^6$ cells per filter. I placed the filter on top of a Pall filter soaked in 1.5 ml of PDF (per litre: 1.5 g KCl, 1.07 g $MgCl{\cdot}6H_2O$, 1.8 g KH_2PO_4, 1.6 g K_2HPO_4, 0.5 g streptomycin sulphate) in a 6-cm Petri dish (Fig. 3.3). I transferred the Petri dishes to a sealed plastic box lined with wet tissues, which I placed in the dark for 48 hours at 22°C to allow fruiting bodies to develop. Following development, I collected the spores in 3 mL of detergent (KK2 + 0.1% IGEPAL and 10 mM EDTA), which dissolved any cells that had not sporulated. I quantified the fraction of both strains in the spores using a BD FACSCanto II flow cytometer (488 nm laser, 513/15 GFP filter). I used a cell counter (Cell Countess II FL, Thermo Fisher) to determine the total number of spores on each filter. In addition to the experimental mixes, which consisted of a labelled and unlabelled strain of two different genotypes (i.e., chimeric mixes), I also developed the unlabelled and labelled cells of each strain by themselves and as a 50-50% mix of labelled and unlabelled cells. I refer to the latter control as the 'control mix'. I treated all three controls (100% unlabelled, 100% labelled, and 50-50% mix of labelled and unlabelled cells) identically to the chimeric mixes.

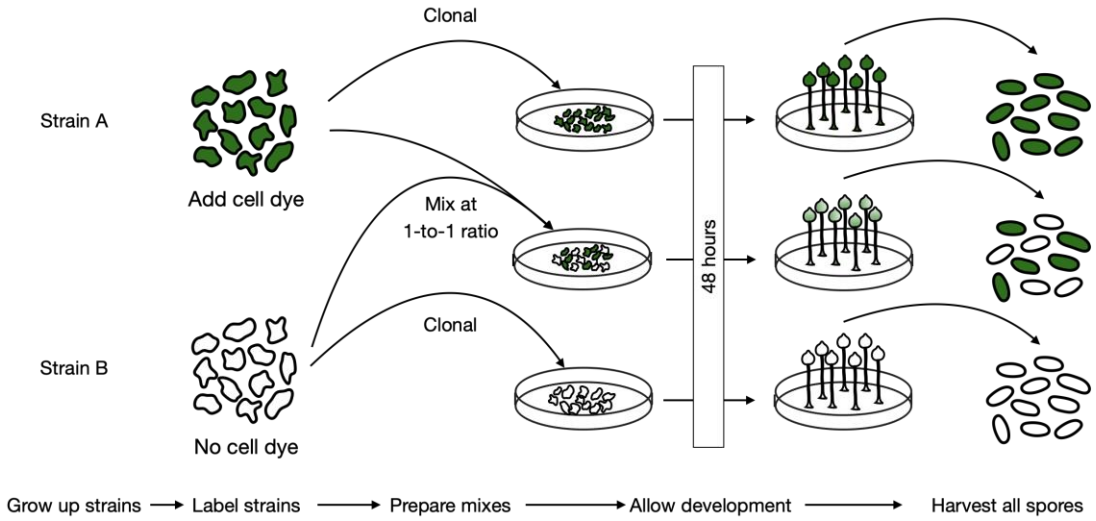


Figure 3.3. Experimental set-up of the developmental assays.

Quantifying segregation

Cheaters can be controlled by ensuring that the benefits of cooperation are directed towards kin (Hamilton, 1964a). One way this can be accomplished is through the recognition and exclusion of non-kin from the social group. Kin recognition and social exclusion have been found in mixes between natural strains of *D. discoideum* (Ostrowski *et al.*, 2008; Benabentos *et al.*, 2009; Flowers *et al.*, 2010). This behaviour is observed as the initial aggregation of two strains which then again sort out to develop into fruiting bodies with greater relatedness than expected by chance (i.e., more clonal), called segregation. Segregation can be assessed by quantifying the proportion of the two strains within individual fruiting bodies, where greater variation in this proportion across fruiting bodies indicates greater segregation (Fig. 3.4).

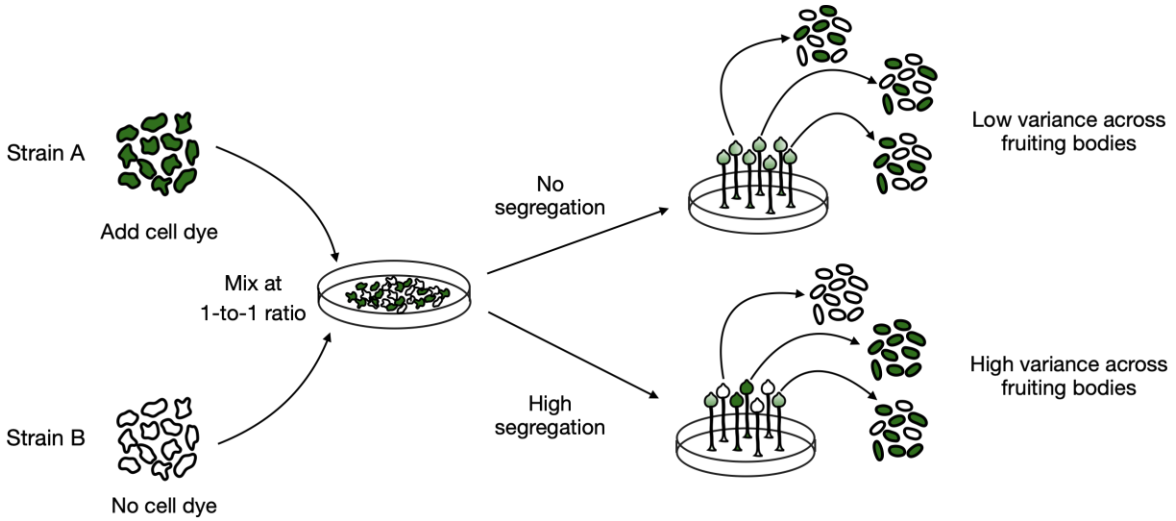


Figure 3.4. Measuring segregation by the fraction of the labelled strain in the spores of the individual fruiting bodies.

I measured segregation according to the methods of Ostrowski et al. (2008). Briefly, following development, I picked twelve fruiting bodies randomly from each filter. I harvested the spores from the individual fruiting bodies in 100 µL spore detergent and quantified the fraction of both strains using a flow cytometer. I calculated the variance in the proportion of labelled spores across the individual fruiting bodies using the standard formula:

$$s^2 = \frac{\Sigma(\mathrm{X} - \bar{x})^2}{n - 1}$$

After sampling the individual fruiting bodies, I collected the remaining fruiting bodies and used them to calculate the filter-wide fraction of labelled spores using the formula:

$$var_{total} = p_{total}(1 - p_{total})$$

where p_{total} is the fraction of the labelled strain in the mix. I calculated the degree of segregation using the formula:

$$r = \frac{s^2}{var_{total}}$$

The value of *r* (akin to relatedness) can range from 0 to 1, where a value of 0 means that the two strains are randomly distributed among the fruiting bodies, and a value of 1 means that each fruiting body consists of only one or the other (clonal, i.e., high relatedness within each fruiting body).

I also measured segregation in the control mixes, which consisted of labelled and unlabelled cells of the same strain. The segregation observed among genetically identical strains should reflect chance effects or environmental factors independent of self/nonself discrimination. It therefore serves as a baseline to which to compare the segregation of genetically different strains.

Genomic DNA isolation for whole genome sequencing and variant calling

I used whole genome Illumina sequencing data to determine the genetic relationships between the strains. Genomic DNA isolation, genome sequencing, and variant calling for the 60 strains from sets 1-3 were performed by Kuzdzal-Fick et al. (2023). I used the same methods to determine the genetic distances for the strains used in set 4. I provide detailed information about DNA isolation and variant calling in the supplementary information.

Statistics

I carried out all statistical analyses in *R* version 2023.03.1 (R Core Team, 2023).

Spore inequity and cheating. For each mix, I calculated the magnitude of spore inequity, as a metric of cheating, as the observed minus the expected fraction of the labelled strain in the spores, where the expected fraction is 0.5 (since the two strains were mixed at equal fractions). To assess if this deviation from equity is greater than expected by chance, I tested if the magnitude was significantly greater than that of the control mixes using a one-tailed t-test.

Spore inequity in local versus foreign strain pairs. I modelled the magnitude of spore inequity as a function of the geographical relationship between the two strains (i.e., local or foreign), with set and block included as random effects. I used maximum likelihood to fit the model (using the 'glmmTMB' function), assuming a beta regression error structure and a logit link function. I tested the significance of geographical relationship using a type II Wald chi-square test.

Variation in clonal sporulation efficiency and its effect on spore inequity. The clonal sporulation efficiency of a strain is the number of spores it produces, divided by the number of starting cells in the clonal controls. To test if strains vary significantly in their sporulation efficiency, I modelled sporulation efficiency as a function of strain ID, set, and block as random effects. I tested the significance of these terms through single deletions of terms and testing the reduced model and full model using a likelihood test that follows a chi-square distribution.

I then tested if differences between strains in their clonal sporulation efficiency are a significant predictor of their fraction in the spores in chimeric mixes. This model tested a strain's fraction in the spores as a function of its predicted fraction (based on its own and its partner's clonal sporulation efficiency), taking into account the geographic relationship between the strains. Here, I used a Gaussian error structure with an identity link function and fit the model using maximum likelihood (using the 'glmmTMB' function). I tested the significance of the predicted spore fraction using a type II Wald chi-square test. I used the 'get_variance_fixed' function from the 'insight' package to determine the proportion of variance explained by the predicted spore fraction (Nakagawa, Johnson and Schielzeth, 2017).

Relationship between genetic distance and spore inequity. Given that both genetic and geographic distance show a non-normal distribution, I tested the relationship between these two terms and the average spore inequity found in a mix using a Spearman's rank correlation test.

Segregation. To examine if strains in a mix exhibit significant segregation, I tested if segregation was greater than that of the control mixes using a one-tailed t-test. To reduce sampling error, I excluded fruiting bodies in which the spore count was lower than 100.

To test if segregation could limit cheating (Ho *et al.*, 2013; Ho and Shaulsky, 2015), I used a one-tailed Spearman's rank correlation to test whether greater segregation is associated with lower spore inequity. Since I also predict that relatedness between strains influences segregation, I additionally tested whether there is a positive relationship between the genetic distance between strains and the level of segregation using a one-tailed Spearman's rank correlation test.

Results

How common are cheaters in nature and do they exist in all populations?

First, I examined how many strains cheat on one or more of their social partners. To do so I tested if the average spore inequity observed in a mix was greater than that observed in the 'control mixes' using a one-tailed t-test (spore inequity in the control mixes: 0.032±0.004 mean±se, N=79). In the control mixes, a given strain is 'mixed' with itself, and these mixes therefore serve as a baseline for determining if the spore inequity in a mix of genetically different strains is greater than expected by chance. Based on this criteria, I identified sixteen strains (20% of all strains) that cheated against their social partner(s): eleven strains cheated against just one other strain, one strain cheated against two strains, two strains cheated against three strains, and two strains cheated against four strains (all P<0.05). These results show that cheaters exist in nature, but also, they are moderately rare. Also, whereas most of these strains only cheat against a single other strain, four strains cheat against more than half of their social partners (every strain is tested against 6-7 partner strains).

The distribution of cheaters was also not uniform across sites; the sixteen cheaters were found in nine of the fifteen sites: HFMA-2, PAMD-A1, NHPA-10, MLBS-B1 and MLBS-B2 harboured one cheater (=20% of strains), MLBS-C1, MLBS-C2 and AFCT-10 harboured two cheaters (=40% of strains), and AFCT-5 harboured four cheaters (=80% of strains). This means that no cheaters were found in the remaining six sites. These results show that the occurrence of cheaters can greatly vary among natural populations, and cheaters and non-cheaters (cooperators) may co-occur in some populations. Finally, the average spore inequity in the mixes that harboured a cheater was 0.11±0.006 (mean±se, range: 0.06-0.14; N=27 mixes), which means that a cheater was able to increase its fraction from 50% in the cells to 61% in the spores.

Does resistance to cheaters arise in nature?

To detect whether resistance to cheaters is present in natural populations, I tested several predictions:

First, if resistance evolves readily, then **cheaters may be suppressed by their local social partners and therefore uncommon within populations** (prediction 1). Moreover, if resistance is specific to local cheaters, then I may also see that **strains can cheat foreign social partners** (prediction 2), since the evolved resistance mechanisms may not be effective against an unfamiliar cheater (Miller, Sidell and Ostrowski, 2023). To test these predictions, I examined if the strains that cheat are more likely to do so against a foreign than a local strain. In addition, if cheaters are uncommon within populations, I should find that the mean level of cheating is lower for local compared to foreign strain pairs (Fig. 3.1). Specifically, whereas local co-adapted strains should exhibit lower levels of cheating (because they are well-matched for cheating and resistance alleles), strains without a shared evolutionary history may be idiosyncratic depending on the cheating-resistance allele combination.

The results showed that of the sixteen identified cheaters, five strains cheated only against a foreign strain. In contrast, seven strains cheated only against a local strain, and four strains cheated against both a local and a foreign strain. The behaviour of the sixteen cheaters across all their mixes, local and foreign, is plotted in Figure 3.5. Notably, of the nine strains that cheated against a foreign strain, in five cases, they cheated against a strain that was isolated less than 3 kilometres away (AFCT-5 against AFCT-10, MLBS-B1 against MLBS-D2, and MLBS-C1 against MLBS-C2). Thus, these results reveal no overall pattern where cheating was less frequent in local compared to foreign strain pairs—in fact, cheating was slightly more common against local social partners.

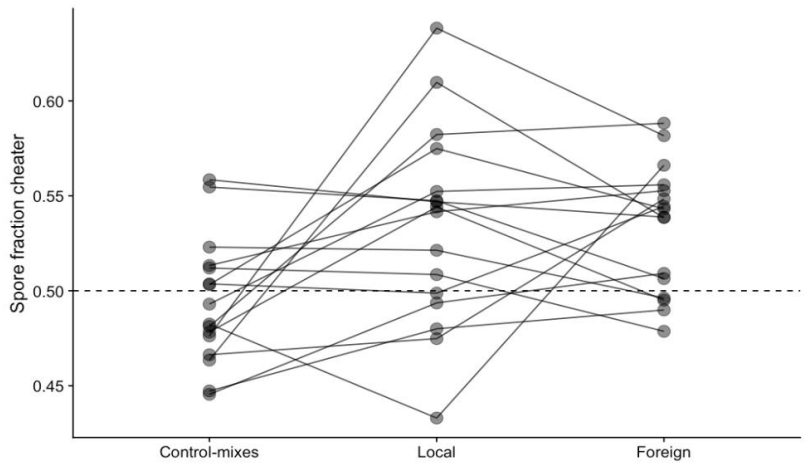


Figure 3.5. The average spore fraction of a cheater in the control mixes, in mixes with local strains, and mixes with foreign strains. In this case, a cheater is a strain that cheats against at least one other strain (this can be a local or foreign strain).

As a consequence of cheaters being suppressed by their local partners, I also predicted that the level of spore inequity would on average be lower in local compared to foreign strain pairs. However, the results showed no significant difference between the average level of spore inequity observed in local (0.053±0.004 mean ± se, *N*=106) versus foreign (0.051±0.004 mean ± se, *N*=128) strain pairs (Fig 3.6A;

χ^2=1.14, *df*=1, *P*=0.29). Together, these results provide no evidence that cheaters are suppressed by their local partners (prediction 1) and may cheat on their foreign partners (prediction 2).

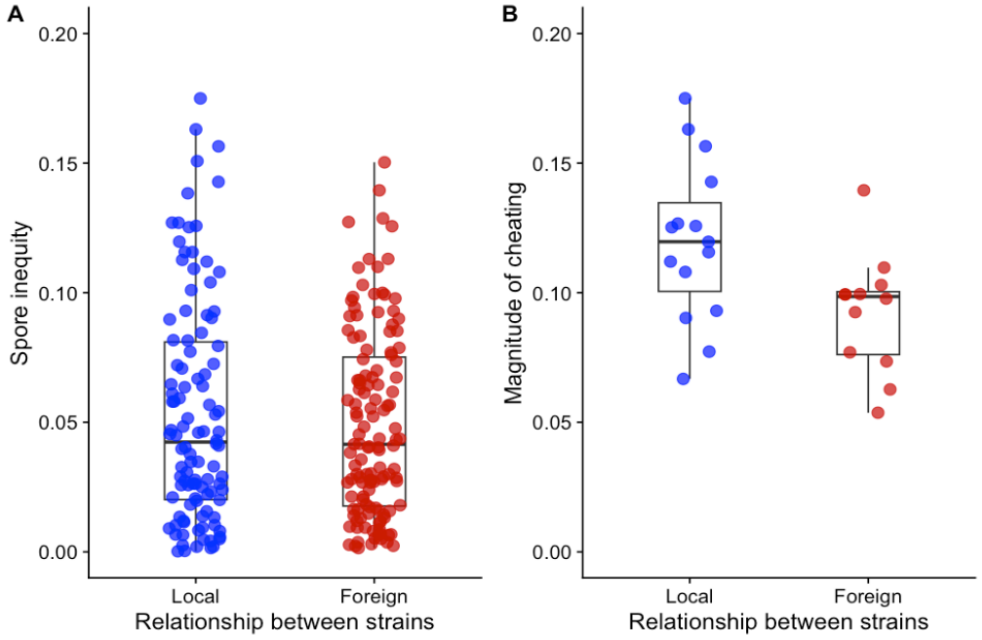


Figure 3.6. (A) There is no significant difference in the mean spore inequity between mixes made up of local and foreign strain pairs (χ^2=1.14, *df*=1, *P*=0.29). (B) Considering only those mixes that did exhibit significant cheating (defined as the spore inequity in a mix that is significantly greater than that found in the control mixes), the spore inequity was higher in local than foreign strain pairs (χ^2=7.85, *df*=1, *P*=0.005).

Finally, although I expect that resistance will limit the success of cheating, there will be time lags between when newly selected cheating mutations arise and the counter-evolution of resistance mutations. Therefore, I also predict that **the strongest examples of cheating would be observed in local strain pairs** (prediction 3). I tested this prediction by comparing the magnitude of spore inequity between local and foreign strain pairs in those pairs that exhibit significant cheating (Fig. 3.1). Indeed, the strongest cheating does tend to be among local strain pairs (Fig. 3.6B; χ^2=7.85, *df*=1, *P*=0.005), thus providing some support for prediction 3.

Does cheating result from other factors?

I will now briefly consider two other mechanisms that can lead to spore inequity: *i.* spore inequity is 'merely' a social incompatibility between genetically diverged strains, and *ii.* spore inequity results from differences in fixed behaviours.

Natural strains of *D. discoideum* show strong isolation by distance, whereby populations that are more geographically divergent are also more genetically divergent (on average, this will not be true for *all* strains) (Kuzdzal-Fick, unpublished). One possible consequence of this lack of gene flow is that populations may diverge in traits important for social interactions. In turn, genetic divergence could result in social incompatibilities, including spore inequity—in which case, social exploitation evolves as a side-effect of divergence in allopatry rather than being directly selected (Ostrowski 2019). If so, I may find a relationship whereby spore inequity increases with greater genetic divergence between strains. Also, I will examine the relationship between geographical distance and the observed spore inequity.

In contrast to the prediction of the social incompatibility hypothesis, however, I found no relationship both between spore inequity and the genetic and geographic distance between strains in a mix (Fig. S3.1; geographic distance: r=-0.03, df=232, P=0.64; genetic distance: r=0.01, df=226, P=0.94).

Differences in fixed allocation partly explain the magnitude of spore inequity

A second possibility is that spore inequity results from inherent differences between individuals in their expression of a cooperative trait. Specifically, prior work found that spore inequity in *D. discoideum* can result from two processes, which are not mutually exclusive (Strassmann, Zhu and Queller, 2000; Buttery *et al.*, 2009; Buttery, Thompson and Wolf, 2010). The first process, called facultative cheating, occurs when strains alter their spore allocation in chimera (Strassmann, Zhu and Queller, 2000; Santorelli *et al.*, 2008; Buttery *et al.*, 2009; Khare *et al.*, 2009). This process requires the ability to change allocation behaviour between the clonal and chimeric states. The second mechanism, called fixed allocation cheating, occurs when strains have different clonal spore-vs-stalk cell allocations, and neither strain alters this ratio when they form a chimera (Hudson *et al.*, 2002; Buttery *et al.*, 2009). In other words, under fixed allocation cheating, the strain with the intrinsically greater spore allocation will become overrepresented in the spores. The latter can still be referred to as cheating, since a strain that allocates fewer cells to the stalk still benefits from the relatively larger stalk produced by its partner in a chimera, without paying the cost (Ghoul, Griffin and West, 2013; Ostrowski, 2019).

To assess the contribution of fixed allocation differences to the observed level of spore inequity, I asked first whether the strains differ in their sporulation efficiency. Specifically, strains that allocate more cells to the spores should produce more spores from a given number of starting cells. Consistent with different allocations, I observed significant variation among strains in their clonal sporulation

efficiency (i.e., spores per starting cell; χ^2=60.52, df=1, *P*<0.001). Using these values, I then calculated a strain's expected spore fraction in a mix as:

$$expected\ fraction\ focal\ strain = \frac{sporulation\ efficiency\ focal\ strain}{sporulation\ efficiency\ focal\ strain + \ sporulation\ efficiency\ parnter\ strain}$$

If differences in clonal spore-stalk ratios explain the observed proportions of the two strains in the spores, then this expected fraction should agree closely with the observed fraction. To see if this were the case, I modelled the observed fraction in the spores as a function of the expected fraction, while also accounting for the geographic relationship between strains (i.e., local or foreign strain pair). I found that the expected fraction was indeed a strong predictor of the observed fraction of the two strains in the spores (Fig. 3.7; χ^2=13.69, *df*=1, *P*<0.001). However, the amount of variation explained by the two strains' clonal sporulation was low (R^2=0.04). This means that clonal sporulation, likely caused by strain differences in spore-stalk allocation, has a significant influence on spore inequity, but other factors also influence the outcome. The remaining variance that cannot be explained by fixed allocation differences might be explained by facultative shifts in the behaviour or other uncontrolled sources of variation. I discuss these ideas in more detail in Chapter 4.

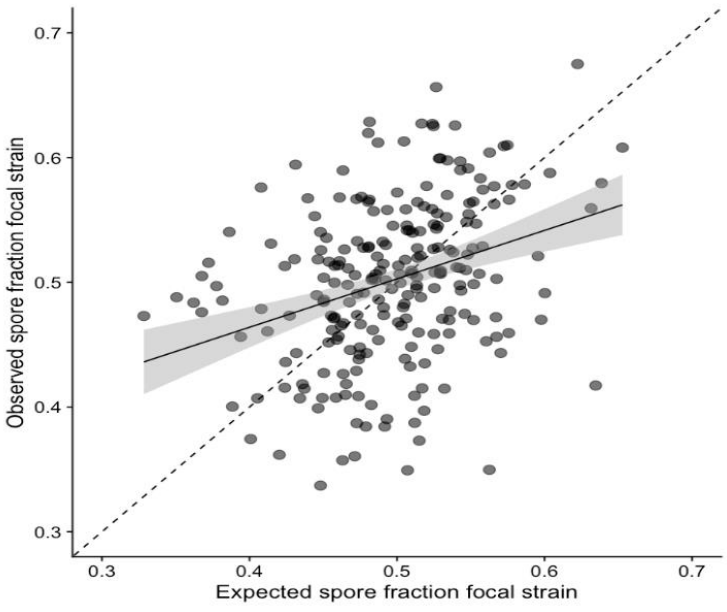


Figure 3.7. Differences in clonal sporulation efficiency between strains can partly predict their fraction in the spores during chimeric development. The x-axis shows a strain's expected fraction in the spores in a mix, based on its own and its partner's clonal spore production. The y-axis shows the observed fraction of a strain in the spores in a mix. Each point represents the mean of a particular mix, based on *N*=3-4 replicates. The dashed line indicates the null hypothesis that the observed and expected fractions are the same.

Testing two other mechanisms that may limit or prevent cheaters

In addition to the possibility of evolved resistance, I test two other mechanisms that might limit the success of cheaters: *i.* they limit their own spread by reducing the fitness of the group, and *ii.* kin discrimination excludes potential cheaters from the social group.

Cheaters may be limited in their spread by the reduced fitness of chimeric compared to clonal groups, referred to as chimeric load (Mendes-Soares *et al.*, 2014). This reduced fitness could result from evolved social incompatibilities between strains from the same and different populations (as discussed earlier). For this reason, chimeric load will potentially increase with genetic or geographic distance between the two strains.

To test if group fitness is lower in chimeric groups, I quantified the pair's observed sporulation efficiency and compared it to its expected value, based on the sporulation of the strains individually. Notably, I found that the sporulation efficiency in chimeras was often *greater* than expected. Specifically, the sporulation efficiency in 189 mixes (=81%) was on average higher than the expected value; this increase was significant in 45 mixes (all $P<0.05$; Fig. 3.8A). In addition, I found a significant positive correlation between the genetic distance and the sporulation efficiency in chimeras (Fig. 3.8B; Spearman's rank correlation: $r=0.30$, $df=232$, $P<0.001$).

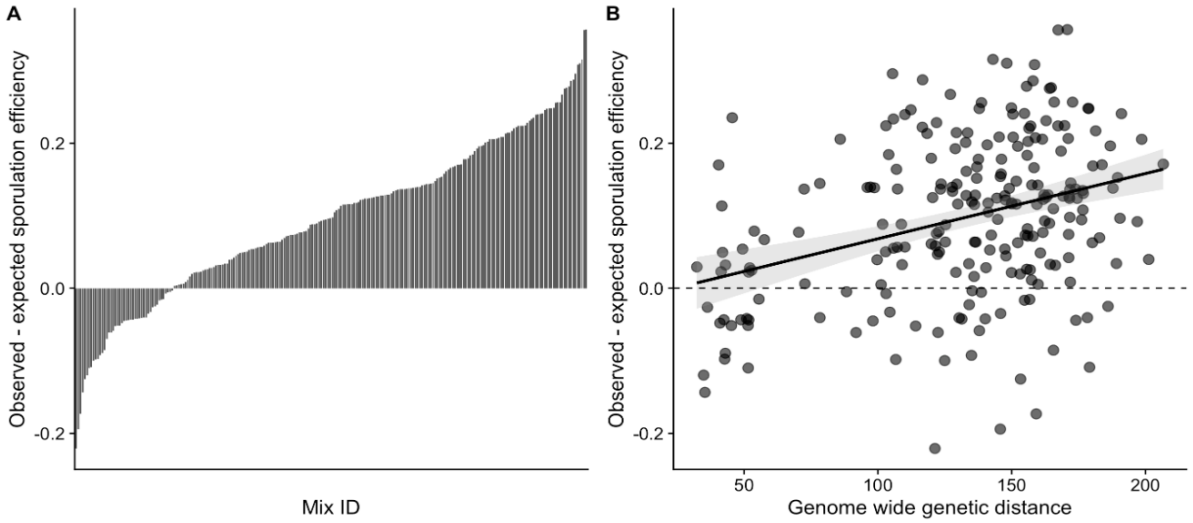


Figure 3.8. (A). The sporulation efficiency of chimeric groups is often greater than expected based on the sporulation efficiencies of the two strains developed clonally. (B). The deviation-from-expected sporulation efficiency of a chimeric group increases as the genetic distance between the strains in a pair increases (Spearman's rank correlation: $r=0.30$, $df=232$, $P<0.001$). In both plots, zero indicates the situation where the observed sporulation efficiency of the chimera is equal to the predicted value, based on the sporulation efficiencies of the two strains when developed clonally.

Kin discrimination is frequent in chimeras but does not protect against spore inequity

Kin selection theory predicts that altruism can be protected from cheating if its benefits accrue disproportionately to kin (Hamilton, 1964a). Prior work has shown that *D. discoideum* exhibits a mechanism of kin discrimination that allows cells to identify and preferential cooperate with kin (Ostrowski *et al.*, 2008; Benabentos *et al.*, 2009; Flowers *et al.*, 2010). Following an initial period of co-aggregation, genetically different strains partially sort out to form fruiting with higher-than-expected relatedness, called segregation. By limiting chimerism, kin recognition might also prevent spore inequity, as it can exclude a would-be cheater from the group (Ho *et al.*, 2013).

Segregation is caused by genetic differences at two highly polymorphic genes, *tgrB1* and *tgrC1* (Benabentos *et al.*, 2009; Hirose *et al.*, 2011, 2017). Both genes encode cell-surface proteins that together function as a ligand-receptor pair that confer recognition during the aggregation stage. Furthermore, the degree of sequence similarity at these *tgr* alleles is positively correlated with the degree of segregation between strains (Benabentos *et al.*, 2009).

To address whether different strains segregate during development and, if so, whether it limits cheating, I examined the proportion of the two strains in individual fruiting bodies (see Methods). To determine if segregation was significant, I tested if the average degree of segregation observed in a mix was greater than that observed in the 'control mixes' using a one-tailed t-test (segregation in the control mixes: 0.010±0.004 mean±se, N=16). The results showed that 44% (=21 out of 48) of the mixes showed significant segregation (all P<0.05). Since segregation is caused by genetic differences at *tgrB1-tgrC1*, I expect that segregation should be a function of the genetic divergence at these loci. Moreover, *tgrB1-tgrC1* divergence is likely to be correlated with genome-wide divergence as well. In other words, more divergent strains are more likely to be divergent at *tgrB1* and *tgrC1*, too.

The results showed a significant positive correlation between a strain pair's genetic distance at the *tgrB1-tgrC1* locus and the degree to which strains segregate from each other in a mix (Fig. 3.9A; Spearman's rank correlation r=0.36, df=48, one-tailed P=0.005). To correct for possible inaccuracies in calculating the genetic distance at the *tgrB1-tgrC1* locus, I additionally tested this relationship using genome-wide genetic distance—and found a similar positive correlation (Spearman's rank correlation r=0.29, df=48, one-tailed P=0.022). Lastly, the relationship between segregation and spore inequity was not significant (Fig. 3.9B; Spearman's rank correlation r=0.16, df=48, P=0.93). These results

confirm that segregation is common between natural strains and increases with genetic dissimilarity at the *tgr* locus; however, they show no evidence that increased segregation limited cheating.

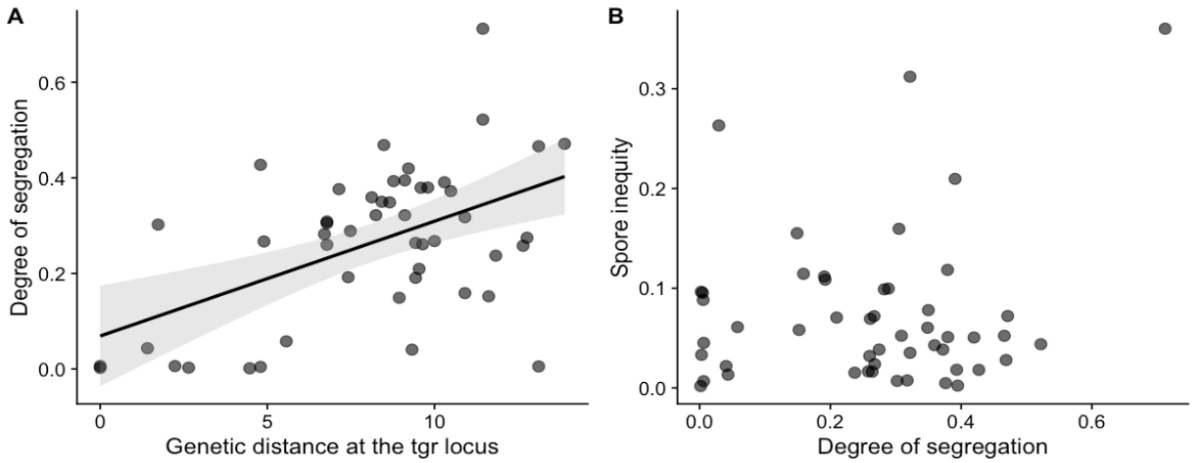


Figure 3.9. I confirmed prior work showing that segregation increases with genetic distance at the *tgrB1-tgrC1* locus but found no evidence that segregation limits cheating. (A) Segregation plotted as a function of genetic distance at the *tgrB1-tgrC1* locus. Segregation ranges from 0 to 1, where 0 means strains are equally distributed across fruiting bodies, and 1 means that fruiting bodies consist of either one strain or the other. **(B) Spore inequity as a function of segregation.**

Discussion

Enforcement is increasingly recognised as an important contributor to the maintenance of cooperation. It may be particularly relevant in cooperative societies where relatedness is low and therefore the effectiveness of kin selection is limited (Frank, 1995; El Mouden, West and Gardner, 2010; Ågren, Davies and Foster, 2019). Enforcement of cooperation occurs in animal societies through policing or punishment of non-cooperators (Ratnieks, 1988; Hauser, 1992; Clutton-Brock and Parker, 1995; El Mouden, West and Gardner, 2010; Singh and Boomsma, 2015). In addition, the plausibility of enforcement has also been repeatedly shown through laboratory evolution experiments in social microbes (Khare *et al.*, 2009; Zhang *et al.*, 2009; Hollis, 2012; Kümmerli *et al.*, 2015; Levin *et al.*, 2015; Miller, Sidell and Ostrowski, 2023). Despite the many advantages to studying cooperation in microbes, however, there is little understanding of whether these behaviours or evolutionary dynamics exist in natural populations.

In this study, I explored how common cheating is in natural populations of the social amoeba *Dictyostelium discoideum*. In addition, I looked for evidence that resistance might evolve to counter cheating, leading to its suppression. If this hypothesis holds, then I expected to find few cheaters (and low levels of cheating) in local strain pairs because the gene that confers cheating is matched by a gene that suppresses its effects (prediction 1). Moreover, when strains are tested against foreign social partners, the cheater and suppressor genes may be mismatched, revealing their existence (prediction 2). The results showed that eleven strains cheated against one or more local social partners. On average, these cheaters increased their fraction from 50% in the cells to 62% in the spores. Notably, in eight populations (=53%), none of the strains cheated against their local partners. These findings suggest that cheaters may exist in natural populations but are not widespread.

To test prediction 2, I compared the findings on cheating between local strains with that observed between foreign strains. The results showed that a similar number of strains cheated against local (11 strains) and foreign (9 strains) partners. In addition, strains that cheated against their foreign social partners on average took up 59% of the spores in these mixes. Furthermore, where cheating existed, it was significantly stronger in local compared to foreign strain pairs. This means that I found no evidence that cheaters are commonly suppressed by their local social partners.

These findings lead to two questions: *i.* why did I not find resistance to cheaters in local strain pairs, and *ii.* why did I not find an explosion of cheaters in foreign strain pairs? One possibility is that cheaters are selectively favoured. This means that when cheaters arise, they can adapt rapidly to exploit their local social partners, and resistance either does not evolve, or it takes some time before mutations emerge that counter this behaviour. One reason cheaters may be successful against local strains is the high genetic similarity between them. This study and previous studies showed that *D. discoideum* exhibits kin discriminatory segregation, where segregation increased with greater genetic distance at the *tgrB1-C1* locus (Benabentos *et al.*, 2009; Hirose *et al.*, 2011, 2017). Closely related strains are likely to show greater similarity at this locus, meaning that cheaters initially may ‘avoid’ kin discrimination by their close relatives. This also may explain why cheaters are uncommon in foreign strain pairs, as they may be easily detected as non-self by foreign strains, and therefore not be successful. Local adaptation of cheaters has also been found in other microbial populations (Bruce *et al.*, 2017; Butaitė, Kramer and Kümmerli, 2021).

The possibility that cheaters may be ahead in the cheater-cooperator dynamics is likewise supported by two other lines of evidence. A genome-scale investigation on a large collection of evolved cheater

mutants demonstrated that single mutations in more than 100 genes involved in different pathways can lead to cheating. These findings suggest that the genetic opportunities for cheating are high and thus cheaters may emerge frequently (Santorelli *et al.*, 2008). In addition, Miller et al. (2023) showed that, while resistance does evolve readily, it was often weaker and did not fully counter the effect of the cheating. Further work that examines the possibility of local adaptation of cheaters may benefit from a time-shift experiment in which cheaters are tested against local social partners from the past, present and future (Thompson, 2005).

The level of cheating, and therefore resistance, that can occur between strains may also be statistically difficult to detect. This can be explained as follows. Say strains A and B both exhibit roughly an 80-to-20 spore-to-stalk ratio and are mixed and allowed to develop fruiting bodies. If strain A contributes no cells to the stalk, then strain B still takes up 44% (=80/80+100) of the spores (assuming it still allocates 20% of cells to the stalk and does not compensate for the reduced contribution of strain A.) This is already 'only' a 6% deviation and might be difficult to detect statistically.

I also observed that average spore production in chimeric groups was often higher than in clonal groups, similar to what has been observed in prior studies (Buttery *et al.*, 2009; Kuzdzal-Fick *et al.*, 2023). On one hand, these results can indicate chimeric vigour, as found in some groups of social insects (Hughes and Boomsma, 2004; Saar *et al.*, 2018). Alternatively, they may indicate an increased propensity for selfishness in response to non-kin detection through the *tgrB1-C1* locus (Kuzdzal-Fick *et al.*, 2023). Specifically, analysis of prior RNA-seq datasets (Parikh *et al.*, 2010; Hirose *et al.*, 2015) revealed that prestalk-biased genes are downregulated in chimeric groups when the strains have non-matching *tgrB1-tgrC1* alleles (Kuzdzal-Fick et al. 2023). In other words, recognition of non-self through the *tgrB1-tgrC1* locus likely influences cell-type proportioning downstream of this locus, causing cells that are unable to avoid chimeric development to behave more selfishly by becoming spores. Unfortunately, as a result of the experimental design, I could not confirm the relationship between the increased sporulation efficiency and the level of segregation in chimera directly. However, I did find that segregation and sporulation efficiency both increased with genetic distance, further supporting the existence of a link between these processes and strengthening the hypothesis that genetic heterogeneity in a group results in more selfish behaviour, observed as an increased spore-to-stalk allocation (DeAngelo, Kish and Kolmes, 1990; Buttery *et al.*, 2009; Madgwick *et al.*, 2018; Kuzdzal-Fick *et al.*, 2023). It remains to be tested empirically how reduced stalk investment influences dispersal success.

In this study, I examined the nature of social interactions between natural strains of *D. discoideum* in a laboratory setting. Whereas using natural strains instead of laboratory-evolved strains is a considerably closer estimate of a natural situation, it remains that I only studied the interactions in a single, constant environment. This environment was specifically chosen to maximize selection for cheating behaviours: under conditions of no food and at equal starting frequencies (Matapurkar and Watve, 1997; Brännström and Dieckmann, 2005; Madgwick *et al.*, 2018). Prior work on microbial populations has demonstrated the influence of both the abiotic and biotic (intra- and interspecific) environment on the nature and intensity of social behaviours (Velicer, Kroos and Lenski, 2000; Greig and Travisano, 2004; Griffin, West and Buckling, 2004; R. Kümmerli *et al.*, 2009; Rivera-Yoshida *et al.*, 2019). Hence, the interactions between strains may vary based on conditions, leading to different outcomes under different experimental setups. Therefore, understanding these dynamics requires careful consideration of the contextual factors that drive these behaviours.

Collectively, the findings of this study showed that cheating is fairly rare in all contexts (12% of mixes; 20% of strains). The magnitude of cheating found in this study is also similar to that found using mutagenesis-generated cheaters (Santorelli *et al.*, 2008, 2013; Khare *et al.*, 2009; Miller, Sidell and Ostrowski, 2023). In addition, similar conclusions are reached using a completely different data set (Chapter 4), suggesting that they are robust and general. For now, it remains unclear why cheating is not common — i.e., whether it is suppressed or simply doesn't happen. However, my results do not provide strong evidence of repression. Nonetheless, I do find a strong response when genetically different strains interact, with kin discrimination commonly observed and strains adjusting their cell allocation patterns in favour of more spores. In addition, as the highest magnitudes of cheating are found between co-occurring strains, this may suggest that cheating evolves more rapidly than resistance. Collectively, these findings suggest that *Dictyostelium* has required and evolved adaptations to control cheating, even if imperfect.

Supplementary information

Genomic DNA isolation for whole genome sequencing and variant calling

To extract genomic DNA for sequencing, I grew cells to late-exponential phase on SM plates together with *K. pneumoniae*. I harvested the cells and washed them free of bacteria four times via differential centrifugation (450 g, 3 min) in KK2. To extract nuclei, I incubated the washed pellet of cells on ice for 10 min with nuclei buffer (40 mM Tris-HCl pH 7.8, 6 mM $MgCl_2$, 40 mM KCl, 0.1 mM EDTA, 5 mM DTT, 1.5% sucrose, 0.4% NP-40). I then pelleted the nuclei at high speed (4500 g, 15 min at 4°C) and dissolved the pellet in 100 µL of 100 mM EDTA, pH 8.0 (Thermo Fisher). To isolate the DNA, I sequentially added 450 µL STE solution (10 mM Tris-HCl pH 8.0, 10 mM EDTA, 400 mM NaCl), 50 µL 10% SDS (Thermo Fisher), and 5 µL 20 mg/ml Proteinase K (Sigma) to the nuclei. I mixed the solution by inversion and incubated it at 60°C for 1 hour. To clean the DNA, I performed three rounds of phenol-chloroform-isoamyl alcohol treatment and a final round of chloroform treatment, each time centrifuging at top speed for 10 min to separate the phases. I precipitated the DNA using $1/10^{th}$ volume 3 M NaOAc and 2x volume 100% ethanol. To improve yield, I incubated the tube overnight at -20°C. After centrifuging, I performed a final wash step with 70% ethanol and resuspended the air-dried gDNA pellet in TE buffer (1 M Tris-HCl, 0.5 M EDTA, pH 8.0) with 100 µg/ml RNase A (Thermo Fisher). Library preparation and whole genome Illumina sequencing were performed by Custom Science (NZ).

For each sequencing sample, I aligned the reads to the *D. discoideum* AX4 reference genome (assembly/GFF3 file generated on 30-Nov-2016 from dictybase.org with the duplication of chromosome 2 masked) using bwa-mem. To sort the sam files, get rid of duplicate reads, convert them to bam files, and obtain metrics of duplication, I used Picard tools SortSam, MarkDuplicates, BuildBamIndex, and CollectWgsMetrics, respectively. I performed variant calling using gatk HaplotypeCaller with -ploidy 1 and -emit-ref-confidence GVCF. I restricted variant calling to the six true chromosomes (DDB0232428, DDB0232429, DDB0232430, DDB0232431, DDB0232432, and DDB0232433) using gatk SelectVariants. I combined the vcf files using gatk CombineGVCFs and performed joint genotyping on the combined vcf file using gatk GenotypeGVCFs -new-qual. I removed multi-allele variants using bcftools norm -d. Following recommended hard filtering options, I filtered SNPs using gatk VariantFiltration with the options “QD > 2.0“, “FS > 60.0“, “MQ < 40.0“, and “SOR > 3.0“, and “isHomVar==1 && DP<4.0”. I only retained biallelic SNPs that had no missing data. I imported this final vcf file to *R* using the package ‘vcfR’. I calculated the Euclidean genetic distances among strains using the ‘poppr’ package. In addition to calculating the genetic distance across the entire genome, I calculated the genetic distances among strains exclusively at the genomic region spanning the *tgrB1-tgrC1* locus. This particular region was chosen because prior work demonstrated that genetic

differences among strains at these genes cause (Benabentos *et al.*, 2009; Hirose *et al.*, 2011, 2017; Ho *et al.*, 2013).

Table S3.1. List of strains used in this study.

Set	Strains	Origin	Location ID	GPS Coordinates
1	EO606, EO608, EO610, EO614, EO617	Great Smoky Mountains, NC	GSNP-15	35°60’852, -83°44’736
1	EO622, EO623, EO627, EO630, EO635	Smith College MacLeish Field Station, MA	SMFS-1	42°44’858, -72°68’129
1	EO1004, EO1005, EO1006, EO1007, EO1033	New Hampshire Proctor Academy, NH	NHPA-24	43°44’23, -71°82’35
1	EO1094, EO1097, EO1100, EO1105, EO1111	Mountain Lake Biological Station, VA	MLBS-B2	37°35’358, -80°53’615
2	EO680, EO681, EO683, EO686, EO689	Aton Forest Connecticut, CT	AFCT-10	42°03’011, -73°13’441
2	EO1337, EO1338, EO1339, EO1341, EO1348	Mountain Lake Biological Station, VA	MLBS-C2	37°37’293, -80°51’875
2	EO1256, EO1260, EO1264, EO1267, EO1270	Mountain Lake Biological Station, VA	MLBS-C1	37°37’292, -80°51’873
2	EO661, EO664, EO667, EO670, EO671	Aton Forest Connecticut, CT	AFCT-5	42°02’848, -73°13’483
3	EO545, EO549, EO550, EO552, EO553	Harvard Forest Massachusetts, MA	HMFA-2	42°53’882, -72°18’185
3	EO1010, EO1032, EO1034, EO1035, EO1036	New Hampshire Proctor Academy, NH	NHPA-24	43°44’23, -71°82’35
3	EO1231, EO1235EO1237, EO1241, EO1243	Mountain Lake Biological Station, VA	MLBS-B1	37°35’365, -80°53’622
3	EO1308, EO1311, EO1312, EO1317, EO1320	Mountain Lake Biological Station, VA	MLBS-D2	37°37’745, -80°52’209
4	EO366, EO368, EO369, EO370, EO371	New Hampshire Proctor Academy, NH	NHPA-10	43°45’207, -71°82’578
4	EO639, EO640, EO642, EO643, EO645	Smith College MacLeish Field Station, MA	SMFS-2	42°44’914, -72°68’204
4	EO1370, EO1371, EO1375, EO1377, EO1378	Smithsonian Conservation Biology Institute, VA	SCBI-8	38°89’344, -78°14’647
4	EO1828, EO1829, EO1830, EO1835, EO1836	Pennsylvania Mount Davis, PA	PAMD-A1	39°78’546, -79°17’421

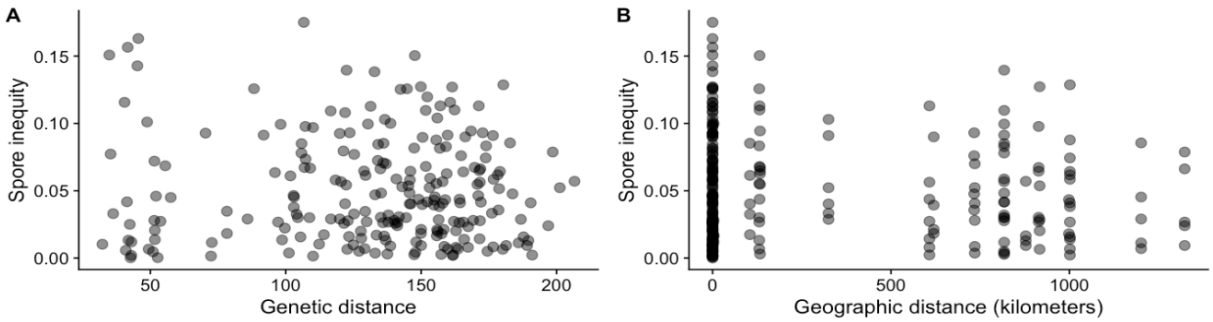


Figure S3.1. Spore inequity is not 'merely' a consequence of evolved social incompatibilities between strains.

The relationship between the (A) genetic distance and (B) geographic distance and spore inequity in a mix is flat.

Chapter 4.

Linear and nonlinear dominance hierarchies in the social amoeba *Dictyostelium discoideum.*

Abstract

Social groups often form dominance hierarchies, and these hierarchies are almost always linear. However, why linear dominance hierarchies emerge is not well understood. In the social amoeba *Dictyostelium discoideum*, cells form a multicellular fruiting body when starved, which consists of a ball of viable spores held aloft by a stalk of dead cells. In genetically mixed ('chimeric') fruiting bodies, conflicts can arise over the equitable sacrifice of cells to the dead stalk, and some strains predictably dominate others in the spores. Using pairwise mixes of strains that co-occurred in small soil samples, we determined the dominance hierarchies in four natural populations of *Dictyostelium*. These hierarchies were significantly linear in two of four populations, but also extremely shallow, indicating that co-occurring strains are competitively similar. We used quantitative genetic analyses to assess the causes of social dominance. Each strain's solo spore production was a significant predictor of its performance in pairs. However, we detected additional genetic contributions of both the focal and partner strain, indicating additional cryptic traits that mediate social competitiveness. In contrast to earlier studies showing strong fitness differences among strains collected over a larger spatial scale, we show that co-occurring strains are remarkably competitively equivalent, resulting in linear yet shallow hierarchies. Our results underscore the importance of biologically relevant spatial scales in assessing fitness interactions among microbes. They also explain why social trait diversity might be observed despite dominance hierarchies that should eliminate this variation.

Introduction

Social interactions within a group frequently result in the formation of a dominance hierarchy, commonly referred to as a "pecking order" (Schjelderup-Ebbe 1922). These social structures emerge from repeated antagonistic interactions between individuals that result in the establishment of a clear dominant and subordinate individual (Drews, 1993). For example, fish and rodents establish dominance through aggressive behaviours that involve chasing and biting, and crustaceans exhibit claw-grasping (Chase *et al.*, 2002). Based on the outcomes of the individual dominance relationships, a rank of all individuals in the group is established. Individual ranks within a hierarchy can significantly impact fitness, with higher-ranking individuals obtaining greater access to limited resources and reproductive opportunities compared to lower-ranked individuals (Wilson, 1975; Clutton-Brock, Albon and Guinness, 1984; Nelissen, 1992; Goessmann, Hemelrijk and Huber, 2000).

The structure of a dominance hierarchy can be characterized by two properties: its linearity and steepness (de Vries, Stevens and Vervaecke, 2006). These properties are assessed by analysing the

collective of pairwise, or 'dyadic', interactions within the group (Drews, 1993; de Vries, 1995; de Vries, Stevens and Vervaecke, 2006). In a perfectly linear hierarchy, one individual dominates all others, the second dominates all but the most dominant, and so on. For example, in a triad of individuals A, B, and C, the hierarchy is linear if the relationships are transitive, e.g., if A>B, B>C, and A>C, where ">" indicates dominance (Fig. 4.1A). In contrast, a hierarchy is increasingly non-linear when one or more intransitive triads exist, e.g., if A>B, B>C, but C>A. The steepness of a dominance hierarchy in turn reflects the differences in dominance ability between adjacently ranked individuals, with a hierarchy being shallow when the differences are small and steep when the differences are large (Fig. 4.1B) (Vehrencamp, 1983; Barrett *et al.*, 1999; de Vries, Stevens and Vervaecke, 2006).

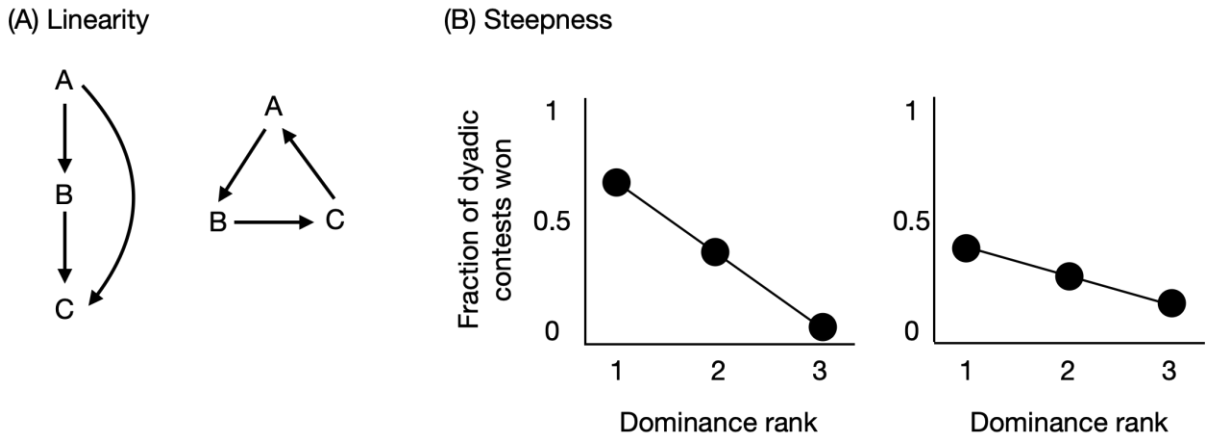


Figure 4.1. Two properties of a dominance hierarchy. (A) Linearity. In a perfectly linear dominance hierarchy, all triads are transitive (e.g., A>B, B>C, and A>C; left side). Alternatively, in a less linear dominance hierarchy, one or more triads are nontransitive (e.g., A>B, B>C, but C>A; right side). **(B) Steepness.** A dominance hierarchy is steep if the differences between adjacently ranked individuals are large and outcomes highly repeatable (left). A hierarchy is flat if the differences between adjacently ranked individuals are small and/or less repeatable (right).

The majority of dominance hierarchies examined in nature have been linear (Hausfater, Altmann and Altmann, 1982; Barkan *et al.*, 1986; Heinze, 1990; LeBrun, 2005; Valderrabano-Ibarra, Brumon and Drummond, 2007; Wittemyer and Getz, 2007; Correa *et al.*, 2013; Vullioud *et al.*, 2019). One major hypothesis to explain this linearity proposes that differences in individuals' pre-existing attributes predict their success in social contests (Chase *et al.*, 2002; Chase and Seitz, 2011). Consequently, the hierarchy can be predicted it has formed, and this scenario is therefore referred to as the 'prior attributes' hypothesis (Jackson and Winnegrad, 1988; Drews, 1993; Chase *et al.*, 2002). These prior attributes may relate to physical (size and weight), personality (aggressiveness), genetic (genotype), social (maternal rank), and physiological (hormone levels) characteristics (Jackson and Winnegrad,

1988; Drews, 1993; Nakano and Furukawa-Tanaka, 1994; Beaugrand and Cotnoir, 1996; Goessmann, Hemelrijk and Huber, 2000; Sundström *et al.*, 2004; Schjolden, Stoskhus and Winberg, 2005; Chase and Seitz, 2011). However, identifying which dominance attributes are of significant influence on dominance and quantifying their relative importance for the establishment of a hierarchy and its linearity remains challenging (Appleby, 1983; de Vries, 1995; Chase and Seitz, 2011; Shizuka and McDonald, 2012; Schmid and de Vries, 2013).

The social amoeba *Dictyostelium discoideum* serves as an effective model system for investigating social dominance and dominance hierarchies. In response to starvation, thousands of unicellular amoebae aggregate to form a multicellular fruiting body, which consists of a stalk of nonviable cells that supports a sorus of viable spores (Huss, 1989; Kessin, 2001). The self-sacrifice of the stalk cells is considered to be an altruistic act, as it is likely to benefit the survival and dispersal of the spores (Kuzdzal-Fick *et al.*, 2007; J. Smith, Queller and Strassmann, 2014). Importantly, when genetically different individuals aggregate to form a single "chimeric" fruiting body, some strains dominate by preferentially forming spores and relying on the stalk formation by their social partner, such that spore production is unfair (Filosa, 1962; Hamilton, 1964a, 1964b; Buss, 1982; Strassmann, Zhu and Queller, 2000). Consequently, chimeric fruiting body formation can involve a dominance relationship with a dominant and subordinate strain, offering a basis for constructing a dominance hierarchy among a group of co-occurring strains (Fortunato, Queller and Strassmann, 2003).

The two studies that examined the dominance hierarchy within a group of natural strains of *D. discoideum* both revealed linearity (Fortunato, Queller and Strassmann, 2003; Buttery *et al.*, 2009). In support of the prior-attributes hypothesis, Buttery *et al.* (2009) showed that pre-existing differences in spore production between strains during clonal development could predict social dominance and linearity in the group. However, both studies used the same seven strains, thus establishing a linear dominance hierarchy only once at a single site in North Carolina. They also found a slightly different order of strains, suggesting that the dominance hierarchies may not be repeatable. Moreover, these strains were collected within a 1 km^2 area. Owing to the large spatial scale over which these strains were sampled, it is unknown whether they interact in nature and, therefore, whether the observed dominance hierarchy is ecologically relevant. Indeed, strains collected over more relevant spatial scales might exhibit lower dominance levels and less linear hierarchies. In other words, co-occurring strains might co-occur *because* they exhibit non-transitive relationships. Thus, assessing the linearity of dominance hierarchies over more realistic spatial scales is a priority.

In this chapter, I examined the dominance relationships among strains in four natural populations of *D. discoideum*. The strains that make up a population were isolated from soil taken from the surface of a 10-by-10 cm plot, such that the maximum distance between strains was 14 cm (i.e., the diagonal of the square plot). Interactions between strains at this distance are reasonable, given that *D. discoideum* slugs routinely travel multiple centimetres from their aggregation sites (e.g., see Jack et al. 2015), and moderate gene flow occurs over distances <0.5 km (Kuzdzal-Fick, unpublished). From the observed dominance relationships, I constructed and evaluated the linearity and steepness of the dominance hierarchy. For those populations that were linear, I asked if pre-existing differences in dominance ability, assessed through the clonal spore production, could predict the dominance outcome and a strain's rank in the hierarchy. Additionally, I performed a quantitative genetic analysis to estimate the genetic basis of variation in social dominance. This analysis examines a focal strain's success as a function of its own genes ('direct genetic effects', or DGEs), those of its partner ('indirect genetic effects', or IGEs), and their interaction (genotype x genotype (GxG) epistasis). IGEs and GxG epistasis are collectively referred to as the social environment, and existing studies have demonstrated that these factors are widespread and can significantly alter evolutionary trait dynamics compared to expectations based solely on DGEs (Moore, Brodie and Wolf, 1997; Wolf *et al.*, 1998; Bijma and Wade, 2008).

Of the four natural populations of *D. discoideum* studied, two populations exhibited a dominance hierarchy that was significantly linear. Conversely, the other two populations did not show significant linearity, with one being marginally nonlinear. Notably, in the cases where linearity was observed, the dominance hierarchies were shallow, indicating minimal fitness differences among co-existing strains, at least in terms of their ability to dominate in the spores in chimera. The lack of linearity in two populations, combined with shallow dominance hierarchies in all populations indicates co-occurring strains that are well-matched for social fitness. Lastly, the quantitative genetic analyses showed the importance of genetic identity and traits of the focal individual but also that of its social partner on the level of social dominance of the focal individual, providing empirical evidence for the importance of the social environment.

Materials and Methods

Strain collection, isolation, and selection

The strains of *D. discoideum* used in this study were acquired between 2016 and 2019 by staff and students of the Ostrowski laboratory. Soil sampling and strain isolation methods are described in detail

by Kuzdzal-Fick *et al.* (2023). The strains and sites of sampling are listed in Table S4.1 and shown in Figure 4.2A.

To ensure that the chosen strains were not clones of one another, I used Illumina genome sequencing to determine the pairwise genetic distances among the strains from each site. Illumina sequence data were provided by Kuzdzal-Fick, and detailed information and methods concerning these data are described in Kuzdzal-Fick *et al*. (2023). I chose strains with an Euclidean genetic distance of >5 (1,756 SNPs), as preliminary analyses indicated that values below this cut-off are within the margin of error for clones for this sequencing methodology. Figure 4.2B shows a genetic distance-based tree based on the Euclidean distance.

Experimental design

I examined all possible pairwise combinations of strains for each of the four sites. For three sites with seven strains each—MLBS, GSNP, and AFCT—this resulted in 21 mixes. For site SMFS, with six strains, this resulted in 15 mixes. The lower number of strains in site SMFS was a result of having excluded strain EO631 because it did not progress beyond the slug stage during the experiments. I tested all combinations between strains from GSNP and SMFS on the same day, and from MLBS and AFCT on a different day. I performed three temporally independent blocks, each initiated from frozen spore stocks.

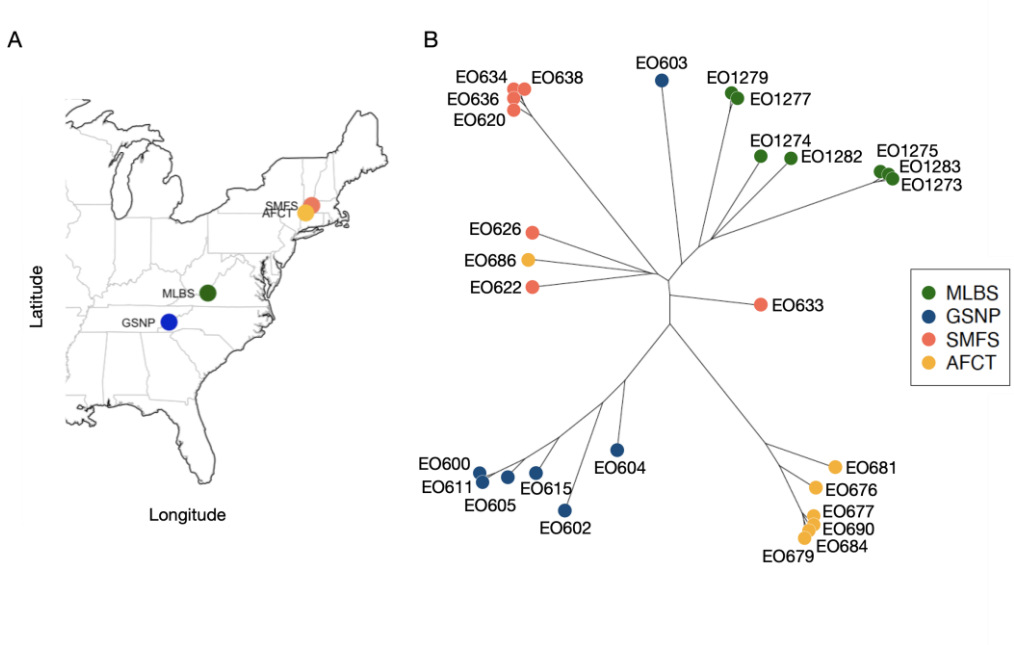


Figure 4.2. (A) The 27 strains of *D. discoideum* in this study were sampled from four sites across the US. (B) A distance-based tree showing the genetic similarity among the strains based on genome-wide SNPs.

Cultivation, cell staining, and developmental assays

At the start of each block, I inoculated the relevant strains from the freezer onto SM-agar plates (Formedium Ltd, 2% agar) with *Klebsiella pneumoniae* as a food source. After fruiting bodies had formed, I collected and replated $5x10^5$ spores with *K. pneumoniae* on fresh SM-agar plates for germination and population expansion. After approximately 40 hours, I harvested the cells during the mid-exponential growth phase and washed them three times via differential centrifugation (450 x *g* for 3 min) in KK2 buffer (14.0 mM K_2HPO_4 and 3.4 mM KH_2PO_4, pH 6.4) to remove the bacteria. After washing, I resuspended the cells to a density of $1x10^7$ cells/ml in KK2.

To distinguish the two strains in a mix, I treated one strain in each pair with the fluorescent dye CellHunt Green CMFDA (Molecular Probes), diluted in DMSO according to the manufacturer's specifications. I added the cell dye at a concentration of 20 µM, incubated the cells on a shaking platform in the dark for 30 minutes, washed them once in KK2, and incubated them again for 30 minutes to allow the efflux of excess dye. I then washed the cells twice in cold KK2 and resuspended them at a concentration of $1x10^8$ cells/mL in cold KK2. I treated the unlabelled strain with DMSO only, and these samples underwent the same treatment as the labelled strains.

For each mix, I combined equal volumes of the labelled and unlabelled cells and deposited a 50 µl aliquot of the mix in a 3-by-3 square (an area of 1 cm^2) of a 47 mm gridded 0.45 µm nitrocellulose filter, resulting in a total cell number of $5x10^6$ cells (Fig. 4.3). I placed the nitrocellulose filter on top of a Pall filter that was moistened with 1.5 ml of PDF (per litre: 1.5 g KCl, 1.07 g $MgCl{\cdot}6H_2O$, 1.8 g KH_2PO_4, 1.6 g K_2HPO_4, 0.5 g streptomycin sulphate) in a 6-cm Petri dish. I transferred the Petri dishes to a sealed plastic box that contained wet tissues and placed the box in the dark for 48 hours at 22°C to allow fruiting bodies to develop.

Following development, I collected the spores in 3 mL of detergent (KK2 buffer + 0.1% IGEPAL and 10 mM EDTA), which dissolved any remaining cells. I measured the fraction of both strains in the spores on a BD FACSCanto II flow cytometer (488 nm laser, 513/15 GFP filter). I used an automated cell counter (Cell Countess II FL, Thermo Fisher) to quantify the total number of spores. For each mix, I developed the labelled and unlabelled cells of each strain by themselves. I also developed mixes that consisted of labelled and unlabelled cells of the same strain, which I refer to as the 'control mix' (Fig. 4.3). All three types of clonal controls (100% labelled, 100% unlabelled and 50-50% control mix) were otherwise treated identical to the chimeric mixes.

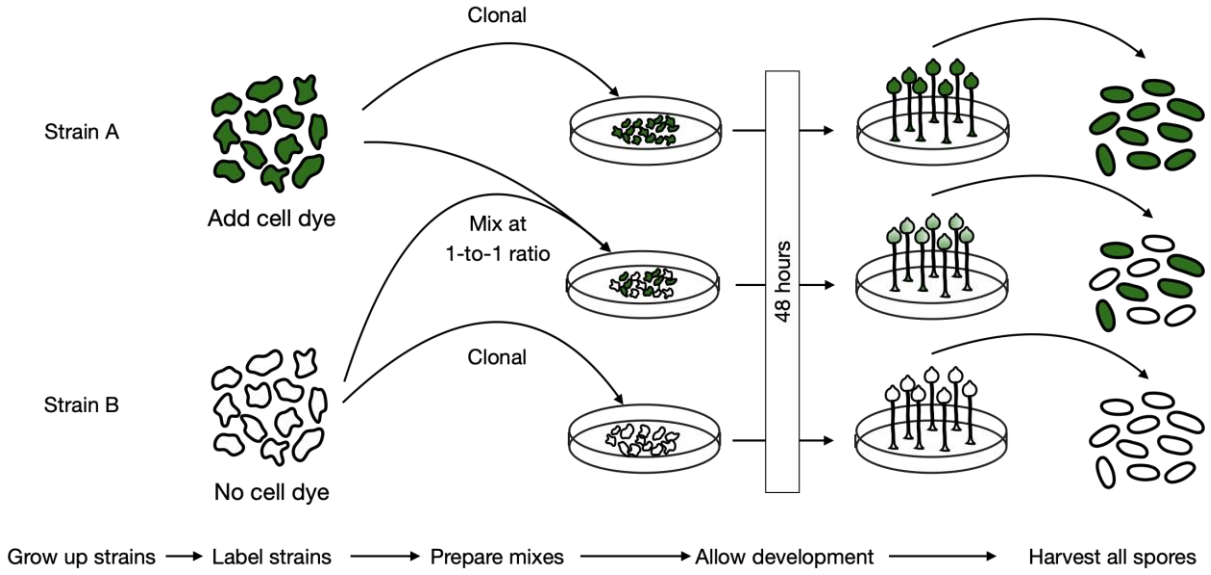


Figure 4.3. Overview of the mixing experiments to assess social dominance. For each mix, the cells of a labelled and unlabelled strain were combined at equal cell numbers, spotted on a nitrocellulose filter, and allowed to form fruiting bodies. After development, the spores were harvested, and the fraction of the labelled and unlabelled strain was determined using a flow cytometer. For each mix, labelled and unlabelled cells of both strains were developed by themselves, as well as in a 'control mix' consisting of labelled and unlabelled cells of the same strain.

Determination of winner and loser strain and quantification of social dominance

For each mix, I determined the 'winner' and 'loser' strain based on their average fraction in the spores (across *N*=3 blocks). Since the strains were combined at equal starting cell ratios, I identified the winner as the strain with >50% of the spores, and the loser as the strain with <50% of the spores. I quantified the winner's extent of social dominance as the magnitude of spore inequity: the increase in the strain's representation in the spores compared to its initial representation in the cells. This measure ranges from 0 to 0.5, where a value of near 0 indicates roughly equal fractions of the winner and loser in the spores, and a value of 0.5 indicates that the winner makes up all the spores. For example, a social dominance value of 0.1 indicates that the winner makes up 60% of the spores in the chimera (i.e., increased by 10% relative to its starting frequency).

Quantification of the linearity of the dominance hierarchy

I calculated the linearity of the dominance hierarchy using standard methods (Appleby, 1983). Briefly, I constructed a win/loss matrix of *N*-by-*N* individuals, with the order of the individuals initially being arbitrary. I assigned an entry in the matrix a value of 0 if the row individual was the 'loser' when mixed with the column individual (<50% of the spores), and a value of 1 if the row individual was the 'winner'

when mixed with the column individual (>50% of the spores). Using the row sums (S_i) derived from the matrix I calculated the observed number of transitive triads (*d*) using the formula:

$$d = \frac{N(N-1)(2N-1)}{12} - \frac{1}{2} \times \sum (S_i)^2$$

I calculated the degree of linearity (*K*) using the formulas:

$$K = 1 - \frac{24d}{N^3 - N} \text{ or}$$

$$K = 1 - \frac{24d}{N^3 - 4N}$$

for even or uneven values of *N*, respectively. To assess the significance of *K*, I determined the degrees of freedom (*df*) and chi-square (χ^2), since the distribution of *d* approaches that of a chi-square distribution with increasing *N*. The formulas for these calculations are:

$$df = \frac{N(N-1)(N-2)}{(N-4)^2}$$

and

$$\chi^2 = \frac{8}{N-4}\left[\frac{N(N-1)(N-2)}{24} - d + \frac{1}{2}\right] + df$$

In the given formula, when the group size is *N*=6, df equals 30, and when *N*=7, df equals 23.3. According to a chi-square reference table, *K* is considered significant at *P*<0.05 if χ^2>43.8 when *df*=30 and χ^2>35.2 when *df*=23.3.

In response to observing a very low estimate of steepness (discussed in the Results), I also computed the triangle transitivity using the 'transitivity' function from the 'EloRating' package. Briefly, this function calculates the proportion of transitive triangles (P_t) using the formula:

$$P_t = \frac{N_{transitive}}{N_{transitive} + N_{cyclic}}$$

in which $N_{transitive}$ is the number of transitive triads in the group, and N_{cyclic} is the number of intransitive triads (Shizuka and McDonald, 2012). The triangle transitivity (t_{tri}) was then calculated as:

$$t_{tri} = 4(P_t - 0.75)$$

t_{tri} is scaled to run from 0 to 1, where 0 indicates the random expectation (0.75; regardless of the *N*) and 1 indicates maximum transitivity. Statistical significance of t_{tri} was assessed via randomization. A

P<0.025 or *P*>0.975 means that the hierarchy is significantly more or less linear than expected by chance, respectively, based on 1,000 randomizations that were used to determine the null distribution.

Quantification of the hierarchy steepness

The hierarchy steepness quantifies the absolute differences in dominance ability between consecutively ranked individuals in a hierarchy. Steepness therefore also serves as a measure of ranking certainty. To calculate hierarchy steepness, I used the 'steepness' function from the 'EloRating' package (Neumann et al. 2011). In contrast to the binary win/loss matrix that we used to calculate the linearity, steepness was calculated using continuous data—specifically, the fraction of a strain in the spores. Steepness values range from 0 to 1, where 0 indicates a shallow hierarchy with small differences between adjacent ranks, and 1 indicates a steep hierarchy with large differences between adjacent ranks, and higher-ranked individuals consistently win against adjacent lower-ranked individuals (Figure 1B). The null hypothesis is that the observed steepness is no greater than a steepness based on random win probabilities (de Vries, Stevens, and Vervaecke 2006). To test this, randomization is used. Here the statistical significance (right-tailed *P*-value) of the observed steepness is determined by calculating the proportion of times that the steepness generated randomly under the null hypothesis is greater than or equal to the observed steepness (based on *N*=1000 iterations).

Determining the ranking of strains in a hierarchy

For those populations that demonstrated significant linearity, I obtained the strain ranking using the 'isi13' function from the 'compete' package (Curley 2016). This function uses randomization to identify the optimal order of strains in a linear hierarchy (based on *N*=1000 iterations). The resulting linear order is that with the fewest inconsistencies (*I*) and the smallest inconsistencies (*SI*) among all potential orders. An inconsistency describes the situation where a lower-ranked individual dominates a higher-ranked individual in the assumed linear order (Schmid and de Vries 2013).

Do strains vary in their prior attribute clonal spore production?

To assess if strains within a site vary in their inherent ability to dominate, I used a generalized linear mixed model (GLMM) that examined the clonal spore production as a function of strain ID and block. Because the response variable was a count, I used a Poisson error structure with a log link function. I included both terms as random effects and assessed their significance through single deletions of terms, comparing the reduced models with the full model using a likelihood ratio test.

Does linearity arise from differences between strains in their ability to produce spores?

To test the hypothesis that linearity arises from a pre-existing hierarchy based on differences between individuals in their inherent ability to sporulate, I first ranked strains within a site based on their clonal spore production. The strain that produced the most spores clonally was ranked first, and so on. I then compared this 'prior attribute ranking' with the ranking based on the observed dominance relationships determined from the mixes above. The expectation, under the prior attribute hypothesis, was that the rankings in the two hierarchies would align.

Quantitative genetic effects analysis

I performed two GLMMs to investigate the effects of clonal spore production, the genotypes of the focal and partner strain, and the interaction between them, on the focal strain's relative (percentage; Model 1) and absolute (total; Model 2) spores in a mix.

In Model 1, I used beta regression since the response variable was a fraction between 0 and 1. Given the paired data (with fractions summing to 1), I randomly selected one strain from each pair as the focal strain and ran the model on this subset. I modelled the *clonal spore production* of the focal and partner strains and assessed their significance through type II Wald chi-square tests. I modelled the *genotype* of the focal strain, the partner strain, and their interaction as random effects. In addition, we included block as a random effect. I assessed the significance of the random effects through single deletions of terms, comparing the reduced models and the full model using a likelihood ratio test. Finding a significant effect of the focal genotype would indicate the presence of direct genetic effects (DGEs), a significant effect of partner genotype would indicate the presence of indirect genetic effects (IGEs), and a significant effect of the interaction between the focal and partner genotype would indicate the presence of genotype-by-genotype (G×G) interactions, or epistasis. To quantify the proportional contributions of the DGE, IGE and G×G interaction terms, I divided the variance components of each random term by the sum of the variance components of all random terms.

Model 2 incorporated several modifications compared to Model 1. Specifically, as the response variable in Model 2 was a count (i.e., the number of spores of the focal strain) and showed overdispersion, I used a negative binomial error structure (family=nbinom2). In addition, I included genetic distance between two strains in a chimera as a covariate and I used the full data set because the total spore production of both strains in a pair was independent. I included mix_id as a random variable to correct for including the spore production of both strains in a mix. I analyzed all other terms and performed tests of significance as described in Model 1.

In summary, with these models, I aimed to *i.* assess if the prior attribute 'spore production during clonal development' serves as a significant predictor of a strain's relative (Model 1) and

absolute (Model 2) dominance over spore production during chimeric development, and *ii.* quantify the relative importance of direct genetic effects (DGEs), indirect genetic effects (IGEs) and genotype-by-genotype (G×G) interactions on the focal strain's spore production during chimeric development.

Results

Dominance hierarchies are mostly linear but shallow

Tables 4.1-4.4 show the dominance matrices for the sites AFCT, GSNP, MLBS and SMFS respectively. Each matrix value indicates the average spore fraction obtained by the strain in that row when co-developed with the strain in the corresponding column (based on *N*=3 blocks). Table 4.5 compares the linearity and steepness estimates for the four sites, along with the previously studied North Carolina (NC) site by Fortunato *et al.* (2003). Fortunato's study, testing all pairwise combinations of seven strains collected from Little Butt's Gap (NC), demonstrated significant linearity, though steepness was not determined. I calculated the steepness for the NC hierarchy using their reported values (Table 2 from Fortunato *et al.* 2003). Similar to the NC site, sites AFCT and MLBS showed significant linearity based on Kendall's *K*. Sites GSNP and SMFS did not show significant linearity (Table 4.5). Steepness for each hierarchy, including NC, did not significantly differ from zero. To address potential issues that may result from calculating linearity at a low steepness (Sánchez-Tójar, Schroeder and Farine, 2018), I additionally calculated the triangle transitivity (t_{tri}). Using this method, I observed a lower degree of linearity, but the hierarchies of sites AFCT and MLBS remained significantly linear (Table 4.5). In addition, using this metric, the hierarchy in site GSNP was marginally nonlinear (observed value is in the 3.4th percentile of the null distribution—i.e., *P*=0.068; explained in the Methods).

Table 4.1-4.4. The dominance hierarchy matrix for sites SMFS, AFCT, MLBS, and GSNP respectively. Each entry indicates the mean fraction of the spores achieved by the strain in that row when co-developed with the strain listed in the corresponding column, based on *N*=3 blocks.

SMFS	EO620	EO622	EO626	EO633	EO634	EO638
EO620	-	0.526	0.515	0.489	0.488	0.518
EO622		-	0.417	0.513	0.461	0.443
EO626			-	0.388	0.527	0.481
EO633				-	0.514	0.520
EO634					-	0.477
EO638						-

AFCT	EO676	EO677	EO679	EO681	EO684	EO686	EO690
EO676	-	0.631	0.596	0.586	0.475	0.450	0.424
EO677		-	0.479	0.505	0.478	0.391	0.335
EO679			-	0.532	0.544	0.508	0.409
EO681				-	0.498	0.458	0.461
EO684					-	0.446	0.493
EO686						-	0.505
EO690							-

MLBS	EO1273	EO1274	EO1275	EO1277	EO1279	EO1282	EO1283
EO1273	-	0.450	0.493	0.478	0.389	0.470	0.461
EO1274		-	0.527	0.533	0.413	0.537	0.512
EO1275			-	0.485	0.405	0.469	0.442
EO1277				-	0.417	0.394	0.464
EO1279					-	0.475	0.610
EO1282						-	0.509
EO1283							-

GSNP	EO600	EO602	EO603	EO604	EO605	EO611	EO615
EO600	-	0.577	0.537	0.628	0.495	0.476	0.501
EO602		-	0.504	0.623	0.572	0.421	0.478
EO603			-	0.623	0.572	0.421	0.478
EO604				-	0.502	0.506	0.467
EO605					-	0.515	0.485
EO611						-	0.603
EO615							-

Table 4.5. Summary of the properties of the four dominance hierarchies from this study and the NC population tested in Fortunato *et al.* (2003)**.** *K* is a metric of linearity, P_t is the proportion of transitive triads, and t_{tri} is the triangle transitivity. See main text for explanations of terms. For site GSNP, t^{tri} is strongly negative and in the 3rd percentile of the distribution based on randomization (i.e., 966/1000 randomizations were more positive than the observed value, and thus *P*=0.034*2=0.068 (the *P*-value is doubled because significance is now tested in opposite direction, i.e., tail); cut-offs for a two-tailed test are the 2.5th and 97.5th percentiles.)

	K	χ^2	df	*P*(*K*)	$P_{t\ (\text{expect 0.75 if random})}$	t_{tri}	$P(t_{tri})$	steepness	$p_{steepness}$
SMFS	0.250	38.7	30	<0.90	0.700	-0.200	0.777	0.030	1.00
AFCT	0.786	40.0	23.3	<0.025	0.914	0.657	0.029	0.064	1.00
MLBS	0.929	45.3	23.3	<0.005	0.971	0.886	0.009	0.060	1.00
GSNP	0.143	16.0	23.3	<0.90	0.657	-0.371	0.966*	0.040	1.00
NC (Fortunato *et al.* 2003)	0.929	45.3	23.3	<0.005	0.971	0.886	0.009	0.304	0.36

Are dominance hierarchies generally linear in populations of *D. discoideum*?

The North Carolina (NC) site, as tested by Fortunato, exhibited the same linearity as site MLBS (Table 4.5; *K*=0.929, $\chi^2_{23.3}$=45.3, *P*<0.005). Despite this similarity, MLBS displayed a very shallow hierarchy, with a steepness approaching zero, a pattern also observed in AFCT, GSNP, and SMFS. Notably, all four steepness values were substantially lower than that of the NC population. The lack of steepness in my study indicates much lower social dominance and, therefore, more equitable spore production. Specifically, in the Fortunato study, the average social dominance was 0.26 (across *N*=21 mixes), indicating a shift from a 50-50% distribution in the cells to 76-34% in the spores. In contrast, in this study, the average social dominance was only 0.045 (across *N*=78 mixes; Figure 4.4). Significant social dominance was observed in only 9% of all mixes: in 3 out of 21 mixes from GSNP, 2 out of 21 mixes from AFCT and MLBS, and none of the 15 mixes from SMFS. Significance was assessed based on a one-sample t-test, where the null hypothesis is that the observed social dominance is equal to that of the control mixes (Figure 4.4). In those mixes where I observed significant social dominance, the magnitude was 0.094±0.011 (mean±se, *N*=7), indicating a shift from a 50-50% distribution in the cells to 59-41% in the spores.

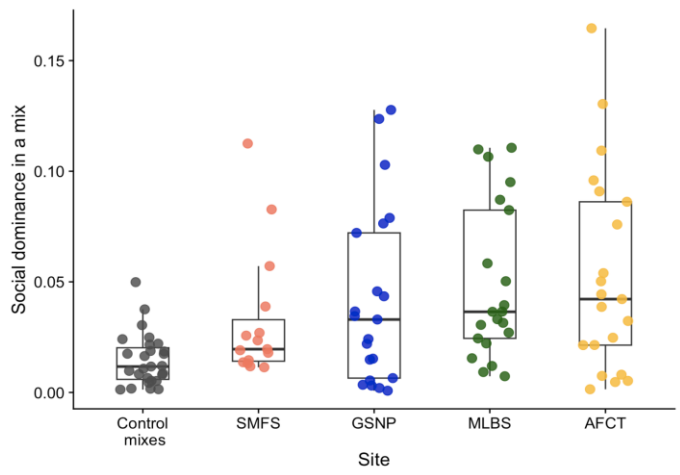


Figure 4.4. The magnitude of social dominance observed in control mixes and mixes of genetically different strains. Each point indicates the mean social dominance across *N*=3 blocks. For example, a value of 0.10 indicates a shift from 50-50% in the cells to 60-40% in the spores. The control mixes are 'mixes' that consist of labelled and unlabelled cells of the same strain; these mixes serve as a baseline against which the mixes of genetically different strains can be compared.

Can differences in the inherent ability to dominate in the spores explain linearity?

Prior work found that social dominance and linearity resulted from inherent differences between strains in their spore production (Buttery *et al.*, 2009). The strains used in this study likewise show significant variation in spore production (SMFS: χ^2=3.8e6, *P*<0.001, GSNP: χ^2=2.9e6, *P*<0.001, AFCT: χ^2=1.4e6, *P*<0.001, MLBS: χ^2=4.4e5, *P*<0.001), allowing me to test this hypothesis for the two sites that showed significant linearity. To do so, I ranked the strains from most to least dominant based on their clonal spore production (termed 'prior attribute' ranking) and compared it to the ranking derived from outcomes of the pairwise strain interactions (termed 'observed' ranking) (Fig. 4.5). If the two rankings are similar, spore production is a robust predictor of social dominance and linearity. Conversely, if the rankings differ substantially, then spore production of the two strains is not a good predictor of social dominance and linearity. Figure 4.5 shows that in both populations the two rankings exhibit multiple differences in order, indicating that the differences between strains in the spore production are not strong predictors of the dominance hierarchy.

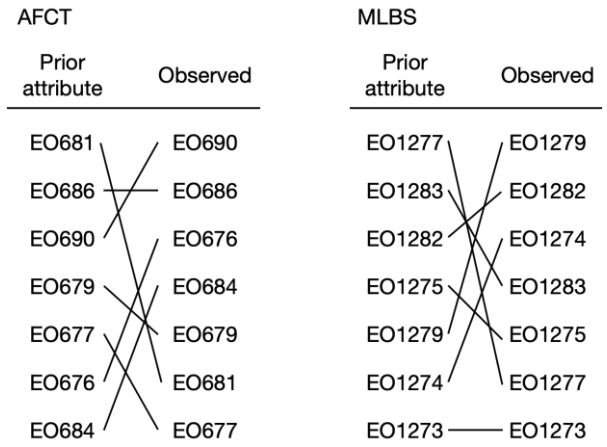


Figure 4.5. Comparison of the expected dominance rank (left), based on prior attribute spore production, and the observed dominance rank (right) in the two populations (AFCT and MLBS) that exhibit significant linearity. To visualize the changes in the order of the ranking, a single strain in the two rankings is connected by a line, and the crossing of two lines indicates changes rank order.

Evidence that prior attributes, direct genetic effects, and social genetic effects influence social dominance

So far, I have examined the variation in social dominance as a product of a strain's own predetermined dominance ability—in this case, its ability to sporulate. However, dominance is likely also determined by traits of the social partner. These traits might not have been identified yet or might only be expressed during the social interaction. The influence of a social partner's genes on the phenotype of the focal individual is called indirect genetic effects (IGEs) (Wolf *et al.*, 1998). Conversely, direct genetic effects (DGEs) describe the direct influence of an individual's own genes on its phenotype. In addition, when the interaction between genotypes is dependent on the specific combination of genotypes (i.e., social context) these effects are called genotype-by-genotype (G×G) interactions. Collectively, IGEs and G×G interactions are referred to as the social environment: the environment created by genes present in social partners (Wolf, 2003).

To estimate the proportional contribution of the individual genetic effects on dominance I performed a quantitative genetic analysis (Bijma, 2014). Since prior work showed that clonal spore production significantly influences a strain's spore fraction (Buttery *et al.*, 2009), the model also included both the focal and partner strain's clonal spore production as explanatory factors. Lastly, the I included site and genetic distance between strains in a mix. I performed two models: Model 1 tested the focal strain's spore *fraction* in a mix as the response variable, and Model 2 tested the focal strain's *total* spore production in a mix (discussed below).

The results of model 1 showed that a focal strain's spore fraction in a chimera was significantly influenced by its clonal spore production, as well as that of its partner strain (Table 6). There was no significant effect of genetic distance, and so this term was removed from the final model (c^2=0.039,

df=1, *P*=0.84). The focal strain's spore fraction was significantly influenced by the direct genetic effect (DGE) of the focal strain, but not by the indirect genetic effect (IGE) of the partner strain and the interaction between the genotypes of the focal and partner strain (G×G interaction). Analysis of the partitioned genetic effects revealed that 53.1% of the random effects variance was explained by DGEs, 28.5% by IGEs, and 17.4% by G×G interactions (Table 6). Because the focal and partner genotypes can influence dominance through two different terms (their clonal spore production and respective random effects), we assessed their relative contributions using an R^2 metric appropriate for mixed models (Nakagawa and Schielzeth 2013). I found that 11.0% of the total variance could be explained by the fixed effects (i.e., clonal spore production) and 63.7% by the fixed and random effects. Thus, although highly significant, the clonal spore production of the two strains explains only a small fraction of the total variance. This result indicates that the prior attributes were not the main drivers of the outcome: rather, these strains must have other, unidentified attributes that largely determine their ability to dominate the spores.

Table 4.6. Results of the model that tested the focal strain's fraction in the spores in chimera as a function of prior attribute clonal spore production and genetic identity of the focal and partner strain, and their interaction. The variance explained by the individual genetic effects (DGE, IGE, and GxG interaction) and block are calculated by dividing each term's variance by the sum of the variances of all random effects.

	Term	df	χ^2	*P*		
Fixed	Focal clonal spore production	1	11.464	<0.001		
	Partner clonal spore production	1	8.551	0.003		
	Term	df	χ^2	*P*	Variance	% Var. explained
Random	Focal genotype (DGE)	1	14.925	<0.001	0.016	53.1
	Partner genotype (IGE)	1	5.268	0.022	0.009	28.5
	Focal-by-partner interaction (GxG)	1	0.422	0.52	0.005	17.4
	Block	1	0.036	0.85	0.0003	1.0
	Total				0.0303	

I found that total spore production in chimeras is often greater than what is expected based on the average spore production of both strains when developed clonally, consistent with other studies (Buttery et al. 2009; Kuzdzal-Fick et al. 2023). The excess spore production found in this study shows, however, no relationship with the spore inequity observed in that mix (Figure S1; Spearman's rank correlation: *r*=-0.08, *df*=76, *P*=0.50). Therefore, modelling variation in a strain's fraction in the spores as a response variable may ignore important G×G interactions. Specifically, if both strains increase their spore allocation, but do so *equally*, then each strain's spore fraction would remain at 0.5 despite

a strong change in behavior in response to one another. For this reason, I examined not only a strain's spore *fraction* (i.e., its dominance, as tested in Model 1) in chimera but also its *total* spore production (Model 2).

The results of Model 2 showed that the focal strain's total spore production in chimera was significantly influenced by the clonal spore production of the partner strain, but not that of the focal strain (Table 7). In addition, there was a significant positive relationship between the genetic distance between the two strains and spore production (estimate=0.003; P<0.001; 95% CI: 0.002-0.004). In other words, the more genetically different the two strains are, the more spores the focal strain produces.

In contrast to the earlier model, the focal strain's spore production was significantly influenced by IGEs, but not by DGEs. Similar to before, there was no significant influence of the GxG interactions. Analysis of the partitioned genetic effects revealed that 8.9% of the total variation in spore production was explained by DGEs, 77.4% by IGEs, 0.4% by G×G interactions, 2.8% by block, and 10.6% by mix id (Table 7). We again estimated the R^2 for the fixed and random effects, which showed that 35.7% of the variance in outcome was attributable to the fixed effects, and 44.1% was attributable to the fixed and random effects together. The small difference means that the outcome is largely explained by the fixed effects—the clonal spore production of the two strains and how genetically different they are. While significant, the random effects are small in magnitude. Finally, the total R^2 is also lower compared to the earlier model (44.1% versus 63.7%), indicating that the total spore production of two strains together is less predictable than their percentages.

Table 4.7. Results of the model that tested the focal strain's total spore production in chimera as a function of prior attribute clonal spore production, genetic distance, genetic identity of the focal and partner strain, and their interaction. The variance explained by the individual genetic effects (DGEs, IGEs, and G×G interactions) and block were calculated by dividing each term's variance by the sum of the variances of all random effects.

	Term	df	χ^2	*P*		
Fixed	Focal clonal spore production	1	64.848	<0.001		
	Partner clonal spore production	1	7.786	0.005		
	Term	df	χ^2	*P*	Variance	% Var. explained
Random	Focal genotype (DGE)	1	0.484	0.49	0.001	8.9
	Partner genotype (IGE)	1	17.488	<0.001	0.006	77.4
	Focal-by-partner interaction (GxG)	1	0.00	1.00	0.000	0.4
	Block	1	0.159	0.69	0.0002	2.8
	Mix id	1	0.187	0.67	0.0009	10.6
	Total				0.015	

Discussion

Here I examined the structure of dominance hierarchies, focusing on their linearity and steepness, in four natural populations of the social amoeba *Dictyostelium discoideum*. In two populations, I identified significantly linear dominance hierarchies. In the third population, the hierarchy was not significantly linear, whereas in the fourth, it was marginally non-linear. Despite this variation, all four hierarchies were shallow, with relatively few significant deviations from spore equity. Together, these results indicate strains with a high degree of competitive similarity for social fitness. The absence of strong hierarchies suggests that the nature of the social interactions in societies of microbes differ from those observed in animal societies in which generally linearity is found.

The finding of less linear and relatively flat social hierarchies (or: *less inequity*) differs from two earlier studies, both of which used a set of isolates collected at varying distances over a ~1 km^2 area of North Carolina (Fortunato, Queller and Strassmann, 2003; Buttery *et al.*, 2009). Notably, the set of strains used here were isolated from small soil samples of 10 g or less, and the tested strains were no more than 14 cm from each other at the time of sampling—and presumably, often much less. They were thus much more likely to consist of strains that do interact in nature. These results underscore the importance of an appropriate spatial scale for the study of microbial cooperation in nature.

I expect co-occurring strains to be well-matched for important fitness characteristics, for several reasons. First, large variance in social fitness can arise when selection is relaxed in the laboratory (Larsen *et al.*, 2023). By similar logic, low variance in social fitness is expected for traits subject to strong past selection. In other words, co-occurring strains might co-occur *because* they do not have large fitness differences. Second, previous studies have shown that spore inequality will drive counter-evolutionary changes to restore fairness (Khare *et al.*, 2009; Hollis, 2012; Levin *et al.*, 2015; Miller, Sidell and Ostrowski, 2023). Thus, both ecological and evolutionary processes should promote the co-existence of well-matched competitors.

In animal societies, dominance hierarchies are often linear, and much work has been devoted to understanding the determinants of a dominance hierarchy and whether it arises from 'prior attributes' (i.e., genetic attributes) or emerges from 'social dynamics', which may include winner or loser effects (Chase and Seitz, 2011). In this study, I did not allow for winner/loser effects, as we re-grew the strains from frozen for each replicate. That said, the variance attributed to block effects was *more than an order of magnitude smaller* than that attributed to the different genetic effects. Overall, these results suggest that the dominance hierarchies, despite being shallow, were repeatable.

In the populations that did exhibit significant linearity, I could investigate the causes of that linearity. I hypothesized that the difference in the representation of the strains in the spores could reflect inherent differences in their spore-stalk allocation—their 'prior attribute'. However, I observed many shifts in rank between the expected performance of strains based on this prior attribute and their actual performance in the mix. Despite this result, the focal and partner strains' clonal spore production were significant predictors of their percentage of the spores, indicating that these prior attributes do contribute to the establishment of the linear dominance hierarchy, at least in part. The statistical analyses also indicate that social dominance was significantly influenced by direct and indirect genetic effects, but not genotype x genotype interactions. Notably, I detected these significant genetic effects *after* accounting for the clonal sporulation of the two strains. These results suggest that the linearity results, in part, from additional, non-quantified dominance traits.

A major focus in this chapter was on the percentage of the two strains in the spores—the reason for this focus is clear: it reflects *relative* fitness differences of two strains. These differences are paramount for the evolutionary dynamics of allele frequency changes. However, I also considered the *absolute* number of spores produced by each strain. In prior work, they showed that a near-universal response to nonself strains is a facultative increase in the differentiation to spore cells (Kuzdzal-Fick

et al., 2023). These findings suggested that strains sense nonself and respond selfishly, increasing their investment in the spores relative to the dead stalk. I again observed this response in this study.

The quantitative genetic analysis lends further support to this finding, as I detect not just IGEs, DGEs, but also G x G interactions when we consider the *total* spores produced by each strain. However, G x G was not a significant term in the model of a strain's percentage of the spores (i.e., compare Table 6 to Table 7). Taken together, this suggests that the shift towards selfishness that occurs in chimerae (i.e., a shift from stalk to spore allocation) is probably occurring to a similar extent for different strains. This would increase the total spores but leave the percentage of the two strains largely unchanged. It thus suggests a selfish response to nonself—but one that is fairly generic.

In summary, this study shows that linearity is indeed a common attribute of social amoeba hierarchies, as determined from four populations at large geographic distances from one another. However, the social amoeba social hierarchies tend to be shallow, indicating low levels of inequity. Nevertheless, there is significant inequity present in natural populations, given that the experimental mixes differed from the clonal controls. I show that prior attributes can predict linearity of the dominance hierarchy. However, I also find substantial contributions of DGEs and IGEs, where the traits were not specifically quantified in advance or predicted. This study adds to a large body of work that indicates social dominance hierarchies are frequently linear. However, the shallowness indicates a relatively fair distribution of the spores among cooperating strains. Whether this substantial cooperation reflects that cheating has not arisen or that it is successfully suppressed is a topic for future study.

Supplementary information

Table S4.1. List of strains with their sampling site and GPS coordinates.

Strain ID	Origin	Site ID	GPS coordinates
EO600, EO602, EO603, EO604, EO605, EO611, EO615	Great Smoky Mountains, NC	GSNP	35°60'852, -83°44'736
EO620, EO622, EO626, EO633, EO634, EO638	Smith College MacLeish Field Station, MA	SMFS	42°44'858, -72°68'129
EO676, EO677, EO679, EO681, EO684, EO686, EO690	Aton Forest Connecticut, CT	AFCT	42°03'011, -73°13'441
EO1273, EO1274, EO1275, EO1277, EO1279, EO1282, EO1283	Mountain Lake Biological Station, VA	MLBS	37°37'293, -80°51'875

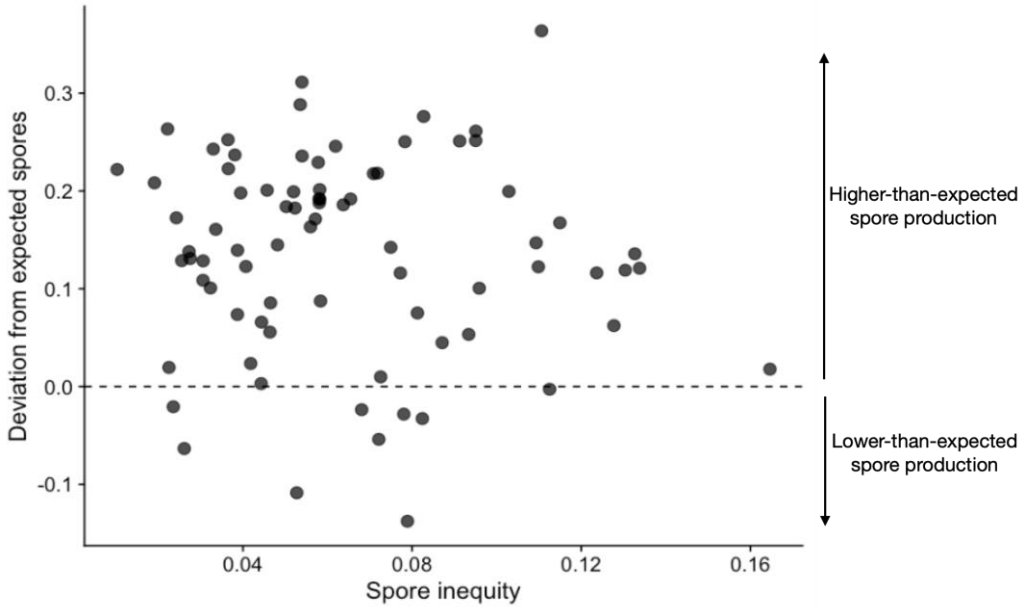


Figure S4.1. The relationship between the spore inequity and the deviation from the expected total spore production in a mix. Positive and negative values of the deviation from expected spores indicate that a mix produced more or fewer spores than expected based on the average spore production of the strains when developed clonally. 69 out of 78 points lie above the dashed line, indicating that spore production was frequently higher in chimera compared to clonal groups. This increase in total spore production showed however no relationship with the spore inequity observed in a mix, meaning that often both strains increased their spore production to equal numbers.

Chapter 5.

Natural variation in fruiting body morphology in the amoeba *Dictyostelium discoideum.*

Abstract

Reproductive altruism, where some individuals reproduce and others do not, is considered one of the pinnacles of cooperative societies. However, the exact level of reproductive altruism will depend on inclusive fitness considerations, including the relatedness of reproducing and non-reproducing individuals, as well as the benefits and costs accruing to each, respectively. In the social amoeba *Dictyostelium discoideum*, thousands of cells aggregate to form a multicellular fruiting body in which some cells die forming a rigid stalk that supports the rest of the cells, which become viable spores. The level of stalk investment by the social group can therefore be considered a metric of altruism investment. Importantly, as genetically unrelated cells can co-aggregate to end up in a single 'chimeric' fruiting body, theory predicts that selection will promote genotypes to cheat by preferentially forming spores and avoiding stalk investment. Because of the extreme differences in fitness consequences of stalk cells versus spores, the level of altruism investment is therefore likely under strong social selection. Here I examined fruiting body morphology in four natural populations to assess the extent to which stalk formation was variable within populations and maintained to different extents among populations. I found variation in stalk investment as well as the size of fruiting bodies, both at a cm-scale and between geographically isolated populations. These findings indicate the widespread potential for cheating, as well as the divergent evolution of altruism investment.

Introduction

In many social groups, individuals specialise in different tasks, called division of labour. In the case of reproductive division of labour, a fraction of individuals reproduce and others aid in their reproduction (Wilson, 1975; Harvell, 1994). Reproductive division of labour is commonly observed in social Hymenoptera (ants, wasps, bees, and termites), where the queen reproduces, and workers do not. Instead, these individuals rear the queen's offspring, defend the nest, or forage for food (Wilson, 1975; Robinson, 1992; Smith *et al.*, 2008). The specialisation on different tasks is thought to enhance colony efficiency and therefore, to have allowed more complex social groups to evolve (Oster and Wilson, 1978; Tsuji, 1994; Reeve and Keller, 1995; Nonacs and Hager, 2011; Van Gestel, Vlamakis and Kolter, 2015; Cooper and West, 2018).

Although less extreme, some measure of reproductive skew is also observed in other societies. Birds, fish, rodents, and primates (including humans) exhibit a continuum of levels of altruism investment, varying from equitable reproduction among individuals in the group (i.e., low reproductive skew) to reproduction being limited to only a few individuals (Sherman *et al.*, 1995; Nonacs and Hager, 2011). Finally, most multicellular organisms show reproductive skew since germline cells specialise in

reproduction, and somatic cells carry out structural and functional tasks but do not themselves reproduce (Michod and Roze, 2001).

Reproductive division of labour in clonal organisms can be easily explained by kin selection, where the fitness interests are aligned through very high relatedness (i.e., clonality) among reproductive and non-reproductive cells (Hamilton, 1964a; Cooper and West, 2018). Here, the somatic cell's direct fitness costs of cooperation are mitigated by inclusive fitness benefits accrued through the reproduction of clone mates. In non-clonal societies, however, reproductive division of labour can be undermined by selfish cheaters who contribute less to non-reproductive tasks and exploit cooperation by other cells (Trivers, 1971; Bull and Rice, 1991; West, Pen and Griffin, 2002; Sachs *et al.*, 2004). Evolutionary theory therefore suggests that the level of reproductive division of labour achieved will be impacted by many factors, including relatedness, the costs and benefits associated with the behaviour, and extrinsic factors (Hamilton, 1964a; West and Cooper, 2016).

Dictyostelium discoideum is a soil amoeba that exhibits reproductive division of labour during its multicellular life stage. Upon starvation, thousands of individual cells aggregate to form a migratory slug and eventually a fruiting body. The fruiting body consists of approximately 80% reproductive spores and 20% non-reproductive stalk cells (Bonner and Slifkin, 1949; Bonner, 1982; Kessin, 2001). Because the cells undergo self-sacrifice to form the stalk, this act is considered altruistic (Buss, 1982; Armstrong, 1984; DeAngelo, Kish and Kolmes, 1990). It is thought that stalk cells provide an advantage to spore cells by lifting them above the soil, promoting their dispersal to better environments (J. Smith, Queller and Strassmann, 2014). The observed spore-to-stalk ratio might thus represent a balance between the inclusive fitness benefits of stalk formation for dispersal and the direct fitness benefits of becoming a spore (Kaushik and Nanjundiah, 2003). Also taking into account the ecological circumstances and biophysical limitations of a fruiting body unit, there may be an optimal ratio of cells performing both tasks where group fitness is maximized (DeAngelo, Kish and Kolmes, 1990; Matsuda and Harada, 1990).

Importantly, the aggregative nature of fruiting body formation in *D. discoideum* allows genetically unrelated cells to end up in a single, 'chimeric' fruiting body (Buss, 1982). Under these conditions, conflict may arise over which cells become viable spores and which cells self-sacrifice to become dead stalk cells (Strassmann and Queller, 2011). Specifically, theory predicts that selection should promote cheaters: genotypes that preferentially form spores and avoid costly stalk fate. One possibility is that the selective pressure of the potential for cheating may promote lower levels of altruism investment (Hamilton, 1964a). Whether this does happen may depend on the mechanisms by which strains cheat, the frequency of chimerism, and the costs of stalklessness. Specifically, if cheating results from fixed

differences among strains in their clonal spore-to-stalk ratio, called fixed cheating, this may drive reduced stalk height (Matapurkar and Watve, 1997; Hudson *et al.*, 2002; Kaushik and Nanjundiah, 2003; Brännström and Dieckmann, 2005). Another possibility is that cheaters will only increase to a certain critical value, after which the altruistic strain will recover and eventually coexist with the cheater in a stably oscillating population, i.e., negative frequency-dependent dynamics (Matapurkar and Watve, 1997; Brännström and Dieckmann, 2005). Alternatively, if cheating results from a facultative shift to spore production, called facultative cheating, cheaters could become fixated in the population; under the assumption that cheaters are equally fit as cooperators, which has not yet been refuted (Ross-Gillespie *et al.*, 2007).

To date, few studies have looked for evidence for these theoretical predictions in nature (Buttery *et al.*, 2009; Votaw and Ostrowski, 2017). This may in part result from the difficulty of directly quantifying stalk allocation, but instead, having to rely on indirect measurements of either stalk height or stalk volume. Buttery *et al.* (2009) estimated the stalk volume based on images of upright fruiting bodies in which the stalk's length and the stalk's width at its base, midpoint, and just below the sorus were measured to calculate the stalk volume using the formula of a sphere. They did not directly report stalk height but did report significant variation in the sorus-to-stalk volume ratio among seven strains sampled from a site in North Carolina, suggesting possible polymorphism in altruism investment.

Votaw and Ostrowski (2017) estimated stalk investment in populations from Texas and North Carolina in two ways. The first way involved measuring the length of the stalk in images of fruiting bodies on their sides. For a subset of strains, they also compared the lengths of the prespore versus prestalk regions of the slug using GFP-reporters that express GFP under the control of the promoter of a prespore gene, *cotB*. Using these methods, they found that strains from Texas produced larger fruiting bodies (i.e., taller stalks that hold aloft more spores) compared to strains from North Carolina, suggesting larger aggregate sizes in Texas strains. After correcting for differences in size, there was also variation in the spore-to-stalk ratio, suggesting that strains have additionally diverged in their level of stalk investment. The finding of significant variation in spore-stalk ratios among co-occurring strains is important, as it means there is an opportunity for fixed allocation cheating where strains with lesser stalk allocations could benefit from the greater stalk formation of partner strains.

In this study, I examined fruiting body morphology across a greater range of strains and sites. Moreover, in contrast to earlier studies, I examined the spore-stalk allocation of strains from the same vs different soil samples. I determined whether co-occurring strains varied in their clonal stalk investment, and I investigated whether fruiting body height and spore-to-stalk investment evolved divergently across geographically distant sites.

These analyses address several questions. First, is fixed allocation cheating likely to be common within populations? The answer to this question requires knowing whether spore-stalk allocation variation exists at the cm-scale in the soil. Moreover, the repeated observation of spore-stalk allocation polymorphism across populations provides evidence that negative frequency-dependent selection is allowing stalk-favouring and stalk-avoiding strains to co-exist. Second, how constrained is fruiting body morphology? How extreme can the relative sizes of the sorus and stalk be? While the ecological causes of any morphological divergence are not investigated, analyses of allometric scaling in fruiting body dimensions can aid in identifying physical constraints on fruiting body size or proportioning that influence the evolution of altruism investment. Third, the ability to examine intraspecific variation in altruism investment (i.e., the proportion of cells that form stalk) allows me to test whether these traits evolve in accordance with evolutionary theory; specifically, to test the prediction that higher altruism investment should be observed in populations where relatedness is high or cheating intensity is low (Cooper and West, 2018; Madgwick *et al.*, 2018).

Materials and Methods

Strain selection

The strains used in this study are a subset of strains used in Chapter 3. The strains were collected between 2016 and 2019 by staff and students of the Ostrowski laboratory. The collection of soil and the isolation of strains are described in Kuzdzal-Fick *et al.* (2023). Information regarding the handling of soil samples and strains during transport between the US and NZ is discussed in Chapter 3. The strains and sites are listed in Table S5.1 and shown in Figure 5.1.

Strain cultivation and measurements

At the start of each block, I inoculated the spores from freezer stocks onto lawns of *Klebsiella pneumoniae* on SM-agar plates (SM broth, Formedium Ltd., 2% agar). After fruiting bodies had formed, I collected the spores and replated 5.0×10^5 spores with *K. pneumoniae* on fresh SM-agar plates. I harvested the cells during mid-exponential phase and washed the cells three times in cold KK2 buffer (14.0 mM K_2HPO_4 and 3.4 mM KH_2PO_4, pH 6.4) via differential centrifugation at 450 x *g* for 3 min to remove the bacteria. I then resuspended the cells at a density of 1×10^8 cells/ml in cold KK2.

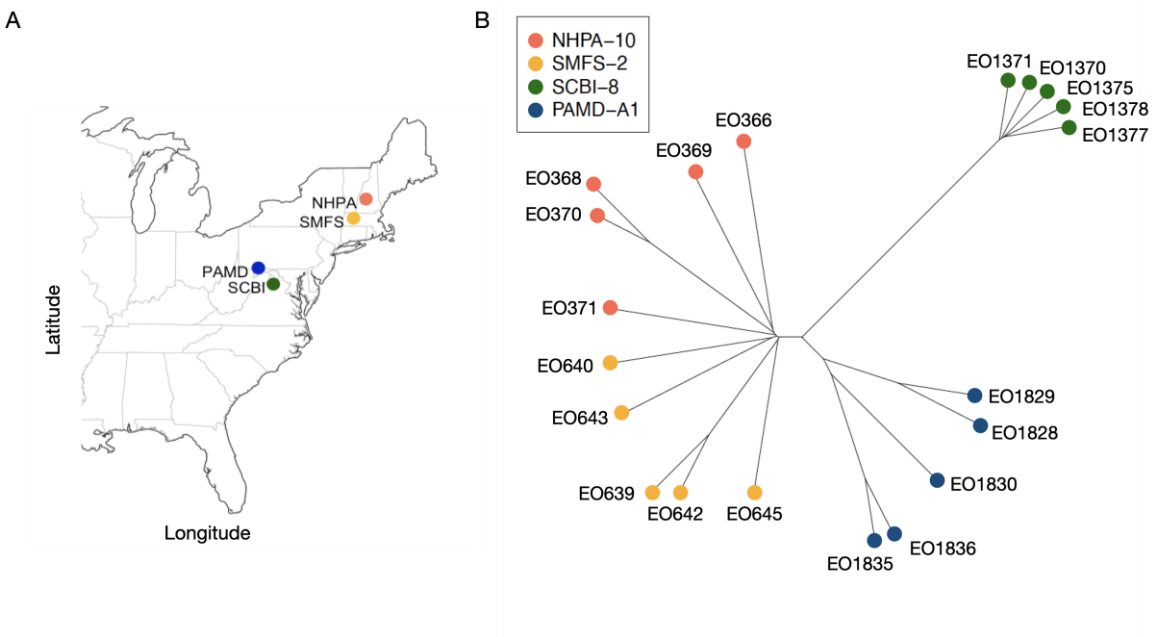


Figure 5.1. (A) Sampling locations of the four sites used in this study. (B) A distance-based tree showing the genetic similarity based on genome-wide SNPs among the twenty strains.

For the development of the strains, I deposited a 50 µL aliquot of the culture in a 3-by-3 square (~1cm^2) of a gridded 0.45 µM nitrocellulose filter, resulting in a total of 5.0x10^6 cells. I transferred this filter to a 6-cm Petri dish that contained a Pall filter moistened with 1.5 mL PDF buffer (per litre: 1.5 g KCl, 1.07 g MgCl·6H_2O, 1.8 g KH_2PO_4, 1.6 g K_2HPO_4, 0.5 g streptomycin sulphate). I transferred the Petri dishes to a plastic box lined with wet paper towels on the bottom and stored the box for 48 hours at 22°C in the dark. Following development, I determined the morphology of the individual fruiting bodies according to methods described in Votaw and Ostrowski (2017). Briefly, I used forceps to collect ten randomly chosen fruiting bodies. I placed each fruiting body in a separate well of a 96-well plate that contained 100 µL spore detergent (0.1 % IGEPAL in KK2 buffer with 10 mM EDTA) and imaged the wells at 50x magnification. From the image, I measured the length of the stalk in ImageJ. Since most stalks were bent, I used the "segmented line" function to measure the length of the stalk from the bottom of the spore head to the top of the basal disk. After imaging, I used a pipette to disperse the spores and lyse any remaining cells. I used an automated cell counter (Cell Countess II, Thermo Fisher) to calculate the number of spores and the average spore size in each fruiting body. In total, I measured the stalk length, the number of spores produced, and the average spore size in 600 fruiting bodies (4 sites x 5 strains x 3 blocks x 10 fruiting bodies).

Statistical analyses

I performed all analyses in R (version 4.2.1). I excluded strain EO640 from the analyses as it showed aberrant fruiting body formation where most aggregates did not progress beyond the slug stage within 48 hours after spotting them on the filter.

Variation in the individual traits

To test for variation in traits, I performed three generalized linear mixed models with dependent variables the stalk height, spore number and average spore size. I included site as a fixed effect and assessed its significance through a type II Wald chi-square test. I included strain id and block as random effects and assessed their significance through single deletions of terms, comparing the reduced models with the full model using a likelihood test that follows a chi-square distribution. I used a Gaussian error structure for the models examining stalk height and spore number, and a Gamma error structure for the model examining spore size.

Relationship between spore number and stalk height

I analysed the relationship between stalk height and spore number using the 'sma' function from the 'smatr' package (Warton *et al.*, 2012). This regression method allows one to examine a scaling relationship between two variables where neither of them is predicting the other. I tested for variation in the slope using the formula *spore number ~ stalk height * group*. I tested for variation in intercept using the formula *spore number ~ stalk height + group* which assumes common slopes. I tested for variation in the shift along the common axis using the formula *spore number ~ stalk height + group, type = "shift"*. In each analysis, *group* was the site and strain id when I tested for variation among sites and among strains within a site respectively. I performed a Sidak correction for multiple comparisons and I set the parameter 'robust=TRUE' to fit the line using Huber's M estimation to downweigh outliers (Warton *et al.*, 2012). I assessed the significance of the slope and intercept using the likelihood ratio statistic from the 'summary' function. I assessed the significance of the position along the common axis using the Wald statistics from the 'summary' function.

Principal component analysis

I carried out a principal components (PC) analysis using the 'prcomp' function from the 'stats' package with centring and scaling.

Relationship between cheating and stalk investment

To test the prediction that higher altruism investment is found in populations where relatedness is high or cheating intensity is low, I examined the relationship between the average spore-to-stalk ratio (quantified in this study) and the average genetic distance or magnitude of cheating found within a site (quantified in Broersma and Ostrowski, in prep.) respectively. Specifically, in a prior study, we quantified spore inequity, as a metric of cheating, in a subset of strain pairs among the set of strains used in this study (*N*=6-7 strain pairs tested per site) (Broersma and Ostrowski, in prep.). I measured the magnitude of spore inequity as the deviation from the ratio in the spores compared to that in the cells before development—which was 0.5 because of equal mixing of strains. For example, a spore inequity value of 0.1 indicates that the ratio of strains shifted from 50-50 in the cells to 60-40 in the spores. I tested the significance of the relationship between spore inequity magnitude and spore-to-stalk ratio within sites using a one-tailed Pearson's correlation test. I used the same test to examine the relationship between the average genetic distance and the spore-to-stalk ratio within sites.

Results

Average fruiting body dimensions

I measured three traits of fruiting body morphology: stalk height, number of spores, and average spore size. Across all strains, the average fruiting body had a stalk height of 3.39±0.07 mm, contained a total of $3.69 \times 10^4 \pm 2.4 \times 10^3$ spores, and the spores had an average size of 1.60±0.03 mm (grand mean across *N*=19 strains). On each filter, I deposited 5×10^6 cells and collected an average of 4.27×10^6 spores following development, which indicates that ~85% of cells became spores.

Between- and within-population variation in fruiting body morphology

There was significant variation in stalk height and spore size, but not spore number, among sites (Figure 2; Table S2). In addition to differences across sites, there was significant variation among strains within sites for all three traits (Figure 2; Table S2). Together, these observations demonstrate both polymorphisms within sites and divergence across sites in fruiting body morphology.

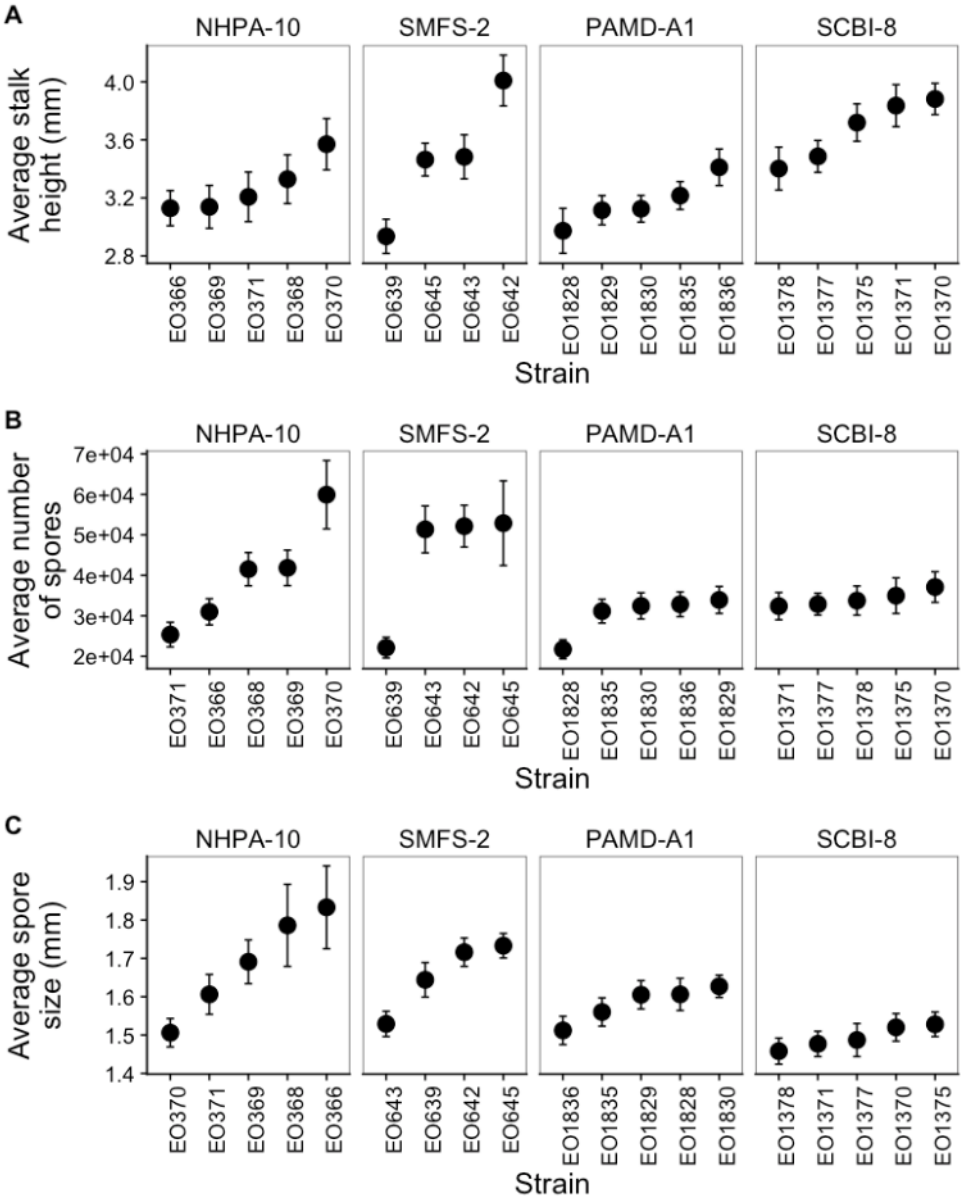


Figure 5.2. Fruiting body traits (A) stalk height, (B) spore number, and (C) spore size for four to five strains from four sites. Each point represents the grand mean for a single strain based on 30 fruiting bodies (=10 fruiting bodies per block x 3 blocks). Error bars represent one standard error of the mean (based on *N*=3 blocks).

Analysis of the scaling relationship between spore number and stalk height

I examined the biological scaling relationship between spore number and stalk height to get an estimate of the level of stalk investment and test if this estimate varies among and within sites. This method allowed me to disentangle absolute changes in size from proportional changes that can reflect differences in spore-to-stalk investment (Warton et al. 2012). For example, one strain might produce fruiting bodies that contain more spores and taller stalks than another strain; this strain might simply produce larger fruiting bodies, without a change in the relative allocation of the spores versus the stalk. This distinction is relevant because I am interested in whether strains vary in their altruism

investment, that is, the fraction of cells allocated to reproductive versus non-reproductive cell fate. Figure 3A illustrates potential differences in the relationship between spore number and stalk height in four hypothetical strains.

Between- and within-population variation in size and stalk investment

Apart from strains EO642 and EO1377, all strains showed a significantly positive relationship between spore number and stalk height (Figure 3B and Table S3). Moreover, I found significant variation in the degree to which stalk investment changes with size (i.e., differences in slope), stalk investment (i.e., differences in intercept), and group size (i.e., differences in the position along the common axis) both within and across sites (Table S4). Specifically, NHPA-10 and PAMD-A1 showed significant variation in spore-stalk allocation among strains. Strains from SMFS-2 showed variation in size but not spore-stalk allocation. Notably, strains from SCBI-8 showed no significant variation in any of the three traits, meaning they were morphologically similar (Table S4).

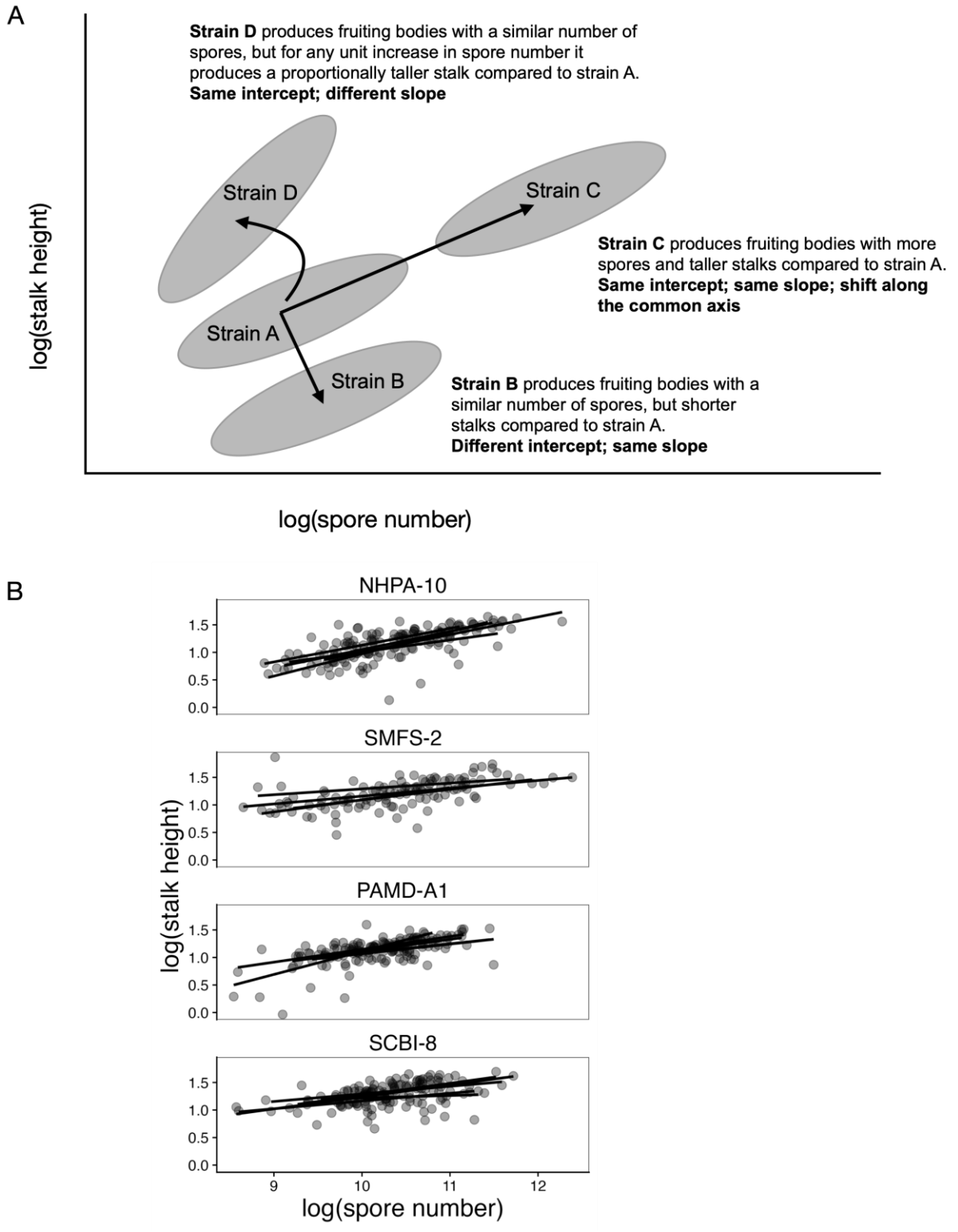


Figure 5.3. (A) The relationship between spore number and stalk height in four hypothetical strains demonstrates how to distinguish between differences in stalk (altruism) investment and fruiting body size. (B) The observed relationship between spore number and stalk height in strains from four sites. Each point indicates a single fruiting body. Lines indicate the best fit (based on *N*=30; 10 fruiting bodies x 3 blocks).

Principal components analysis

To quantify the relative contributions of stalk investment versus fruiting body size to variation in fruiting body morphology, I performed a principal component analysis (PCA). PCs are orthogonal, allowing for examination of different morphological attributes independently. Given the strong positive relationship between spore number and stalk height (Fig. 5.3B), I predicted PC1 would capture size variation, allowing me to then examine subsequent PCs for variation in stalk investment.

The factor loadings of the PCA are presented in Table 5.1. The analysis showed that PC1 was influenced most strongly by stalk height and spore number, both in a positive direction, and explained 56.0% of the variance. As expected, this suggests that PC1 predominantly captured the variation in size. Interestingly, spore size was negatively related to spore number and stalk height (albeit more weakly), suggesting that spore size might trade off with spore number. PC2 was influenced most strongly by spore size and explained 31.0% of the variance. PC3 was influenced most strongly by stalk height and spore number, but in opposite directions, and explained 13.0% of the variance. Thus, PC1 primarily captured the variation in size, PC2 primarily captured the variation in average spore size, and PC3 captured the variation in spore number versus stalk size *after controlling for size.* Therefore, high values of PC1 represent fruiting bodies of greater size (i.e., more spores *and* taller stalks), whereas high values of PC3 represent fruiting bodies with reduced stalk investment (i.e., more spores but shorter stalks). In short, variation in fruiting body morphology among strains resulted both from variation in size and stalk investment.

Table 5.1. Factor loadings of the principal component analysis.

Trait	PC1	PC2	PC3
Spore number	0.647	-0.359	0.672
Stalk height	0.683	-0.118	-0.721
Average spore size	-0.338	-0.926	-0.169
% Variance	56.0	31.0	13.0

Is greater cheating associated with decreases in stalk investment?

Populations are likely to vary in their cheating load, reflecting their different ecological and genetic circumstances. For example, populations where relatedness is high or low should permit cheating to different extents (low and high, respectively). Where cheating is pervasive, clonal stalk investment may be lower, reflecting a different evolutionary balance between the benefits of stalk production and the costs of cheating (Hudson *et al.*, 2002). Put another way, I expect that populations with high relatedness and/or low intensity of cheating might maintain higher stalk investment. To see whether this is true, I examined the relationship between average relatedness, cheating intensity, and altruism investment. For cheating intensity, I used the estimates of mean spore inequity from Chapter 3, and for altruism investment, I used PC3 (as spore-stalk ratio) from this chapter. The relationship between spore inequity and the spore-to-stalk ratio was not significant (Fig. 5.4A; one-tailed Pearson's correlation r=-0.59, df=2, P=0.79), but the correlation was moderate in magnitude and negative, as predicted. In addition, the relationship between genetic distance and the spore-to-stalk ratio was not significant (Fig. 5.4B; one-tailed Pearson's correlation r=-0.87, df=2, P=0.07), but, again, the correlation was strong in magnitude and negative, as predicted.

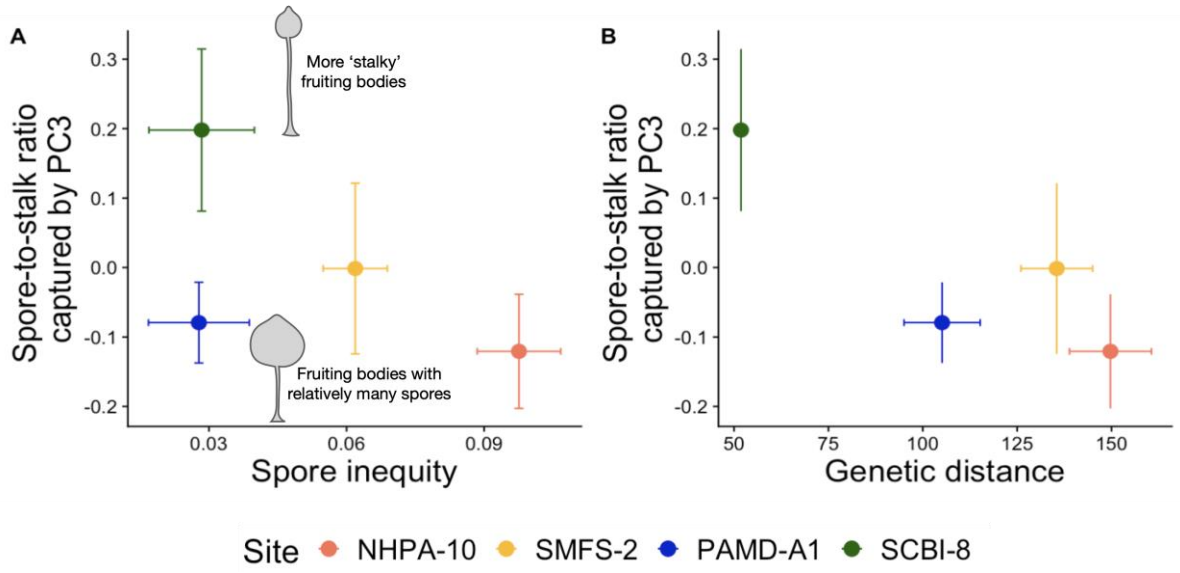


Figure 5.4. (A) Relationship between mean spore inequity and clonal spore-to-stalk ratio for four populations. Spore inequity is the degree to which two strains deviate from an equal percentage of the spores in a pairwise mix (see Chapter 3) and is averaged across all mixes from Chapter 3. Principal component 3 (PC3) from this chapter is used as a proxy for greater spore investment (see text). High values of PC3 indicate fruiting bodies with relatively tall stalks after controlling for overall size. Error bars represent one standard error of the mean. (**B) The relationship between genetic distance and spore-to-stalk ratio for four populations.**

Discussion

Species that live in social groups and exhibit reproductive division of labour show a continuum in the level of altruism investment, i.e., the fraction of individuals that forgo reproduction themselves to promote the reproduction of others in the group. Evolutionary theory predicts that the level of altruism investment is influenced by many factors, including the relatedness between the non-reproductive and reproductive individuals, as well as the costs and benefits associated with either subgroup. Here I measured spore-to-stalk allocation as a proxy for altruism investment in four populations of the social amoeba *Dictyostelium discoideum*. I found variation in spore-to-stalk allocation among strains from geographically different populations as well as among strains isolated from a 10-by-10 cm plot of soil. These results suggest both population divergence in the level of altruism investment and polymorphism in traits important in social interactions.

There was a general positive correlation between stalk height and spore number, meaning that variation in stalk height was in part attributable to variation in multicellular size. One possible explanation for increased fruiting body size may result from the need to produce taller stalks in some environments, as discussed by Votaw and Ostrowski (2017). Here, the production of larger fruiting bodies accomplishes lifting spores further off the ground without requiring a greater per capita investment in stalk production. Put differently, selection for taller stalks may indirectly select for larger size. Further work may benefit from examining fruiting body size across environments in which taller or shorter stalks could be favoured, for example, in more or less humid environments, or environments of high or low relatedness (Bonner and Shaw, 1957; Armstrong, 1984).

The variation in stalk investment among strains within two populations is in line with the result from the two prior studies that examined the same set of traits (Buttery *et al.*, 2009; Votaw and Ostrowski, 2017). Collectively, these findings suggest that stalk-avoiding and stalk-favouring strains may coexist within a population. This possibility is consistent with two other lines of evidence. First, theoretical studies showed that under certain conditions cheaters and cooperators may coexist in a stably oscillating population (Matapurkar and Watve, 1997; Brännström and Dieckmann, 2005). Second, genomic analysis found that genes that mediate cheating behaviours showed genetic signatures consistent with negative frequency-dependent selection where multiple alleles are maintained in the population (Ostrowski et al. 2015).

Fixed allocation differences among strains were shown to greatly influence the outcome of social interactions in chimera in terms of spore representation (Buttery et al. 2009; but also see Chapter 4). However, so far, only a single study examined if this variation exists at the centimetre scale at which

strains are likely to interact in the soil (Votaw and Ostrowski, 2017). Hence, this study significantly expands the number of populations in which this variation is examined, and together these studies demonstrate that there is substantial variation on which selection could act.

Importantly, the above discussion also highlights that these dynamics depend on the frequency of chimeric fruiting body formation in nature and the relationship between stalk height and fitness (dispersal success). Unfortunately, both factors are not known with certainty. However, genotyping of fruiting bodies from small soil samples (0.2 g) showed that 63% of fruiting bodies consisted of more than a single genotype (Fortunato et al. 2003a), suggesting that chimerism may be common in nature. Concerning the importance of stalk height to dispersal success, it was found that spores from intact fruiting bodies are better dispersed by flies compared to spores from knocked-over ('stalkless') fruiting bodies (J. Smith, Queller and Strassmann, 2014). However, no studies have yet tested the effect of varying stalk height on dispersal success. Though this will likely be challenging to test, future work would benefit from better estimates of the costs associated with reduced stalk investment, which are both observed in some clonal groups (as shown in this study) and frequently in chimeric groups (Votaw and Ostrowski, 2017; Kuzdzal-Fick *et al.*, 2023).

Assessing the extent to which social traits vary across and within natural populations is important for understanding how these traits may evolve in nature. In this study, I have not addressed what evolutionary forces may drive and maintain intraspecific diversity. Nonetheless, the results of this study revealed that less and more cooperative phenotypes may coexist at a cm-scale in nature and that geographically distant populations have diverged in their level of altruism investment.

Supplementary information

Table S5.1. List of strains and their sampling sites and GPS coordinates.

Strain ID	Origin	Location ID	GPS Coordinates
EO366, EO368, EO369, EO370, EO371	New Hampshire Proctor Academy, NH	NHPA-10	43°45'207, -71°82'578
EO639, EO640, EO642, EO643, EO645	Smith College MacLeish Field Station, MA	SMFS-2	42°44'914, -72°68'204
EO1370, EO1371, EO1375, EO1377, EO1378	Smithsonian Conservation Biology Institute, VA	SCBI-8	38°89'344, -78°14'647
EO1828, EO1829, EO1830, EO1835, EO1836	Pennsylvania Mount Davis, PA	PAMD-A1	39°78'546, -79°17'421

Table S5.2. Summary of the results of the mixed models that examined variation in fruiting body morphology across and within sites.

Effects	Stalk height			Spore number			Spore size		
Fixed effects	*df*	χ^2	$P(\chi^2)$	*df*	χ^2	$P(\chi^2)$	*df*	χ^2	$P(\chi^2)$
Site ID	3	13.126	0.004	3	2.678	0.44	3	18.827	<0.001
Random effects	*df*	χ^2	$P(\chi^2)$	*df*	χ^2	$P(\chi^2)$	*df*	χ^2	$P(\chi^2)$
Strain ID	1	23.095	<0.001	1	33.8	<0.001	1	9.546	0.002
Block	1	113.71	<0.001	1	6.523	0.011	1	48.88	<0.001

Table S5.3. The slope, intercept, and correlation coefficient (*r*) of the relationship between spore number and stalk height.

Strain ID	Site	Slope	Intercept	*r*	*P*
EO1370	SCBI-8	0.294	-2.503	0.49	0.006
EO1371	SCBI-8	0.436	-2.483	0.51	0.004
EO1375	SCBI-8	0.354	-2.513	0.74	<0.001
EO1377	SCBI-8	0.395	-2.592	0.10	0.60
EO1378	SCBI-8	0.403	-2.602	0.39	0.035
EO1828	PAMD-A1	0.329	-2.607	0.67	<0.001
EO1829	PAMD-A1	0.386	-2.703	0.49	0.006
EO1830	PAMD-A1	0.337	-2.687	0.46	0.013
EO1835	PAMD-A1	0.284	-2.632	0.72	<0.001
EO1836	PAMD-A1	0.382	-2.597	0.67	<0.001
EO366	NHPA-10	0.430	-2.668	0.79	<0.001
EO368	NHPA-10	0.494	-2.721	0.82	<0.001
EO369	NHPA-10	0.418	-2.774	0.49	0.006
EO370	NHPA-10	0.385	-2.743	0.85	<0.001
EO371	NHPA-10	0.456	-2.576	0.56	0.001
EO639	SMFS-2	0.314	-2.588	0.61	<0.001
EO642	SMFS-2	0.403	-2.597	0.24	0.10
EO643	SMFS-2	0.403	-2.738	0.47	0.009
EO645	SMFS-2	0.200	-2.658	0.70	<0.001

Table S5.4. A summary of the models that tested if sites (top table) and strains (bottom table) within a site showed significant variation in the slope, intercept, and position along the common axis, of the relationship between spore number and stalk height.

Term	*df*	χ^2 or *F*	*P*(χ^2 or *F*)
Slope	3	9.593	0.022
Intercept	3	55.95	<0.001
Position along the common axis	3	21.36	<0.001

Term	Site	*df*	χ^2 or *F*	*P*(χ^2 or *F*)
Slope	NHPA-10	4	3.29	0.51
(=the degree to which stalk	SMFS-2	3	14.00	0.003
investment changes with size)	PAMD-A1	4	4.10	0.40
	SCBI-8	4	3.65	0.46
Intercept	NHPA-10	4	25.14	<0.001
(=stalk investment)	SMFS-2	3	4.39	0.22
	PAMD-A1	4	14.48	0.006
	SCBI-8	4	8.07	0.09
Shift along the common axis	NHPA-10	4	8.28	0.08
(=size)	SMFS-2	3	26.69	<0.001
	PAMD-A1	4	5.07	0.28
	SCBI-8	4	4.71	0.32

Chapter 6.

Concluding remarks.

Evolutionary theory predicts that cooperative systems are vulnerable to selfish cheats, who gain the benefits of cooperation without paying the associated costs; even if this comes at a cost to others or the group (Hamilton, 1964a; Axelrod and Hamilton, 1981; Szathmáry and Smith, 1995; Frank, 2003; Bourke, 2011). Both theoretical and empirical studies have sought to understand the forces and mechanisms that allow cooperation to be maintained despite the potential for selfish behaviour (Hamilton, 1964b; Hurst, Atlan and Bengtsson, 1996; Queller, 1997; Frank, 2003; Nowak, 2006; El Mouden, West and Gardner, 2010; Bourke, 2011; Ågren, Davies and Foster, 2019). Collectively, these studies demonstrated that cheaters can be prevented through mechanisms that limit cooperation to relatives (kin selection) and mechanisms that repress competition, which both unite the interest of the group and eliminate within-group conflict.

While early studies primarily focussed on cooperation and cheating in animal societies, it became increasingly apparent that these behaviours extended to other biological levels of organisation, including at the level of the cells and the organisms, and across diverse taxa, including microbes (Hurst, Atlan and Bengtsson, 1996; Crespi, 2001; West *et al.*, 2007; Gardner and Úbeda, 2017). This led to questions about whether similar principles may explain the maintenance of cooperation in animal and non-animal societies.

Dictyostelium discoideum has become an established model system in social evolution studies because of its remarkable life cycle (Strassmann and Queller, 2011). When starved, genetically unrelated cells can co-aggregate to form a 'chimeric' fruiting body in which some cells altruistically die to form a supportive stalk and others become viable spores (Kessin, 2001). Consistent with evolutionary theory, chimeric fruiting body formation can select for cheaters: genotypes that reap the benefits of stalk formation by others but fail to contribute their fair share to its production (Strassmann, Zhu and Queller, 2000; Fortunato, Queller and Strassmann, 2003; Buttery *et al.*, 2009). While progress has been made in elucidating the causes and consequences of cheating and mechanisms that may limit cheating by using laboratory-generated mutants, few studies have examined these behaviours in natural strains.

In this thesis, I aimed to bridge the gap between the laboratory and nature. I focussed on three key questions: *(i)* Does cheating occur in nature, *(ii)* What adaptations exist in nature that limit or prevent cheating, and (*iii*) Have these adaptations led to evolutionary divergence in social behaviours within and across populations? Below I highlight the main findings of the individual chapters.

In **Chapter 2** I reviewed the literature on the evolution and function of stalked fruiting body formation in taxa that exhibit aggregative multicellularity. Stalked fruiting body formation has evolved independently in five of the six supergroups of the Eukarya. While the general structure, a stalk that holds aloft a spore-producing head, is similar across groups, there is substantial morphological diversity across and within the groups. This diversity in the formation and appearance of stalked fruiting bodies likely leads to variations in the associated costs, benefits, and functional constraints of these structures—though it remains mostly speculative as to what these might entail.

I then focussed on *Dictyostelium discoideum* and I discussed prior work that examined the possible evolutionary implications and stability of altruistic stalk formation in genetically mixed groups. These studies confirmed the importance of relatedness for the maintenance of stalk formation and demonstrated the possibility that social conflict over cell fate may influence phenotypic diversity in stalk and fruiting body morphology within and across natural populations.

Importantly, this review also highlighted some gaps in knowledge. While prior studies demonstrated proof-of-principle that strains and populations may diverge in fruiting body morphology, do we find this variation in nature, and if so, does it influence the outcome of the social interactions between strains? In addition, because of the few strains examined so far, little is known about possible functional constraints imposed by development or physics that likewise influence fruiting body and stalk formation in *D. discoideum*. I addressed some of these gaps in **Chapters 4** and **5**.

In **Chapter 3**, I focussed on two questions: *i.* how common is cheating in natural populations, and *ii.* does resistance to cheating arise in nature? The results showed that 20% of strains tested showed behaviour indicative of cheating against at least one other strain, and cheaters were found in half of the sites (populations). The results also showed that cheaters could increase their frequency in the spores by roughly 10% (from 50-50 in the cells to 60-40 in the spores)—a magnitude of spore inequity similar to that found in experiments with mutagenesis-generated cheaters. These findings suggest that cheaters may be moderately common in nature, cheaters are not found in all populations, and populations vary in the number of cheaters they harbour.

Using cross-inoculation experiments where strains are tested in the presence of local (same site) and foreign (different site) strains, I then tested two possible predictions that could support the possibility that resistance can evolve to suppress cheating: few cheaters and low levels of cheating in local strain pairs, and more cheaters and higher levels of cheating in foreign strain pairs. However, the results

showed no evidence for these predictions. Instead, limited evidence showed that where cheating existed, it was significantly stronger in local compared to foreign strain pairs. These findings may suggest that cheaters are selectively favoured, i.e., are ahead in the cheater-cooperator dynamics.

I also found that strains showed a strong response to development with non-self. This was the case both before fruiting body formation, where during aggregation strains in part avoided development with non-self (i.e., segregate), and during fruiting body formation, where strains increased their spore production (and likely reduced stalk allocation, but I did not quantify this trait). While both processes showed no relationship with spore inequity (cheating), they are in line with evolutionary theory that predicts that genetic heterogeneity should promote selfish behaviour.

The results of **Chapter 3** thus demonstrated that while the social interactions between natural strains are likely diverse and complex, they, however, infrequently result in cheating. These findings therefore suggest that various mechanisms exist that limit cheating in social groups of *D. discoideum*, but these mechanisms are imperfect.

In **Chapter 4**, I examined if, similar to animal societies, a group of strains of *D. discoideum* sampled from the same site (i.e., co-occurring) form a linear dominance hierarchy. And if so, whether this linearity resulted from differences between strains in their inherent ability to dominate, quantified as their clonal spore production. Of the four populations tested, two populations showed significant linearity and two populations did not show significant linearity, of which one was marginally nonlinear. All hierarchies were however flat, meaning that strains exhibit minimal fitness differences and may be competitively similar. The existence of nonlinear, or linear but flat, hierarchies may serve as processes that maintain the high genotypic and phenotypic diversity that is observed at a very small scale in nature.

In the two populations that showed significant linearity, there was little evidence that social dominance and consequently linearity resulted from pre-existing differences among strains in their clonal spore production. To examine more thoroughly what drives variation in a strain's fraction in the spores in chimera, I performed a quantitative genetics analysis that included both direct genetic effects (DGEs) of the focal strain, indirect genetic effects (IGEs) by the partner strain, and the interaction between the DGEs and IGEs (genotype-by-genotype (GxG) interactions). This type of analysis allows one to quantify the proportional contribution of the traits inherent to the individual itself, to its social partner, and the interactions between them, to the observed phenotype, without

the need to identify these traits. The analysis revealed that roughly half of the variation in social dominance was explained by direct genetic effects (53%). The other half of the variation was explained by indirect genetic effects (29%) and genotype-by-genotype interactions (17%), which together make up the social genetic environment. These findings suggest that while social dominance is foremostly dependent on the identity and traits of the individual itself, the social genetic environment additionally has a considerable influence.

Evolutionary theory predicts that the level of altruism investment in a social group is influenced by the relatedness between individuals and the costs and benefits associated with the behaviour. In **Chapter 5** I examined the fruiting body morphology in four natural populations of *D. discoideum* to investigate if strains and sites have diverged in their level of altruism investment, quantified as spore-to-stalk ratio. I found variation in spore-to-stalk allocation among strains isolated from a 10-by-10 cm plot of soil as well as among strains from geographically different populations, suggesting both within-site polymorphism and population divergence.

In addition to variation in stalk investment, I found variation in overall fruiting body size, i.e., group size. Specifically, greater stalk investment was often associated with greater spore production. This finding may suggest selection for taller stalks which could be accomplished through greater size without greater altruism investment. One notable exception here was the very stalky fruiting bodies produced by strains from site SCBI-8. This set of strains additionally may demonstrate that stalk height has a limit, where at certain stalk heights it cannot maintain the spore-to-stalk ratio found in smaller groups.

The findings of substantial diversity in phenotypic traits could suggest the existence of evolutionary processes that promote and/or maintain this variation. Additionally, existing within-site polymorphism in spore-to-stalk allocation may greatly impact the outcome of social interactions between these strains, as demonstrated in **Chapter 4**.

In summary, in this thesis, I examined the social interactions between natural strains of *D. discoideum* to test various aspects regarding the maintenance of cooperation in non-animal societies. In **Chapters 1** and **2** I discussed what is known about cooperative fruiting body formation in *D. discoideum* and highlighted the need to test various theoretical predictions in a more natural setting. In **Chapter 5** I demonstrated that there is substantial variation in social traits within natural populations upon which selection can act, as well as between populations, potentially leading to divergence in social

behaviours. In **Chapter 4** I demonstrated that behaviours dependent and independent of the social environment contribute to unequal fitness outcomes in social interactions. Lastly, in **Chapter 3** I demonstrated that cheaters and moderately common in nature, suggesting that mechanisms exist that limit this behaviour, however, these are imperfect.

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Statements of Contribution

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