Intragenomic epistasis negatively impacts within-species coevolutionary arms races

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Sean Lewis McFadyen

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Abstract

Cooperation is susceptible to exploitation by selfish individuals. These individuals, referred to as cheaters, gain the benefits of cooperation without paying the costs. Cheating can select for individuals who can resist or prevent cheating, and resistance can in turn select for individuals that overcome the resistance. Thus, cheating and resistance potentially coevolve and lead to an arms race of adaptations and counter-adaptations.

Within a species, the existence of such an arms race is complicated by sexual reproduction among individuals that harbor alleles encoding the different strategies. Sexual reproduction will generate recombinants that harbor both cheating and resistance alleles. The impact of this recombination should depend on the extent of epistasis: that is, how the combined effects of these mutations differ from their effects individually. The goal of this thesis is to address how sexual reproduction impacts a within-species coevolutionary arms race, in this case, between cheating and resistance.

The model organism *Dictyostelium discoideum* provides the perfect model to answer these questions. It has a unique life cycle, consisting of a unicellular (amoeba) stage, cooperative multicellular development (resulting in the formation of a fruiting body), and facultative sexual reproduction (resulting in the formation of a resting structure, termed a macrocyst). During the formation of the multicellular fruiting body, some cells are sacrificed to build a rigid stalk of dead cells that supports the rest. Cheaters exploit this altruistic sacrifice, and resistors prevent the cheaters from cheating. Resistance may in turn select for more efficient cheating, and this reciprocal selection can potentially spark a within-species arms race. During the sexual reproduction phase, recombination can then mix and match alleles encoding conflicting strategies into a single genetic background, allowing for epistatic effects to occur.

Here I use the model organism *Dictyostelium discoideum* to understand how coevolution of cheating and resistance is impacted by sexual reproduction. To do so, I carried out a long-term evolution experiment to select for cheating and resistance. To understand the impact of recombination, I evolved replicate populations with or without periodic recombination. To address experimental difficulties, I also carried out an experiment to

determine the optimal germination method for macrocysts produced during the *Dictyostelium* sexual cycle. Finally, I used computer simulations of the co-evolutionary process in *Dictyostelium discoideum* to explore how recombination and epistasis impact co-evolution, which allowed me to vary parameters of interest over a broader range of values than was possible in the laboratory. My findings suggest that recombination and resulting epistatic effects between conflicting strategies may prevent or slow within-species co-evolutionary arms races.

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Chapter 1: Introduction

1.1 Cooperation is widespread

Cooperation is observed across all levels of biological organization, from large, complex, multicellular organisms to single-celled microbes (Szathmáry & Smith, 1995). Multicellular organisms often form social groups that cooperate in various ways, including for predator detection (Santema & Clutton-Brock, 2013; Sorato et al., 2012) or pack-hunting (Jordan et al., 2023). In microbes, cooperation can take the form of the production of communal goods (Guerinot, 1994; Ratledge & Dover, 2000; West & Buckling, 2003), or the cooperative construction of multicellular biofilms (Nadell et al., 2009) and fruiting bodies (Kessin, 2001).

Although cooperation can bring many benefits to a group, it often entails costs for the individuals. An evolutionary problem can arise when the cost to the individual is greater than the benefits – how is such a trait able to be selected for? Hamilton tackled this issue by introducing the concept of 'inclusive fitness'. An individual's inclusive fitness is composed of two parts: direct and indirect fitness (Hamilton, 1964). Direct fitness is the fitness an individual achieves through its own reproduction, whereas indirect fitness is that achieved by the reproduction of one's relatives, discounted by their relatedness (Hamilton, 1964; West et al., 2007). Altruism, defined as an act that is costly to its bearer but benefits others, can thus evolve by natural selection when the cost to an individual's direct fitness is offset by the benefits for its indirect fitness – in other words, as long as there are overall inclusive benefits of the behavior (Hamilton, 1964; Kerr et al., 2004).

1.2 Cooperation is prone to exploitation

Hamilton's Rule helps to explain how altruistic behaviors can evolve, particularly when relatedness among the interacting individuals is high. However, when the relatedness among group members is not sufficiently high, cooperation can be vulnerable to selfish individuals. These individuals gain the benefits of cooperation without incurring the associated costs (Bourke, 2011). In social interactions, individuals that benefit from a public good without paying the costs of its production are referred to as "cheaters" (Ghoul

et al., 2014). Owing to the fitness advantage of avoiding the costs of cooperation, cheating should be strongly selected. In some cases, cheating can lead to the extinction of the cooperative group (Axelrod & Hamilton, 1981; Frank, 1994; Rainey & Rainey, 2003).

Cheating can manifest in bacterial communities during the production of communal goods (Bruce et al., 2017; Griffin et al., 2004; Jiricny et al., 2010). In *Pseudomonas fluorescens,* these communal goods are siderophores; iron-scavenging molecules able to be utilized by all local bacteria. Cheaters do not produce their fair share of the goods, avoiding the cost, while still consuming them and reaping the rewards of their production (Bruce et al., 2017; Griffin et al., 2004; Jiricny et al., 2010). Thus, these individuals are more fit than their producing counterparts and can become over-represented in subsequent generations.

In eusocial insects (bees, ants, and termites), cheating occurs when a lineage produces excess queens or workers lay their own eggs (Hamilton, 1972; Oldroyd & Ratnieks, 2000). Typically, workers in eusocial insect societies are sterile while only the queen retains the privilege of reproduction. A lineage that overproduces queens can overtake a population owing to the reproductive advantage of being a queen (Bourke & Ratnieks, 1999; Hamilton, 1972). Similarly, egg-laying workers are able to spread their genes through an avenue besides the queen (Oldroyd & Ratnieks, 2000; Ratnieks & Visscher, 1989; Ratnieks & Wenseleers, 2005).

In all of these examples, selfish individuals are able to exploit the cooperation of the group for their own benefit. When these cheaters flourish, their selfish genes proliferate thus endangering the future of cooperation (Axelrod & Hamilton, 1981; Frank, 1994; Rainey & Rainey, 2003).

1.3 How is cooperation maintained?

Despite the evolutionary pressures promoting the appearance of cheaters, cooperation remains widespread, and explaining this is an important challenge in evolutionary biology. Many multicellular organisms undergo a single-cell bottleneck at the start of each generation, which can reduce internal conflicts of interest by ensuring high

relatedness between somatic and germline cells (Fisher et al., 2013; Grosberg & Strathmann, 1998). As somatic and germline cells are clonal, somatic cells which don't directly pass on their genes are not under pressure to selfishly proliferate, as identical copies of their genes are being propagated through genetically identical germline cells. This system promotes cohesion, as every cell within a multicellular organism proliferates together.

However, single-celled organisms routinely cooperate with others of different genotypes and thus have strong incentives to cheat the group and act selfishly. When relatedness is low, the incentive to cheat is high as the inclusive fitness benefit is absent (Hamilton, 1964). Thus, cheating should be strongly selected for during cooperation with distantly related social partners.

Some mechanisms are thought to allow for the inhibition of cheaters in a cooperative scenario – cheaters may have inherently lower fitness (Ennis et al., 2000), the population may be highly related (Gilbert et al., 2007), or individuals may choose to only cooperate with others that are closely related, ensuring that beneficiaries pass on their genes (Ostrowski et al., 2008).

1.3.1 Kin selection

Population structure can increase relatedness, and thereby limit the interactions between cooperators and cheaters and thus minimize the effects of a cheaters exploitation while enabling any fitness-lowering effects of cheaters to be selected against (Gilbert et al., 2007). Similarly, the separation of cheaters and cooperators may not be due to simple passive population structure and instead due to active kin-discrimination whereby cooperators preferentially cooperate with related individuals instead (Ostrowski et al., 2008). For example, in *Dictyostelium discoideum* kin-discrimination is likely facilitated by the cell-cell adhesion protein encoding genes *tgrB1* and *tgrC1* (formerly known as *lagC1* and *lagB1*) (Benabentos et al., 2009; Hirose et al., 2011). These highly polymorphic genes encode matching transmembrane proteins that are required for social development and interact in a ligand-receptor pair - only a particular set of *tgrC1*

ligands are capable of binding to a given *tgrB1* receptor (Hirose et al., 2017). The high polymorphism in these genes leads to many variations of ligand and receptor allowing *Dictyostelium discoideum* to effectively discern genotypes and avoid cooperating with distantly related individuals.

Another example of kin discrimination can be seen in bacterial iron scavenging. Bacterial populations are frequently iron-limited, because most iron in the environment is insoluble (Guerinot, 1994; Ratledge & Dover, 2000; West & Buckling, 2003). During times of iron deficiency, bacteria secrete siderophores, which are released extracellularly and enable iron uptake and use (Guerinot, 1994; Ratledge & Dover, 2000).

Siderophores are considered a public good: their production is costly to the individual and the benefits of siderophores can be accrued by both producers and non-producers. Non-producers are thus considered cheaters, since they reap the benefits of cooperation without incurring its costs (Bruce et al., 2017; Griffin et al., 2004; Jiricny et al., 2010).

Some bacteria have developed methods to limit the impact of cheaters. For example, the bacterium *Pseudomonas fluorescens* can privatize its public good, making a siderophore (called pyoverdine) that can only be used by other producers. This mechanism essentially involves directing public goods to relatives, who share the gene for cooperation, in this case, pyoverdine production. An alternative approach is to direct harm to non-relatives. For example, the same bacterium can also produce bacteriocins which target siderophore receptors. These bacteriocins are toxins which kill non-producing cells, limiting the benefits of siderophore production to closely related individuals through an alternative avenue (Bruce et al., 2017).

These examples show how the active exclusion of distantly related individuals allows the benefits of cooperation and thus the increase in inclusive fitness to be shared only by those with the same genes, enabling an avenue for selection to act upon (Hamilton, 1964). In both active kin-discrimination and passive population structure scenarios, a higher relatedness among cooperators limits the extent of exploitation and prevents cheaters from driving cooperation to extinction.

1.3.2 Enforcement

1.3.3.1 Policing

Eusocial insects cooperate by both caring for their young in a group-fashion and by performing a division of labor within the colony (Hamilton, 1972). Most notably this division of labor extends to the restriction of reproduction, with one or a few queens reproducing and workers being sterile (Hamilton, 1972; Wilson, 1971). The vast majority of the colony thus do not reproduce themselves, but perform tasks necessary to ensure reproduction of the queen. Because the workers are the daughters of the queen, they gain an inclusive fitness benefit through raising the queen's offspring.

Despite eusocial insects showing extreme cooperation and altruism, there are opportunities for conflict, especially when females mate with multiple males, leading to offspring with higher or lower relatedness. In some cases, male lineages arise that 'cheat' by producing offspring that preferentially adopt the queen fate (and avoiding the sterile worker fate) (Bourke & Ratnieks, 1999; Hamilton, 1972; Wenseleers et al., 2003). In doing so, they increase their genetic representation in the next generation while benefiting from the altruism of the sterile workers (Bourke & Ratnieks, 1999; Hamilton, 1972). Excess queen production can come at a cost to the colony overall - queens require increased nutrition as they develop, and more individuals adopting the queen fate results in fewer workers with which to care for them (Bourke & Ratnieks, 1999).

Another form of selfishness occurs when female workers—which are typically sterile—develop ovaries and lay unfertilized eggs (Ratnieks & Visscher, 1989; Ratnieks & Wenseleers, 2005). Unfertilized eggs are haploid, as opposed to the diploid eggs laid by queens. These worker-laid eggs thus always result in male offspring as a consequence of the haplodiploid sex-determination system seen in eusocial insects. Similar to the excess production of queens, overproduction of workers can entail fitness penalties for the colony. It can disrupt the division of labor and hamper the colony's effectiveness (Ratnieks & Wenseleers, 2005).

In eusocial insects, cheating can be suppressed by 'policing', whereby workers cull immature queens during times of excess queen fate adoption (Dobata, 2012; Wenseleers

& Ratnieks, 2006; Wenseleers et al., 2003). Egg-laying workers are also subject to this violent enforcement of cooperation. For example, in the honeybee (*Apis mellifera)* cooperative workers will destroy worker-laid eggs and may attack the selfish worker themselves (Oldroyd & Ratnieks, 2000; Ratnieks & Visscher, 1989).

1.3.3.2 Counter-evolution of resistance

Slime molds are single-celled social amoebae capable of multicellular development (Raper, 1984). Multicellular development is induced by starvation and high cell densities. The cells aggregate in groups of ~10^5 cells to form mounds. The mounds give rise to slugs which then further develop into fruiting bodies. These fruiting bodies consist of a stalk of hardened, dead cells that holds aloft a sorus containing live spores. Every individual amoeba involved in the construction of these fruiting bodies may differentiate into either a stalk cell or a spore cell. Cells that adopt the stalk fate die and are thus unable to pass on their genetic information, while cells that adopt the spore fate benefit from their sacrifice through the construction of the fruiting body. The fruiting body structure enhances the spores ability to disperse via passing invertebrates, conferring a fitness benefit (Smith et al., 2014). The construction of these multicellular fruiting bodies is thus altruistic, in that the amoebae that become stalk cells and die give up their own fitness to help others in their population (Kerr et al., 2004).

Sacrifice on the part of the stalk cells can be explained by inclusive fitness theory if the stalk cells are benefitting relatives (Hamilton, 1964). However, aggregations can include cells of variable relatedness, and this low relatedness means that cells potentially gain an advantage by behaving selfishly (Strassmann et al., 2000). Specifically, amoebae can behave selfishly by preferentially adopting the spore fate, ensuring their (cheating) alleles are passed on and improving their own fitness at the cost of the group (Ennis et al., 2000; Khare & Shaulsky, 2010; Santorelli et al., 2008; Strassmann et al., 2000). This can produce fruiting bodies with shorter stalks less able to disperse spores, or if the proportion of cheaters is high enough, no stalk at all.

Prior work has shown that the cellular slime mold (aka social amoeba) *Dictyostelium discoideum* can evolve resistance to cheating (Hollis, 2012; Khare et al., 2009; Levin et al., 2015; Miller et al., 2023). Khare and colleagues performed a selection screen using a known cheater mutant (*chtC*) to identify potential resistors (Khare et al., 2009). They identified and described *rccA,* a mutant that resists cheating by the *chtC* mutant. When chimeric fruiting bodies were formed from equal starting ratios of the mutants *rccA* and *chtC,* the *rccA* mutant was able to achieve an equal representation in the spores, an achievement unobtainable by the wild-type strain. This resistant mutant was not resistant to exploitation by cheaters other than the *chtC* mutant, however, indicating that its resistance was specific to the specific cheating method employed by the *chtC* mutant. Furthermore, this resistant mutant was not a cheater itself – when co-developed with the wild-type strain in a chimera, it did not form more spores than expected. This experiment shows that the invasion of a population by cheaters can select for resistance, and that resistance is specific.

The experiment performed by Khare and colleagues (Khare et al., 2009) utilized a non-evolving cheater. Levin and colleagues (Levin et al., 2015) set out to determine whether the counter-evolution of resistance could be achieved in populations where both parties are coevolving. They repeatedly developed a wild-type strain with an obligate cheater, allowing both to coevolve throughout the duration of the experiment. At the end, equal ratio chimeric fruiting bodies were formed between the evolved cheater and both the ancestral wild-type strain and the evolved wild-type strain. Levin and colleagues found that the evolved strains were able to resist the cheating of their counterpart, retaining a much larger representation during sporulation than their ancestors. This experiment demonstrated that resistance is able to be selected for even with a coevolving cheater and that resistance is a viable method for cheater suppression in natural populations. If cheating selects for resistance, as these examples show, then resistance might also select for mechanisms to overcome that resistance. Thus, one possible outcome of this conflict is a long-term coevolutionary arms races between cheating and resistance.

1.4 Within-species coevolution and intragenomic arms races

Coevolution occurs when an antagonistic adaptation in one species spurs the evolution of a counter-adaptation in another (Thompson, 1994). This coevolution can potentially result in arms races – a runaway escalation of the coevolving parties (Dawkins & Krebs, 1979). Most studies of coevolution have focused on two different species: for example, hosts and pathogens (Frank, 1991; Gagneux, 2012; Papkou et al., 2019), predators and prey (Brodie III & Brodie Jr, 1999; Wilson et al., 2018), or plants and herbivores (Petschenka & Agrawal, 2016). However, in principle, within species arms races could occur in response to different types of conflict, such as male-female conflict over optimal levels of mating (Arnqvist & Rowe, 2002; Chapman & Partridge, 1996; Rice, 1996), parent-offspring conflict over optimal provisioning (Haig, 1993; Summers & Crespi, 2005; Trivers, 1974), queen-worker conflict over colony sex ratios (Oldroyd & Ratnieks, 2000; Passera et al., 2001; Trivers & Hare, 1976), or social conflict over public goods.

In the case of male-female conflict over mating, adaptations that increase a male's reproductive success can come at the expense of female fitness. For example, in water striders, conflict arises between the sexes over optimal mating rates (Arnqvist & Rowe, 2002; Rowe et al., 1994). These matings are characterized by violent struggles, where females attempt to escape excessive mating attempts made by males (Arnqvist & Rowe, 2002). In response to this conflict, males evolved exaggerated clasping genitalia, while females evolved long abdominal spines and a tilting abdominal tip to resist the male's armaments (Arnqvist & Rowe, 2002; Rowe et al., 1994). The reciprocal evolution of male and female armaments seen in this species is an example of intragenomic arms races.

Such arms races may also occur between cheating and resistance strategies in socially cooperative species. As cheating selects for resistance (Hollis, 2012; Khare et al., 2009; Levin et al., 2015; Miller et al., 2023), resistance may also select for more effective cheaters, laying the groundwork for an intragenomic coevolutionary arms race.

1.5 Recombination and epistasis

In between-species arms races, the two entities do not mate—by definition, since they are different species. In contrast, within-species arms races are subject to an additional process: recombination. Within species, however, sexual reproduction generates new combinations of alleles, which can disrupt co-adapted gene complexes and other types of linkage disequilibrium (Felsenstein, 1974; Keightley & Otto, 2006; McDonald et al., 2016). In the specific case of a coevolutionary arms race, recombination will mix and match alleles encoding competing strategies into a single genetic background (Fig 1.1).

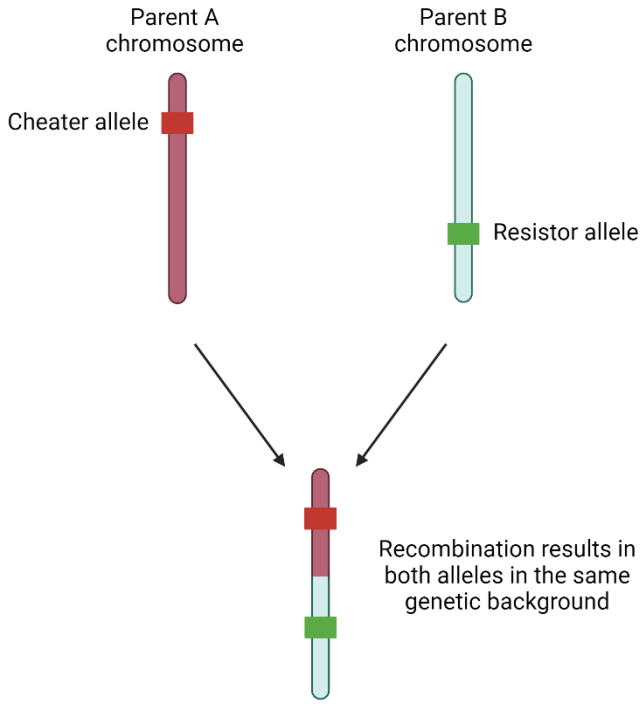

Figure 1.1 Example of recombination. Parent A harbors a cheater allele on one chromosome. Parent B harbors the allele that confers resistance to the cheating method of Parent A. When these parental genotypes are recombined during meiosis, progeny with both cheating and resistance alleles can be produced. The phenotype of the resulting progeny is thus uncertain.

This mixing and matching effect can occur when conflicting alleles are located on different chromosomes, but also when the alleles are located on the same one as a result of 'crossing over' between homologous chromosomes (Mather, 1938).

The effect of recombination on a within-species arms race will likely depend in large part on: (1) the fitness effects of particular allele combinations in *different* genetic

backgrounds (i.e., between social partners), which provides their initial selective advantages, (2) the fitness effects of particular allele combinations in the *same* genetic background (i.e., within an individual) following recombination, and (3) whether there is a consistent relationship between (1) and (2). For example, if mutations arise that confer cheating, counter-mutations that confer resistance may be favoured in that cheater's *social partners*. Subsequent mating between individuals that harbour cheating and resistance mutations might then produce individuals that have both, as well as individuals that have neither (i.e., regenerate the wild-type genotype). The key question is therefore what the fitness effects of individuals are that have these mutation combinations.

This difference in phenotypic or fitness effects of alleles (or mutations) at one locus, dependent on alleles (or mutations) at another locus is known as epistasis (de Visser et al., 2011; Wolf et al., 2000). Classically, epistasis occurs among different loci within the same individual, which can be detected by comparing the phenotypic or fitness effect of the two single mutants to their combined effect in the double mutant. Specifically, epistasis is defined as a nonindependence of effect: if the effect of one allele is not predictable without knowledge of the value of another allele, then its effect is epistatic (Wolf et al., 2000). However, epistasis can also arise between individuals or genomes: specifically, when the fitness effect of an allele in one individual depends on the presence or absence of alleles in another individuals. These two forms of epistasis are known as intragenomic and intergenomic epistasis, respectively (Wolf et al., 2000).

In the case of social conflict, we expect intergenomic epistasis, in that the fitness effects of a cheating allele in an individual should depend on the presence or absence of a resistance allele in its social partners. However, in the case of within-species arms races, recombination will mix and match these alleles, and intragenomic effects (i.e., phenotypic or fitness effects of these mutations in the same genetic background) become important. For example, cheating-resistance double mutants might be subject to antagonistic epistasis (Desai et al., 2007; Wolf et al., 2000), nullifying their effects. Alternatively, they could be subject to synergistic epistasis (Whitlock & Bourguet, 2000; Wolf et al., 2000), which exaggerates their effects. Or they could have the same effects

singly as in combination (no epistasis). However, the existence of these epistatic effects is likely to impact the selective advantage of the cheating and resistance alleles and therefore impact the dynamics of a coevolutionary arms race. However, there has been no work examining how recombination and epistasis influence the plausibility of a within-species coevolutionary arms race.

To answer this question, I utilized the unique life cycle of the social amoeba *Dictyostelium discoideum*. As I describe in more detail below, its unique life-cycle has all the necessary ingredients for testing how recombination might influence a coevolutionary arms race. First, it has a unique form of cooperation that is susceptible to cheating (Buttery et al., 2009; Ennis et al., 2000; Khare & Shaulsky, 2010; Santorelli et al., 2008; Strassmann et al., 2000). Second, cheating can select for resistance to cheating (Hollis, 2012; Khare et al., 2009; Levin et al., 2015; Miller et al., 2023). Third, these dynamics potentially give rise to an arms race (Hollis, 2012; Levin et al., 2015; Ostrowski et al., 2015). Finally, it undergoes facultative sexual reproduction, meaning that recombination may mix and match cheating and resistance alleles (Francis, 1998; Wallace & Raper, 1979).

The goal of this research is to address this possibility experimentally and theoretically. To do so, I (1) performed laboratory experimental evolution of *D. discoideum* with and without sexual reproduction, and (2) developed a simulation, based on the *D. discoideum* life cycle, to explore how periodic sexual reproduction influences the invasion and fixation of cheating and resistance alleles, given assumptions about how these mutations, selected for their benefits across genetic backgrounds, might interact when recombination brings them together into a single genome

1.6 *Dictyostelium* as a model organism

Dictyostelium discoideum is a social amoeba with three distinct phases of its life cycle: (1) asexual cell division, (2) multicellular development, and (3) sexual reproduction (Fig. 1.2).

During the unicellular (or vegetative) stage of the life cycle, haploid amoebae live in the surface soil of forests. In this stage, they engulf and kill bacteria and other small microbes by phagocytosis and reproduce by asexual mitotic cell divisions, giving rise to genetically identical daughter cells (Kessin, 2001; Raper, 1984). When food runs out and starvation begins, the cells enter the 'social' cycle and undergo a transition to a multicellular form. This process is initiated by aggregation of 10^4-10^6 cells, which form a mound and eventually a multicellular worm-like structure called a 'slug', capable of long-range migration (Strassmann et al., 2000). Eventually, the cells in the slug terminally

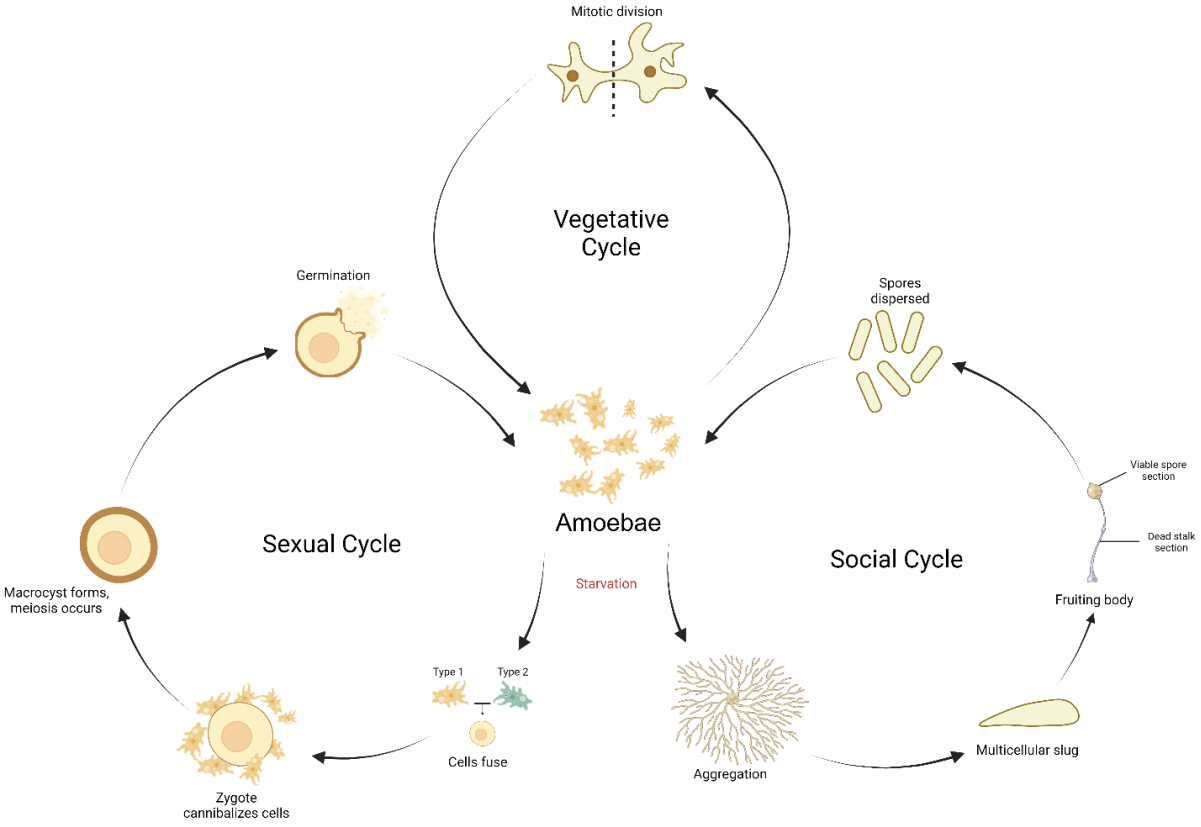

Figure 1.2 ***Dictyostelium discoideum*** **social development cycle.** Top: Dictyostelium discoideum divides mitotically as amoebae. Right: As starvation occurs, cells aggregate to form a multicellular slug that then develops into a fruiting body with a stalk and spore section. The spores germinate back into amoebae, restarting the cycle. Left: Upon starvation, cells of compatible mating types fuse to form a zygote. The zygote attracts and cannibalizes nearby cells to form a macrocyst. Meiosis occurs inside the macrocyst before germinating back into amoebae and restarting the cycle. Made with BioRender.com

differentiate and transform into a fruiting body, consisting of a sorus containing spores held aloft by a rigid stalk.

Approximately 80% of the cells (from the posterior of the slug) will form the live spores, whereas the remaining 20% (from the anterior of the slug) form the stalk and die (Kessin, 2001). Their death is thought to provide an advantage to the spores by lifting them out of the soil, increasing the chances that passing invertebrates will disperse them to favorable locations (Smith et al., 2014). Death of the stalk cells is thus thought to be an example of altruism because these individuals sacrifice their own fitness to help other members of their population (Axelrod & Hamilton, 1981; Kerr et al., 2004).

In *Dictyostelium*, multicellularity is achieved by aggregation of the cells, rather than clonal division from a single starting cell, as it occurs in many other organisms (Bonner, 2015; Kessin, 2001). Thus, fruiting bodies can contain unrelated cells and occasionally social amoebae of other species (Buttery et al., 2009; Fortunato et al., 2003; Jack et al., 2008; Strassmann et al., 2000). Multicellular structures – slugs and fruiting bodies – that contain multiple genotypes are referred to as "chimerae", in reference to the creature of Greek mythology that was a monstrous hybrid of different animals.

Dictyostelium's chimerism can lower the relatedness of the cooperating cells, potentially making multicellular development vulnerable to exploitation (Buttery et al., 2009; Strassmann et al., 2000). Specifically, genotypes that preferentially adopt the spore fate (or manipulate others into adopting the stalk fate) should have a large fitness advantage (Ennis et al., 2000; Khare & Shaulsky, 2010; Santorelli et al., 2008). These individuals are referred to as cheaters, because they gain the advantages of the stalk without paying their fair share of the cost to produce it.

The first described *Dictyostelium discoideum* cheater mutant was *chtA* (Ennis et al., 2000), which is unable to produce fruiting bodies in a clonal population. This is due to the mutant lacking a developmental protein which signals for the development of pre-stalk cells, resulting in the sole production of pre-spore cells during the social development phase. This particular cheater mutant is thus an 'obligate' cheater, in that the mutant is unable to alter its cheating behavior depending on the behavior of its social partners.

Khare and Shaulsky describe a mutant which expresses an alternate form of cheating – 'facultative' cheating (Khare & Shaulsky, 2010). A facultative cheater has the option (but not the obligation) to cheat, and may alter its behavior depending on the parties involved. Khare and Shaulsky describe *chtC,* a mutant that forms normal fruiting bodies in a clonal population of *chtC* mutants, but preferentially forms spores when co-developed with a wild-type strain. In the case of the *chtC* mutant, pre-stalk cells are unable to maintain the pre-stalk fate and transdifferentiate into pre-spore cells. This mechanism does not majorly affect the morphology of *chtC* clonal fruiting bodies. When cultivated with wild-type cells, however, the wild-type cells compensate for the deficiency of *chtC* pre-stalk cells by allocating a greater proportion to the stalk. This mechanism causes the *chtC* mutant to produce more than its fair share of spores in chimerae, which increases its representation over time - effectively, causing it to cheat its cooperative social partner.

Furthermore, such facultative cheaters are thought to be common in nature (Buttery et al., 2009; Fortunato et al., 2003; Noh et al., 2018; Santorelli et al., 2008; Strassmann et al., 2000). Santorelli and colleagues performed a large-scale screen for facultative cheating mutants and discovered 31 capable mutants (Santorelli et al., 2008). This study showed that due to the large number of pathways through which facultative cheating can be achieved, it is likely to occur in natural populations. Buttery and colleagues provide further support by showing that within chimeric fruiting bodies formed from natural isolates, unequal spore allocation often favors one genotype due to facultative cheating and differences in fixed spore allocation (Buttery et al., 2009).

If starvation occurs in dark and wet conditions, *D. discoideum* will instead enter its sexual cycle, which results in the formation of large, triple-walled resting structures termed 'macrocysts' (Clark et al., 1973; Gregory W. Erdos et al., 1973; Saga et al., 1983). First described by Blaskovics and Raper (Blaskovics & Raper, 1957) in the related species *Dictyostelium mucoroides* and *Dictyostelium minutum,* these structures provide an alternative response to starvation. Macrocysts are much more resistant to environmental hazards than the spores that formed during multicellular development (Fukui, 1976). Combined with long maturation periods, these structures may serve as a 'hibernation'

function whereby Dictyostelids can enter a prolonged dormant state and await more favorable environmental conditions before germination.

During the sexual cycle, instead of aggregating to form a slugs and fruiting bodies, two haploid parental cells of opposite mating types fuse to form a diploid zygote, referred to as a giant cell (Bloomfield et al., 2010; Saga et al., 1983; Saga & Yanagisawa, 1982). The zygote signals to the surrounding haploid cells, which aggregate around the giant cell in much smaller numbers than during slug aggregation (~200 cells per macrocyst compared to ~100,000 per slug) (O'Day, 1979; O'Day & Keszei, 2012). These attracted cells are phagocytosed and aid in forming external layers of cellulose around the giant cell (Filosa & Dengler, 1972; Lewis & O'Day, 1985). As the macrocyst matures, these engulfed cells disappear and the contents of the cyst become homogenous.

Within the macrocysts, meiosis and several rounds of mitosis occur over a long period of incubation before they ultimately germinate and release haploid recombinant progeny (Francis, 1998; Ishida et al., 2005; Okada et al., 1986; Wallace & Raper, 1979). The progenies of any given macrocyst are genetically identical, but across a population of macrocysts, many different recombinant genotypes are observed (Bloomfield et al., 2019; Francis, 1998; Francis & Eisenberg, 1993; Wallace & Raper, 1979).

The exact trigger inducing germination of *Dictyostelium discoideum* macrocysts remains unknown. Despite this, low levels of germination (<1%) can be achieved by plating macrocysts on starvation plates in ambient light and temperature (Bloomfield et al., 2010; Francis, 1998; Francis & Eisenberg, 1993; Wallace & Raper, 1979). While the conditions required to trigger germination in the laboratory are not known, *Dictyostelium discoideum* populations appear to undergo recombination frequently in nature, based on the low levels of genome-wide linkage disequilibrium in natural populations (Flowers et al., 2010; Ostrowski et al., 2015). This observation indicates that recombination may be taking place frequently as the mixing and matching of alleles prevents piggy-backing effects between closely-associated alleles (Felsenstein, 1974; Keightley & Otto, 2006; McDonald et al., 2016).

In *Dictyostelium*, there are three different mating types, and parent strains must be of different mating types for macrocyst formation (Bloomfield et al., 2010). Each mating

type is determined by the combination of genes present at the *mat* locus; *matA*, *matB, matC, matD, matS,* or *matT* (Fig 1.3) (Bloomfield et al., 2010). Mating type 1 strains have the *matA* gene. Mating type 2 strains have the *matB, matC* and *matD* genes. Mating type 3 strains have the *matS* and *matT* genes. Each mating-type is compatible with either of the other two, but not itself – aside from rare 'homothallic' strains, for which the mechanisms of their self-compatibility are not yet fully understood (Bloomfield et al., 2010).

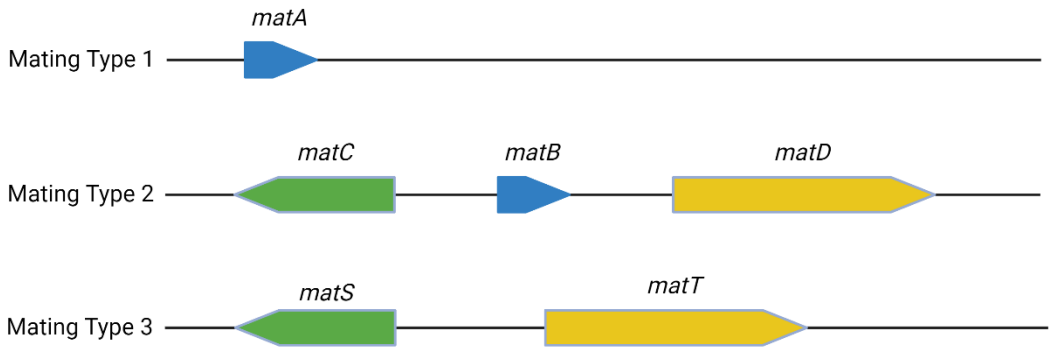

Figure 1.3 *Dictyostelium discoideum* mating type locus. Mating type one strains are characterized by only a single mating type gene, *matA*. Mating type two strains harbor three mating type genes, *matC, matB,* and *matD*. Mating type three strains carry two mating type genes, *matS* and *matT*. Homology between genes is indicated by color; 1) *matA* and *matB* colored blue, 2) *matC* and *matS* colored green, and 3) *matD* and *matT* colored yellow. Figure not to scale. Made with BioRender.com, adapted from Bloomfield, 2010 (Bloomfield et al., 2010)

1.7 Research hypothesis and objectives

I hypothesize that recombination could slow or halt an ongoing within-species arms race owing to epistatic interactions between cheater and resistor alleles when introduced into the same genetic background through recombination. To test this hypothesis, I performed a long-term evolution experiment over ~140-160 generations, where replicate populations, found from a mix of two ancestral strains, evolved in the presence or absence of occasional recombination. Prior work has shown that laboratory experimental evolution readily results in reciprocal adaptations of the strains (Hollis,

2012; Khare et al., 2009; Levin et al., 2015; Miller et al., 2023). However, the impact of recombination on this process has not been previously examined.

As described below, analyses during the evolution experiment suggested that the macrocysts were not germinating. For this reason, I carried out a follow-up experiment to assess different methods of macrocyst germination. I compared three different germination methods (UV light, freeze-thaw cycles, or disruption with glass beads), testing their effectiveness after 2, 4 or 6 weeks of maturation. Finally, I used a simulation of the co-evolutionary process in *Dictyostelium* to assess how periodic recombination and other factors, such as costs of cheating and resistance, mutation rates, and epistasis, influence co-evolutionary dynamics. These simulations allowed me to vary more factors than I could using laboratory experiments and should inform future empirical studies.

Chapter 2: Long-term evolution experiment

2.1 Introduction

To assess the impact of recombination on an ongoing within-species arms race, I performed a long-term evolution experiment, consisting of replicate populations evolved with or without period recombination. To do so, I initiated 24 replicate populations, each consisting of a 50-50 mix of a mating type I and a mating type III strain. In half of these populations, I induced macrocyst formation after the 10th round of development, whereas the other 12 populations, which were otherwise identical, did not undergo periodic macrocyst formation. There were a further 6 populations consisting of either one strain or the other, but not both (three populations of the mating type I strain, and three populations of the mating type III strain). These populations were subjected to the conditions that induce mating, but did not mate, since they lacked their partner of the opposite mating type.

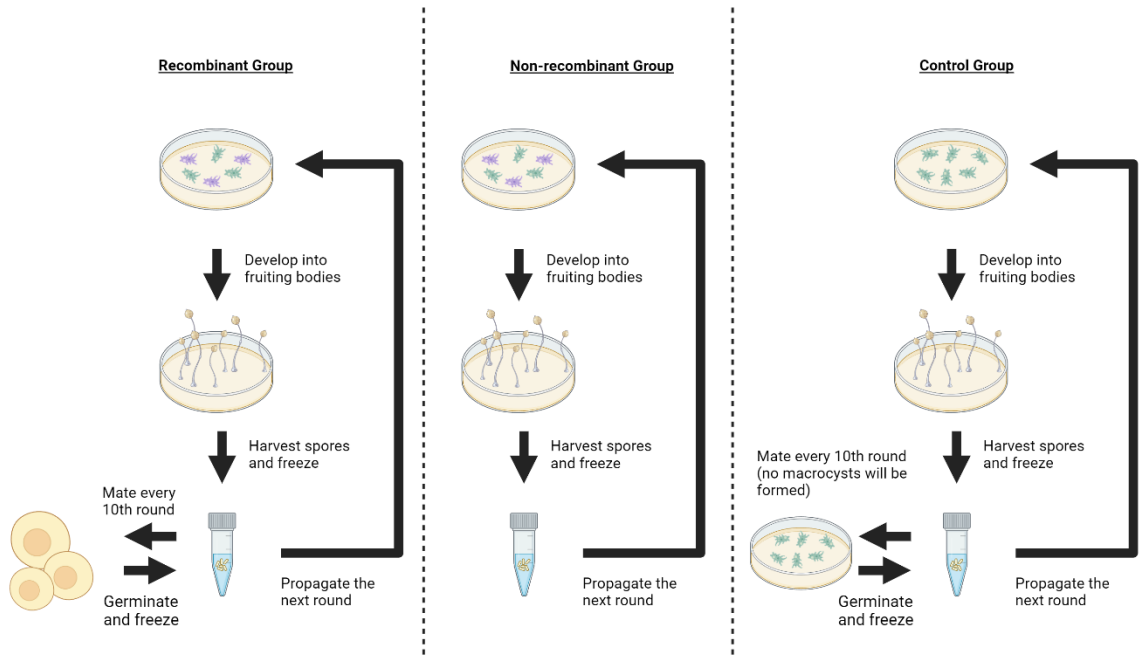

Figure 2.1 Overview of long-term experimental evolution procedure. Left: The recombinant group starts with a 1:1 mixed population of EO1336 (mating type I) and EO1273 (mating type III). These are developed into fruiting bodies and harvested of spores which are stored frozen at -80°C and used as the starting population for the next round of development. Every tenth round, spores are instead used to form macrocysts, which are germinated and put back into the cycle as the starting population. Middle: The non-recombinant group mimics the recombinants, except never undergoes macrocyst formation. Right: The control group starts with only one strain, otherwise mimicking the recombinant group. The control group is developed for both strains. Made with BioRender.com

2.2 Method

2.2.1 Ancestors

The strains used for this experiment were natural isolates EO1273 (mating type III) and EO1336 (mating type I), which were isolated from soil samples collected at Mountain Lake Biological Station in Virginia, USA. The two strains were sampled from different soil samples that were approximately 10 meters apart.

2.2.2 Treatment Groups

The 12 recombinant populations were initiated from a 1:1 mix of strains EO1273 and EO1336. The 12 non-recombinant populations were also initiated from a 1:1 mix of EO1273 and EO1336; however, this group did not experience mating conditions. The six control populations were clonal, consisting of three replicate populations of EO1336 and three replicate populations of EO1273. The control populations experienced the same recombination-inducing treatment as the recombinant populations, but do not form macrocysts owing to the lack of a compatible mating type strain.

2.2.3 Developing populations

All mixed populations were initiated from the same frozen spore stock consisting of the two strains in a 1:1 ratio. Single strain populations were initiated directly from the ancestor frozen spore stocks.

At the start of each round, $4x10^6$ total spores were spread evenly over a 350 µL *Klebsiella pneumoniae* (SM broth; Formedium Ltd) lawn in 10-cm petri dishes containing 30 ml of SM/5 agar (24.9g/L Formedium™ SM Agar/5 powder - 1.7% agar). The plates were then incubated at 22°C for ~72 hours to allow for spore germination, expansion of the population, and multicellular development, which resulted in a lawn of fruiting bodies.

The spores from these fruiting bodies were harvested using a metal inoculation loop dipped in KK2 (diluted 1:10 from the following 10x stock; 22 g/L KH_2PO_4, 7 g/L K_2HPO_4) +

20% glycerol and counted. Aliquots containing $4x10^6$ spores were aliquoted into a fresh 1.5 mL Eppendorf tube and frozen at -80°C. To commence the next round, these aliquots were defrosted and plated on bacterial lawns on SM/5 agar media, as described above. This process of germination, population expansion, and multicellular development was repeated for 10 rounds of development, at which point the recombinant and control populations were subjected to macrocyst formation.

2.2.4 Recombination rounds

Macrocyst Induction: Forty fruiting bodies were picked from each population and mixed via pipetting into 10-cm Petri dishes containing 10 mL of LP agar (1 g/L lactose, 1 g/L Bacto™ Peptone, 15 g/L agar powder), topped with 10 mL of SS buffer (0.6g/L NaCl, 0.75 g/L KCl, 0.3 g/L $CaCl_2$) and 150 µL of stationary phase *Klebsiella pneumoniae*. These plates were wrapped in aluminum foil and incubated at 22°C for 14 days.

Macrocyst Cleaning: After 14 days, the resulting macrocysts were dislodged by rinsing the plates with SS buffer and transferred to 50 mL falcon tubes. They were then incubated at 45°C for 30 minutes to induce germination of any spores that were present and incubated overnight at 22°C.

The next day, the supernatant was discarded, leaving the macrocysts, which settled at the bottom of the tube. The macrocysts were resuspended in 1 mL 10% NP-40 to kill any remaining amoebae and transferred to 2mL Eppendorf tubes. To ensure the macrocysts were free of incorporated spores or amoebae, I washed the macrocysts several times. This process consisted of pipetting the suspension vigorously 5-10 times using a P1000 pipette and vortexing briefly at maximum speed. The macrocysts were centrifuged at 100 x g for 2 minutes, the supernatant discarded, and the macrocyst pellet resuspended in 2 mL 10% NP-40. The wash process was repeated until no spores or amoebae were present when a 10 µl sample was viewed at 100X magnification. Following the final wash, the macrocysts were washed several times in KK2 to remove the NP-40 detergent by centrifuging at 100 x g for 2 minutes and resuspending in 2 mL of KK2.

Macrocyst Germination: 2mm glass beads were added to the 0.3 mL mark on a 2 ml Eppendorf tube, and KK2 was added until the beads were covered. The sample was vortexed at the maximum speed for eight 15-second bursts. A 10 µL sample was inspected at 100X magnification to verify that the macrocysts had been disrupted (Fig 2.1b).

The macrocyst-glass bead suspension was then transferred to a SM/5 agar plate with 350 µL of *Klebsiella pneumoniae*. Once the plates had dried, the beads were removed, and the plates were incubated at 22°C to allow the amoebae to divide and form fruiting bodies, which usually appeared within 72 hours.

Non-recombining treatments: In lieu of macrocyst induction, the 12 non-recombining populations underwent one additional round of development to equalize for the extra cycle provided by the recombination round. The six control populations were subjected to macrocyst induction conditions—as expected, no macrocysts formed. These populations were immediately centrifuged at 400 x g for 3 minutes and $4x10^6$ spores were plated onto a lawn of 350 µL *Klebsiella pneumoniae* on SM/5 agar for a round of multicellular development.

2.3 Results

2.3.1 Macrocyst formation and germination

Macrocysts were induced directly after the 10th round of development in all recombinant populations. The bead germination treatment was successfully applied and produced free-floating cells originating from these macrocysts in all recombinant populations (see Fig 2.2b for an example). These cells appeared to be mostly circular, with a smaller number of cells taking on an amoeboid appearance. Macrocysts were not able to be produced in any control population.

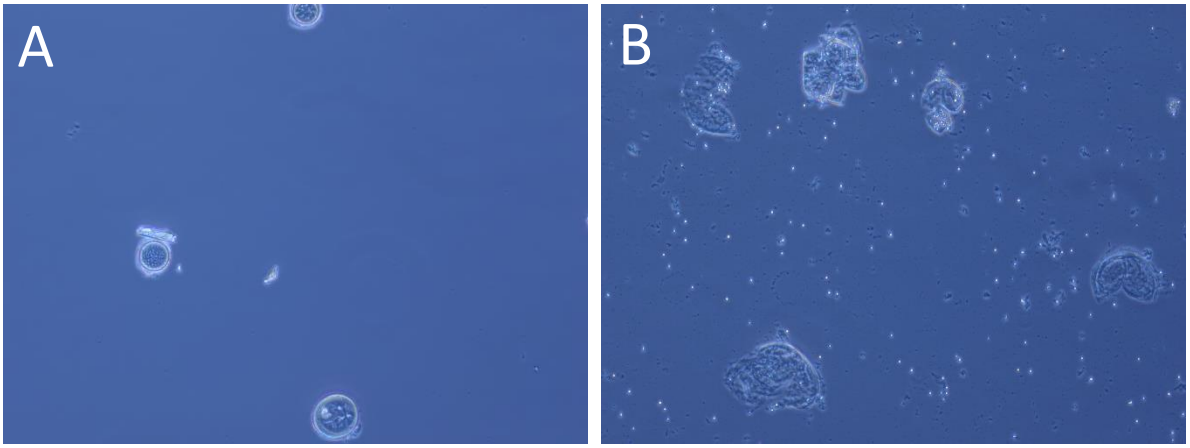

Figure 2.2 Disruption of macrocysts using glass beads. (A) Cleaned macrocysts from one population, prior to vortexing with glass beads. (B) The same population, following the bead treatment. Note that the macrocysts in (A) are round and intact, with no external spores or amoebae. In (B), the macrocysts are not intact and released cells are visible in the supernatant.

However, macrocysts were unable to be produced after the 20th round of development in these same populations. Several attempts were made to induce macrocyst formation; however, none were successful. This unexpected issue rendered the experiment unfeasible to continue past the 20th round of development. Without the ability to produce macrocysts, the effects of recombination were unobservable using the proposed experimental setup and thus the experiment was suspended.

Multiplex PCR using primers for both mating types (Table 2.1) was performed to investigate whether the issue was caused by the fixation of a single mating type (Fig 2.3). DNA was isolated from all recombinant populations directly from frozen stocks using a 5% Chelex 100 resin bead mixture.

Initial denaturation was run at 95°C for 4 mins. Denaturation, annealing and extension phases were run at 94°C for 30 seconds, 51°C for 30 seconds, and 65°C for 1 and a half

minutes respectively. Denaturation, annealing and extension were repeated a total of 40 times before final extension at 65°C for 10 minutes. To visualize bands, 5µL of the PCR product was run on a 0.8% agarose gel for 35 minutes at 100 volts alongside a 1kB DNA ladder. This PCR revealed that *matS* was the only mating type present in all recombinant populations directly preceding macrocyst forming conditions (Fig 2.3), explaining why macrocysts were unable to be formed. Without a partner of a complementary mating type, the sexual cycle was not able to initiate despite favorable macrocyst forming conditions.

Table 2.1 Multiplex mating type PCR primers

Primer Name	Sequence (5'-3')	Mating Type	Product Size
*matA*_forward	CACACTAAACATGGACCCAC	I	360bp
*matA*_reverse	CCCCTAAATCTTTACCAAGTCA	I	360bp
*matS*_forward	CGATCAGTTGGAAAACATTAC	III	650bp
*matS*_reverse	GGATACCAAAAAACTAGTTT	III	650bp

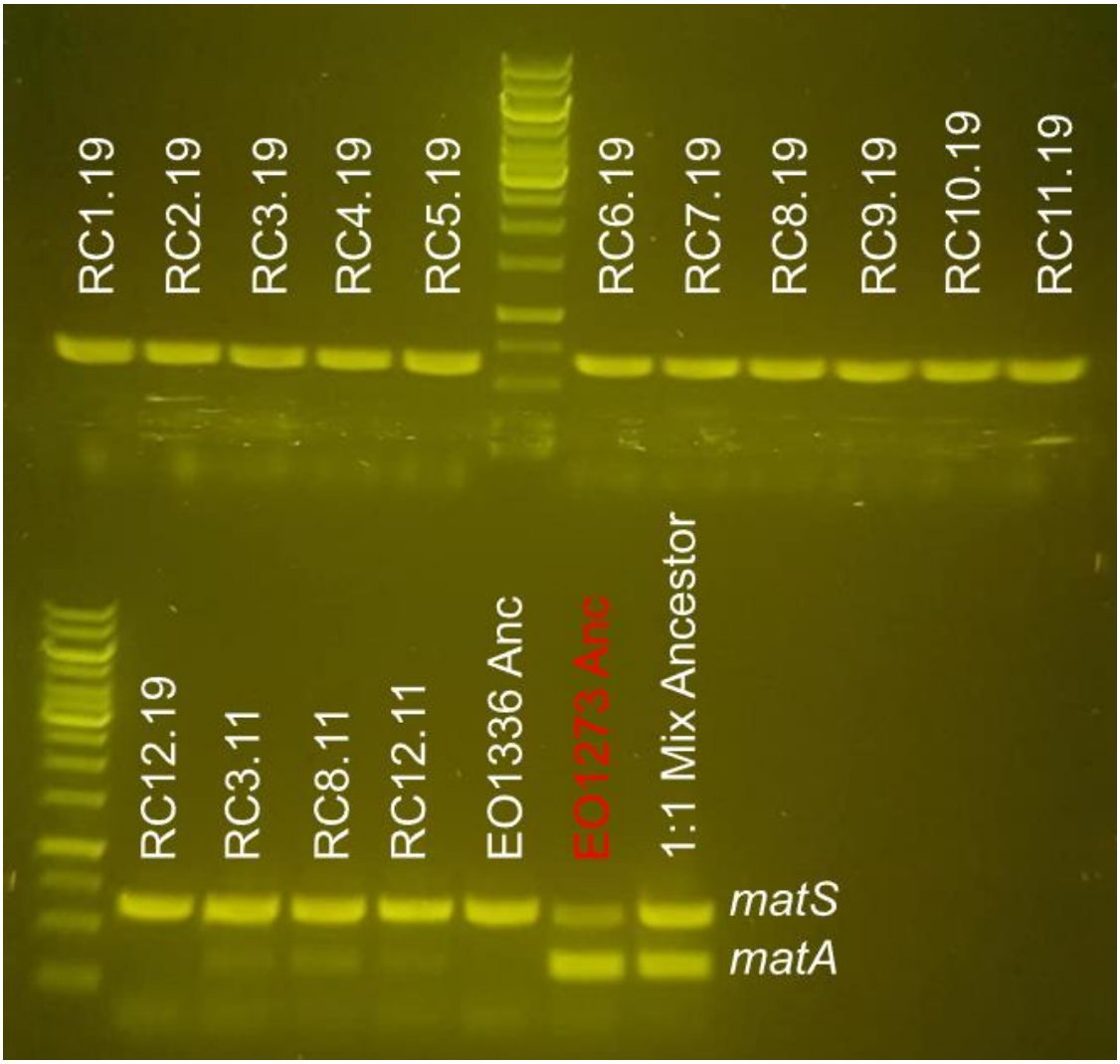

Figure 2.3 PCR of the mating type locus to monitor the outcome of the evolution experiment. I carried out a multiplex PCR on genomic DNA extracted from the two ancestors, the initial populations (with 1:1 ratio of the ancestors), and the evolved populations. I used a mix of two pairs of primers, one specific to the *matA* (mating type I) locus and the other to the *matS* (mating type III) locus. The lower band indicates matA, with the higher band indicating matS. For each label, 'RC' indicates a replicate evolving population in the recombination treatment. Numbers before the decimal indicate the replicate population, and numbers after the decimal indicate the round. The results show that both mating types were present after the first recombination round (Round 11; two bands present in all populations). However, only one band was detected in all populations at Round 19, indicating loss of one mating type. Note that the ancestor EO1273 shows two bands instead of the expected one band. However, PCRs from this same frozen stock produced only one band, suggesting there was carryover between the wells in this gel.

2.4 Discussion

2.4.1 Outcome of experimental evolution

The experimental evolution protocol was successful, in that I was able to complete 20 cycles of germination, growth, and multicellular development for 30 replicate populations. Until the 20th round, the populations for the most part had sporulated within 72 hours, ensuring that I could complete two rounds per week and all populations largely stayed in lockstep. However, following Round 20, I was not able to get 8 populations to complete development, and re-starting these populations from Round 19 did not ameliorate the problem (Supplementary Figure 1.6). Increasing the developmental timeframe failed to encourage full development of these populations.

2.4.1.1 Phenotypic changes during evolution

All populations underwent phenotypic changes over the course of experimental evolution. Fig 2.3 shows ancestral populations (left) and their evolved counterparts (right) 48 hours after plating on growth media (SM/5 agar lawned with 350 µL *Klebsiella pneumoniae*). All ancestral populations from the non-recombinant, recombinant and control groups were still in the late slug/stalk phase of fruiting body development 48 hours post plating, while most evolved populations presented mature fruiting bodies. Fruiting bodies are able to be identified by the regular, round spore heads (sori), whereas slugs/stalks appear more random and varied in shape and size.

The increased rate of development could be explained by adaptation to the growth media, resulting in increased fitness. These adaptations may have caused evolved individuals to have shorter vegetative growth cycles, resulting in more active amoebae than the ancestral populations at a given time point. More amoebae would thus exhaust the bacterial food source faster and induce the starvation state that signals the start of social development earlier, effectively giving more time for fruiting bodies to mature. Alternatively, faster development of the evolved populations may be due to mutations which increase the rate of fruiting body construction directly.

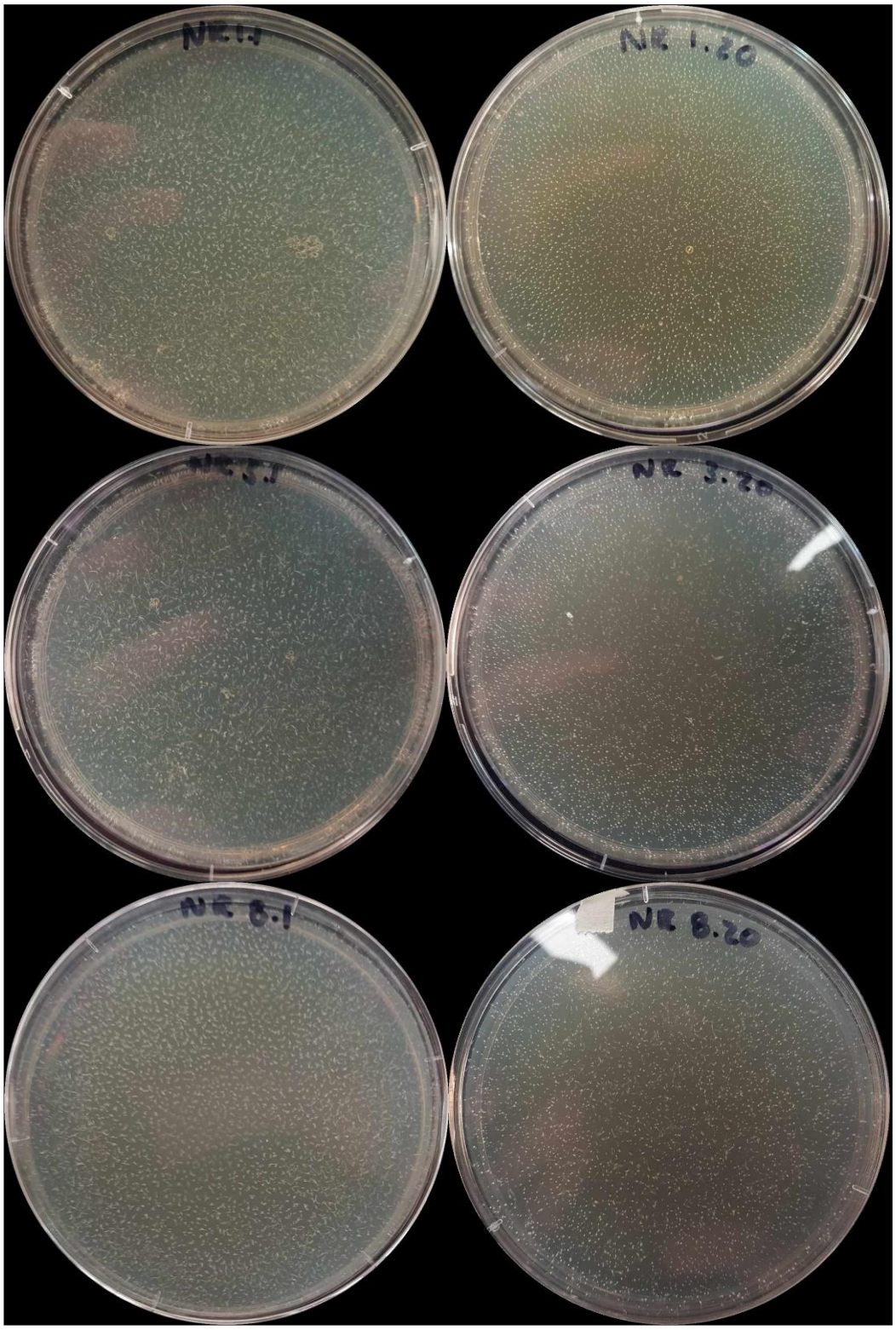

Figure 2.4 Development speed comparison between ancestral and evolved populations. *Dictyostelium discoideum* populations plated on growth SM/5 growth media lawned with *Klebsiella pneumoniae*, 48 hours post plating. Left half: Ancestral populations are in the stalk development phase and full fruiting bodies have not yet developed. Right half: The complementary evolved populations, displaying full fruiting bodies by the same time point. Fruiting bodies can be identified by the circular morphology of the sorus (spore head).

2.4.1.2 Recombination rounds

Macrocysts formed in all recombinant populations after Round 10, and they appeared free of contaminating spores and amoebae after the cleaning process (Fig 2.1a). The use of glass beads after two weeks to rupture macrocysts also resulted in the release of amoebae into the supernatant (Fig 2.1b), suggesting that this method might offer a major improvement in the speed and efficiency of germination. Prior work showed that germination of *Dictyostelium discoideum* macrocysts required >6 weeks of maturation, followed by a 2–4-week germination period (Bloomfield et al., 2019; Francis, 1998; Francis & Eisenberg, 1993; Wallace & Raper, 1979), meaning that a single trial takes several months to complete. Furthermore, this traditional method resulted in extremely low germination rates (<1%).

However, several observations during this experiment make it unclear if the glass bead disruption worked as expected. First, no macrocysts formed during the second round of recombination (after Round 20). In addition, multiplex PCR of the evolved populations showed that the mating type I locus (*matA*) was undetectable in all populations. If so, the failure to find a compatible mate could explain the inability to form macrocysts.

In principle, the loss of one of the mating type loci should have been prevented by sexual recombination (Fisher, 1930). The ratio of mating types and sexes are balanced by frequency dependent selection: as the frequency of any mating type outgrows the frequency of the other, the benefits of that specific mating type decrease as there is more competition for mates (Fisher, 1930). These loci are expected to be maintained under balancing selection at approximately equal ratios if (1) progeny can only be produced from successful mating of strains of opposite mating types, and (2) there is no genetic drive at the mating type locus (or linked genes) or differential investment in the dead cells of the macrocyst that would cause progeny with one mating type to disproportionately survive over others (Hamilton, 1967). Consistent with these conditions, previous studies have shown the mating type locus to be under balancing selection in *Dictyostelium discoideum* (Douglas et al., 2016).

There are a few possible explanations for why a single mating type may have become fixed by the later rounds of this long-term evolution experiment.

1. Recombinant progeny of one mating type harbor a selective advantage over recombinants of the other mating type. In other words, mating and recombination occurred, but selection during the sexual stage led to the fixation of one mating type locus.

2. Cells of one ancestor had a selective advantage over cells of the other ancestor, and recombination did not occur (or did not occur sufficiently often to counteract this effect). If so, then the mating type locus will hitchhike to fixation with the alleles that confer high fitness in that genetic background. This hypothesis assumes little recombination took place, which could occur if: (1) too many contaminating spores or amoebae were present, swamping out the recombinants resulting from macrocysts, (2) macrocyst germination was ineffective or caused progeny death.

2.4.2 Future research

The issue of one strain quickly outcompeting the other and thus one single mating type rising to fixation and preventing any attempts of macrocyst formation could have been controlled by using strains that were identical in all aspects but the mating type locus. Such strains have been utilized in previous studies (Bloomfield et al., 2010). By ensuring an identical genetic background between the parental strains, differences in inherent fitness are minimized, preventing selection-driven fixation of any single mating type.

It is possible to rule out selection during the sexual stage as a possible reason behind the fixation of mating types by performing a multiplex PCR using mating type specific primers (see Table 2.1) after each recombination round. By running the same PCR on a known 50:50 mixture of the two mating type strains, one is able to compare the brightness of the resulting bands and approximate the ratio of mating types directly after the sexual cycle. If these bands appear to be in a roughly 50:50 ratio, Fisher's principle (Fisher, 1930) is likely holding and balancing selection is maintaining the mating types.

Chapter 3: Germination Experiment

3.1 Introduction

As the long-term evolution experiment had failed to produce the anticipated results due to sub-par macrocyst germination, I performed a follow-up experiment to explore more efficient germination methods.

In short, five separate crosses of different strains of *Dictyostelium discoideum* were performed, and the resulting macrocysts incubated for 2, 4 or 6 weeks. Macrocysts from every cross were then washed to remove spores and unincorporated cells, imaged and treated with four separate germination methods for each time point. Fruiting bodies were counted two weeks post-treatment and germination efficiency calculated as the number of macrocysts plated divided by the number of fruiting bodies observed.

The germination methods examined were a light irradiation method based on a similar study in a closely-related species (*Dictyostelium mucoroides*) (Abe & Maeda, 1986), the mechanistic bead method used in the long-term evolution experiment (North & Cotter, 1991) and freeze-thaw cycling.

Light-irradiation – Abe and Maeda (Abe & Maeda, 1986) performed a similar germination assay where the effects of pH, temperature, time and light exposure were examined for their relationship to germination rates. They found that a period of short-wavelength light irradiation significantly improved germination rates of *Dictyostelium mucoroides* macrocysts. Although this experiment was performed with a different species, light-irradiation still remains a strong candidate for improving germination rates in *D. discoideum*.

Bead rupturing – North & Cotter (North & Cotter, 1991) describe a method with which to break and fractionate *Dictyostelium mucoroides* macrocysts using glass beads. While their method was not intended to produce live progeny, a few alterations to the method may be enough to generate an efficient, reproducible way to germinate *D. discoideum* macrocysts. Ideally, this method would have disrupted the mature macrocyst structure without damaging the progeny, consequently releasing the amoebae where they may resume normal vegetative functions.

Freeze-thaw cycling - Repeated freeze-thaw cycles were intended to both mimic seasonal temperature shifts and possibly disrupt the outer macrocyst walls through the formation of ice crystals within and surrounding the structures. Freeze-thaw cycles are a known method to combat hard-seededness (where seeds are unable to absorb water and cannot germinate) and induce germination in certain plants (Acharya et al., 1993; Shibata et al., 1995). As the hardy walls and low germination rates of macrocysts resemble those seen such hard-seeded plants, there was potential for this method to induce the same results.

Control method – The Wallace and Raper method (Wallace & Raper, 1979) was used as the control method in this experiment. This is the method typically used to germinate macrocysts, with a <1% germination rate. Other methods would be compared to the rates observed using the Wallace and Raper method to determine if they produced a different result.

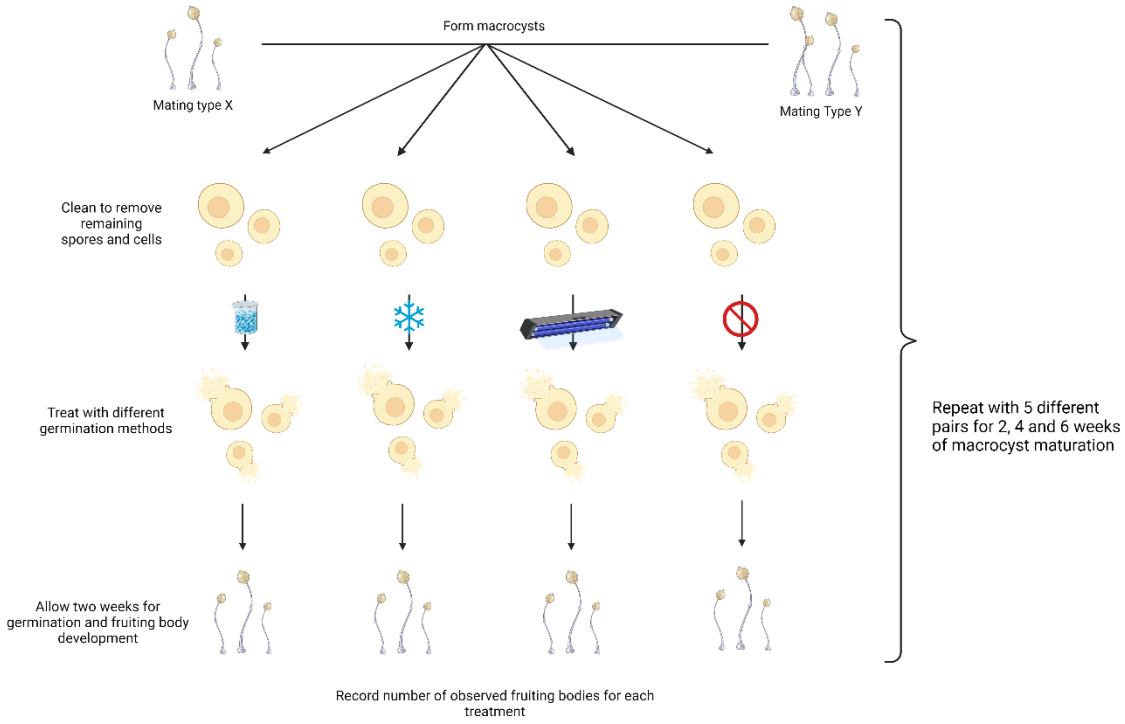

Figure 3.1 Experimental design. Each of five pairs of compatible strains were mixed and incubated under macrocyst-inducing conditions for 2, 4 or 6 weeks (see Methods). Mature macrocysts were then washed to remove any remaining spores or amoebae and subjected to different treatments to induce germination: glass beads, freeze-thaw cycles, UV light, or no treatment (control). Following treatment, the macrocysts were deposited on non-nutrient agar. After two weeks, the number of macrocysts and fruiting bodies were recorded. The experiment was performed for 5 pairs of strains simultaneously, and the entire experiment repeated three times independently. Figure made with BioRender (BioRender.com).

3.2 Method

3.2.1 Strains

Table 3.1 Strain pairs used to test different germination methods

Strain Pair	Strains (Mating types)	Location
1	EO56 (I), EO57 (III)	Claytor Nature Science Center (Virginia, USA)
2	EO603 (II), EO604 (I)	Great Smoky National Park (North Carolina, USA)
3	EO685 (III), EO686 (II)	Afton Forest (Connecticut, USA)
4	EO686 (II), EO688 (III)	Afton Forest (Connecticut, USA)
5	EO1307 (II), EO1308 (I)	Mountain Lake Biological Station (Virginia, USA)

The five pairs are shown in Table 3.1. These pairs were chosen following preliminary experiments concluding that they consistently form numerous macrocysts within a two-week period, ensuring that we had enough macrocysts to proceed with germination. Each strain pair were co-isolated from the same small surface soil sample (<10 g; maximum distance of 14 cm). Thus, these strains co-occur in close proximity in nature, increasing the chances that they have mated in the past and reducing the probability of genetic incompatibilities that could limit germination.

These pairs had been confirmed as macrocyst-forming through two separate macrocyst formation assays in which a single fruiting body of each strain in a cross was added to two wells in a 24 well plate with 0.5 mL LP agar topped with 0.5 mL SS buffer and 5 µL *Klebsiella pneumoniae*. The 24 well plate was wrapped in foil and incubated at 22°C for 14 days before observation for macrocyst formation. Crosses were considered compatible and 'macrocyst forming' if both wells of a cross contained macrocysts - crosses in which only a single well contained macrocysts were not considered suitable for use in this experiment. Macrocyst formation was consistent in all selected crosses across both assays (not pictured).

3.2.2 Macrocyst induction

$4x10^6$ spores of each strain (for a total of $8x10^6$ total spores) were combined and added to a 10-cm petri dish containing 10 mL of LP agar, topped with 10 mL of SS buffer and 100 µL of stationary phase culture of *Klebsiella pneumoniae*. The suspension was mixed well by pipetting, and the plates were wrapped in foil and incubated at 22°C for 2, 4 or 6 weeks prior to the germination treatments. The entire experiment was carried out using a complete block design, with all five pairs tested across all three maturation periods and all four germination methods per block. The entire experiment was then repeated three times independently, with each block initiated from frozen spore stocks.

3.2.3 Harvesting macrocysts

Following maturation, macrocysts were collected from the LP agar plates into 15 mL Falcon tubes. This process involved transferring the 10 mL of SS buffer overlay and then washing the agar surface with an additional 5 mL of SS buffer, for a total of 15 mL SS buffer.

3.2.4 Washing macrocysts

During the macrocyst production process, some spores fail to germinate into amoebae and linger in the media. These spores mix with amoebae which do not incorporate into macrocysts and form a sticky mucus-like substance. Many macrocysts will have this mucus clinging to their exterior walls, and so must be cleaned before germination to avoid contaminating the macrocyst progeny with unwanted cells.

To achieve this, the macrocysts were heat-shocked at 45°C in a water bath for 30 minutes to induce germination of any leftover spores. The tubes were then incubated overnight at 22°C. The following day, the macrocysts were cleaned according to the method of Wallace and Raper (Wallace & Raper, 1979). The macrocysts were centrifuged at 300 x g for 3 minutes at 4°C, the supernatant was decanted and discarded, and the macrocyst

pellet was resuspended in 5mL SS buffer before being mixed via pipetting. This was repeated a further two times, for a total of three SS buffer washes.

After the final SS buffer wash, macrocysts were resuspended in 10 mL of 0.005% SDS and transferred to 50 mL falcon tubes. The tubes were incubated at 22°C with shaking at 180 rpm for 3.5 hours, after which the shaking was turned off and the macrocysts were incubated overnight at 22°C.

The following day, when the macrocysts, which form large clumps visible by eye, had settled, the supernatant was decanted and discarded, being careful not to disturb the macrocyst 'pellet'. The macrocysts were resuspended in 5 mL SS buffer and mixed by pipetting. The mixture was then centrifuged at 300 x g for 3 minutes at 4°C, and the supernatant again decanted and discarded. This process of resuspension, mixing, centrifugation and decanting was repeated for a total of three washes before the macrocyst pellets were resuspended in 10 mL 25mM EDTA (pH 7.1) and left shaking at 180 rpm for 150 minutes at 22°C. Tubes were centrifuged at 300 x g for 3 minutes at 4°C, and the supernatant once again decanted and discarded. The macrocyst pellet was then resuspended in 5 mL 0.0025% streptomycin solution (250 g/L streptomycin sulfate salt) and incubated overnight at 22°C.

Finally, suspensions were centrifuged at 300 x g for 3 minutes at 4°C with the supernatant being decanted and discarded. The remaining macrocyst pellet was resuspended in 5 mL SS buffer, mixed via pipetting, and centrifuged at 300 x g for 3 minutes at 4°C again. This was repeated for a total of three washes before the macrocysts were transferred to 2 mL Eppendorf tubes. Images were taken of each in a 96-well plate at 50x and 100x magnification before any treatments were performed to check for remaining spores and amoebae.

3.2.5 Germination Treatments

Glass Beads - Macrocysts to be bead-treated were centrifuged at 300 x g for 3 minutes at 4°C. The supernatant was then decanted and discarded, and the 2 mL Eppendorf tubes filled to the 0.5 mL mark with 2 mm sterile glass beads. The tubes were then filled with

0.0025% streptomycin solution until the beads were barely covered and vortexed at max speed for eight fifteen-second bursts, giving a short pause between each. This 2 mm bead vortexing mechanically ruptures the macrocysts and releases progeny amoebae into the solution.

The resulting solution was transferred to a 96-well plate and imaged at 50x, 100x and 200x magnification. After imaging, the solution was transferred back to 2 mL Eppendorf tubes and centrifuged at 450 x g for 3 minutes at 4°C. The supernatant was carefully decanted until only a small amount of liquid remained (~50-100 µL) and resuspended via pipetting. This step was very carefully performed as at this stage the pellet contains progeny amoebae from the ruptured macrocysts. The full amount of remaining liquid was then transferred to a 24-well plate with 1 mL streptomycin agar (250 g/L streptomycin sulfate salt, 15 g/L agar) per well and incubated for two weeks at room temperature in ambient light levels.

Freeze-thaw cycles - Macrocysts to be freeze/thawed were centrifuged at 300 x g for 3 minutes at 4°C and the supernatant decanted and discarded. Macrocyst pellets were resuspended in 1 mL streptomycin solution and thoroughly mixed via pipetting. The tubes were then frozen at -20°C overnight and thawed at room temperature over a 2-hour period before being refrozen and thawed again the next day. Three freeze/thaw cycles were performed before centrifuging at 300 x g for 3 mins at 4°C, discarding the supernatant and resuspending in 50 µL streptomycin solution, mixing via pipette. Macrocysts were plated on a 24-well plate with 1 mL streptomycin agar per well, incubated at room temperature and ambient lighting for 11 days on the bench, for a total of 2 weeks since germination treatments began.

UV irradiation - Macrocysts to be irradiated were centrifuged at 300 x g for 3 minutes at 4°C and the supernatant discarded. Macrocysts were resuspended in 50 µL 0.0025% streptomycin solution, mixed by pipette and plated on a 24-well plate with 1 mL streptomycin agar per well. These were then enclosed in a cardboard box with a grow light (combination of blue and red LEDs of wavelengths 460-470nm and 620-630nm respectively) and incubated for 72 hours at room temperature. To ensure no other light entered the box, the box was taped shut. After the 72-hour incubation, macrocysts were

moved to the bench where they were incubated at room temperature and ambient lighting for 11 days, for a total of 2 weeks since germination treatments began.

Control - The control macrocysts were treated according to the Raper method (Wallace & Raper, 1979). The macrocysts were centrifuged at 300 x g for 3 minutes at 4°C with the supernatant decanted and discarded. The macrocysts were then resuspended in 50 µL 0.0025% streptomycin solution, mixed by pipetting and plated on a 24-well plate with 1 mL streptomycin agar per well. This 24-well plate was then incubated at room temperature and ambient lighting on the bench for two weeks.

After cleaning, the plates were incubated at room temperature on non-nutrient streptomycin agar after their respective germination treatments to allow for social development to occur. Ideally, after macrocysts had germinated and released progeny amoebae, the lack of available bacteria due to the antibiotic-infused media would immediately force the social developmental cycle to begin before any rounds of cell division could occur. This allowed for approximation of germination rates based on the number of visible fruiting bodies after allowing 2 weeks for macrocysts to germinate and fruiting bodies to develop.

3.2.6 Recording results

After two weeks of development on streptomycin agar, wells were inspected for fruiting bodies and macrocysts. An image was taken of each well and the number of visible macrocysts were manually counted using the Cell Counter plugin (https://imagej.net/ij/plugins/cell-counter.html) for ImageJ (Schneider et al., 2012). Fruiting bodies were counted by eye under a dissecting microscope.

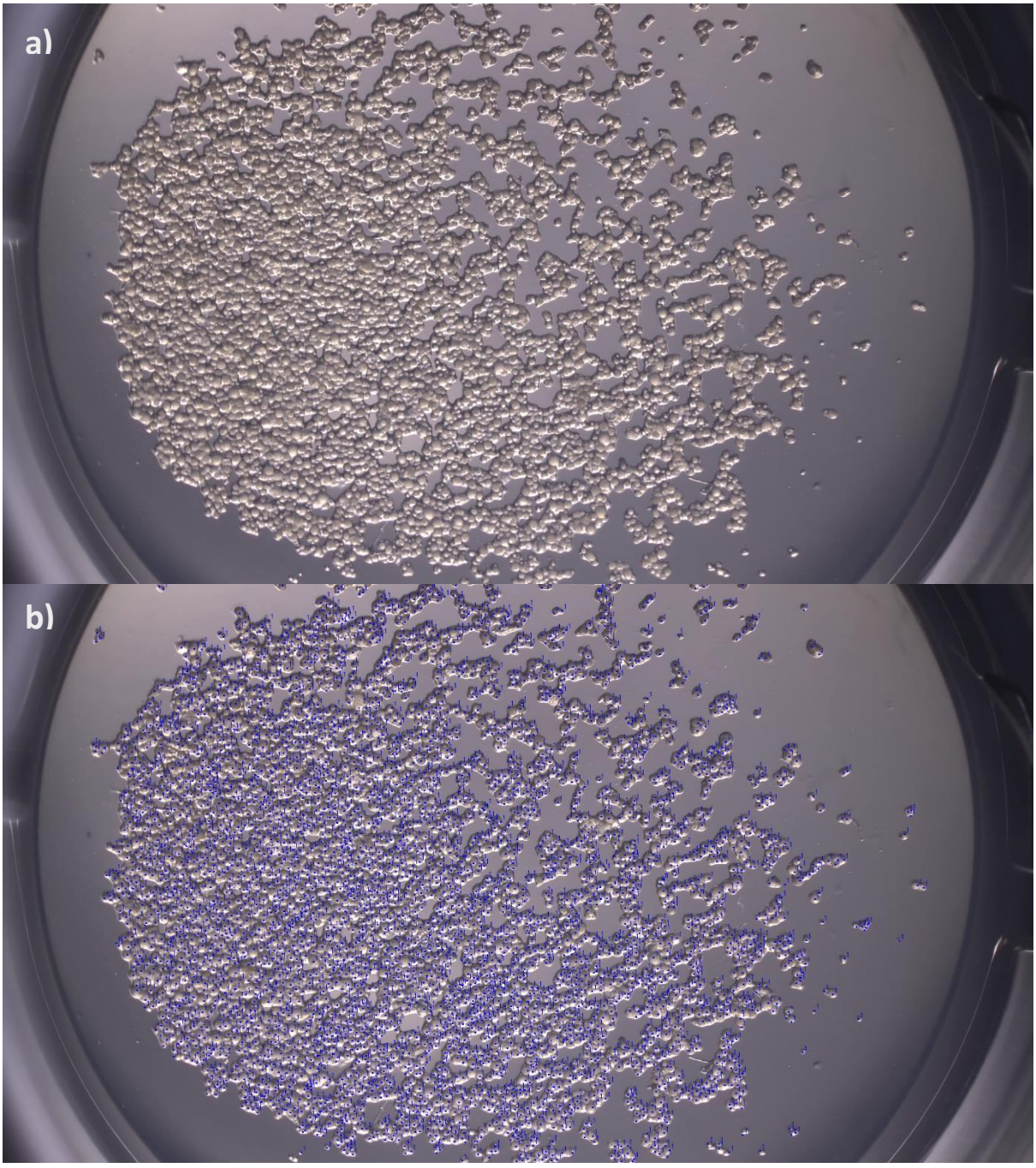

Figure 3.2 Macrocyst germination plates counting method. A: Strain pair 2 (EO603, EO604) replicate 3 week 6 macrocyst germination plate (90mm diameter) before being counted. B: The same image after manual counting using ImageJ's Cell Counter plugin (https://imagej.net/ij/plugins/cell-counter.html). Manual counting was employed to ensure a high degree of accuracy during the counting procedure.

3.3 Results

After macrocysts and fruiting bodies were counted for each timepoint and treatment, the results were analyzed using R to determine the resulting effects. Fig 3.4 shows the relative germination efficiencies of each treatment by timepoint, while Fig 3.5 shows the amount of macrocysts produced by each individual cross.

3.3.1 Statistical analysis

All analyses were performed using R Statistical Software (v4.3.2; R Core Team 2023). Further packages used in the analysis included dplyr (v1.1.4; Wickham et al. 2023), ggplot2 (v3.4.4, Wickham, 2016), tidyverse, lme4 (Bates et al., 2015) and lmerTest (Kuznetsova et al., 2017).

To investigate the influence of both fixed and random effects on macrocyst germination rates, I employed linear mixed models (LMM) using the lme4 (Bates et al., 2015) package in R. The fixed effects included treatment, timepoint and replicate while the random effect accounted for variability across separate crosses. This approach allowed me to appropriately model the structure of the data and control for intra-group correlations.

The linear mixed model was specified as:

$$fb \sim tp * treat * (1|pair) + rep$$

Where:

- *fb* is the number of observed fruiting bodies
- *tp* is the length of incubation as an ordinal variable; 2, 4, or 6 weeks
- *treat* is the treatment method as a categorical variable; beads, freeze-thaw cycles, grow light, or standard
- *pair* is the cross of strains as a random categorical variable
- *rep* is the replicate as a fixed categorical variable

The fixed effects of incubation time and treatment were hypothesized to have a significant effect upon the number of fruiting bodies observed, however ANOVA analysis of the linear mixed model revealed that neither incubation time nor treatment produced any statistically significant effects.

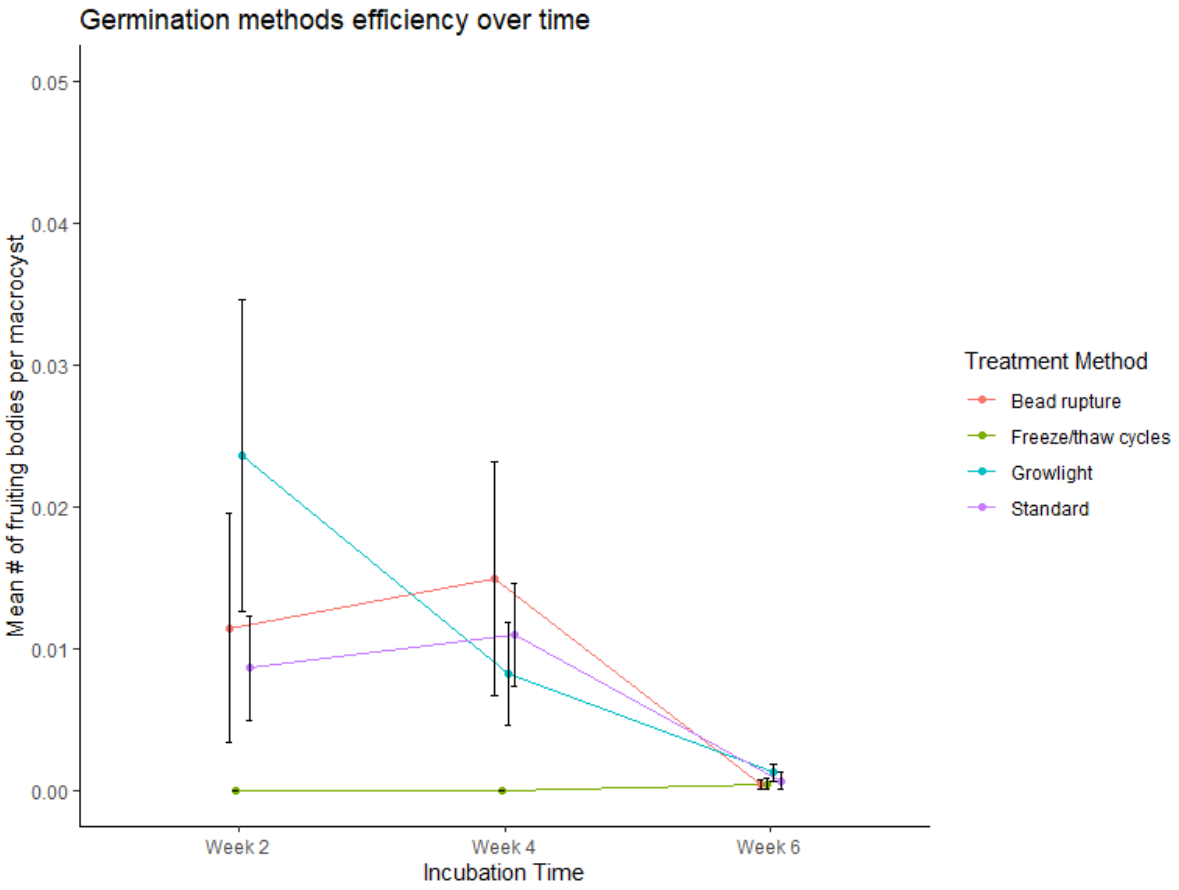

Figure 3.3 Mean germination efficiency over time by treatment. Germination efficiency was measured through proxy using the number of observed fruiting bodies per macrocyst plated after two weeks of incubation at room temperature and ambient lighting. Four treatment methods were tested; mechanical rupturing of macrocysts via 2mm beads, repeated freeze/thaw cycles, extended exposure to a grow light and the standard Raper method. Each point represents data from three replicates. Error bars represent the standard error for each mean.

3.3.2 Macrocyst production varies between crosses

After macrocysts were counted, it became clear that cross 1 (EO56 crossed with EO57) produced a significantly higher number of macrocysts than any other cross (Fig 3.5, 3.6). This high macrocyst production cross results in far more numerous, but much smaller macrocysts than those produced by other crosses (Fig 3.6). However, analysis of the linear mixed model indicated that this increased macrocyst production did not correlate with any increase in macrocyst germination efficiency.

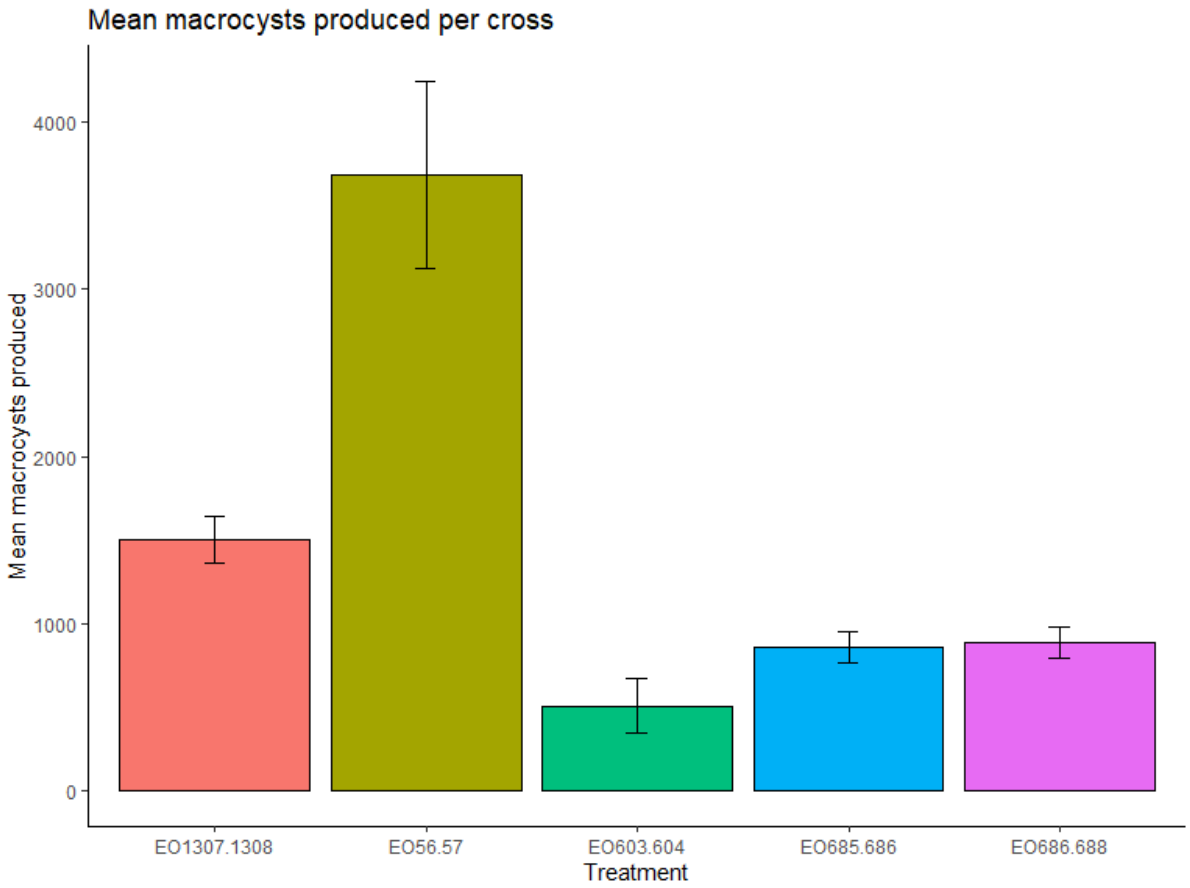

Figure 3.4 Mean macrocysts produced per cross. Bar chart showing the mean number of macrocysts produced for each cross in the experiment. EO56.57 exhibited a significant increase in macrocyst production compared to other crosses. Error bars show standard error.

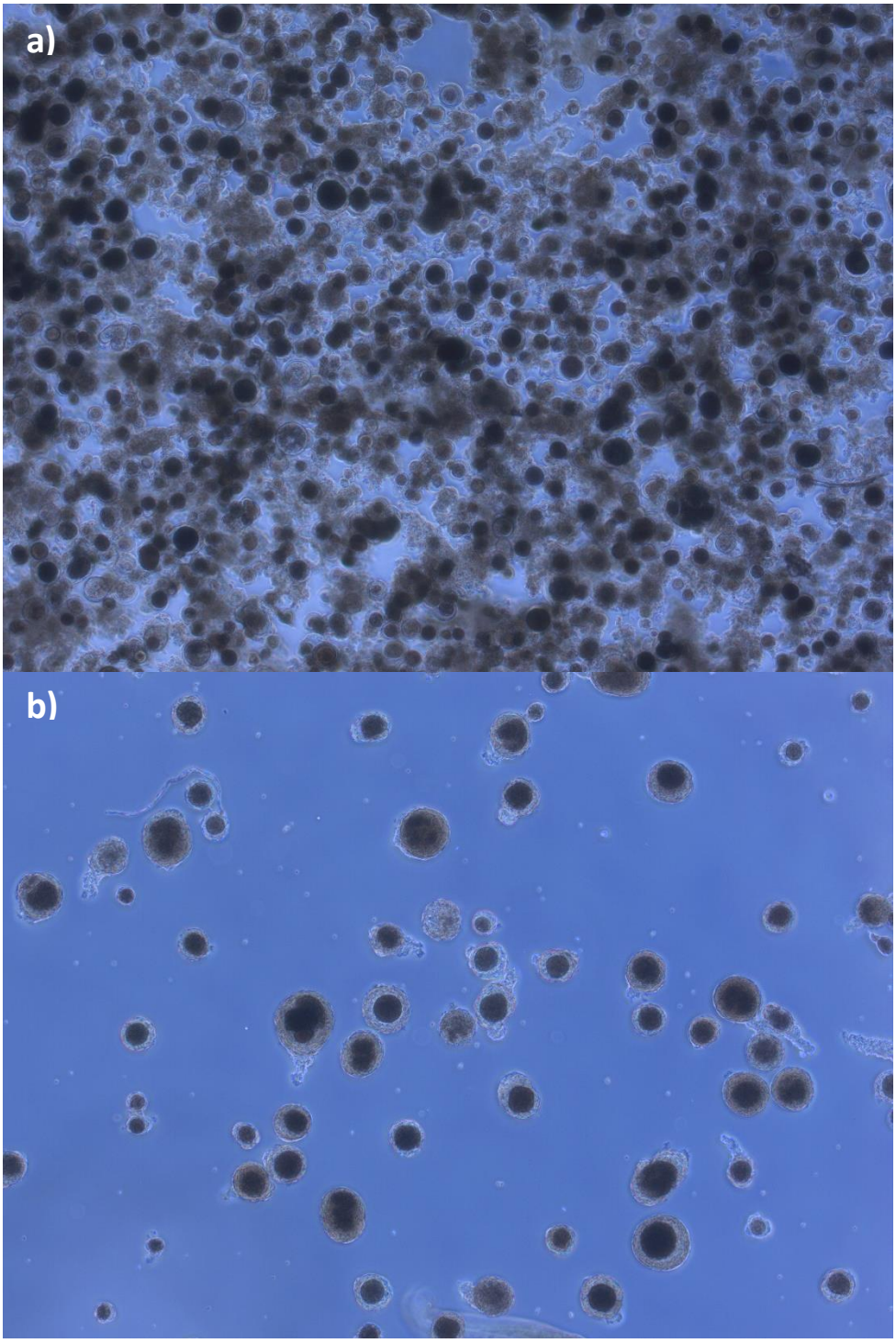

Figure 3.5 Comparison between a high production cross and regular cross. A: High production strain pair 1 (EO56, EO57) macrocysts, replicate three after 6 weeks of incubation, showing vast quantities of macrocysts. B: Regular strain pair 4 (EO686, EO688) macrocysts, replicate three after 6 weeks of incubation showing larger, but much fewer macrocysts than the super producer. Both images were taken in 24 well plates (16.5mm well diameter) at 50x magnification, intended solely for visual size comparison. Both groups were initiated under the same starting conditions.

3.4 Discussion

3.4.1 Low germination rates

All tested treatments failed to produce any significantly higher germination rates than those previously described in literature (Bloomfield et al., 2010; Francis, 1998; Francis & Eisenberg, 1993; Raper, 1984; Wallace & Raper, 1979). This suggests that all treatments had a negligible effect upon germination rates, apart from the freeze-thaw treatment which failed to produce almost any fruiting bodies.

3.4.2 Freeze-thaw treatment has a negative impact on germination rate

Macrocysts often appear entirely impenetrable to outside stimulus due to their sturdy cellulose walls. Whilst these defences provide exceptional hardiness, even to the point of rendering them immune to some detergents (e.g. NP-40), these same walls may also be the driving factor behind the low germination rates observed in the sexual cycle of *Dictyostelium discoideum*. *D. discoideum* macrocysts display very similar behaviour to that of hard-seeds produced by some plant-life. These plants produce seeds with exceptionally tough outer shells which are water-impenetrable and thus become unable to germinate despite favourable conditions (Acharya et al., 1993; Shibata et al., 1995). Thus, to investigate possible methods of inducing germination in macrocysts, I turned to methods known to increase the germination rates of such hard-seeded plants, namely repeated freeze-thaw cycles (Acharya et al., 1993; Shibata et al., 1995). In such plants, repeated freeze-thaw cycles produce ice crystals which degrade and breach the protective outer wall of these seeds, allowing water to permeate into the seeds resulting in their subsequent germination (Shibata et al., 1995). Ideally, a similar mechanism may encourage the germination of *Dictyostelium discoideum* macrocysts through degradation of the outer macrocyst walls, enabling a better perception of environmental conditions.

Investigation into the efficacy of repeated freeze-thaw cycling as a means to enhance the germination of *Dictyostelium discoideum* macrocysts yielded insightful, although somewhat disappointing results. Contrary to the hypothesis that freeze-thaw cycling

would facilitate a greater degree of macrocyst germination, I found that this treatment rendered macrocysts almost entirely unable to germinate (Fig 3.4). While this effect was not statistically significant, this treatment stood alone as the only treatment to produce almost no fruiting bodies whatsoever. This outcome suggests that the freeze-thaw process and subsequent formation of ice crystals may inflict irreparable damage to critical biological processes essential for germination.

3.4.3 Notable germination efficiency decrease past six weeks of incubation

Previous research on the topic of macrocyst germination has shown that germination rates increase with time (Abe & Maeda, 1986), however my results indicate that the optimal macrocyst incubation period is between 2-4 weeks. At 6 weeks of incubation all germination treatments produced almost no fruiting bodies, a result consistent across all replicates.

However, other experiments involving *Dictyostelium discoideum* macrocysts often incubate for >6 weeks and leave them for 2-4 weeks to germinate without any indication of significantly decreased germination rates (Bloomfield et al., 2010; Francis & Eisenberg, 1993; Wallace & Raper, 1979). One possibility is that as incubation time increases, so too does the period over which amoebae emerge. Fruiting bodies were counted 2 weeks after plating macrocysts on the germination media – if any fruiting bodies were formed past this point, they were not included in the results.

3.4.4 High macrocyst production cross

The discovery of a highly compatible cross in this experiment may prove invaluable to future research requiring the use of *Dictyostelium discoideum* macrocysts. One consideration when speculating the cause of the observed macrocyst production rates is that both strains from this cross originated from the east coast of the United States as opposed to the west coast for all other crosses. Geographical distance has been shown to have large impacts on genetic distance due to reductions in gene flow (Hutchison &

Templeton, 1999; Slatkin, 1993; Wright, 1943), and pronounced genetic differentiation between strains of *Dictyostelium discoideum* isolated from differing locations has been shown previously (Douglas et al., 2011). Although full genome sequencing of these strains was beyond the scope of this project, it may prove useful for determining the exact cause of the drastic increase in macrocyst production displayed by this cross. Identification of genetic variations associated with increased macrocyst production could lead to greater usage of the *Dictyostelium* sexual cycle in future research. Of particular interest is the *mat* locus responsible for mating compatibility in *Dictyostelium discoideum*.

Chapter 4: Simulation

4.1 Introduction

Experimental evolution can be immensely useful for investigating the dynamics of evolution in real time. Some experimental evolution studies begin with populations polymorphic at a specific locus and measure changes in allele frequencies (Murray & Cutter, 2011), while others start with identical populations and examine *de novo* mutations generated under experimental conditions (Khare et al., 2009; Lenski et al., 1991; Levin et al., 2015). In both scenarios, what can be learned from such experiments is not limited by existing preconceptions, and completely novel insights are able to be gleaned. Whether through the identification of important genes, new protein structures, or alternate pathways through which a trait can be acquired, experimental evolution carries the promise of exciting and potentially unexpected results.

However, experimental evolution has some significant limitations. Propagation of large numbers of populations under different conditions can be labor-intensive or prohibitive, while the long time periods required to observe evolutionary processes can limit how many factors can be examined or varied within a single study. For example, in my long-term evolution experiment, I induced the sexual cycle after 10 developmental cycles. This length was arbitrarily selected – would intervals of 8 or 12 cycles yield different results? To test this question empirically would require three times as many populations, which was not feasible. Similarly, I was limited to a single type of medium, a single pair of mating types, a single population size, method of germination, and maturation time. All of these decisions potentially impact the outcomes of the experiments, but varying them all is not feasible.

Mathematical models and computer simulations can serve as a useful guide for examining how a large number of parameters might affect an evolutionary outcome. As major factors that influence adaptation can be readily varied (e.g., population sizes, mutation rates, etc.), these approaches offer researchers a glimpse into the possible outcomes, which can shape and focus future empirical studies. Indeed, models and simulations have frequently been used to examine co-evolutionary dynamics and bridge

the gap between theory and empirical studies (Dieckmann & Law, 1996; Dobata, 2012; Kopp & Gavrilets, 2006; Liu et al., 2022; Nuismer & Doebeli, 2004).

Some models focus entirely on mathematical predictions, such the one presented by Dobata, who presents quantitative mathematical model predicting the consequences of caste-fate conflict and potential arms races in eusocial Hymenoptera (Dobata, 2012). Doabata's model examines two traits – those expressed in larva and those expressed in workers that impact whether a specific larva develops into a queen. However, as individuals are not simulated, this model stops short of investigating the underlying individual genes and genotypes that cause the modelled phenotypes.

Other models include both mathematical and simulation components, allowing examination of the underlying genes contributing to a given trait. For example, Liu and colleagues present both a mathematical model and accompanying simulation to investigate the mechanisms contributing to the evolution of manipulative cheaters (Liu et al., 2022). This model reveals oscillating coevolutionary dynamics between manipulative traits and the suppression strategies used to counter them. Interestingly, they found that the addition of recombination to their model severely impacted their results, eliminating oscillation entirely. Where this model incorporates both individual simulation and multiple avenues of manipulation and suppression, it does not include the simulation of epistasis between strategies.

Given the limitations of my experimental approaches to determining how sexual reproduction influences a coevolutionary arms race (Chapter 2), I decided to explore these topics using a simulation where I could vary key factors of interest, including: population size, frequency and efficiency of sexual reproduction, cheating and resistance mutations, and associated costs of these behaviors for other fitness components, such as germination or sporulation.

Briefly, I simulate a population of amoebae that can undergo asexual cell division and multicellular development, with or without bouts of sexual reproduction. As opposed to more general models, integrating the specific biology of this organism allows for model predictions to be easily observed and tested empirically in the laboratory.

Genotypes are simulated at the individual level using a matching alleles model (Gomulkiewicz et al., 2003; Luijckx et al., 2013). Specifically, an individual's genotype consists of several 'gene-pairs' (Fig 4.2). Each gene-pair consists of a cheating locus (with a cheating and non-cheating allele) and complementary resistance locus (with a resistance and non-resistance allele). Nullifying epistasis is able to be toggled within the simulation, dictating the phenotypic effects of carrying both cheating and resistance alleles in a single genetic background.

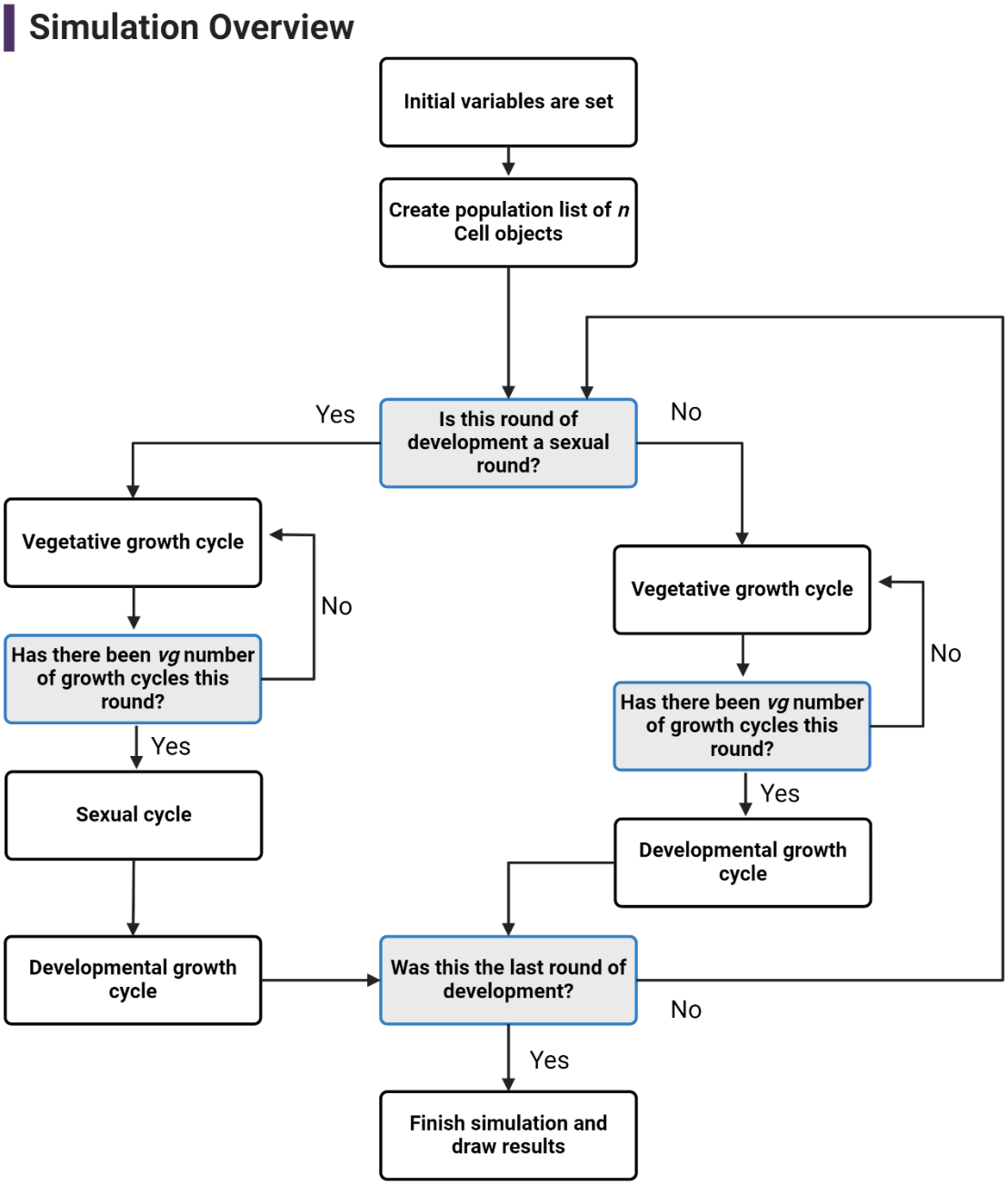

Figure 4.1 Flowchart overview of simulation functions. After initial variables are set a new list is populated with *n* Cell objects to be passed to various functions. This population then enters a loop of vegetative growth, sexual and developmental cycles before completing and producing a graph of the results. Made with BioRender.com

4.2 Model Overview

4.2.1 Dependencies

The simulation source code and generated data files used to produce figures are available from https://github.com/PrimodialSoup/DictyosteliumCheaterSimulation. The python packages NumPy (Harris et al., 2020), matplotlib (Hunter, 2007), SciPy (Virtanen et al., 2020) are required to run this program. NumPy was used for various mathematical functions throughout the simulation, while SciPy was used to calculate confidence intervals and other statistics. Matplotlib is used for plots and other visual representations of the data once the simulation concludes.

4.2.2 The Cell class

Each individual is haploid and represented by a Cell class object. This object has three properties: genotype, mating type, and fitness.

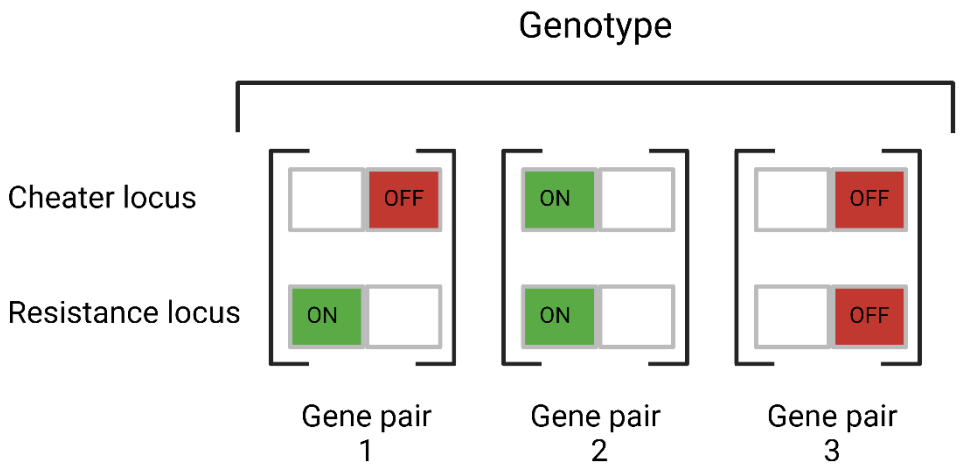

Figure 4.2 Genotype property. The genotype property of all Cell objects contains a list of gene pair objects as shown. Each gene pair has two loci which are either on or off resulting in four total available states for each gene pair object – Resistor only, cheater only, wild-type or both cheater and resistor loci active. Made with BioRender.com

The genotype property is represented by a list of gene pair objects (Fig 4.2). Each gene pair consists of a cheating locus and a resistance locus, which can be in either the active or inactive state. This leads to four possible states for each gene pair – both loci inactive (wild-type at both loci), only the cheater locus is active (cheater allele, but no cognate resistance allele), only the resistance locus active, or both loci active. These four states are represented by the integers 0, 1, 2, and 3, respectively.

The mating type property of the cell class is represented by the integers 1, 2 or 3, representing *Dictyostelium*'s mating types I, II, or III, respectively. The mating type value is used determine the compatible pairings of cells during the sexual cycle. Each mating type can only partner with a cell of a different mating type. For example, a cell of mating type 1 can only form a macrocyst with a cell of mating type 2 or 3.

Finally, an individual's fitness property determines its probability of dividing during the vegetative growth cycle. This property is represented by a floating-point number between 0 and 1, with 0 indicating a cell is unable to divide and 1 indicating a cell has full division capability. Whether the cell does divide (if able to) is determined by its genotype and other variables that dictate the various fitness costs of each genotype. By default, all cells will start with a fitness value of 1 and costs are then applied, lowering the value.

4.2.3 Population initialization

The list of parameters is provided in Table 4.1. The simulation starts by initializing the population. First, a list is populated with *n* cells. The mating types are assigned randomly to cells in the proportions specified by *mt_1*, *mt_2*, and *mt_3*. Every cell is then assigned a genotype based on initial values (*ch_start* and *res_start*, the starting frequencies of cheating and resistance alleles). This list constitutes the overall population, which is passed to each function sequentially to simulate the three life stages of Dictyostelium discoideum: vegetative growth (asexual cell division), multicellular development (fruiting body formation), and sexual reproduction (macrocyst production; Fig. 1.2). All functions both take and return a list of cell objects. Therefore, functions can be called in any order, ensuring modularity.

Table 4.1 List of simulation variables

Variable name	Function	Default Value
n_runs	Number of runs to simulate	10
n_dev	Number of social development cycles to simulate per run	500
i_macrocyst	A list containing the cycles on which the sexual cycle is simulated. Incompatible with sex_cycle_interval	0
sex_cycle_interval	Number of developmental cycles between sexual cycles. Incompatible with i_macrocyst	0 (10 for runs with sexual cycles)
vg	Number of rounds of vegetative growth per developmental cycle	10
n	Total population size	10000
sl	Number of cells per slug formed in the developmental cycle	1000
sp	Fraction of spores per slug	0.8
m_ch	Mutation rate of the cheating allele in each gene pair	0.001
m_re	Mutation rate of the resistance allele in each gene pair	0.001
c_ch	Cost of the cheating allele	0.01
c_res	Cost of the resistance allele	0.01
ch_germ	Cheater spore germination rate penalty	0
ch_start	Starting cheating allele frequency	0
res_start	Starting resistance allele frequency	0
ch_res_dist	Whether cheating and resistance alleles are initially distributed randomly (1) or without co-occurrence (0)	1
ch_eff_rc	Cheater allele effectiveness when paired with complementary resistance allele (Boolean, 0 = non-functional, 1 = fully functional)	0
res_eff_rc	Resistance allele effectiveness when paired with complementary cheater allele (Boolean, 0 = non-functional, 1 = fully functional)	0
mc_count	Number of macrocysts formed per sexual cycle	100
mc_germ_count	Number of progeny cells per germinated macrocyst	100
recomb_chance	Chance of recombination	1
gene_pairs	Number of gene pairs to simulate	3
mt1_start	Proportion of mating-type 1 cells at simulation start	0.5
mt2_start	Proportion of mating-type 2 cells at simulation start	0.5
mt3_start	Proportion of mating-type 3 cells at simulation start	0

4.2.4 Vegetative growth function

At the start of the vegetative growth cycle, a fitness value is assigned to each cell based on its genotype, incorporating any costs associated with that genotype.

During the vegetative growth stage, *n* cells are sampled with replacement from the population, with the probability of being selected weighted by the fitness values. Prior to placing progeny in the population, each gene pair can mutate from the active state to the inactive state, or vice versa according to the parameters *m_ch* and *m_re*, the mutation rates at the cheating and resistance loci, respectively. The vegetative growth cycle is repeated *vg* times, at which point the population undergoes multicellular development.

4.2.5 Multicellular development

During multicellular development, I simulate the existence of mutations that generate manipulative, facultative cheaters. Briefly, a manipulative cheater is defined as a genotype that gains a numerical advantaged by inducing its partners to form the dead stalk. A facultative cheater adopts the spore fate when victims are present in the slug, but forms a normal-sized stalk when clonal (Strassmann et al., 2000). In *Dictyostelium*, obligate cheaters do not form the stalk, regardless of their partner strain. Thus, obligate cheaters produce stalkless fruiting bodies when they are present at high frequencies (Ennis et al., 2000), whilst facultative cheaters produce fruiting bodies of standard proportions, regardless of their frequency (Strassmann et al., 2000). In *Dictyostelium discoideum*, facultative cheating occurs readily in the lab and in nature (Buttery et al., 2009; Santorelli et al., 2008; Strassmann et al., 2000), and so I implemented this form of cheating in this model.

First, all cells in the population randomly aggregate into slugs of a fixed (*sl*) size. After a slug is formed, all cells in a slug have a chance to adopt the pre-stalk fate. This chance is configurable, but defaults to 20% in accordance with the 80-20% pre-spore-to-pre-stalk percentages observed in *Dictyostelium discoideum* slugs (Kessin, 2001). Any cells that are not selected to become pre-stalk are instead flagged as pre-spore. Not all pre-stalk cells become stalk cells; if they cheat (and their partner is not resistant), then another

individual will take their place. Each pre-stalk cell is first checked for cheating alleles at each gene-pair. If a designated pre-stalk cell is capable of cheating based on its genotype, a random cell is chosen from the pool of pre-spore cells and checked against the cheating pre-stalk cell. If the first cell is capable of exploiting the second cell (i.e., the first cell has a cheating allele and the second cell lacks the cognate resistance allele), then their fates are swapped: the first cell (which was originally a pre-stalk cell) becomes a spore, and the second cell (originally a pre-spore cell) becomes a stalk cell. Alternatively, if a pre-stalk cell is not capable of cheating or is not capable of cheating the second cell, it becomes a stalk cell as originally fated.

This implementation simulates the frequency-dependent selection that may be involved in the dynamics of cheats and cooperators (Adin Ross-Gillespie et al., 2007; Gore et al., 2009; Patel et al., 2019). Specifically, at high frequencies of resistors, cheaters are likely to encounter an unexploitable cell, eliminating their fitness benefit. Thus, cheaters have an advantage when rare. Similarly, resistors have the greatest fitness when they encounter cheaters they can resist – that is, when their cognate cheaters are common.

Furthermore, epistasis can be toggled for either locus in a gene pair using the *ch_eff_rc* and *res_eff_rc* variables (Table 4.1). When a locus has epistasis enabled, a cell which has both cheating and resistance alleles at a single gene pair loses the phenotypic effects of that allele. For example, when epistasis is enabled at both loci and a cell has both alleles, it will behave as a phenotypically wild-type cell incapable of both cheating and resisting.

This system simulates manipulative cheating, in that cheaters manipulate a vulnerable individual into performing a sub-optimal action that benefits the cheater. It also simulates facultative cheating, in that the cheating behavior changes depending on the composition of the population. Specifically, all fruiting bodies retain the 80:20 spore stalk ratio regardless of the number of cheaters present by ensuring that fate substitutions are 1:1.

4.2.6 Sexual cycle

Macrocyst formation and recombination are simulated by selecting a fixed number of random cells from the population to become starting cells for macrocysts. For each starting cell, a random cell of a different mating type from the remaining population is selected to become the partner cell for the sexual cycle. All other cells are removed, simulating the cannibalization of the surrounding cells by zygotes during macrocyst formation, as well as other causes of death of non-incorporated cells (O'Day, 1979; Wallace & Raper, 1979). These cells and their corresponding partners will become the parental cells of recombinant progeny.

From each parental pair, only a single progeny genotype is produced. This process mimics the real-life observations of *Dictyostelium* macrocysts, where each macrocyst releases recombinant progeny of a single genotype. In a population of compatible cells, however, different macrocysts will produce different genotypes, leading to genotypic diversity at the population level (Bloomfield et al., 2019; G. W. Erdos et al., 1975; Francis, 1998; Wallace & Raper, 1979). Depending on the value of *recomb_chance*, the probability of recombination, the progeny genotype will be either one of the parents' genotypes or it will undergo recombination, with all possible recombinant genotypes being equally likely. The progeny cell undergoes asexual cell division before germination and release of the cells, which are added to a new population. The new population is then passed to the developmental cycle function. This process simulates what is observed during macrocyst germination in the lab: each macrocysts releases recombinant progeny, which immediately aggregate and form fruiting bodies.

4.4 Results

This model was run under various parameter settings to investigate the role of each variable on the maintenance or fixation of cheating and resistance alleles (Supplementary Figs 1.3 – 1.5). Each run consisted of 500 developmental cycles with 10 rounds of asexual cell division per development cycle—thus, comprising 5,000 generations.

Each figure depicts the frequency of cheater and resistor alleles as a proportion of the total possible alleles, summed over all loci. For example, a value of 1.0 means that every cell in the population has an active allele at every gene pair, while a 0.33 value indicates 33% of the loci in the entire population have an active-form allele. Note that this does not mean that every allele is *phenotypically* active. For example, when epistasis is enabled, some of these alleles do not confer their encoded effects, owing to the presence of an allele at a different locus.

4.4.1 Cheating selects for resistance to cheating

Laboratory experiments with *Dictyostelium* have shown that the presence of cheaters selects for resistors (Hollis, 2012; Khare et al., 2009; Levin et al., 2015; Miller et al., 2023), and indeed this model indicates the same (Fig 4.1). Fig 4.1 shows the results of the model when both alleles are allowed to mutate (solid lines) or only cheating or resistance alleles alone are allowed to mutate (dashed lines) with otherwise default parameters. This results in populations with both alleles present in the population or either of the alleles by themselves, and allows us to observe whether reciprocal selection occurs. When only resistors are present in the population, the allele frequency stabilizes at ~20%. This baseline frequency level is a product of the chosen mutation rate and vegetative growth cost for the allele (Supplementary Fig 1.1, 1.2). However, the presence of cheater alleles in the population selects for the evolution of resistor alleles, driving the stable frequency up to ~40%. Conversely, a population with only cheater alleles present quickly results in near fixation of the allele – introducing resistor mutations prevents this meteoric frequency increase and lowers the cheater allele stable frequency to ~50%.

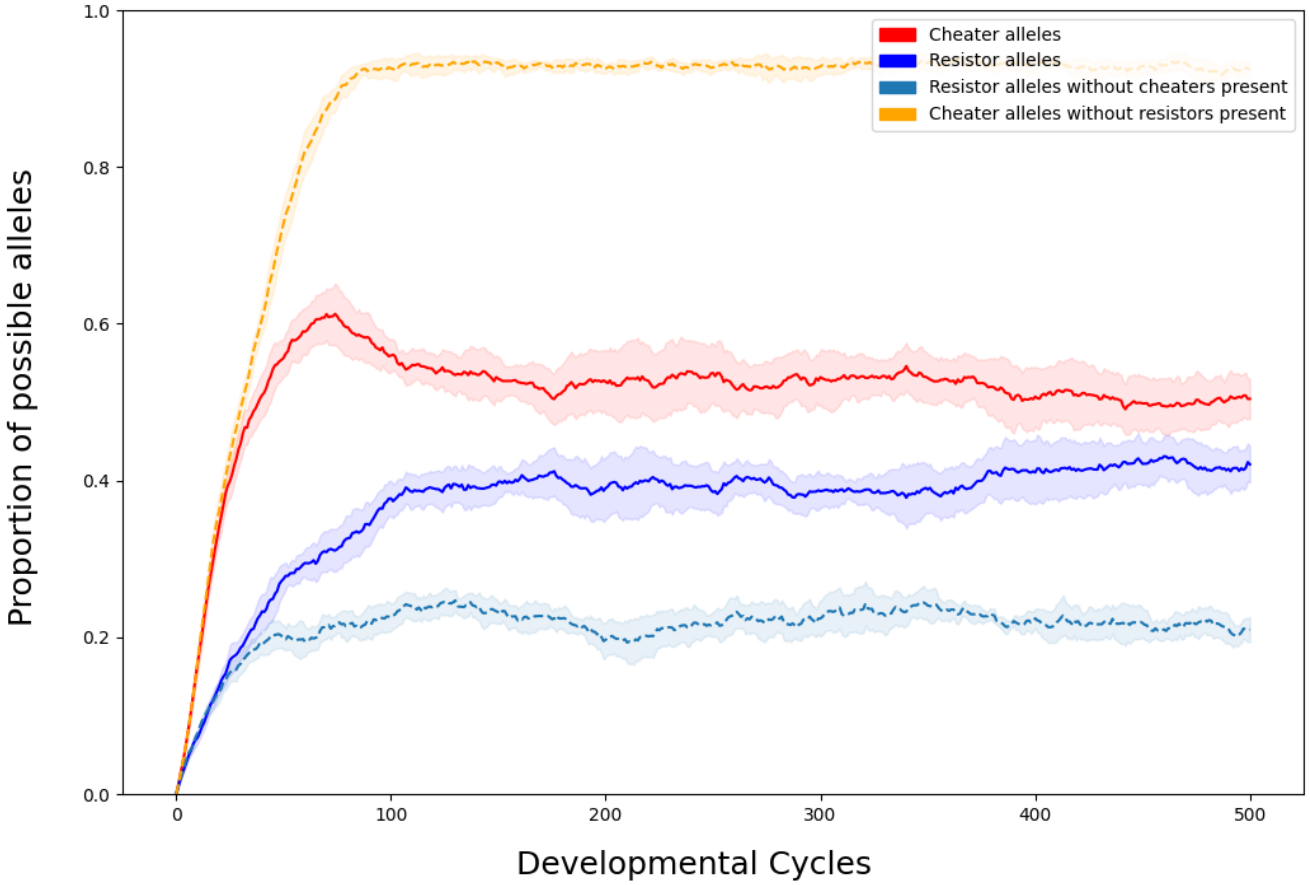

Figure 4.3 Cheater alleles increase the frequency of resistor alleles. All simulations were run with default values (see Table 4.1), unless otherwise stated. Lines indicate the mean value across 10 runs, and shading indicates the 95% confidence interval. Solid lines indicate a run with full default parameters, while dashed lines indicate a run where only a single set of alleles were allowed to evolve. Resistor alleles are present at significantly higher frequencies in populations where cheater alleles are present than where they are not (teal dotted line vs blue solid line).

4.4.2 Sexual cycles maintain mating types at equal frequencies

A macrocyst is created by the fusion of two cells of compatible mating types (Bloomfield et al., 2010). The resulting progeny of this macrocyst will always carry one of the two parental mating types, regardless of whether recombination has occurred. Therefore, in a two-mating-type experiment where only the macrocyst progeny are used to seed the next generation, the mating types present should return to a roughly 1:1 ratio after each sexual cycle (Douglas et al., 2016; Fisher, 1930; Hamilton, 1967). This is shown to be the case in Fig 4.4.

Fig 4.4a shows that without periodic recombination, mating types are selectively neutral and therefore deviate from the starting ratio owing to genetic drift. Fig 4.4b shows that, when starting with two mating types in a 1:1 ratio and a recombination rate of 100%, each round of sexual reproduction returns the mating type proportions back to 1:1. This is also the case in Fig 4.4c, where the recombination rate was set to 0%. Specifically, the mating types return to equal frequencies after each sexual cycle regardless of whether recombination occurred. The mating types in this model are thus under balancing selection when sexual cycles occur, as shown previously in natural isolates of *Dictyostelium discoideum* (Douglas et al., 2016).

4.4.3 Epistasis between alleles strongly affects stable equilibrium

Figure 4.5 shows one potential outcome of a coevolutionary arms race: the stable equilibrium of the coevolving parties (Dawkins & Krebs, 1979). In this scenario, reciprocal selection results in an evolutionarily stable level of the coevolving alleles (Fig 4.5, Supplementary figure 1.7). This model shows that whereas stable polymorphism of cheating and resistance alleles is achieved in the absence of recombination (Fig 4.5A), its addition alters this balance and results in a much higher frequency of cheater alleles when there are epistatic interactions between complementary alleles (Fig 4.5B).

Epistasis is critical to this arms race disruption (Fig 4.5B, Fig 4.5D). When recombination results in a cell with both cheater and resistor alleles at the same gene pair, epistasis renders the cell phenotypically wild-type (i.e., according to the selected epistasis rules,

a C-R double mutant will be phenotypically wild-type). This process thus re-generates exploitable cells, which selects for cheating alleles, allowing them to increase to higher frequencies. Thus, the impact of sexual reproduction on the frequencies of cheating and resistance alleles depends critically on the phenotypic effect that the alleles have in the same genetic background – that is, on the form and extent of epistasis.

Figure 4.6 shows that recombination is the source of the divergence between the coevolving alleles. In Figure 4.6, we can observe that repeated sexual cycles are not sufficient to cause significant divergence in the absence of recombination – in other words, divergence is not a side-effect of the population bottlenecks that occur concomitant with the sexual cycle. Instead, as the probability of recombination increases, so too, does the divergence, indicating that recombination itself (and resulting epistasis) is the driving force behind the divergence in allele frequencies. While both the cheating and resistance loci maintain polymorphism in my simulations, I note that in small populations subject to greater stochasticity, the chance of fixation of cheating alleles (or loss of resistance alleles) is greatly increased by recombination, which would lead to loss of diversity.

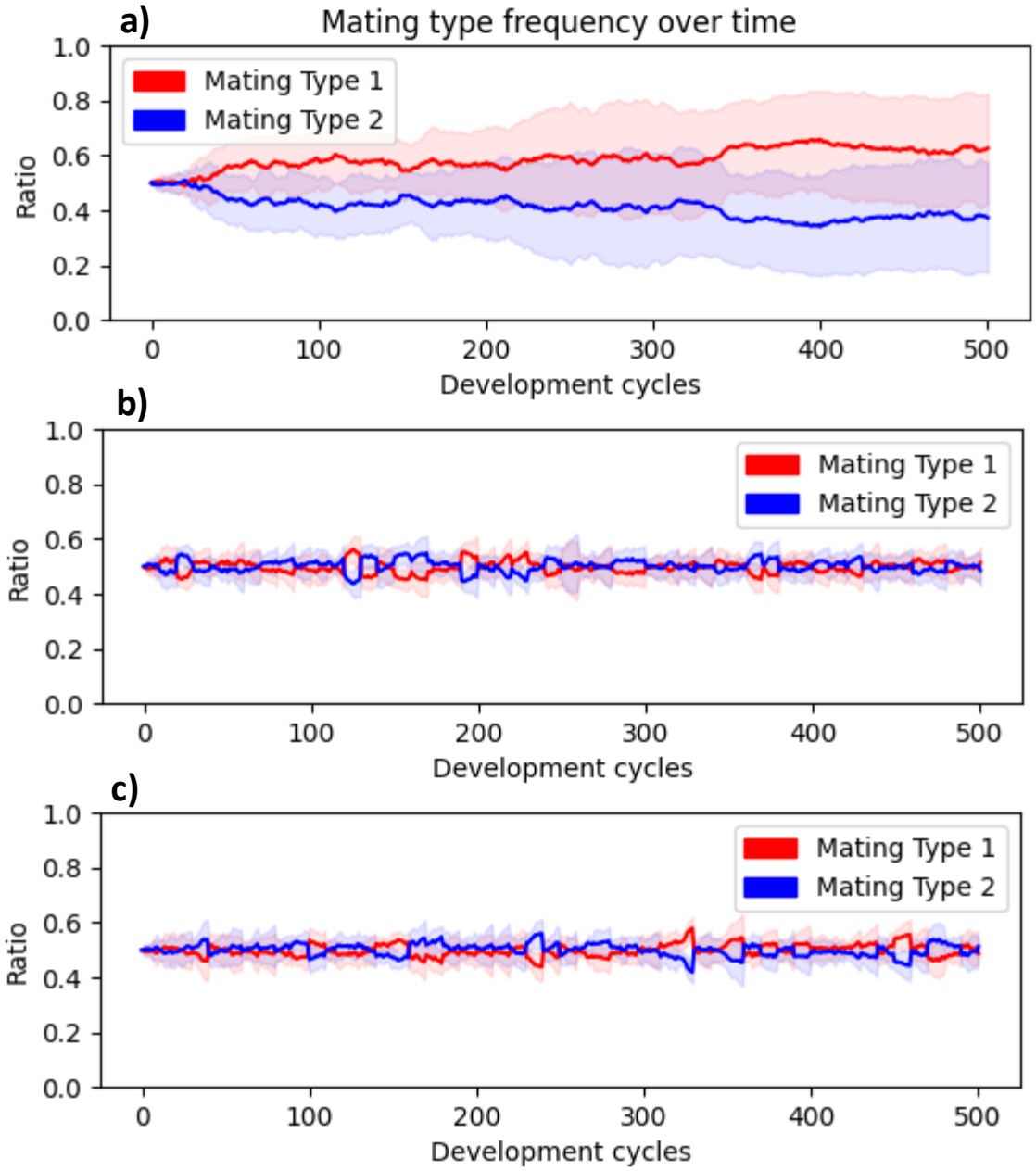

Figure 4.4 Balancing selection on the mating type locus. All simulations were run with default values, with sexual cycles every 10 developmental cycles. Lines represent the average of 10 runs. Mating types 1 (blue) and 2 (red) are initiated at a 1:1 ratio. Error bars indicate the 95% confidence interval based on 10 runs. (A) When no sexual cycles occur, mating types are subject to drift and deviate from the 1:1 ratio, indicated by the increasingly large error bars. (B) When the recombination rate is set to 100% (i.e., all progenies are recombinant), the mating types are maintained at equal frequencies. (C) When the recombination rate is set to 0% (i.e., all progenies are an identical copy of one parent), the same pattern is observed. This result demonstrates that the key feature for maintenance of the two mating types is that survivors have a 50% chance of being one mating type or the other.

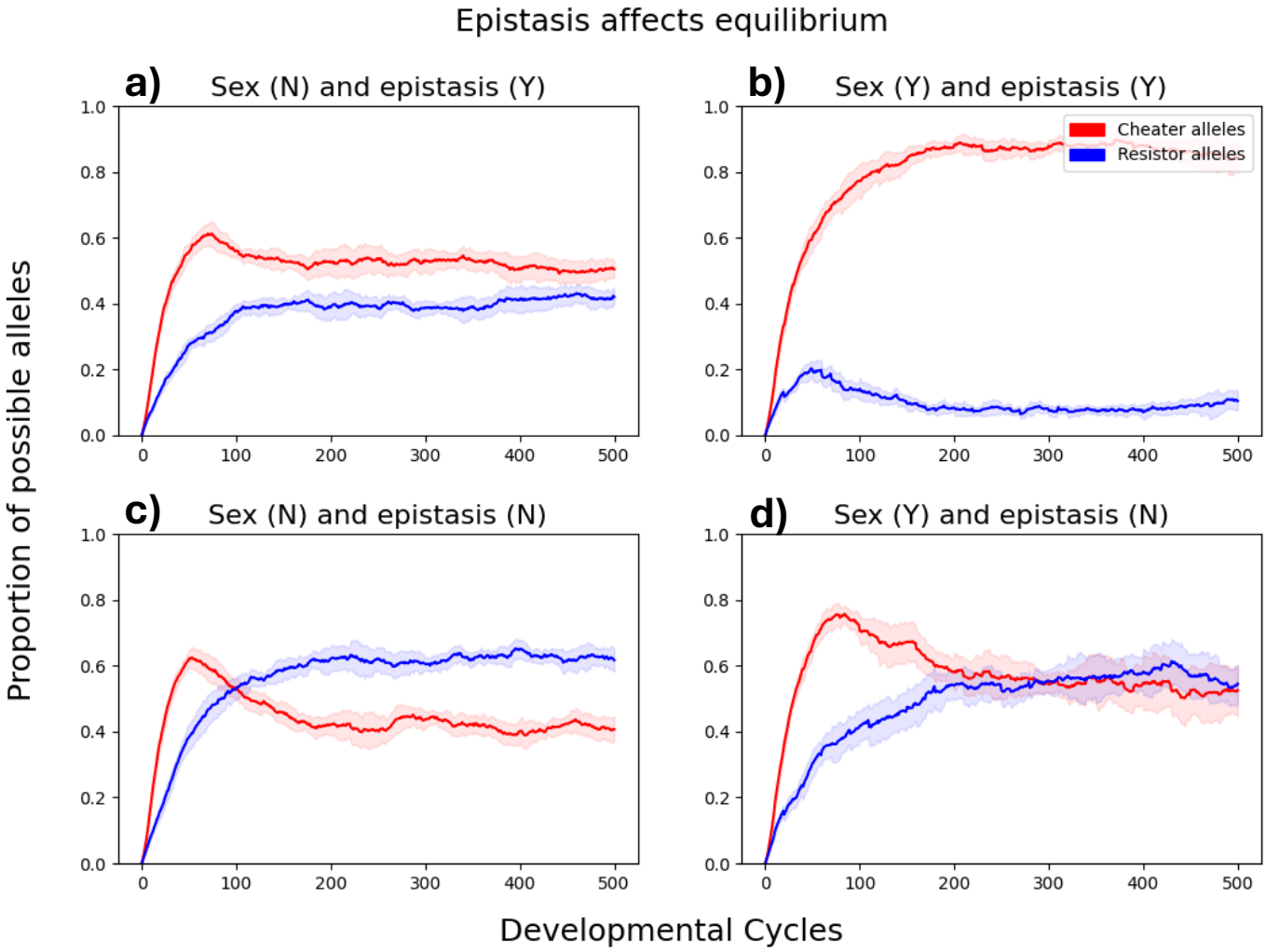

Figure 4.5 Epistasis has a substantial effect on equilibrium. All simulations were run with default values unless otherwise stated. Graphed lines indicate the mean value of 10 runs, while the shaded areas surrounding each line indicate a 95% confidence interval. (A) Standard run with all default values, showing an initial spike in cheater allele frequency but quickly transitioning into a stable equilibrium. (B) A run with the same parameters, except with sexual cycles occurring every 10th developmental cycle. Instead of transitioning to a ~0.5 cheater, ~0.4 resistor allele equilibrium, cheater allele frequency continues to climb and stabilizes at a much higher value while resistor allele frequency drops and stabilizes at a much lower value. (C) A run with default parameters except epistasis is turned off. This results in an almost identical equilibrium as when epistasis is active, except that the stable values for cheaters and resistors are reversed. (D) Default values, with a sexual cycle every 10th developmental cycle and epistasis disabled. Equilibrium is maintained similarly to when sexual cycles are not included, but with equilibrium being achieved at closer to 0.5, 0.5 cheater to resistor allele frequencies.

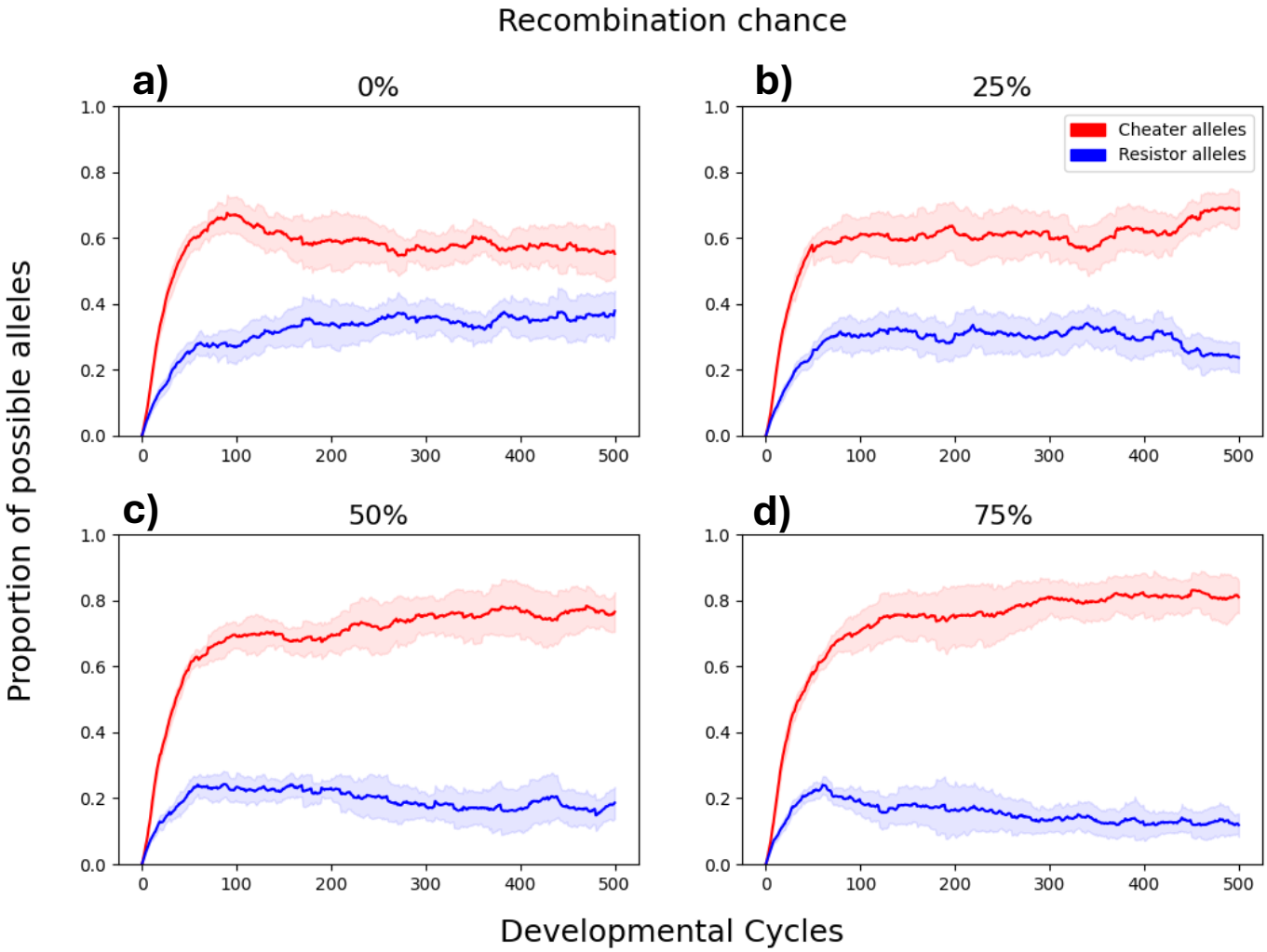

Figure 4.6 Higher probability of recombination results in a stronger divergence of coevolving alleles. All simulations were run with default values unless otherwise stated. Lines indicate the mean of 10 runs, and shading indicates the 95% confidence interval. Sexual cycles were initiated every 10 developmental cycles. (A) 0% recombination rate showing stable equilibrium between cheater and resistor alleles. This indicates that the sexual cycle itself has little effect on the divergence of cheater and resistor alleles. (B) 25% recombination rate. (C) 50% recombination chance. (D) 75% recombination rate. As the probability of recombination increases, cheating alleles increase in frequency and resistance alleles decrease in frequency.

4.5 Discussion

4.5.1 The role of epistasis in maintaining cooperation

If resistance and cheating mutations affect the same genetic pathway, epistatic effects may occur, where the combined impact of both mutations together differs from their individual effects (de Visser et al., 2011; Wolf et al., 2000). When this occurs in an individual, it is referred to as intragenomic epistasis; interactions between genes in the *same* genetic background alter the genes phenotypic effects. Intergenomic epistasis is also a possibility; interactions between genes in separate genomes may impact what effects a particular gene has. This model simulates both forms of epistasis – the resistor phenotype interferes with the expression of the cheater phenotype in its social partner, while co-occurrence of both alleles in a single genome can nullify both, one or the other, or neither of the alleles phenotypic effects (depending on the chosen variables).

Epistasis is especially important in *Dictyostelium*, which has a facultative form of sexual reproduction. As sexual reproduction is optional in this species, cheating and resistance strategies are able to coevolve during periods where sexual reproduction is not taking place. The initiation of the sexual cycle after such periods can thus lead to strong epistatic effects not seen in obligate sexual reproducers, as conflicting alleles may not have been present in the same genetic background for some generations.

In my simulations, I found that nullifying epistasis results in higher frequencies of cheating alleles and lower frequencies of resistance alleles (Fig 4.5, 4.6). Furthermore, only nullification of the resistance allele is required to produce this effect (Supplementary Fig 1.8). Epistasis is not caused by recombination—for example, a sufficiently high mutation rate will also create new combinations of alleles that have a similar, albeit smaller effect (Fig 4.5A, Fig 4.5C). However, these results show that occasional recombination, in the presence of antagonistic epistasis, can be an important determinant of the dynamics of coevolutionary arms races within species.

Previous work has also shown that recombination may occur relatively frequently in natural populations of *Dictyostelium* (Flowers et al., 2010*)*. Flowers and colleagues sequenced 24 wild strains of *Dictyostelium discoideum* and observed a rapid decay of

linkage disequilibrium and the presence of recombinant strains, indicating that the sexual cycle is often utilized in this species (Flowers et al., 2010). Furthermore, they found that the observed pattern of linkage disequilibrium exhibited by this organism was very similar to those seen in other sexually reproducing species such as *Saccharomyces cerevisiae* and *Saccharomyces paradoxus*.

My results indicate that the evolutionarily stable strategy (ESS) under the simulated conditions was a stable equilibrium (Supplementary Fig 1.7). Negative frequency-dependent selection causes the benefits associated with both cheating and resistance alleles to decrease as their frequency increases. In the case of cheating alleles, this is because as cheaters increase in frequency in a population there are fewer victims to exploit. Resistance alleles, however, only carry a benefit when there are cheating alleles to resist. Therefore, when resistance alleles are common, cheating is not, limiting the fitness gained from harboring them. In both cases, when a vegetative development cost is associated with carrying these alleles, harboring them becomes less beneficial as their frequency increases.

Previous empirical studies indicating that candidate cheating genes may be under balancing selection support my model predictions (Ostrowski et al., 2015). Ostrowski and colleagues sequenced the genomes of 20 natural isolates of *Dictyostelium discoideum* and examined whether previously identified candidate cheating genes displayed differing signatures of molecular evolution to those from the rest of the genome (Ostrowski et al., 2015). They found distinct signatures of balancing selection acting upon these genes. Notably, they did not find evidence of an escalating arms race occurring at these loci, instead concluding that the arms race observed was rather in a stalemate scenario – similar to that predicted by this model.

While cheaters are thought to be common due to the large number of potential pathways that cheaters can utilize to exploit cooperation (Santorelli et al., 2008), these results suggest that epistasis also reduces the effectiveness of resistance strategies, allowing cheating to prosper. Epistasis and recombination thus provide an additional avenue by which the prevalence of cheaters is able to explained (Buttery et al., 2009; Strassmann

et al., 2000), despite resistance evolving readily (Khare et al., 2009; Levin et al., 2015; Miller et al., 2023).

4.5.2 Future research

Epistasis as a continuous variable - In this model, epistatic effects are implemented as discrete variables. This means that epistasis at any particular gene-pair is either on or off. Future work could consider epistasis as a continuous variable – a sliding scale rather than a yes or no. Through testing all combinations of nullifying epistasis possible with this simulation (Supplementary Fig 1.8), I observed that the maximal cheating allele frequency is obtained through nullification of the cognate resistor allele. Introducing epistatic nullification of the cheater allele alongside this does not appear to influence this observation. By modelling epistasis in a continuous manner, synergistic epistasis (where the effects of each allele increase in the presence of its complement) would also be able to be simulated, a dynamic not possible using the presented discrete model.

Alternative resistance strategies - There exists many different avenues through which one individual can cheat another and consequently, many avenues through which resistance may be achieved. In this model, I examined a 'passive' resistor which is unable to be exploited by its complementary cheater. Future research could look at modelling alternate forms of resistance – for example, suppression or counter-cheating.

Policing is a form of cheating suppression can be seen in eusocial insects (Ratnieks & Wenseleers, 2005; Wenseleers & Ratnieks, 2006), where workers will kill off excess queens and worker-laid eggs, preventing the spread of selfish genes. The active suppression of cheating strategies as opposed to passive resistance may result in entirely different coevolutionary dynamics. Suppressive resistance could be modelled by changing the effect of a resistance allele to nullify cheating attempts against all cells in the local slug rather than solely preventing cheating attempts against the resistor.

Counter-cheating is a resistance strategy whereby the avenue of resistance is to cheat the cheater. In *Dictyostelium discoideum*, this is manifested as a strain which acts fairly

when developed in a chimera with wild-type cells, but cheats when developed with other cheaters (Khare et al., 2009).

Implementing alternate resistance pathways and allowing them to occur in a single genetic background simultaneously could help to shed light on the complicated dynamics at play in intragenomic arms races.

Cheater information – In this model, cheaters have access to incomplete information about their social partners. When a cheater becomes fated as a stalk cell and subsequently selects another cell in its slug to exploit, it does not 'know' the genotype of the cell it selected. This results in cheaters sometimes selecting resistant cells, thus failing their attempt at exploitation. This allows for frequency dependent selection to be simulated as the more resistors there are in a population, the more likely a cheater will inadvertently attempt to exploit an unexploitable cell. This also ensures that wild-type cells are still able to persist at low frequencies in a high cheater population – when there are very few exploitable cells, they have a low chance to be successfully cheated.

An alternative form of victim-selection would be to select from the pool of exploitable cells only, ignoring those which are resistant. This method, however, implies a high level of within-slug cell-cell communication, as any cheater would effectively 'know' the genotype of all others in the local slug.

Whether the implemented mechanism is accurate to the biology of *Dictyostelium discoideum* is unknown and would be dependent on the cheating strategy employed. For example, if a cheater cheats by excreting a pheromone that induces stalk-fate adoption of victim cells, then the implemented version of cheating would be incorrect – all cells in a slug would expect to be cheated if possible. However, if cheating was achieved through a cell-cell adhesion protein similar to the *tgrB1/tgrC1* pair, then it would be expected that only neighboring cells in a slug could be cheated and the model presented here would be more accurate. Ultimately, *in silico* simulations always carry assumptions about the organism it models, and the correct assumption can only be validated by empirical studies.

Population structure - Population structure likely plays a large role in the maintenance of cooperation. As cooperators gain an inclusive fitness advantage by cooperating with themselves, pockets of cooperators may form within an overall population despite the presence of cheaters. These cooperative pockets may gradually outcompete the larger population as a whole by solely interacting with each other and thus eventually rise to fixation. However, this simulation is unable to simulate the effects of population structure. The addition of locational information to the simulation may result in deeper insights into within-species coevolutionary dynamics.

Chapter 5: Limitations, conclusions and future research

5.1 Limitations and future research

This project was severely impacted by the inability to consistently germinate macrocysts and produce recombinant progeny. The failure of the long-term evolution experiment due to the fixation of a single mating type indicates that recombinant progenies were not produced during the experiment. Experimental work in this thesis attempted to discover a technique to more consistently germinate macrocysts compared to the traditional method, but failed to identify any viable alternatives.

The *Dictyostelium* sexual cycle has high potential for classical genetic manipulation, and if harnessed correctly would allow for the cheap and easy production of transgenic strains (Hospital & Charcosset, 1997). As an example, classical methods such as backcrossing have been used to great effectiveness in yeast for many years, allowing easy introgression of genes-of-interest into a target background (Marullo et al., 2009; Romano et al., 1985). However, the extremely low germination rates observed using traditional germination methods (<1%) hamper the effectiveness of the *Dictyostelium* reproductive cycle for such use cases. Therefore, in order for use of the *Dictyostelium* sexual cycle to become widely adopted, the importance of further research into effective germination methods for *Dictyostelium* macrocysts cannot be overstated.

Due to the issue posed by problematic macrocyst germination in my long-term evolution experiment, simulation became necessary to answer the research objectives set out in this thesis. While simulations can prove invaluable for bridging the gap between empirical and theoretical studies, simulation naturally comes with many assumptions about the model organism on which it was based, potentially limiting the accuracy of results. For example, the model presented here assumes that: (1) cheaters have incomplete information regarding the genotype/phenotype of social partners, (2) cheating and resistance alleles are paired in such a way that allows for epistasis to occur, (3) resistance is 'passive', and does not involve actively suppressing cheaters, and (4) in a given population, there is an equal chance for cooperation to occur between any two individuals (i.e. no kin discrimination).

Future work focusing on the effects of recombination and epistasis on coevolutionary dynamics should investigate whether the predictions made in this model can be replicated empirically. This could be achieved through long-term experimental evolution, similar to the experiment presented in Chapter 2, but instead utilizing strains which differ only at the mating type locus. Such strains have been used in other studies (Bloomfield et al., 2010), and should have very little difference in relative fitness, thereby minimizing the chances of repeating the mating type fixation that was observed here. Other factors could also be varied to avoid mating type fixation, such as increasing the frequency of recombination rounds, increasing the macrocyst incubation period, or transitioning to a more effective germination method.

A successful long-term evolution experiment comparing the cheating and resistance phenotypes of populations both with and without periodic recombination would serve to verify and validate the predictions made by the presented model. Critically, an empirical study in this vein may answer whether antagonistic epistasis between cheating and resistance alleles occurs in natural populations of *Dictyostelium* and is essential to ensure the accuracy of the theoretical work presented.

5.2 Conclusions

I utilized *Dictyostelium discoideum's* unique life cycle to investigate the effects of recombination on within species coevolutionary dynamics. By leveraging the model organism's distinctive biological processes, this study offers implications for understanding within-genome conflicts in a wider context.

First, I described a long-term evolution experiment to determine the effects of periodic recombination on an intragenomic coevolutionary arms race between cheaters and resistors. Unexpected mating type fixation unfortunately halted this investigation before any meaningful results could be obtained pertaining to the initial experimental aims. Likely, sub-par germination efficiency was the cause of this development – an issue which has long plagued the use of the *Dictyostelium* sexual cycle in research. However, this experiment did result in noticeable adaptations of the evolved populations. A significant increase in social development speed was observed when comparing

ancestral and evolved populations, the exact mechanics of which are unknown at present but provide exciting avenues for future research.

As a follow up to the difficulties experienced in the initial long-term evolution experiment, I performed a germination assay to assess the efficacy of various germination methods. While no particularly effective macrocyst germination method was discovered, repeated freeze-thaw cycles were found to have a highly deleterious effect on germination rates. The cause of the observed effects may be due to the formation of ice-crystals within or around the macrocyst structure that disrupt essential germination processes, similar to the effect that causes increased germination in hard-seeded plants (Acharya et al., 1993; Shibata et al., 1995). These findings may serve to inform future inquiries into the germination process of the *Dictyostelium* sexual cycle.

Finally, I used simulation of the co-evolutionary process in *Dictyostelium* to assess how periodic recombination and other factors, such as costs of cheating and resistance, mutation rates, and epistasis, influence the co-evolutionary dynamics of cheating and resistance to cheating.

My model has identified intragenomic epistasis as a potential avenue through which within-species coevolutionary arms races may be regulated within natural populations. This has been achieved by simulating populations of *Dictyostelium discoideum* with and without periodic recombination and nullifying epistasis.

The model suggests that stable equilibrium as a result of a within-species arms race would be severely impacted by the introduction of recombination and consequent antagonistic epistasis between coevolving alleles (Fig 4.5). Epistatic effects between cognate alleles facilitated by periodic recombination results in a constantly refreshing pool of victims for selfish individuals to exploit, allowing for the continued selection of cheaters despite the presence of resistors. Furthermore, my results suggest that only nullification of the resistance allele is required to cause the observed increase in cheating allele frequency (Supplementary Fig 1.8).

Sexual cycles alone (and the population bottlenecks they incur in *Dictyostelium discoideum*) are not enough to recreate this effect (Fig 4.6). Recombination must be

present to mix and match conflicting alleles into a single genetic background to allow for antagonistic epistasis to act and disrupt an otherwise stable equilibrium.

As recombination appears to occur frequently in natural populations of *Dictyostelium discoideum* (Flowers et al., 2010; Ostrowski et al., 2015), my findings may help to explain why facultative cheaters are common in natural isolates (Buttery et al., 2009; Strassmann et al., 2000) despite their presence selecting for the counter-evolution of resistors (Hollis, 2012; Khare et al., 2009; Levin et al., 2015; Miller et al., 2023).

While *Dictyostelium discoideum* was used as the model organism for this study, the conclusion that epistasis may be a driving factor in preventing or slowing within-species arms races is widely applicable. When both within-species intergenomic conflict and sexual reproduction are present in a population, epistasis between alleles encoding conflicting strategies as a direct result of recombination may serve to prevent the emergence of a within-species arms race.

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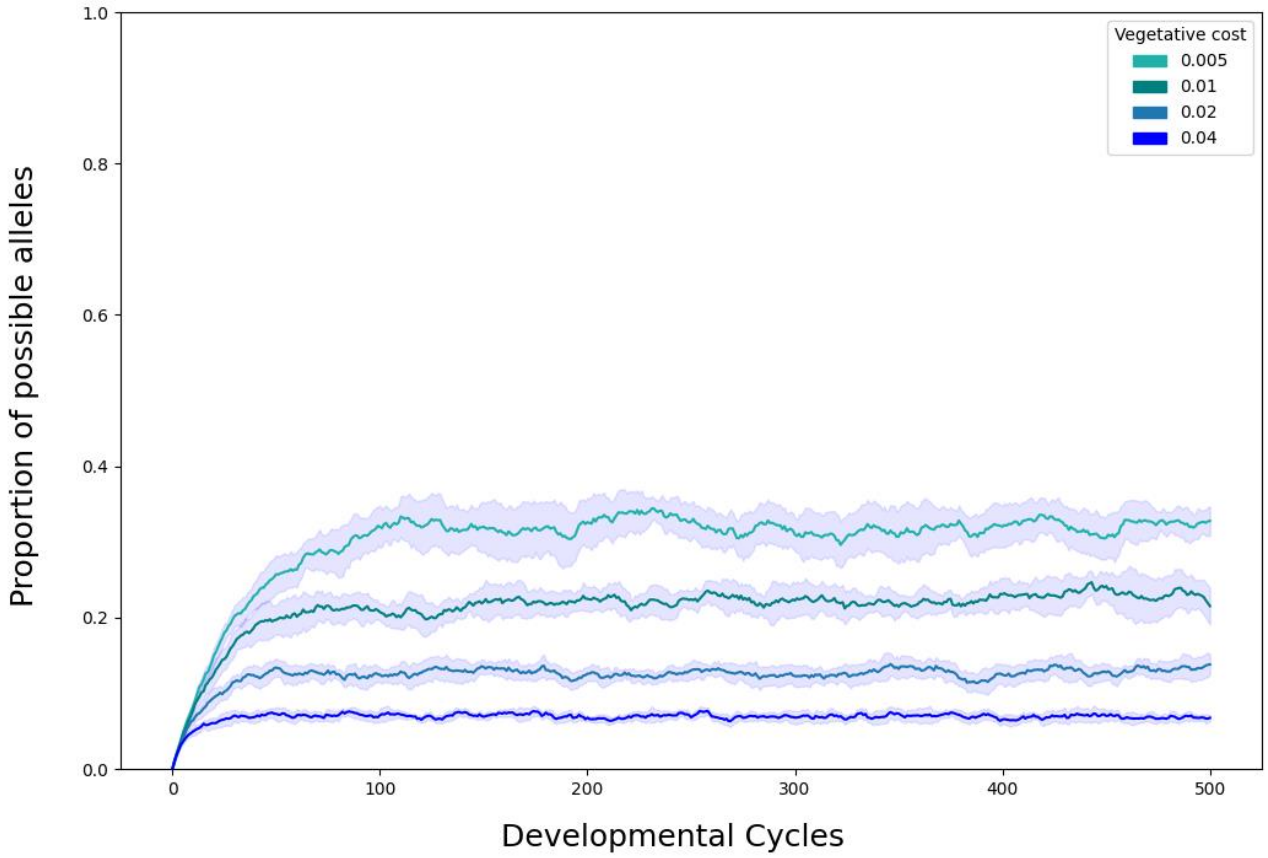
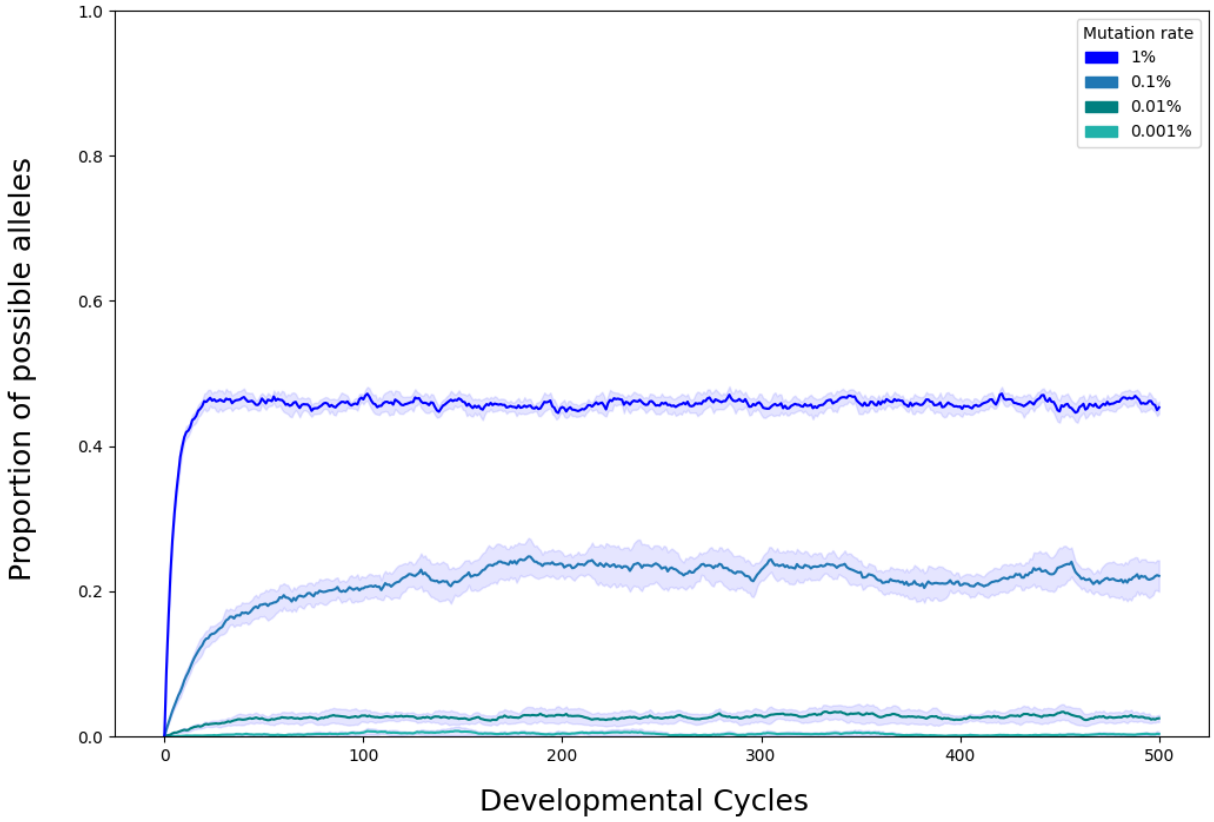
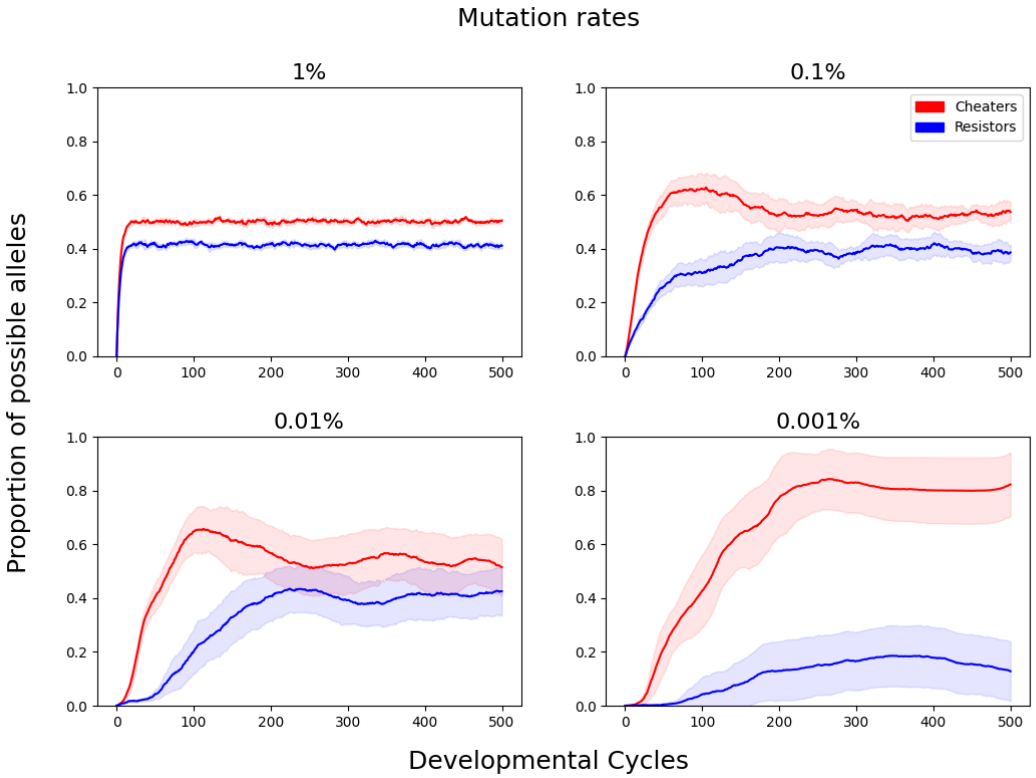
Supplementary Figures



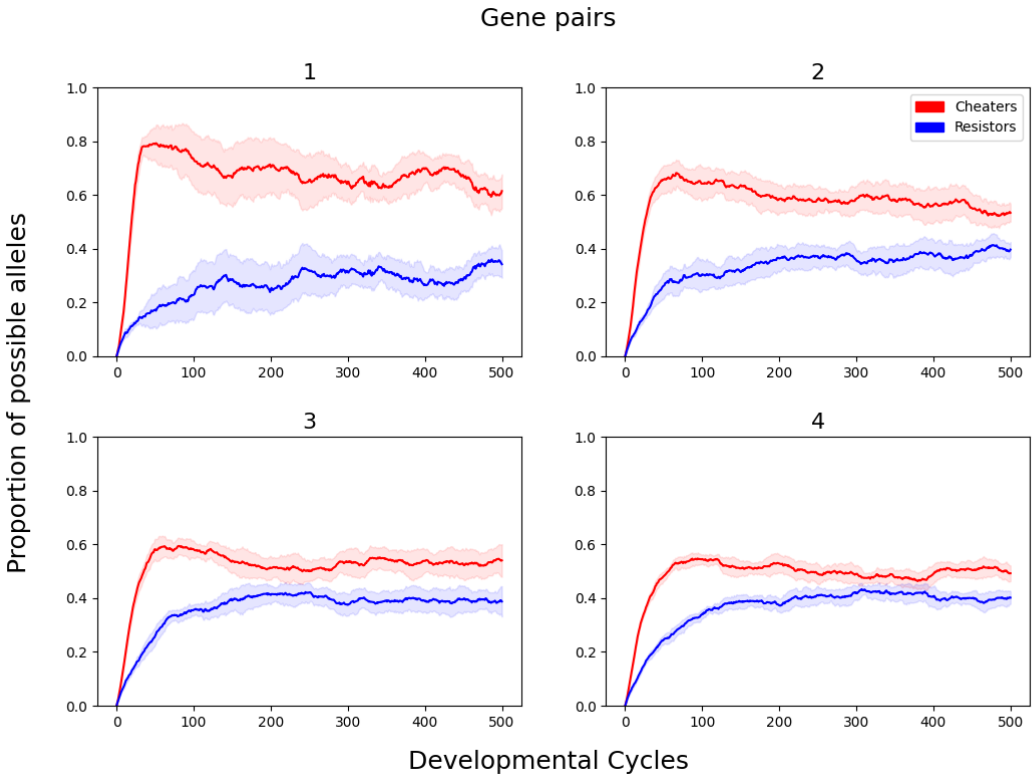
Supplementary Figure 1.1 Vegetative cost effect on resistor alleles stable frequency. All simulations were run with default values except only resistance alleles were allowed to mutate. Each line shows a simulation with a different vegetative cost associated with the resistance allele. Graphed lines indicate the mean value of 10 runs, while the shaded areas surrounding each line indicate a 95% confidence interval. As the vegetative cost of the allele increases, the baseline frequency at which it is present decreases.



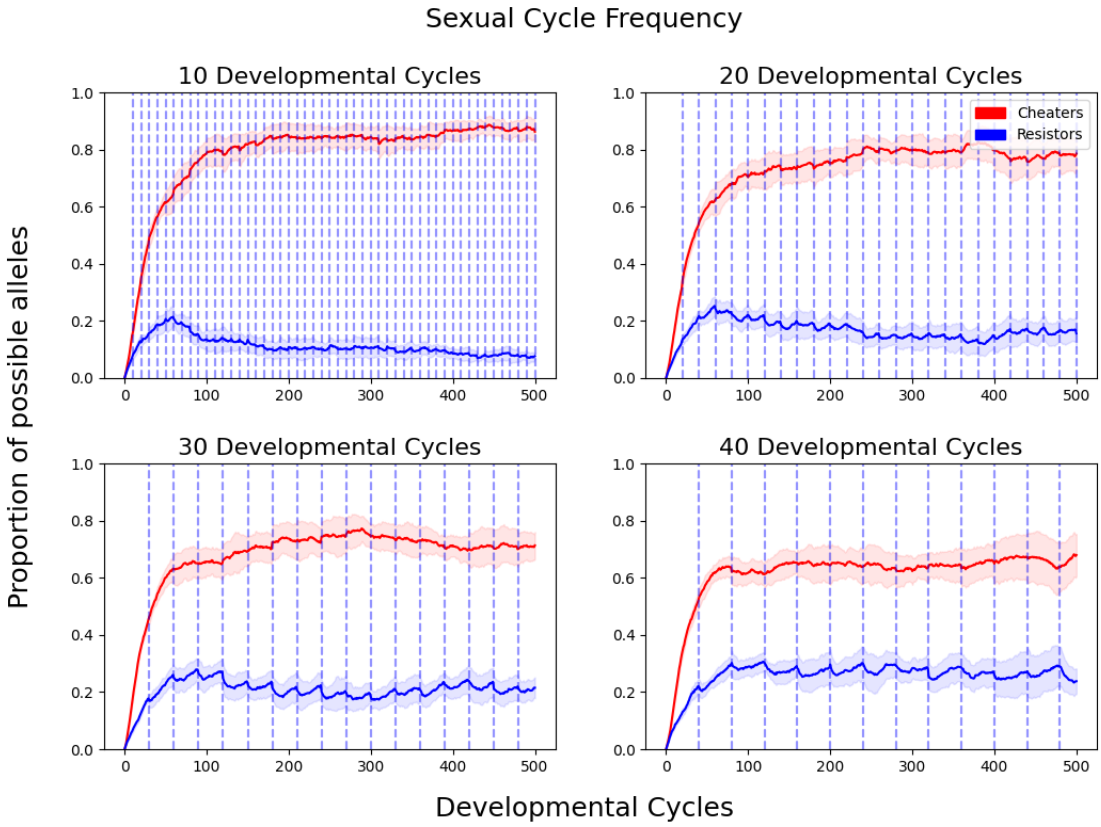
Supplementary Figure 1.2 Mutation rate effect on stable resistor frequency. All simulations were run with default values except only resistance alleles were allowed to mutate. Each line shows a simulation with a different mutation rate associated with the resistance allele. Graphed lines indicate the mean value of 10 runs, while the shaded areas surrounding each line indicate a 95% confidence interval. As the mutation rate of the allele increases, the baseline frequency at which it is present also increases.



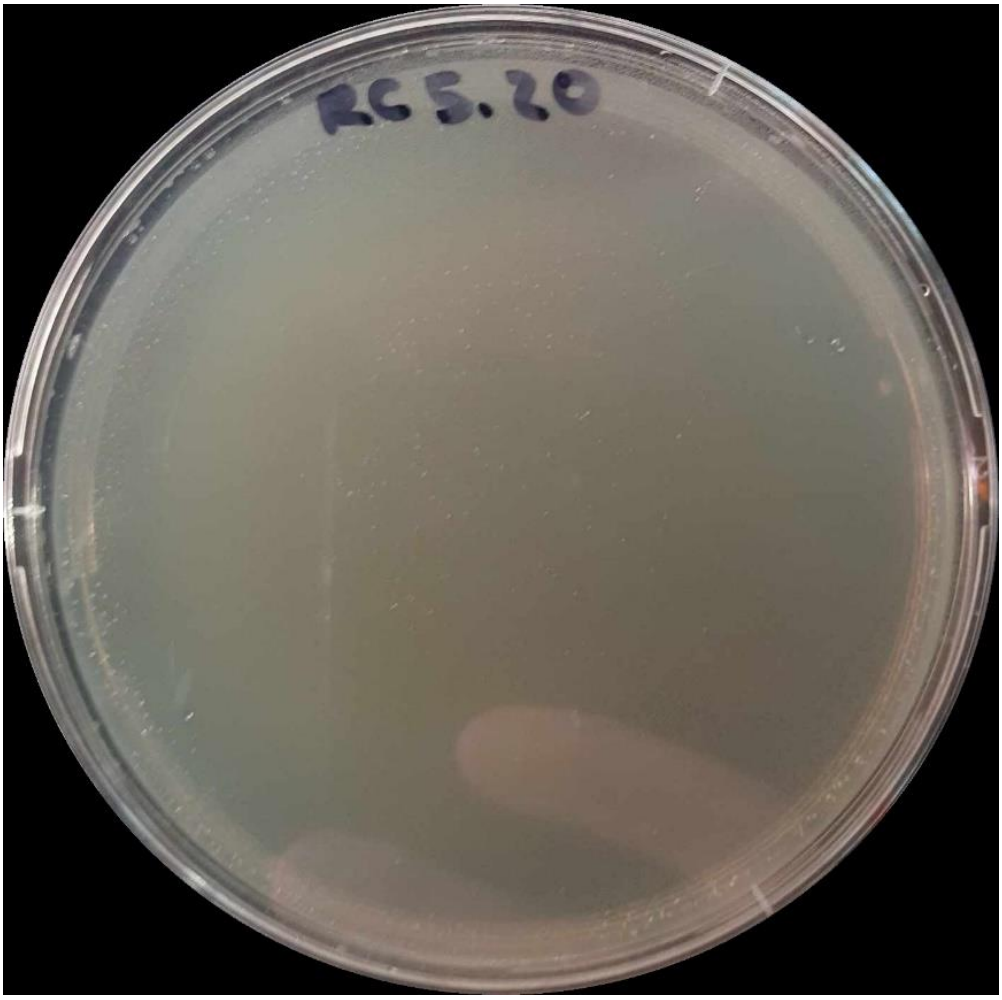
Supplementary Figure 1.3 Higher mutation rates increase stability. All simulations were run with default values unless otherwise stated. Graphed lines indicate the mean value of 10 runs, while the shaded areas surrounding each line indicate a 95% confidence interval. Upper left: At a 1% mutation rate for both allele types, cheater and resistor alleles quickly fall into equilibrium, with extremely little variance between runs. Upper right: At a 0 .1% mutation rate, cheaters alleles show an initial spike in frequency before dropping back into equilibrium. Lower left: At a 0.01% mutation rate, the initial cheater spike lasts longer and spikes higher than 0.1%. Lower right: At 0.001% mutation rate, the initial cheater spike does not return to the equilibrium displayed at higher mutation rates, instead plateauing at ~80% of the possible alleles.



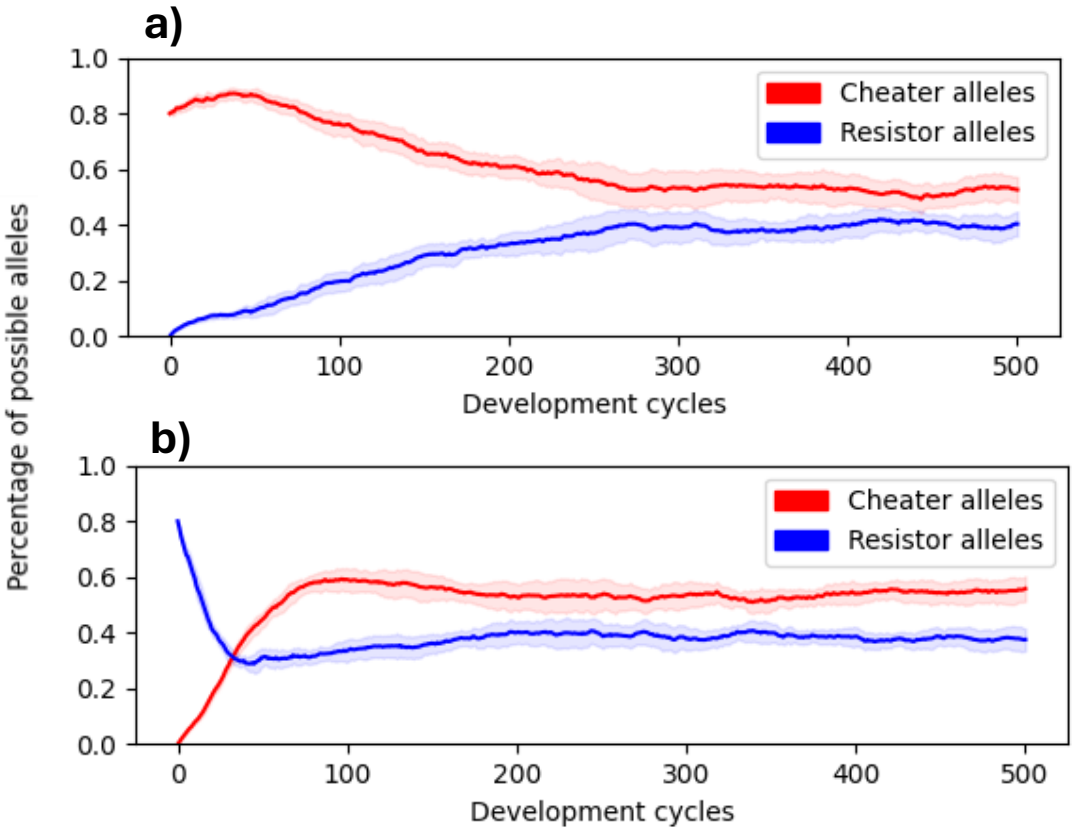
Supplementary Figure 1.4 The effect of gene pairs on coevolutionary dynamics. All simulations were run with default values unless otherwise stated. Graphed lines indicate the mean value of 10 runs, while the shaded areas surrounding each line indicate a 95% confidence interval. As the number of simulated gene pairs increases, the stability of both alleles also increases as indicated by smaller error bars over 10 replicates. Cheater and resistor allele frequencies reach their stable values faster the more gene pairs there are.



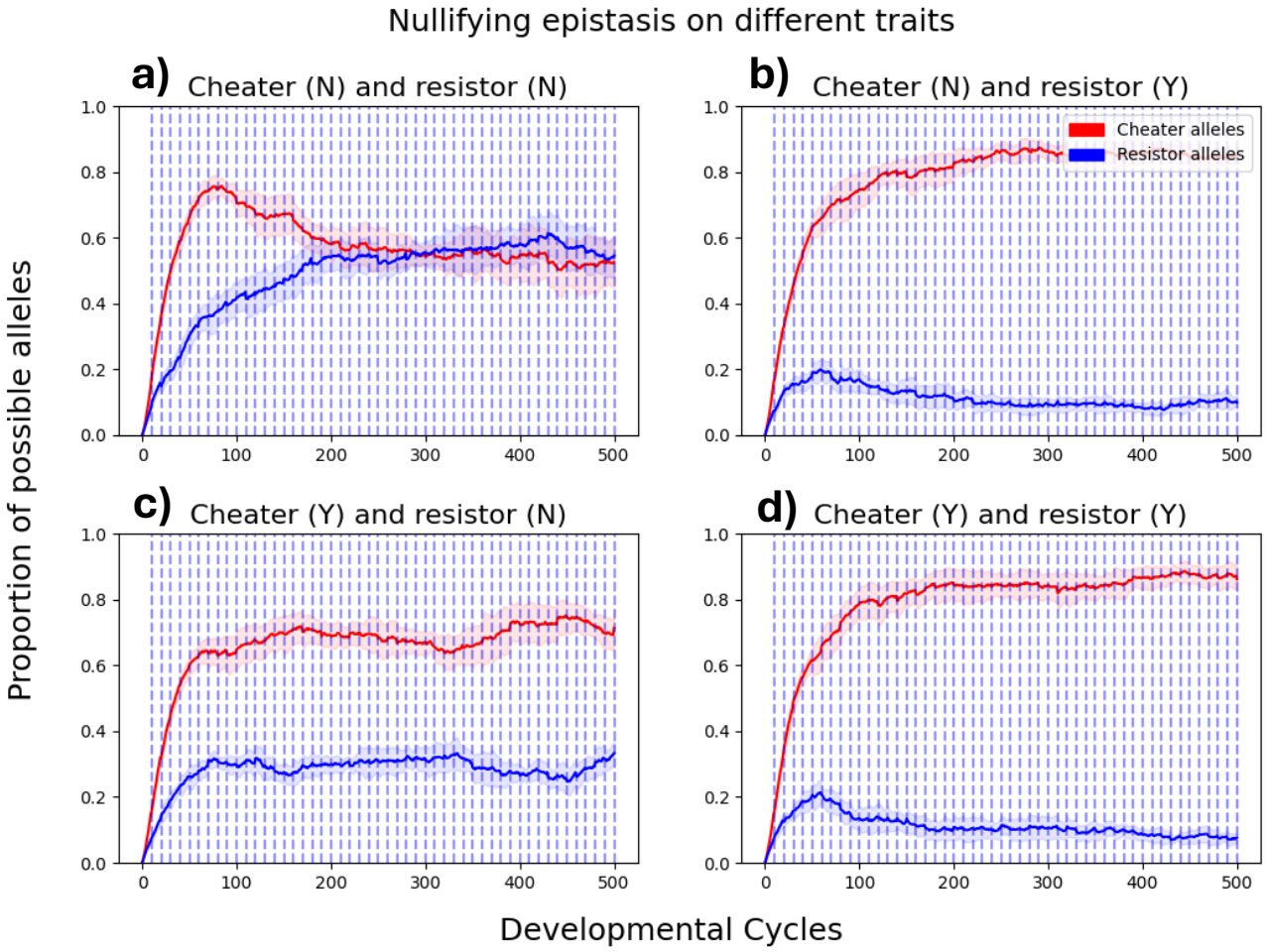
Supplementary Figure 1.5 The effect of sexual cycle frequency on coevolutionary dynamics. All simulations were run with default values unless otherwise stated. Graphed lines indicate the mean value of 10 runs, while the shaded areas surrounding each line indicate a 95% confidence interval. Vertical blue lines indicate sexual cycles. As the frequency of recombination increases, the divergence between cheater and resistor allele frequencies increases.



Supplemental Figure 1.6 Example of non-developing population. *Dictyostelium discoideum* population plated on growth SM/5 growth media lawned with *Klebsiella pneumoniae*, 48 hours post plating. Almost no development is visible. A small number of slugs can be observed near the edges of the plate, but are much smaller than would be expected from *Dictyostelium discoideum*. Further developmental time did not increase the number or size of slugs/fruiting bodies.



Supplementary Figure 1.7 Stable equilibrium is the evolutionarily stable strategy. All simulations were run with default values unless otherwise stated. Graphed lines indicate the mean value of 10 runs, while the shaded areas surrounding each line indicate a 95% confidence interval. (A) When beginning with a high cheater frequency (80%) and no resistors, resistors increase in frequency to the evolutionarily stable levels (0.6 cheater, 0.4 resistor). (B) When the simulations begins with the equivalent level of resistors (80%) and no cheaters, the same is observed – resistor frequency quickly drops back to the expected stable levels while cheaters increase in frequency.



Supplementary Figure 1.8 Nullification of the resistance phenotype causes divergence. All simulations were run with default values unless otherwise stated. Graphed lines indicate the mean value of 10 runs, while the shaded areas surrounding each line indicate a 95% confidence interval. Blue vertical lines indicate sexual cycles. (A) With periodic recombination and no epistasis, allele frequences of both strategies reach equilibrium at ~50%. (B) When the resistor phenotype is nullified through epistasis, cheater frequency greatly increases to ~80% while resistor allele frequency drops to ~10%. (C) When only the cheater phenotype is nullified, alleles stabilize at ~70% cheater, 30% resistor. (D) When both phenotypes are nullified by epistatic effects, the same pattern can be seen as in B, indicating that only nullification of the resistor allele is required to produce the maximal increase in cheater frequency.