Ecological-evolutionary feedback in evolved lineages of *Pseudomonas fluorescens*

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Abstract

Multicellularity cannot proceed to subsequent stages without the evolution of collectives. Thus, it becomes essential to understand the evolution of cooperation before the evolution of multicellularity. However, understanding the evolution of cooperation presents a problem. This is because natural selection rewards selfish behavior; yet, in nature, cooperation is apparently common. Here arises a conflict between individual and collective interest and thus raises questions concerning the evolution of cooperative behavior. Assortment between cooperating types has been identified as the underlying mechanism behind theories for the evolution of cooperation. However, these theories assume the environment to be stable and fail to acknowledge interactions are density and frequency dependent (two components of the environment), capable of generating co-evolutionary interactions. In this regard the feedback between ecology and evolution (eco-evo feedback) is of likely importance.

In a previous experiment, a rudimentary life cycle was established in model bacterial populations where lineages were repeatedly cycled between a cellulose producing, group living cooperator type, termed WS, and solitary, free living cheater types, termed SM. The results of the experiment showed that collective level fitness increased in evolved lineages compared to baseline lineages. I believe that the eco-evo feedback is likely to have occurred on the WS-SM interactions and that this is responsible for the increased fitness of the evolved lineages.

The aim of this thesis was to identify the presence of the feedback in evolved lineages. I compared the *evolutionary dynamics* of frequency dependent interactions between WS and SM, and *population dynamics* due to density dependent factors on the interactions between WS and SM. I also report the joint influence of evolutionary and population dynamic patterns via *eco-space diagrams* of the ancestor and evolved lineages.

The results showed that the interactions between WS and SM are both frequency and density dependent and the joint influence of the above two factors reveals the presence of an eco-evo feedback. The nature of the feedback is suggested to be a reduced transition capacity of SM to switch to WS in evolved lineages. The tendency of evolved SM to produce few WS suggest a strategy on the part of the SM to save the metabolic cost of production of cellulose, by WS, and to trade-off this cost with an increase in fitness of the evolved lineages.

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Chapter 1. Introduction

1.1 Evolution of multicellularity

Evolution of multicellularity from unicellular entities is regarded as one of the major evolutionary transitions (Maynard Smith & Szathmary 1995). The first signs of this transition comes from Cyanobacteria-like organisms which dates back to 3 to 3.5 billion years (Knoll, 2011). Other examples of transitions include, evolution of the eukaryotic cell from two bacterial-like cells, compartmentalisation of cells from individual replicating units like DNA, and formation of superorganisms like honey bee colonies.

Unlike the other transitions, the evolution of multicellularity has developed through successive stages. It is hypothesized that multicellularity progressed through three broad stages (Rose, 2015).

First stage is the **evolution of collectives (groups)** (Figure 1.1), where individual replicating units like unicellular cells come together to form collectives.

Second stage is the **evolution of individuality** (Figure 1.1), where the collectives acquire properties similar to that of cells and in the process become individual replicating units themselves.

Third stage is the **evolution of complexity** (Figure 1.1), where the individual collectives, capable of collective reproduction, come under natural selection to evolve into complex organisms.

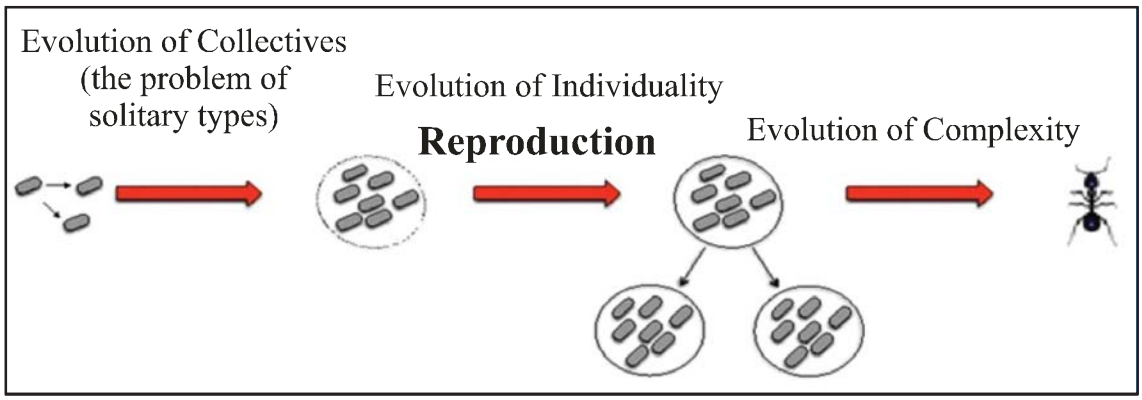

Figure 1.1 Three stages in the evolution of multicellularity. 1 Evolution of groups: individual cells cooperate to form collectives. 2. Evolution of individuality: collectives gain properties similar to individual cells and become replicating unit themselves 3. Evolution of complexity: collectives come under natural selection and evolve into complex organisms.

Multicellularity cannot proceed to subsequent stages without the evolution of collectives, which happens to be the foremost stage in the evolution of multicellularity. Evolution of collectives requires cooperation among cells for the maintenance of early collectives. Thus, it becomes important to address the problem of evolution of cooperation first, before gaining any insight into the further stages of multicellularity.

1.2 Evolution of cooperation

Darwin's theory of natural selection argues for the "survival of the fittest". The statement implies that there is competition among organisms for survival in the next generation and that organisms with higher relative fitness will survive, while others will perish with time. Accordingly, given the importance of persistence, organisms are expected to evolve traits that enhance individual fitness (reproductive success). However, nature is replete with examples of benevolent acts of cooperation, where individuals appear to have evolved traits that enhance the fitness of other types. Why an organism should exhibit a cooperative act, when it's not beneficial for its own fitness is puzzling and leads to an evolutionary question, as to how a non-selfish cooperative behaviour can evolve and become established in a population of inherently selfish individuals (Hill, 1971).

1.2.1 Defining cooperation

Cooperation is a form of social behaviour. Social behaviours are those that have an effect on both the actor and the recipient. Defining any social behaviour has always been a challenge because of the different terminologies used by various authors to describe the same behaviour and because of the amount of interdisciplinary research prevailing in the area.

Cooperation is generally defined as a behaviour that provides a benefit to another individual (recipient), and which is selected for because of its beneficial effect on the recipient (West *et al*., 2006, 2007). The individual who performs the behaviour is called an actor and the individuals affected by the behaviour, are recipients. There are two prominent features of cooperation that need to be made explicit.

Firstly, cooperation should be selected for its benefit on the recipient.

For example, production of dung by an elephant which is feasted upon by the dung beetle cannot be termed as cooperation because the elephant is not purposely excreting the waste to benefit the beetle but is doing as a part of its usual metabolism, meaning that the behaviour is not selected for its benefit upon the recipient. Thus, in order for the behaviour to be classed as cooperation, it should be selected for its benefit upon the recipient.

And secondly, the benefit to the recipient comes at a cooperation cost to the actor.

As discussed an act of cooperation incurs a cost on the part of the actor, which makes the behaviour relatively costly and non-sustainable, but still cooperation persists. Much of the literature on cooperation is thus concerned with determining ways by which this cost can be compensated through direct or indirect fitness benefits (West *et al*., 2006).

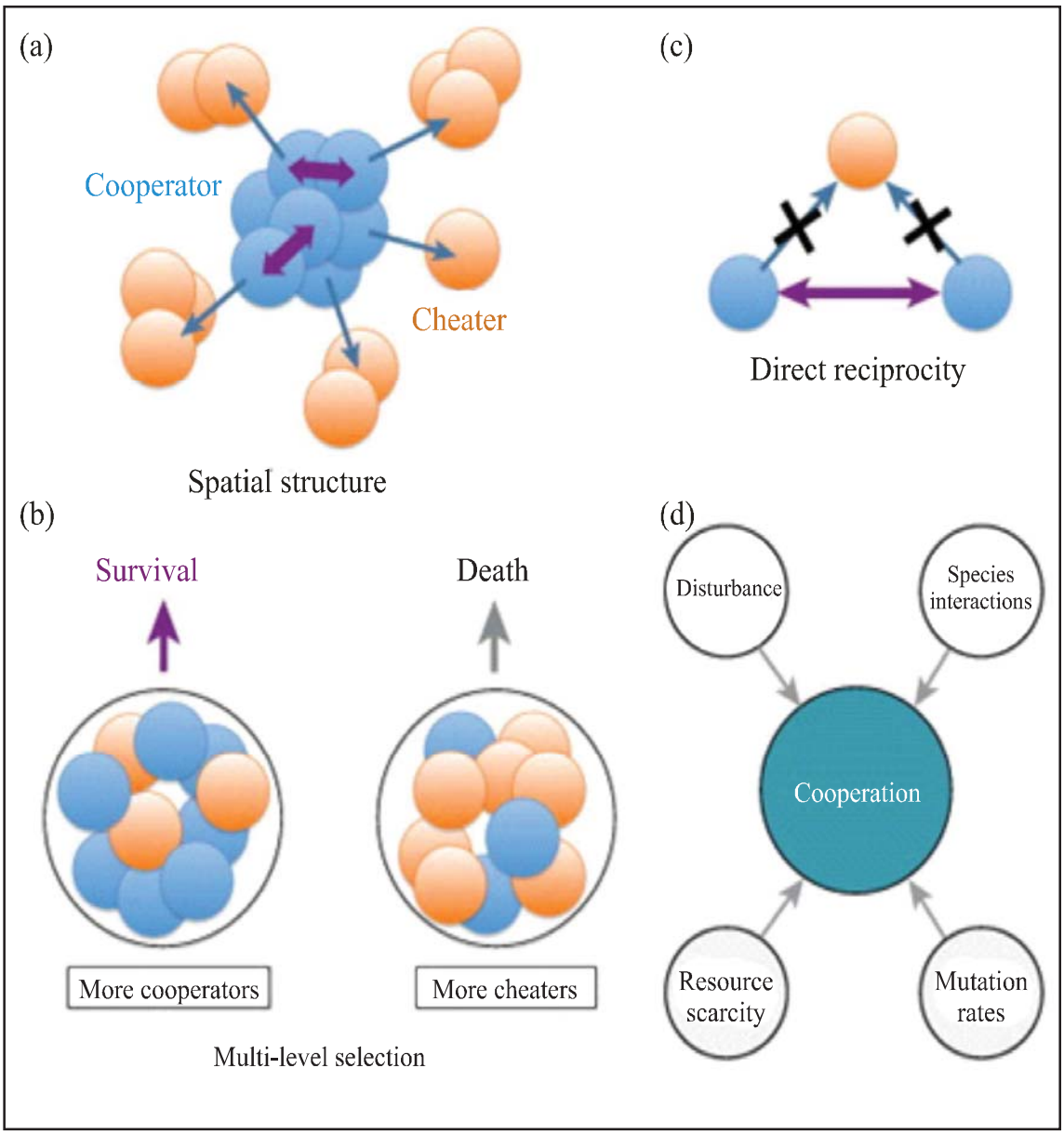

Figure 1.2 Maintenance of cooperation by different mechanisms. (a**)** Spatial heterogeneity: promotes cooperator cells to stay together and benefit preferentially from the resource (b**)** Group selection: groups with a high frequency of cooperators proliferate and survive, whereas cheater-dominated groups go to extinction. (c**)** Direct reciprocity: cooperating cells recognize other cooperating cells, they can discriminate between cooperators and cheaters and keep cheaters away from taking benefits. (d**)** There are other factors which affect the evolution of cooperation. For instance, ecological disturbances: that changes the environment transiently and results in cooperation depending on the frequency and size of the disturbances. Limiting resources and competing species can also influence the interaction between cooperators and cheaters. Also, traits that have evolved due to stochastic environmental disturbances can have influences on cooperative population dynamics. In all the above approaches to the maintenance of cooperation, the central theme is assortative interactions. Blue, cooperator cells; orange, cheater cells. In (a) and (c): purple arrows, cooperative interaction; blue arrows, cheating interaction. Figure adapted from (Celiker & Gore, 2013).

1.3 Theories explaining the maintenance of cooperation

Different theories have been put forward to explain the evolution of cooperation and these can be broadly categorised based on the criteria of direct and indirect fitness benefits. At a fundamental level, these different theories simply provide different explanations for non-random assortment – cooperation can be maintained provided that cooperators have a greater chance of interacting with cooperators than with selfish types.

1.3.1 Theories based on direct fitness gains

Direct fitness gains are ones that increase the fitness (increase in the number of offspring produced) of the bearers of the cooperative trait (actor). Mutualism within species, is an example of one such social behaviour that leads to an increase in the direct fitness of the actor and is classed as a +/+ interaction in the Hamiltonian scheme (Hamilton, 1964). It means both the actor and the recipient derive a benefit from the interaction. Direct fitness benefits can further be classified into two, i.e., non enforced by-product benefits(West *et al*., 2007) and enforced benefits.

a) Non enforced by-product benefits

Non enforced by-product benefit is the direct benefit gained by an individual as a by-product of being a part of a large group. The individuals within such a group are not forced to cooperate. For instance, consider cooperative breeding in meerkats where members in the community help in the rearing of other individual's offspring by defending the territory of other individuals. Such acts increase the productivity of the group, which leads to an increase in overall group size of the community. Increased group size in turn helps individual members of the community in activities crucial for survival, like foraging and winning over other group (Clutton-Brock *et al*., 2001). Thus,

individuals pay a cost for assisting the reproductive fitness of others and receives a direct benefit from increased group size.

b) Enforced benefits

Enforced benefits are direct fitness gains where there is either a reward for cooperation or a punishment for non-cooperation and can be achieved by mechanisms like reciprocity and punishment.

Reciprocity is an act where same pair of individuals (like cooperator-cooperator) cooperates depending on their past history of interactions. The model of reciprocity is based on iterated prisoner's game, also known as tit for tat strategy (Trivers, 1971). Reciprocity works for small group sizes but fails as a theory to explain the evolution of cooperation when groups attain large size. For example, large groups like human societies have a large amount of social interactions, where it becomes intractable for an individual to keep account of numerous social interactions and decide its response (cooperation or defection) accordingly, based on other individuals action.

Punishment is another kind of behaviour under enforced benefits which mandates cooperation by targeting the non-cooperators specifically and punishing them for their non-cooperative behaviour. Punishment entails an additional cost on the cooperator, thus, for punishment to sustain as a stable strategy, two conditions should be met. First, the cost of being punished should be huge, such that it forces others to cooperate and sustain cooperation. Second, the long term benefits of punishing non-cooperators should be more than the cost of maintaining cooperation (Boyd & Richerson, 1992).

1.3.2 Theories of cooperation based on indirect fitness gains

Cooperation can also exist in communities if there is an indirect fitness gain associated with the act. Indirect fitness benefit is gained by cooperator through propagation of the cooperative gene among those who possess the cooperative gene. There are several theories put forward to explain the evolution of cooperation through indirect fitness benefit, some of them are mentioned below.

a) Kin selection theory

It is understandable why organisms which are related to each other should cooperate among themselves. It is because they share ancestry (kin) and the cost of cooperation can be balanced by the propagation of the shared genes between the relatives. Thus there is an indirect fitness benefit associated with the act. Mathematically, Kin selection theory can be expressed by Hamilton's rule (Hamilton, 1964).

$$c\, b < r$$

Where,

c : cost of cooperation to the actor

b : benefit received by the recipient

r : the factor of genetic relatedness

It implies that an act of cooperation will persist as long as the cost of cooperation (c) times the benefit to the related individual (b) is less than the factor of genetic relatedness (r) between the members of cooperating unit.

Kin selection has been used as a general mechanism by empiricists to explain the maintenance of cooperation (Queller & Strassmann, 1998; Foster *et al*., 2006). But recent studies have highlighted examples of many organisms showing high degree of relatedness, not showing cooperation and vice versa. These examples thus indicate that Kin selection is not a driving force for cooperation. It has no mechanistic basis to it and

explains nothing about the causes of non-random assortment, which is all that is required for cooperation to spread (Nowak *et al*., 2010; Doebeli, 2010).

b) Group selection

The current accepted version of group selection is the "new group selection", which proposes that group selection arises due to the tension between two levels of selection, individual and groups (Rainey & De Monte, 2014). The first level is the level of individuals, where selection acts on the variance of individuals cells within groups and the second level is that of the groups themselves, where selection acts on the variance between groups. When variance between groups exceed the variance within group, group selection occurs (West *et al*., 2006) (Figure 1.2(b)). However, it is only possible when the groups can participate directly in the process of evolution by natural selection.

The old group selection theory (Wynne-Edwards, 1962), on the other hand , was based upon the idea of selection acting only at the group level; which included group proliferation and group extinction events. It proposed group extinction of groups with more cheaters, due to exhaustion of resources and group proliferation for groups with more co-operators, because of judicial use of resources.

Theory suggests that the old type of group selection will work only under very restrictive conditions (Smith, 1964). Also, empirical work showed that organisms didn't use the resources judicially, as hypothesized earlier (Krebs & Davies, 2009). Therefore, the new group selection theory is currently used to explain the evolution of cooperation.

c) Indirect reciprocity (IDR)

Indirect reciprocity is reciprocity where the help is given to individuals based on reputation. Here, the individual's cost of cooperation is compensated by an indirect

benefit that it derives from helping others , that in turn cooperate (Rankin & Eggimann, 2009).

First proposed by Alexander (1987), IDR provides an explanation for the evolution of cooperation in social situations where the probability of meeting the same pair of individuals is low enough to predict the vote towards cooperation or defection. In such cases, reputation among individuals which is based on past actions towards others, help individuals to decide whether or not to cooperate. For IDR to be a stable strategy, the probability of knowing the recipient should exceed the cost to benefit ratio of the cooperative act (Nowak & Sigmund, 1998).

d) Limited dispersal

Limited dispersal promotes cooperation by limiting the movement of newly generated individuals who are likely to be the kin of each other, thus reinforcing the spatial aggregation of similar phenotypes and enhancing cooperation (Kümmerli *et al*., 2009).

e) Spatial structure

Spatial structure promotes cooperation by limiting the access of resources to co-operators (producers of the resource) (Figure 1.2(a)). It can be generated by physical segregations as in the case of biofilms. For example, the physical structure of the biofilms promotes the cells to grow in aggregates which create micro- colonies. These micro-colonies create a patchy environment which enhances the growth of cooperators by generating a positive feedback between resource utilisation and growth (Kreft, 2004; Pfeiffer *et al*., 2001).

f) Assortment – a general mechanism for the evolution of cooperation

Assortment is a broader classification under the umbrella of which all the theories of evolution can be explained (Fletcher & Doebeli, 2009). It is the most general mechanism which lies at the core of all the theories discussed above (Fletcher & Doebeli, 2009; Rosas, 2010). Cooperation is promoted, when local interactions within the group generate and enhances an assembly of individuals bearing similar kind of trait (cooperation or defection), such that they interact more with each other (Queller, 1984). The similarity or dissimilarity is expressed by a mathematical parameter 'r', called the factor of relatedness which indicates the degree of assortment.

The working of different theories based on direct and indirect fitness gain can be explained by assortment (Doebeli & Hauert, 2005). For example, reciprocity based on tit-for-tat strategy creates assortment by generating behaviour of cooperation in return of cooperation and defection in return of defection. Also in kin selection, the factor that generates assortment is the genetic relatedness between the individuals (Fletcher & Doebeli, 2009).

The maintenance of cooperation by assortment asserts that similar interactions within a given collective would promote cooperation. This implies that dissimilar interactions between a cheater and a cooperator, would decrease cooperation. This is because cooperation incurs a cost and a direct interaction with a cheater which doesn't pay a cost, reaps the benefits. This leads to invasion of the population of cooperators by cheating types. However, there can exist dissimilar interactions between cheaters and cooperators that not only maintain cooperation but also enhance cooperation. For example, it was shown that mixed (cheater and cooperator) populations of *S. cerevisiae* showed higher productivity than pure populations of cooperators (Maclean *et al*., 2010).

The coexistence of cooperators and cheaters is maintained due to the change in the relative cost and benefit functions of the two types such that, either the cost of cooperation on the part of cooperator is reduced or there is a decrease in the benefit to the cheaters, thus balancing the relative fitness to a point which support coexistence. The change in the relative fitness is brought about by environment. Environment has a strong impact in modulating relative cost to benefit ratio, which makes cheating a context dependent plastic behaviour (Ghoul *et al*., 2014; Zhang & Rainey, 2013). We can see that environment has the capacity to change the expected outcome of mixed interactions to various other possibilities (discussed in section 1.4.1). Therefore, it becomes imperative to give full consideration to the role of the environment in the evolution of cooperation.

g) Environment dependent cooperation

Generally, when the environment is harsh it promotes cooperation (Strassmann *et al*., 2000; Callaway *et al*., 2002). This is because harsh environments create resource limitation which cooperators are better able to cope up with in the long run. Albeit, in such situations cheaters have a short term advantage of not cooperating and instead feeding on the limited resource but this strategy is non-advantageous in the long run and leads to their own demise due to exhaustion of resources (Smaldino *et al*., 2013) (Figure 1.2(d)).

Harsh environments are identified with a lot of variation in the frequency of cooperators and population density. It is through these tools that the environment exercises its impact on cooperation (Doebeli *et al*., 1997; Buckling *et al*., 2009; Lehmann & Rousset, 2010; Lehmann & Keller, 2006). I will now consider more closely how these two forces affect cooperation.

1.4 Frequency and density dependent effects on cooperation

1.4.1 Frequency dependence

Different alleles may contribute differently to the fitness of the organism. The given allele that contributes more is said to be favoured by natural selection and thus would contribute more to future generations. In a given environment, this contribution is thought to be constant and is believed to be the function of an allele's relative fitness and its environment (adaptive or selection coefficient). Likewise an allele for cooperation, is often assumed to have a constant selective value. However, in reality, there exist a relationship between adaptive/selective/fitness values of alleles and the frequency of alleles in a given population. Selection of alleles as a function of the frequency of the alleles in the population is called frequency dependent selection. Similarly, selection on cheater-cooperator interactions can also be affected by the starting frequency of each type in the population.

1.4.1.1 Possible outcomes of frequency dependence

In order to understand the effect of frequency on the fate of cooperation, consider this example of a microbial system consisting of two behavioural types. One is cooperator and the other the cheater. Further, assume that the growth rates or the fitness function of the two types is not constant and is dependent on the number of cooperators present in the mixed population, i.e., it is frequency dependent. In that case, if we mix the two types together in competition, there could be innumerable interactions that these types can adopt with mainly four possible outcomes (Damore & Gore, 2012).

a) Cooperator dominance

The first outcome be that the cooperating strains always dominate in the mixed population at all cooperator frequencies. For example, in cooperative breeding, the benefit to the cooperator increases linearly with the increase in the number of cooperators such that cooperator's relative benefit becomes greater than that of the cheater and it goes to fixation.

b) Cheater dominance

The cheater strain always dominates in the population at all cooperator frequencies. For example, in prisoner's dilemma (Rapoport & Chammah, 1965), cheaters have a high relative fitness and they go to fixation. The prisoner's dilemma also represents a situation of cheater invasion (discussed in section 1.5.2) in a population consisting of cooperators.

c) Coexistence

Both coexist, such that at high cooperator frequency, cheaters are favoured, whereas at low cooperator frequency, cooperators are favoured. Coexistence comes as a consequence of negative frequency dependence (NFD) – a given type is favoured when rare in the population. The microbial world is full of examples where the frequency of the strains is regulated by NFD (Pollitt *et al*., 2014; Ross-Gillespie *et al*., 2007; Rainey & Rainey, 2003).

For instance, in the *P. fluorescens* cheater-cooperator system (described in detail in section 1.5.2), cooperators pay an individual cost that is offset by a group-level advantage, while cheats derive benefit from being part of the collective. Both are maintained in the population by negative frequency dependence (Rainey & Rainey,

2003), although in this case the frequency dependence is time-lagged and oscillatory (Libby & Rainey, 2013).

d) Bistability

The fourth strategy is bi-stability, in this the cooperators get fixed at some high cooperator frequency and cheaters get fixed at low cooperator frequency. Bi-stability arises as a consequence of positive frequency dependence – the given type is favoured when it is relatively high in the population.

An example of bi-stability is the hypothetical green beard gene concept. This concept says that the cooperators can recognise each other by expression of a hypothetical gene which is called "green-beard". The expression of the gene requires a cost which when balanced by benefit from recognition, results in cooperators becoming fixed. The benefit from recognition in turn depends upon a given frequency of cooperators. If the cooperator frequency is below a given threshold, then cheaters will outcompete the cooperators, eventually becoming fixed in the population.

1.4.2 Density dependence

There are mixed suggestions on how density can affect cheater-cooperator interactions.

a) High density leads to high cooperation

In an experiment with yeast populations, it was noticed that in nutrient deficient conditions (low concentration of sucrose in the environment), three evolutionary strategies were available of choosing between, sucrose retention, more production of invertase *(*enzyme which breaks down sucrose) and clumping. 11 out of 12 populations chose clumping with all of them having increased invertase production in the

background (Koschwanez *et al*., 2013).This is because clumping increases local cell density such that benefits of cooperation stay close among cooperators in microbial populations (Pfeiffer *et al*., 2001; Koschwanez *et al*., 2013).

In another study it was shown that increasing resource supply increases cooperation in two of the microbial species of *P. fluorecens* and *P. aeruginosa*. Both species are used as model systems to study cheater-cooperator dynamics, former for biofilm formation and latter for siderophore production. The argument posed is that with increasing resources, density also increases, which brings down the cost of cooperation and increases the proportion of co-operators (Buckling *et al*., 2007).

In the *P. fluorescens* system, although high densities support more cooperators and thus cooperation, it was also found that high densities also support high cheat density which reaches a peak at intermediate resource level. The authors suggested that high oxygen tension generated in the broth phase, because of increased density of biofilm, makes the liquid phase unfavourable, selecting for a cheater population in biofilm. At the same time, the cheater population in the biofilm reaches saturation due to exhaustion of the resources; this is because of the biofilm's spatial environment, which favours cooperators over cheaters.

b) Quorum sensing leads to high density and high cooperation

Quorum Sensing (QS) is a microbial communication system, use to direct the expression of certain genes via feedback to population density. QS is mediated by extracellular molecules – autoinducers which accumulate during cell growth and when molecules reach a threshold concentration, the expression of certain genes is activated. These genes can be ones that help in cooperation. When this is the case, cooperation is said to be QS regulated and and high cell density (high QS) leads to greater cooperation.

For example, it was found that *Candida neoformans*, a type of fungus, has a QS enabled growth system that enables the cells to increase in growth and reach high cell densities both in planktonic and biofilm states. High cell densities in turn leads to high levels of secretion of metabolites that help cells to spread virulence (a cooperative trait) and improve persistence within the host (Albuquerque *et al*., 2014). In yet another example, a similar observation was reported with *P. aeruginosa*, a gram-negative human pathogen. In this system, it was shown that greater levels of QS activity is achieved with high cell densities which in turn increases the metabolites (a cooperative trait) and thus the inclusive growth of the cells (Darch *et al*., 2012).

c) High population density leads to lower levels of cooperation

In contrast to the above view high cell densities can also lead to decreased cooperation. For example, study conducted by (Ross-Gillespie *et al*., 2009) found that in *P. aeruginosa* populations high cell densities promote the cheaters ability to exploit cooperators because of greater direct interaction between the two. They demonstrated a negative density dependence relationship between the initial density and the level of cooperation. But the authors argued that this relationship can be changed by two factors,

(1) If the cooperators are practicing self-restraint, such that cells tend to use their resource efficiently, then increasing cell density will lead to a decrease in the amount of resource available per cell. In such a resource limited environment, cooperators practicing self-restraint will be selected and will find themselves closer to cooperators practicing a similar strategy, which will then further enhance cooperation in a positive feedback loop manner (Pepper & Smuts, 2002).

(2) The other way in which high density can be beneficial, is if the population structure is such that increase in cell density leads to increase in the factor of

genetic relatedness. For instance, a study conducted in yeast populations found that cooperation is positively correlated with density (Maclean & Gudelj, 2006).

In section 1.3.2 we learnt about the different theories given for the evolution of cooperation. These theories assume the environment to be stable and fails to acknowledge the role of density and frequency (the two important forces through which environment manifest the changes) that are capable of generating feedbacks (Heininger, 2015). For this study, we particularly focus on the role of environment in supporting cooperation using *Pseudomonas fluorescens* as a model system. The bacterial model system has been known to show environment dependent cooperation which is discussed in detail in the following sections (section 1.5 and 1.6).

1.5 Environment dependent cooperation in *P. fluorescens*

P. fluorescens has been an excellent model system for studying cheater-cooperator interactions and an even excellent system to study the effect of environment on cheater-cooperator interactions (Brockhurst *et al*., 2010). The organism shows different dynamics of cheater-cooperator interaction in two different environments, the section below (section 1.5.1 and 1.5.2) describe the two interactions.

1.5.1 Environment-1, nature of interaction: cheat as an invader (invasion)

The system consists of a ‘‘wrinkly-spreader’’ (WS) morph, which forms a biofilm at the air–liquid interface in static glass vials by production of cellulosic polymer (Rainey & Travisano, 1998). Although, production of cellulosic polymer is individually costly (Rainey & Rainey, 2003), it provides a group benefit to WS because of formation of the biofilm at the air–liquid interface, which allows access to oxygen (limiting resource) (Rainey & Rainey, 2003). These biofilms formed by WS types are subjected to invasion by the “smooth” (SM) morph that arises by mutation from WS over the course of many days. In this case, WS are the cooperators paying the cost for

the group, while SM are cheats, deriving benefit from being in the biofilm. However, SM contributes nothing to the production of the polymer, such that their presence weakens the mat (Rainey & Rainey, 2003). In this environment, the WS and SM denote the typical cheater-cooperator relationship with the cheater deriving benefit (effect is positive) at the expense of the cost paid by the cooperator (effect is negative).

1.5.2 Environment-2, nature of interaction: cheat as a propagule (coexistence)

Rainey and Kerr in their 2010 paper (Rainey & Kerr, 2010) – based on ideas presented earlier (Rainey, 2007) – proposed a unique hypothesis which suggested the role of cheat as a germ cell. They proposed that cheat as germ cell provided appropriate environmental conditions. It not only helped the collectives to reproduce that allowed them to become units of selection but also allowed the collectives to directly participate in the process of evolution by natural selection , which in turn gave them an opportunity to progress further to the second stage of multicellularity (section 1.1). The paragraph below illustrates the point in detail. Also see Figure 1.3.

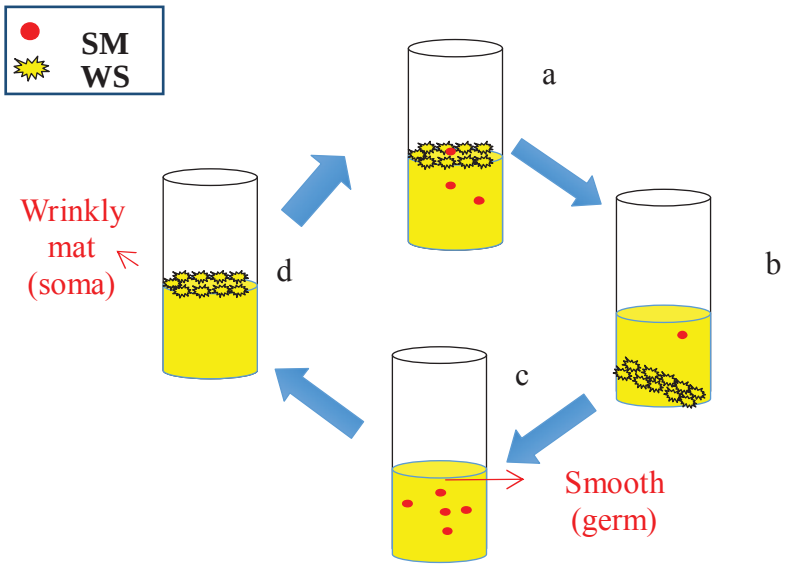

Figure 1.3 Schematic view of the life cycle. (a) a WS mat (collective) with SM types (b) The collapse of the WS mat (c) The SM grows and replete the oxygen, act as germ and give rise to WS cell (d) WS cell divides to form a mat, from where the cycle begins again.

Normally, the biofilm formed by WS types experiences a natural collapse after a few days of growth. After the collapse, it's an evolutionary dead end for the WS occupants within the mat. This is because they are non-motile and unable to move from the biofilm, which ultimately results in death of the cells from anaerobisis (deep down in the broth phase of the glass vial).

On the contrary, if the biofilm has SM types (Figure 1.3(a)), it is not necessarily an evolutionary dead end (Rainey & Kerr, 2010). This is because the collapse of the biofilm would lead to restoration of oxygen (Figure 1.3(b)), even though it would have led to an early collapse of the mat than in the former case (clonal WS mat with no SM types). Collapse of the mat is seen as an opportunity for SM types to escape the mat (because they are motile and not "glued" to WS cells) and obtain oxygen (Figure 1.3(c)). Once re-established, populations of SM cells stand to give rise once again to WS types thus completing the cycle (WS biofilm-SM propagule-WS biofilm) (Figure 1.3(d)) and maintaining coexistence by negative frequency dependence (discussed in section 1.4)

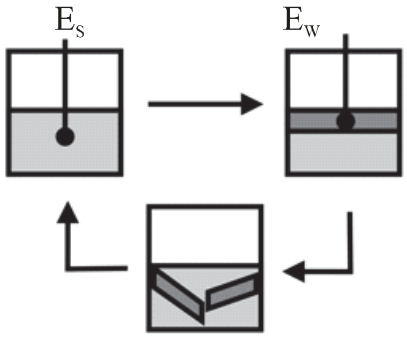

Figure 1.4 Feedback between environment and genotype. E_S state is replete with oxygen and favours the growth of SM. The SM exhausts the resources and creates chance for the WS to arise. The oxygen depleted environment is suitable for the emergence of WS because of its capacity to form WS mat at the air-liquid interface, due to WS cells producing glue. In the E_W state, the WS mat matures and collapses under its own weight, returning back to the original state of E_S. This represents a feedback between the environment and the WS and SM types, which is unique for each set of WS-SM system. Adapted from Libby and Rainey 2013(Libby & Rainey, 2013).

The tuning between the environment and the frequency of WS and SM types is noteworthy in this interaction. As the WS cells in the collective approaches carrying capacity, it creates an environment unsuitable for its growth which leads to collapse of the mat. However, this new environment (E_S) replete with oxygen creates a selection pressure that favours the emergence of SM. The SM divides and depletes all new resources which was available to the cells and creates an environment unsuitable for itself. But, at the same time creates an environment (E_W) that favours the emergence of WS (Figure 1.4). Thus the environment oscillates between two states E_S and E_W as the two types reach their carrying capacity in their respective environments (Libby & Rainey, 2013).

Even though the feedback phenomenon between the genotype and environment is common and will be exhibited by every pair of WS and SM. Each feedback pattern will be different in terms of:

1. The frequency of the given type (SM or WS) in a given state (E_S or E_W)
2. Population density in each state
3. The capacity to switch: (i) from WS to SM (ii) from SM to WS.

Thus, we see that feedback between environment and genotype, generates the potential for a new interaction between the SM and the WS.

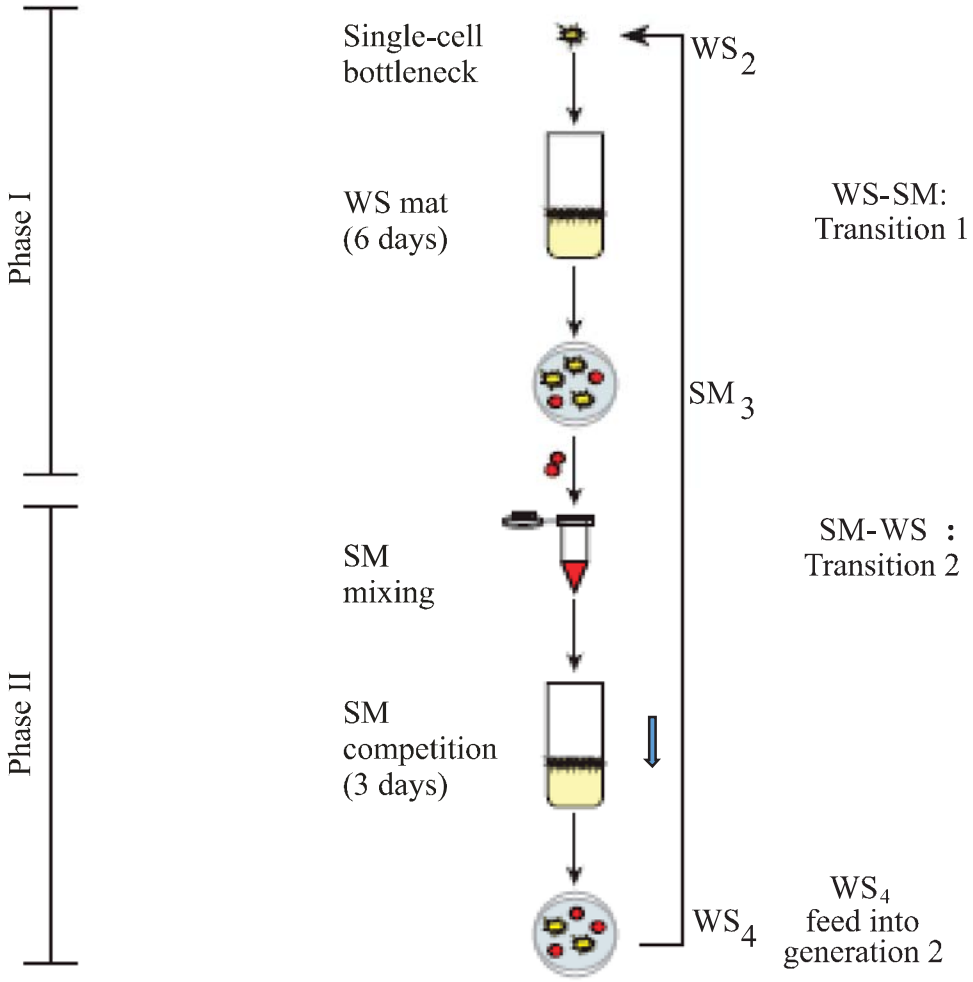

Figure 1.5 A representation of 'generation 1' of the life cycle experiment. Each generation consists of two phases and each phase with one transition. Generation 1 starts from phase I WS_2, which is obtained from ancestral SM_1- SBW25 after 3 days of static growth, harvesting and plating (not shown in fig-method resembles phase II of the life cycle).WS_2 then transitions to SM_3 after 6 days (phase I). The end of phase I mark the first transition: from WS to SM. The collective pool of SM, referred to in the figure as SM_3 , transitions back to WS_4 after 3 days of static growth (phase II). End of phase II marks the second transition from SM to WS and also denotes the end of the current generation, in this case generation 1. The WS (WS_4 in fig) obtained at the end of the generation (gen-1), feeds to the succeeding generation (gen-2). Adapted from Hammerschmidt *et al*. (2014).

1.6 Development of interaction between WS-SM in a long term evolution experiment with *P. fluorescens*

Within the life cycle (described above in section 1.5.2), the transition from WS to SM and vice versa, is mutation dependent, because of which there is likely a limit to the number of times the transition can take place between stages of the life cycle. However, through evolutionary time, selection on the capacity to switch between phases

of the life cycle may result in the evolution of mechanisms that facilitate switching – even the possibility of switching becoming free of mutational requirement.

Recently, an experiment was conducted that selected directly on the capacity of experimental Pseudomonas populations to repeatedly transition between phases of the life cycle (Hammerschmidt *et al*., 2014). Central to the experiment was the imposition of a death-birth process at the level of lineages: whenever a lineage failed to produce the next phase of the life cycle it was deemed extinct and this allowed a successful lineage to split (to export its success), and thus increase its frequency in the overall population (Figure 1.5).

At the end of the experiment, the fitness of derived lineages was compared to the ancestral types. Overall fitness of collectives increased. Subsequent studies showed that the trait explaining enhanced fitness was improved capacity to transition between SM and WS (and vice versa). However, transitioning capacity was not the only responsible factor for tuning the system, as not all evolved lineages had high transitioning capacity but nonetheless, could still make it through the life cycle. Thus, it was considered that different lines may have adopted different strategies to reach the same goal, even though they had the same ancestor to begin with.

The desire to understand more fully the traits improving the evolutionary success of lineages motivates the work described here.

1.7 Motivation

1.7.1 Impact of ecology on evolution

The regime of the life cycle experiment (Hammerschmidt *et al*., 2014) meant a drastic change (propagation of lines with cheater as a propagule for 10 and a half generations) in the ecology of the system.

It is known from previous studies that changing environmental conditions often results in an evolution in morphology, physiology or a change in life history properties. There are numerous such examples highlighting the impact of changing ecology on evolution. The famous example of Galapogos ground finch, *Geospiza fortis,* illustrates the change in morphological character in beaks of this species when it was exposed to long dry periods. After a number of dry years, associated with the availability of big seeds, birds with bigger beaks were more abundant and during wet years, which was associated with the availability of small seeds, the birds with smaller beak size became more abundant (Grant & Grant, 1992).

Like how the change in environment led to an evolutionary change in beak size. Similarly, the change in ecology in the WS-SM system, could have led to an evolutionary change

1.7.2 Impact of evolution on ecology

Evolutionary change is also capable of impacting ecology. Thompson (Thompson, 1998) noted that when evolution is rapid, the changing phenotypes of organisms can bring simultaneous ecological change. Evolution is considered rapid in an ecological sense if the heritable phenotypic change occurs rapidly enough to impact the trajectory of ecological process (Hairston *et al*., 2005). Organisms with shorter

generation times, like microbes, have been known to show an effect on ecology due to rapid evolutionary change.

The evolution experiment also involved microbes that went through rapid evolutionary change which was a quick change in the frequency of WS-SM types, this rapid phenotypic change was capable of affecting the ecology of the system.

1.8 Hypothesis

During the evolution experiment, there evolved an interaction between the cheater and cooperator. The interaction which supported both the survivability (intact WS collective) and reproducibility (production of propagules) of the collective, leading to the evolution of cooperation. These requirements to complete the life cycle would have led to following sequence of steps:

1. **Change in ecology I-** there was strong selection for 10 life cycle generations which required the lines to switch between the phenotypes (Figure 1.6(a))
2. **Evolution due to change in ecology-** this could have led to an evolutionary change in the frequency of WS-SM types (Figure 1.6(b))
3. **Change in ecology II-** This change in WS-SM frequency in turn could have led to an ecological change in population density (Figure 1.6(c))
4. **Eco-evo feedbacks-** Change in ecology leading to evolution and evolution occurring at timescales fast enough to respond to that ecological change such that it impacts the ecology of the system in turn, results in an eco-evo feedback (indicated by loop arrows in Figure 1.6(c)).

This led to the formation of the hypothesis that ecological and evolutionary changes, during the evolution experiment, would have led to the development of an eco-evo feedback loop in evolved lineages which are responsible for maintenance of cooperation.

A signature of this eco-evo feedback, which has likely occurred both on the WS-SM interaction and switching rate, can be traced by comparing the population dynamic pattern of the ancestor and evolved lineages (Yoshida *et al*., 2003).

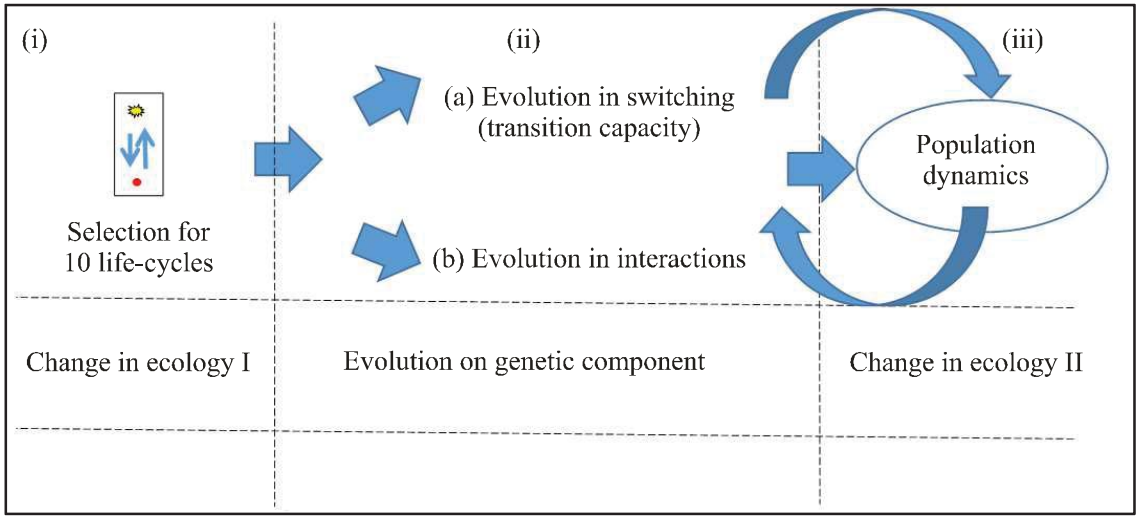

Figure 1.6 Steps in the development of an eco-evo feedback. 1. Change in ecology I: In the evolution experiment there was a selection for 10 life cycle generations which involved switching between phenotypes 2. Evolution on genetic component: The selection in each generation acted upon two life history traits. (a). Transition capacity: tendency for WS to switch to SM and vice versa. (b). Nature of the interaction: the reliability to complete the life cycle in each generation called for a development of a reliable interaction between the WS and SM 3. Change in ecology II: Evolution on the genetic component of the system led to a change in ecology, this change in ecology is likely to manifest at the level of population density. This change in population density in turn induces a genetic change. This loop (indicated by curved arrows) leads to the development of an eco-evo feedback.

1.9 Aim of the study

The overall aim of this thesis is to determine the presence of an eco-evo feedback and to study its effects on the evolutionary success of evolved lineages.

The main aim is structured in the following sub aims and presented in the form of three chapters:

1. To investigate frequency dependent interactions between WS and SM.
2. To investigate density dependent interactions between WS and SM.
3. To obtain frequency versus population density plots (eco-space diagrams).

Trajectories will be obtained from the three parameters of frequency dependence, density dependence and eco-space diagrams. Those obtained will be compared between the evolved and ancestral lineages, to check signs of an eco-evo feedback.

1.10 Thesis structure

The sub aims discussed in the previous section (section 1.9) are presented in the form of three chapters. Chapter 3, describes the results for the frequency dependent interactions between WS and SM. Chapter 4, describes the results for the density dependent interactions between WS and SM, and Chapter 5, describes the results for eco-space diagrams. In Chapter 5, the joint influence of frequency and density is considered. Chapter 6, presents the conclusion; it summarizes the key findings of the results and contains a concluding discussion on each key finding. The chapter also discusses future work opened up from this study.

Chapter 2. Material and Methods

The section below (section 2.1) gives a brief overview of the life cycle experiment conducted in the study done by Hammerschmidt *et al*. (2014). It is important to know about the life cycle to understand the approach behind the experiments and also to know the evolutionary history of the lineages used in this study.

2.1 Life cycle experiment

The life cycle experiment (Chapter 1, Figure 1.5) consisted of a two-phase life cycle. The life cycle was founded with phase II in which a single ancestral SBW25 colony (ancestral SM) was inoculated in a microcosm (25-ml glass vials) and was kept for 3 days in static growth. After plating, a single colony of the most abundant wrinkly spreader (WS) morphotype was picked from each plate to inoculate a fresh microcosm. This led to the beginning of phase I. Lines which had intact mat at day 6 were carried forward to phase II, while those which didn't were considered extinct. The lines were checked for the presence of SM colony types, after being vortexed, mixed and plated. The lines that lacked SM were considered ineligible to proceed to the next generation (phase II). SM colonies from rest of the lines were individually put into 200 ml liquid medium, and incubated for 24 hours under static conditions to get sufficient numbers for inoculation in the next phase. Subsequently, SM from all of the lines were pooled and 6ml was taken to inoculate the microcosms for phase II. For further details on the methods of this evolution experiment, please refer to Hammerschmidt *et al*. (2014).

2.1.1 Transition Capacity (TC)

Transition capacity gives an indirect measure of the switching ability of the lineages. The life cycle experiment described above generated 14 evolved lineages and 14 ancestral lineages – also called baselines lineages – from the experiments performed by Hammerschmidt *et al*. (2014); SBW25 is the ancestor of all of these. The lineages used in this study were selected based on their varied transition capacity. Two baseline lineages – 71, 70 and two evolved lineages – 14 and 43, each of high and reduced transition capacity respectively, were chosen for this study.

Each lineage consist of a wrinkly spreader (WS) and a smooth (SM) pair. Therefore, transition capacity is given in two directions: from WS to SM and vice versa. Table 2.1 shows the transition capacity for the both the evolved lineages and the baseline lineages used in this study. The colour coding of transition capacity profile is based on the proportion of microcosms containing the alternate type. For instance in the case of transition capacity from WS to SM if one out of three microcosms contained the SM type it was colour coded as light red. Likewise if two out of three microcosms contained SM, it was colour coded a darker shade than in the previous case. And finally if three out of three microcosms contained SM, it was colour coded as dark red. The evolutionary history of the lineages selected on the basis of their TC is discussed in detail in the following section.

Table 2.1 Evolutionary history and transition profiles for all of the WS-SM pairs (used in this study).

	Strain name	The lineage type	Characteristics of the lineages	Steps of evolution from ancestor SBW25	Proportion of microcosms with alternate type for 6 days*
A.					
WS to SM	71 WS	BL	Highest TC among BL	WS 2	
	17 WS	EV	Highest TC among EV	WS 26	
	70 WS	BL	Reduced TC among BL	WS 2	
	43 WS	EV	Reduced TC among EV	WS 22	
	WS¶	ANC	Ancestral WS,	WS 2	
B.					
SM to WS	71 SM	BL	Highest TC among BL	SM 3	
	17 SM	EV	Reduced TC among EV	SM 27	
	70 SM	BL	Reduced TC among BL	SM 3	
	43 SM	EV	Reduced TC among EV	SM 23	
	SBW25 SM	ANC	Ancestral wild type SM¥	SM 1	

SM: smooth cell type, WS: wrinkly spreader. BL: baseline lineage, EV: evolved lineage, ANC: ancestor.*White: absent, yellow: the proportion WS type, red: the proportion of SM type, extinction. Colour intensity shows the proportion of microcosms containing a new type: the more the intensity, the higher the proportion. ¥The strain used in the Rainey and Rainey, (2003). ¶ The WS derived from ancestral SM SBW25.

Panel A shows the transition capacity from WS to SM and the evolutionary history of the WS, while panel B shows the transition capacity of SM to WS and the evolutionary history of SM. The last column for both panels contains colour bands representing the transition profile. Each band contains six divisions, and each division is representative of the proportion of microcosms – with SM for panel A and with WS for panel B. The colour gradation in each division represents proportion of microcosms containing a given type: the more the colour intensity, the higher the proportion.

2.2 Evolutionary history of lineages

In this study one ancestral, two evolved and two baseline lineages are used. Each lineage consists of two morphotypes: (i) wrinkly spreader (WS) type (ii) and smooth (SM) type; thus, making 10 morphotypes for 5 lineages. The section below describes the background of the WS and SM types used in this study.

2.2.1 WS and SM of the ancestral lineage – SBW25

The SM of the ancestral lineage is a wild type *P. fluorescens* strain that is isolated from the leaf surface of a sugar beet plant (Silby *et al*., 2009). All other lineages used in this study are obtained from the ancestral SM SBW25 strain including the ancestral WS that is obtained from ancestral SM SBW25 after 3 days of static growth (static growth described in section 2.5.2).

2.2.2 WS and SM of evolved lineages – Line 17 and Line 43

As mentioned before, 14 evolved lineages were generated in the life cycle experiment conducted in study performed by Hammerschmidt *et al*. (2014). From those, 2 evolved lineages – Line 17 and Line 43 – are chosen for this study.

The WS and SM of Line 17 and Line 43 were derived after ten and a half generations of life cycle, in the following manner:

Line 43 WS: During the life cycle, the first WS (labelled as WS_2) was derived from the ancestral SM SBW25 (labelled as SM_1) after three days of static growth. This WS_2 was then transitioned to SM (labelled as SM_3). The transition from SM to WS, and then from WS to SM, constituted 1 generation. Like this the WS was passed through ten and a half generation of life cycle, altogether undergoing 20 transitions (10 generation) + 1 transition (half a generation). In this manner, the WS for all the evolved lineages,

including Line 43 and 17, is 21 transitions/steps away from the first SM, i.e. of ancestral SBW25 (SM_1) and so it gets a number of WS_{22}.

Line 43 SM: The SM for Line 43 used in this study is derived from Line 43 WS_{22} after 48 hours of growth in static, and so is labelled as Line 43 SM_{23}, meaning it is 23 steps away from ancestral SM SBW25.

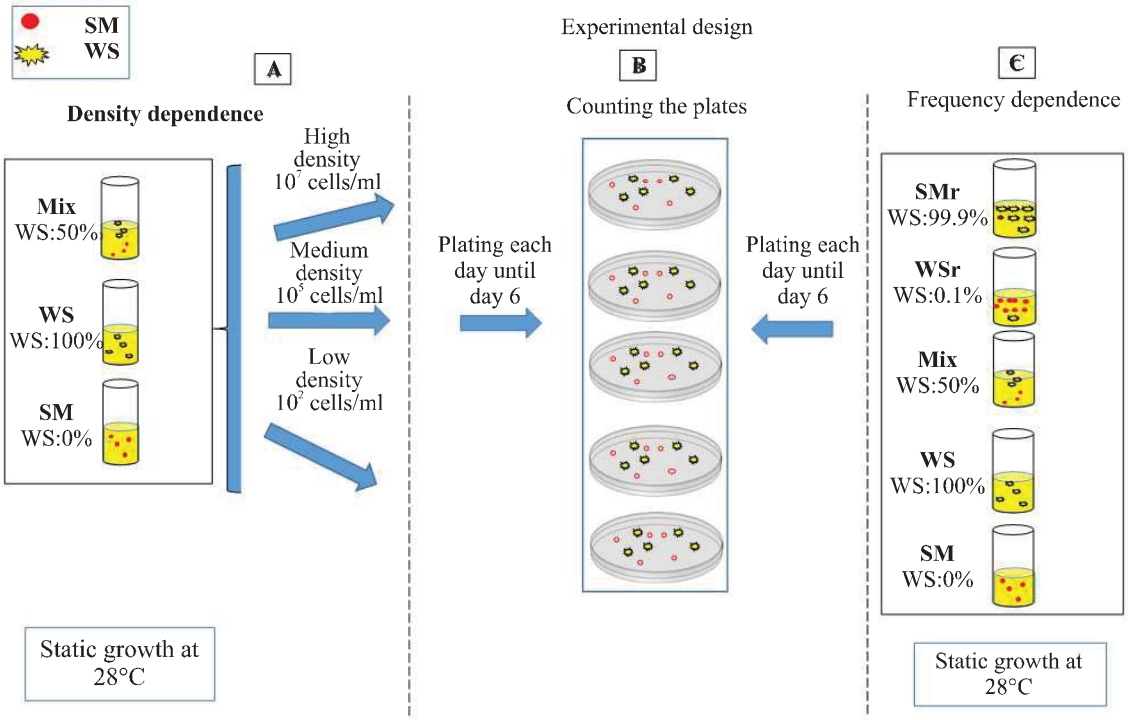

Figure 2.1 Experimental design for frequency and density dependent interactions (i) Frequency dependence. C and B together represent the design employed in frequency dependence, where the mixed –1000:1; 1:1000; 1:1, and pure populations of WS and SM were inoculated at a constant density of 10^6 cells/ml. For each frequency treatment three replicates were used. The microcosms from all the treatments were grown in static for six days. Each day three replicates from each treatment were destructively harvested and subsequently dilution plated on solid agar plates. After 2 days of incubation, the frequency of WS and SM types, and total cell density was recorded. **(ii) Density dependence:** A and B together represent the experimental design employed in density dependence, where each of the treatments: mixed (1:1 WS: SM); pure WS; and pure SM population, were founded at three initial densities: high – 10^7 cells/ml, medium – 10^5 cells/ml and low – 10^2 cells/ml. For each density treatment three replicates were used. The microcosms from all the treatments were grown in static for six days. Each day three replicates from each treatment were destructively harvested and subsequently dilution plated on solid agar plates. After two days of incubation, the frequency of WS and SM types, and total cell density was recorded.

Line 17 WS: In case of Line 17, a mutS (A1489C) mutation is responsible for its high transition capacity (Hammerschmidt *et al.*, 2014). The transition capacity of the line is

so high that within hours of inoculation of a WS or SM type, an alternate type emerged, and the pure cultures of WS and SM contained both types, even at day 0. In order to get rid of this problem, the WS_{22} mutS (A1489C) was reverted to wild type WS_{22} $mutS^{WT}$. This WS_{22} $mutS^{WT}$ has gone through two more generations (although with a cycling period of 48 hours). The WS obtained thereafter, which is WS_{26} $mutS^{WT}$ is used for this study. The WS_{26} $mutS^{WT}$ has a reduced transition capacity compared to the WS_{22} mutS (A1489C); however, the former still retains high transition capacity compared to all the evolved lineages, and therefore is believed to have a non-significant impact on the nature of interactions.

Line 17 SM: The SM for Line 17 used in this study is derived from Line 17 WS_{26} $mutS^{WT}$ after 48 hrs of static growth, and so is labelled as Line 17 SM_{27} $mutS^{WT}$, meaning it is 27 steps away from ancestor SM SBW25.

2.2.3 WS and SM of baselines lineages – Line 70 and Line 71

Among the 14 baseline lineages, Line 70 was chosen for its reduced transition capacity and Line 71 was chosen for its high transition capacity. The WS of Line 71 and Line 70 used in this study were obtained in the study performed by Hammerschmidt *et al*. (2014), by growing SBW25 in a static culture for three days, at the end of which the microcosms were destructively harvested and plated. The single most dominant WS type from one of the densely populated plate of one of the replicates was then picked as representative of baseline lineage WS. Thus the WS obtained for baseline lineage is one transition away from the ancestral SBW25 genotype (SM_1), and so is labelled as Line 70 WS_2 and Line 71 WS_2 respectively.

The SM for Line 70 and Line 71 were derived from Line 70 WS_2 and Line 71 WS_2 respectively in the following manner. The first SM that emerged from WS_2 of

respective lines was picked. This was done by incubating bacterial cultures for six days at 28°C under static conditions and destructively harvesting three microcosms per line per day until the appearance of the first SM phenotype. It was important to pick the first SM to avoid accumulation of mutations in lineage with time. For Line 70 the first SM emerged after 120 hours of inoculation, while for Line 71 it emerged, only after 24 hours. Since the SM obtained for both the lines is derived from WS_2 of respective lines, and is 2 transitions away from ancestral SM SBW25 (SM_1), the SM is labelled as SM_3.

2.3 Experimental set-up

The experimental set up is depicted in Figure 2.2. In the Figure, column C and B, depict the experimental set-up for frequency dependent interaction while column A and B, depicts the experimental set-up for density dependent interactions between WS and SM.

2.3.1 Experimental set-up to study frequency dependent interactions

1. WS and SM types for each lineage were obtained from -80°C freezer stocks and revived following the procedure mentioned in section 2.5.3.
2. Post revival, three colonies each from WS and SM plate (representing three independent biological replicates) were picked and inoculated in 6 ml of King's B media contained in microcosms for overnight growth in shaken conditions (section 2.5.1).
3. On the day of the experiment, i.e. day 0, three WS: SM combinations – 1:1, 1:1000 and 1000:1, were created from overnight cultures for each lineage. These combinations were created in proportion to the optical density (OD) values of the WS and SM types.

4. Together with pure cultures of WS and SM, each lineage had 5 treatments – pure WS, pure SM, and 1000:1 WS: SM, 1:1000 WS: SM and 1:1 WS: SM.
5. On day 0, 6µl inoculum from each treatment was inoculated in triplicates in microcosms containing 6ml of King's B media, to obtain a density of 10^6 cells/ml. This was done for each lineage and the set-up was created for six days.
6. For all the lineages combined, the total numbers of microcosms on day 0 were: 5 lineages* 5 treatments* 3 replicates for each treatment* 6 days=450 microcosms.
7. To know how many cells were present on the day of the experiment, day 0 samples were plated by serially diluting and then plating on solid media, from one set of the replicates. After obtaining day 0 readings, all the microcosms were then incubated in static condition at 28°C.
8. After 24 hours, three replicate microcosms for each lineage and for each treatment were destructively harvested (microcosms were vortexed for one minute, time sufficient to disintegrate the mat) and subsequently plated after serial dilution. After two days of incubation at 28°C, frequency of WS and SM colony types were counted. Approximately 500 colonies were counted from each replicate. This corresponded to day 1 reading.
9. Similarly, after 48 hours, another set of microcosms reserved for day 2, were put through the same procedure as mentioned in step 8th. In this manner, the data for relative WS/SM frequency and total cell density was obtained for all six days. The experiment was repeated two times.

2.3.2 Experimental set-up to study density dependent interactions

1. For density dependent experiments, the first two steps are the same as discussed in the above section 2.3.1.

2. On the day of the experiment, a 1:1 combination of WS: SM was created from overnight culture, for all the lineages. The combination was created in proportion to each lineage's WS to SM OD values.
3. The 1:1 mix along with pure WS and pure SM population, constituted 3 treatments.
4. On day 0, each treatment for each lineage were diluted, and inoculated in microcosms in triplicates (3 replicates) containing 6ml of King's B media to achieve three different densities–high-10^8 cfu/ml, medium -10^5 cfu/ml and low-10^2 cfu/ml. The total numbers of microcosms on day 0 were: 5 lineages* 3 populations* 3 different densities* 3 replicates* 6 days= 810 microcosms.
5. The rest of the experimental procedure was the same as in the case of 'frequency dependent interactions' and the experiment was repeated two times.

Note: There is no experimental set-up for eco-space diagrams. They are a plotting scheme and not an experiment in itself (Sanchez & Gore, 2013). The plots used the data obtained from the frequency and density dependent experiments.

2.4 Approach taken to study interactions

As discussed in the introduction (Chapter 1, section 1.8), the presence of the eco-evo feedback, which has likely occurred on the WS-SM interaction can be identified by comparing the evolutionary dynamics (frequency dependent interactions), population dynamics (density dependent of interactions) and the joint evolutionary and population dynamic patterns (eco-space diagrams), of the ancestor and evolved lineages. The approach is discussed in detail in the following paragraphs.

2.4.1 Approach taken to study frequency dependent interactions

In order to visualize the evolutionary change that would have occurred in the evolved lineages, frequency dependent interactions between WS and SM was observed. The interaction between WS and SM consisted of two components (i) the effect of WS on SM, and (ii) the effect of SM on WS. The effect of WS on SM was determined by comparing the growth profiles of SM when grown on its own and when grown in presence of WS at varying frequencies of WS – 0.1%, 50% and 99.9%. Similarly, effect of SM on WS was determined by comparing the growth profiles of WS when grown on its own and when grown in presence of SM at varying frequencies of SM – 0.1%, 50% and 99.9%. The trajectories obtained were then compared between baseline and evolved lineages, for signs of an evolutionary change.

2.4.2 Approach taken to study density dependent interactions

In order to visualize the impact of ecology, density dependent interactions between WS and SM were observed. The interaction between WS and SM consisted of two components (i) the effect of WS on SM and (ii) effect of SM on WS. The effect of WS on SM was determined by comparing the growth profiles of SM when grown on its own and when grown in presence of WS at varying population densities – high, medium and low. Similarly, the effect of SM on WS was determined by comparing the growth profiles of WS when grown on its own and when grown in presence of SM at varying population densities – high, medium and low. The trajectories obtained were then compared between baseline and evolved lineages, for signs of an ecological change.

2.4.2.1 Log transformation and normalisation of density dependent data

To compare interactions between high, medium and low density, data normalization was performed. In this procedure, the scales of the two growth profiles

which are compared to assess the interaction are made to vary between 0 and 1 such that the units become arbitrary and the profiles can be compared. So quantitatively, one would not be able to determine the absolute growth value of the population by looking at the graphs. However, qualitatively, it would allow the interaction between the two growth profiles to become much more apparent. At the same time, comparison can be made between the interactions performed at different densities.

Note that before normalisation was done, the data was first log transformed – log base 10.

The formula used for normalisation of growth profiles is:

$$x_{new} = \left(\frac{x - x_{min}}{x_{max} - x_{min}}\right)$$

Where,

x_{new} = normalised value

x = non normalised value

x_{min} = minimum value of the data for a given growth profile

x_{max} = maximum value of the data for a given growth profile

2.4.3 Approach taken to study joint effect of frequency and density–eco-space diagrams

Frequency versus population density plots is called eco-space diagrams. These were plotted for each of the different combinations of frequency and density. They assisted to visualize the joint effect of frequency and density on the interaction between SM and WS.

2.4.4 Effect of transition capacity on the trajectory of the plots

Transition capacity (TC) gives an indirect measure to account for switching rate of the lines. A given trajectory generated from an eco-space diagram would essentially be a function of its respective TC. This would be true for ancestral and baselines that haven't undergone an evolution in interactions. However, evolved lineages, which we believe have undergone a change in interactions, would be influenced by both its TC and some other factor-X.

The given trajectory thus, will therefore be representative of two factors:

1. TC
2. X factor – which we hypothesize to be an ecological (eco) and evolutionary (evo) feedback.

The idea is that the trajectory, if it reveals anything other than the pattern expected under TC, then that change in pattern would be possibly due the presence of the eco-evo feedback.

2.5 Media, growth conditions and other methods

The following section describes the materials required and enlists the conditions under which the present study is performed.

All the strains for the experiment were grown at 28°C in 25-ml static glass microcosms having 6 ml of King's Medium B (10 g/l glycerol; 20 g/l Tryptone; 1.5 g/l Mg_2SO_4; 1.5 g/l K_2 HPO_4) (loose caps). For growth on solid media, King's Medium B agar plates were used and incubated at 28°C for 48 hours.

2.5.1 Growth in shaken conditions

A single colony was picked to inoculate a microcosm, containing 6ml of liquid KB media. The microcosms were then incubated at 28°C on a shaker (150 rpm) with access to oxygen for 16 hours.

2.5.2 Growth in static culture

A single colony was picked to inoculate a microcosm, containing 6ml of liquid KB media. The microcosms were then incubated at 28°C in a static environment with access to oxygen for 24 hours.

2.5.3 Revival of freezer stocks

Bacterial strains were revived from freezer stock by streaking with the help of bacterial loop onto King's Medium B (KB) agar plates (10 g/l glycerol; 20 g/l Tryptone; 1.5 g/l Mg_2SO_4; 1.5 g/l K_2 HPO_4, 15g/l agar) and then incubating the plates for 48 hours at 28°C.

2.5.4 Preparation of stock cultures

Bacterial cultures were grown in shaken conditions for 16 hours, after which, 800µl of this culture was mixed with 1 ml of 45% (v/v) glycerol saline solution (8.5 g NaCl, 300 ml H_2O, glycerol to 1 litre) in 2ml cryogenic tubes. The tubes were stored at -80°C.

Chapter 3. Frequency dependent interactions between WS and SM

3.1 Introduction

The hypothesis for this study is that during the life cycle experiment, the interaction between the SM asocial (cheater) type and the WS social (cooperator) type was the subject of evolutionary change (Chapter 1, section 1.8). The necessity of both survival (maintenance of an intact WS collective) and reproduction of WS collectives (production of propagules by WS types) in the life cycle experiment imposed a strong ecological challenge (Chapter 1, Figure 1.3). This led to a change in the interaction between WS and SM at timescales fast enough to respond to ecological feedbacks (Hammerschmidt *et al.*, 2014).

I predict that the signature of this ecological-evolutionary (eco-evo) feedback, which has likely occurred both on the cheater-cooperator (WS-SM) interaction and their switching rate/transition rate, can be identified by comparing the evolutionary dynamics, population dynamics and the joint evolutionary and population dynamic patterns of the ancestral and evolved lineages (Chapter 1, Figure 1.6).

These interactions can be categorized in terms of:

(i) Frequency dependence: an investigation of evolutionary dynamics

(ii) Density dependence: an investigation of population dynamics

(iii) Eco-space diagrams: an investigation of the joint influence of evolutionary and population dynamics [on X due to Y] of the ancestral and evolved lineages.

Chapter 3 describes the frequency dependent nature of interactions. The results for density dependent and eco-space diagrams follow afterwards and are presented in Chapter 4 and Chapter 5, respectively.

For this study, two ancestral lineages (lines 71 and 70) – referred to as baseline lineages – two evolved lineages (lines 17 and 43) and one ancestral SBW25 line were used (Chapter 2, section 2.2). Each line represents a cheater-cooperator system (WS-SM). Lines 71, 70, 17 and 43 derive directly from the experiment of Hammerschmidt *et al*. (2014); SBW25 was the ancestor of all. The interactions are compared between the ancestral/baseline lineages – 71, 70 and evolved lineages – 17, 43.

The baseline lineages represent variation in the ancestral state. In the experiment of Hammerschmidt *et al*. (2014), 14 baseline lineages were obtained after three days of static growth from ancestral SBW25 (Chapter 2, section 2.2.3). The baseline lineages are one mutational step away from the ancestral SBW25 genotype. To take into account variability in transition capacities, baseline lineages manifesting different capacities to transition between each WS and SM phase of the life cycle were chosen for this study. Line 71 has a high capacity to transition, whereas Line 70 has a reduced capacity to transition.

The two evolved lineages experienced ten and a half generations of life cycle evolution (Hammerschmidt *et al*., 2014). In the Hammerschmidt *et al*. (2014) experiment, *Pseudomonas* populations repeatedly transitioned between phases of the life cycle. Central to the experiment was the imposition of a death-birth process at the level of lineages: whenever a lineage failed to produce the next stage of the life cycle it was deemed extinct and this allowed a successful lineage to split (to export its success) and thus increase its frequency in the overall population (of lineages) (Chapter 1, Figure

1.5). The experiment resulted in the generation of 14 such derived lineages out of which two are chosen for this study. Line 17 has a high capacity to transition, whereas Line 43 has a reduced capacity to transition (Chapter 2, section 2.2.2).

3.2 Results

To investigate the effects of changing initial frequency on the growth of WS and SM types, growth profiles of the given type alone and in the presence of the alternate type, at varying frequencies – high (99.9%), medium (50%) and low (0.1%) – is observed for ancestral SBW25, the baseline lineages – 70, 71 – and the evolved lineages – 17, 43. Later, the interactions are compared between the baseline and evolved lineages.

3.2.1 Frequency dependent interactions in ancestral SBW25

From earlier experiments in which the ancestral SM *P. fluorescens* genotype was allowed to compete with the derived WS type (both founded at a 1:1 ratio), it was found that the SM type rapidly increased in frequency compared to the case when SM was grown alone.

This indicated a positive effect of WS on SM. Also, it was seen that the WS mat collapsed prematurely in a mix than a WS type growing alone that indicated a negative effect of SM on WS (Rainey & Rainey, 2003).

I expected the interaction in ancestral lineage SBW25 to be frequency dependent, since wild type *P. fluorescens* SBW25 has been known to show frequency dependent effects (as discussed in Chapter 1, section 1.4). In order to investigate this, the interaction between ancestral SBW25 (SM) and a WS type derived by one mutational event from the SM type, as was done in Rainey and Rainey (2003), was examined, although with some modifications.

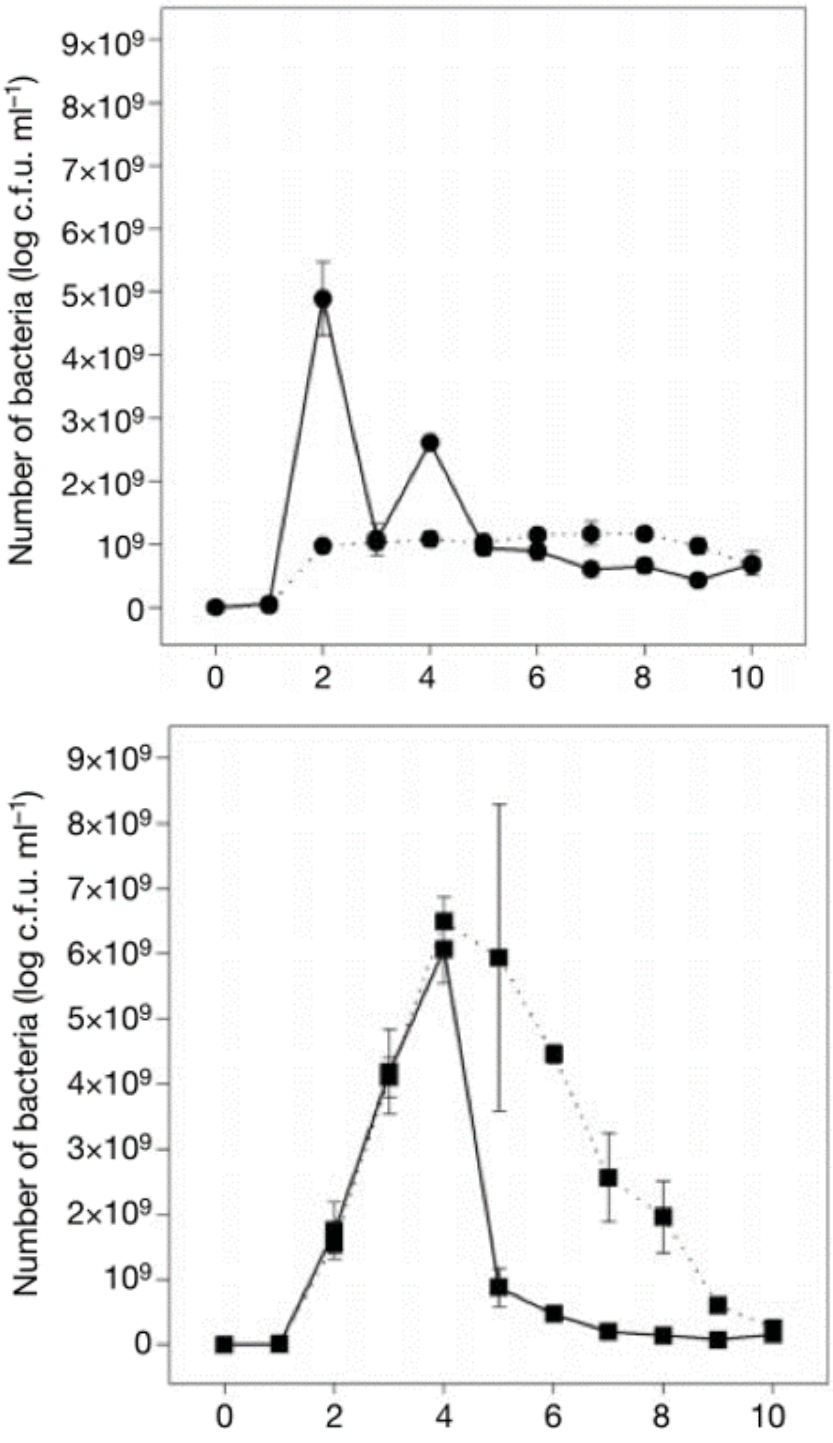

Figure 3.1 Interaction between ancestral SBW25 (SM) and WS. Data are from Rainey and Rainey (2003), (a) shows the effect of WS on SM and (b) shows the effect of SM on WS. Each plot shows comparison between two growth profiles: 1. a given type starting from 100% frequency (dotted line) and 2. a given type in the presence of the alternate type (solid line). The difference between the solid and the dotted lines gives the degree of interaction. Values are ± s.e.m. (n = 3).

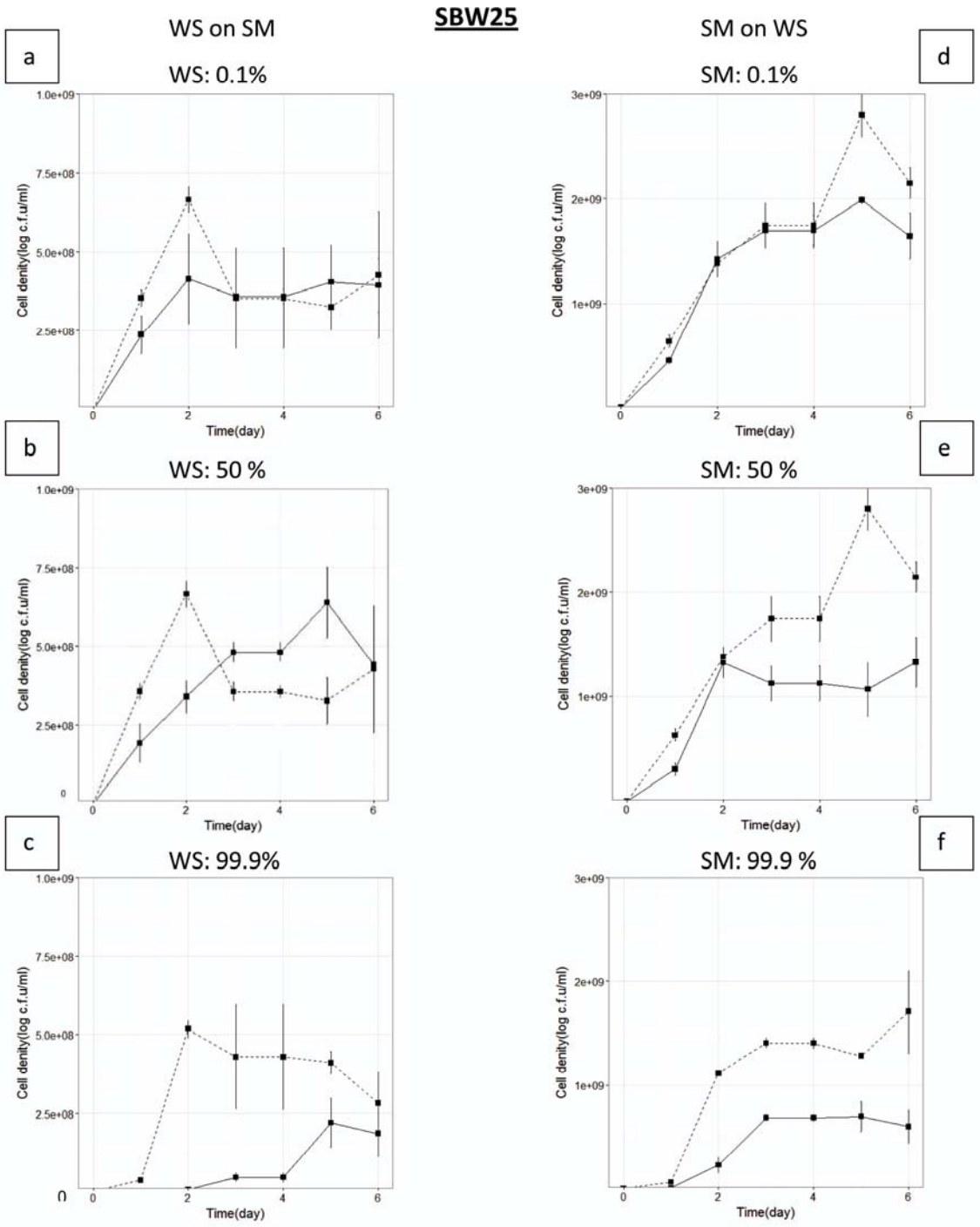

Figure 3.2 Frequency dependent interactions in Line SBW25. Left panel shows the effect of WS on SM at varying founding frequencies of WS (a) WS is 0.1 % (b) WS is 50% (c) WS is 99.9%. While the right panel shows the effect of SM on WS at varying founding frequencies of SM (a) SM is 0.1 %(b) SM is 50% (c) SM is 99.9%. Each plot shows comparison between two growth profiles: 1. a given type starting from 100% frequency (dotted line) and 2. a given type in the presence of the alternate type (solid line). The difference between the solid and the dotted lines gives the degree of interaction. Values are mean ± s.e.m. (n = 3).

Firstly, I not only examined interactions in populations of SM and WS founded by a 1:1 of the two types, but I also examined the dynamic of interactions in populations founded by unequal ratios of the types. The two combinations 1:1000 (WS: SM) and 1000:1 (WS: SM) were chosen to see whether the interaction remains the same at varying founding frequencies. Secondly, in my study, interactions were examined over a six day time scale as opposed to ten day period in Rainey and Rainey (2003).

The interaction was assessed by the same methodology as in Rainey and Rainey (2003). The interaction consisted of two components,

1. Effect of WS on SM and
2. Effect of SM on WS.

The effect of a given type on the alternate type can be determined by comparing the growth of the alternate type when grown alone with growth in the presence of the other type at varying frequencies. For instance, the effect of WS on SM can be determined by comparing the growth profiles of SM when grown on its own and when grown in presence of WS at varying frequencies of WS.

The effect of a given type is called positive/facilitative when the alternate type has a higher relative growth in the presence of the given type compared to growth alone. Otherwise, the effect of a given type is deemed negative, when the alternate type has a lower relative growth in the presence of the given type then just by itself.

For example, the effect of WS on SM would be positive when SM in the presence of WS (represented by bold line in Figure 3.1(a)), would have higher relative growth than SM grown alone (represented by a dotted line in Figure 3.1(a)). On the

other hand, the effect would be negative if SM has a lower relative growth profile in the presence of WS than SM by itself.

Graphs both from Rainey and Rainey (2003) (Figure 3.1(a) and (b)) and this study (Figure 3.2(b) and (e)) are presented for comparison.

a) Effect of WS on SM

(1) Results from Rainey and Rainey (2003)

In Figure 3.1(a) the effect of WS on SM is facilitative, SM is at a significant advantage from the presence of WS because of its capacity to hitchhike within the WS mat and gain access to oxygen. The changes in the trajectory can be explained as follows: decrease in SM between days 1 and 2 is because of the death of SM cells within the broth phase. The increase between days 2 and 3 is due to hitchhiking within the mat. Increase between days 3 and 4 is due to sustained benefit from hitchhiking. The asymptotic decrease after day 5 is because of collapse of the WS mat (Rainey & Rainey, 2003).

(2) Results from this study

From the plots obtained in this study, the effect of WS on SM appears to be different in each case but overall the interactions are confirmed to be frequency dependent. The results are discussed case-wise.

Case 1: populations are founded with an equal ratio of SM and WS, so WS is 50% of the initial inoculum. This amounts to a repeat of the experiment of Rainey and Rainey (2003) (Figure 3.2(b)). The data shows an overall positive effect of WS on SM, although only after day 3. The effect seems to be divided into two parts: in the first part – i.e. from day 1 and 2 – the effect of WS on SM is negative. This may reflect death of

SM cells in the broth phase by anoxia caused by maturation of the mat. In the second part – i.e. from day 3-6 – there is a positive effect. However, this effect is marginally positive compared to what was observed by Rainey and Rainey (2003), where the effect of WS on SM was amplified – especially between days 3-6.

Case 2: populations are founded with a 1000:1 ratio of SM and WS, so WS is 0.1% of the initial inoculum.The case provides no evidence of a positive effect of WS on SM from day 3-6. This can be seen in the data depicted in Figure 3.2(a), where there is no difference in the two trajectories. The only difference is between day 1 and 2, where the effect of WS is negative. This is once again likely to be a consequence of death of SM cells in the broth phase.

Case 3: populations are founded with a 1:1000 ratio of SM and WS, so WS is 99.9% of the initial inoculum. It is expected that an advantage in numbers for WS would benefit WS in the early stages of mat formation, which would positively affect SM. On the contrary, instead of a positive effect of WS on SM, a negative effect of WS on SM is observed (dotted line above solid line from day 1, Figure 3.2(c)).

b) Effect of SM on WS

(1) Results from Rainey and Rainey (2003)

Rainey and Rainey (2003) reported no effect of SM on WS until day 5, after which there was a sudden decline in WS cells due to collapse of the WS mat (Figure 3.1(b)). The early collapse of the mat was brought by the presence of SM, which doesn't contribute to the formation of the mat but weakens it. This leads to an early collapse of the mat compared to a mat of WS alone which collapses but a little later.

(2) Results from this study

Case 1: populations founded with an equal ratio of SM and WS, so SM is 50% of the initial inoculum. There is a negative effect of SM on WS on day 1. On day 2, there is no difference between the two trajectories. From day 3-6, there is a significant difference between the two trajectories, where SM has a negative effect on WS (Figure 3.2(e)). Comparison with Rainey and Rainey (2003) (Figure 3.1(a)) shows that there is no sudden collapse events; however, there is still a negative effect of SM on WS.

Case 2: populations founded with a 1:1000 ratio of SM and WS, so SM is 0.1% of the initial inoculum. There is no difference in the two growth profiles of WS alone and WS in the presence of SM (solid line and the dotted line coincide) until day 4 (Figure 3.2(d)). After day 4, a difference is observed in the two profiles (Figure 3.2(d)). The effect of SM on WS is negative on day 5 and day 6.

Case 3: populations are founded with a 1000:1 ratio of SM and WS, so SM is 99.9% of the initial inoculum. There is a difference in the two growth profiles from day 1 and the difference lasted until day 6. SM has a strong negative effect on WS (Figure 3.2(f)).

3.2.2 Frequency dependent interactions in baseline lineages

The baseline lineages are derived from SBW25 after 3 days of static growth as mentioned before (see Material and Methods, Chapter 2, section 2.2.3). These represent variation in the ancestral state in the life cycle experiment and were used to make direct comparison with evolved lineages. The expectation was that the baseline lineages would behave similarly to ancestral SBW25 and its paired WS type in terms of their interactions. This is because, baseline lineages are genetically close to the ancestral state, and so, little variation is expected in terms of interactions.

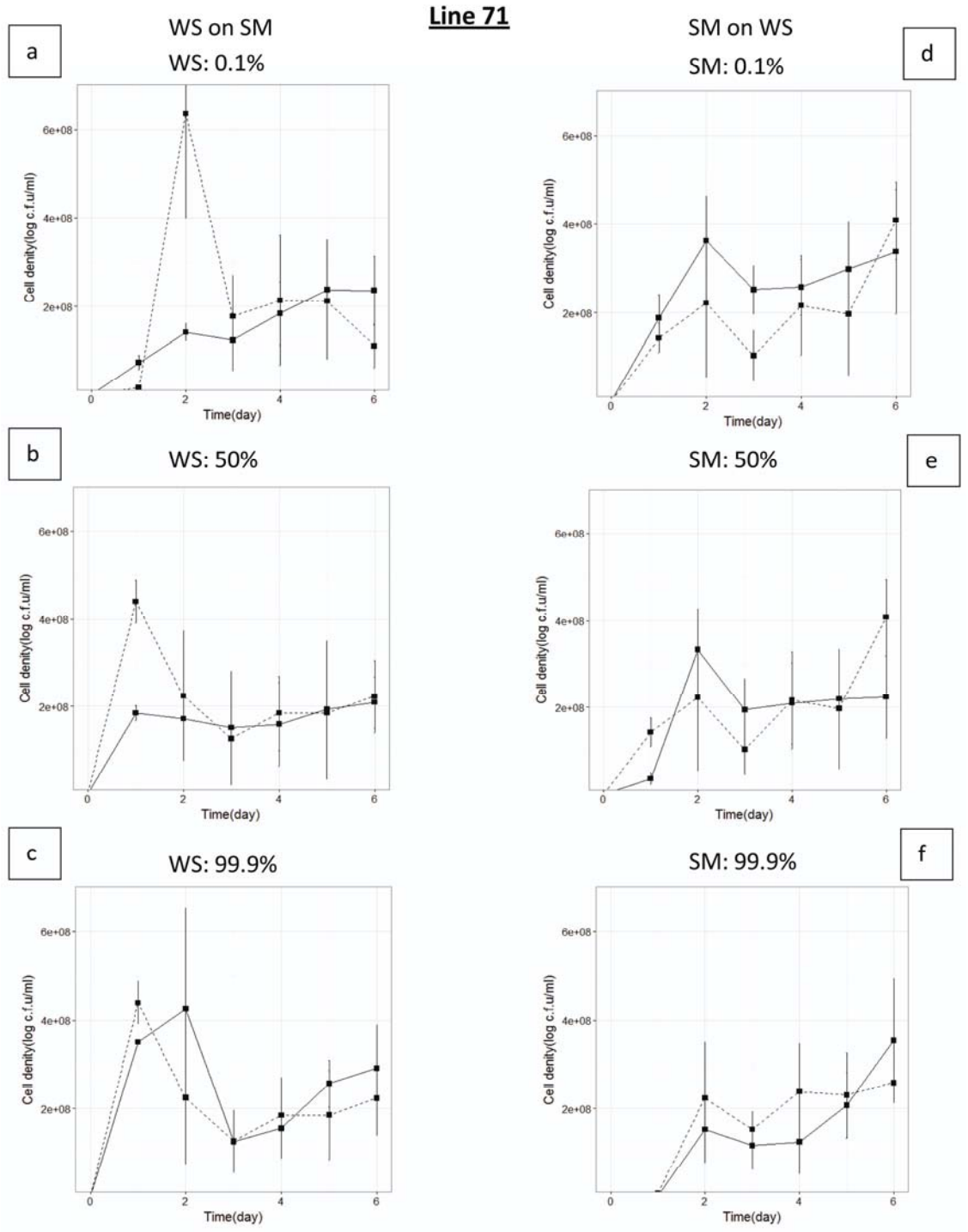

Figure 3.3. **Frequency dependent interactions in Line 71.** Left panel shows the effect of WS on SM at varying founding frequencies of WS (a) WS is 0.1 % (b) WS is 50% (c) WS is 99.9% .While the right panel shows the effect of SM on WS at varying founding frequencies of SM (a) SM is 0.1 %(b) SM is 50% (c) SM is 99.9%. Each plot shows comparison between two growth profiles: 1. a given type starting from 100% frequency (dotted line) and 2. a given type in the presence of the alternate type (solid line). The difference between the solid and the dotted lines gives the degree of interaction. Values are mean ± s.e.m. (n = 3).

However, the fact that every new mutation (of which baseline lineages are a result) is capable of creating a new WS or SM type, that in turn could generate ecotypes very different in character to the original ancestor. This also develops an alternate expectation that the interactions may differ between the baseline lineages and the ancestor.

3.2.2.1 Frequency dependent interactions in baseline lineage – Line 71

a) Effect of WS on SM

Case 1: populations are founded with a 1000:1 ratio of SM and WS, so WS is 0.1% of the initial inoculum. WS has a negative effect on SM on day 2 and no effect afterwards (dotted and solid line coinciding) (Figure 3.3(a)).

Case 2: populations founded with an equal ratio of SM and WS, so SM is 50% of the initial inoculum. WS has a significant negative effect on the growth of SM on day 1, and no effect afterwards (Figure 3.3(b)).

Case 3: populations are founded with a 1:1000 ratio of SM and WS, so WS is 99.9% of the initial inoculum. WS has a negative effect on SM on day 2, and again no effect afterwards (Figure 3.3(c)).

Overall, there is a significant negative effect of WS on SM on day 2, when WS is 0.1% (Figure 3.3(a)). The effect is also negative on day 1 when WS is 50% and 99.9%. The negative effect of WS on day 1 (when WS is 0.1%) and day 2 when (WS is 50%, WS is 99.9%), is possibly because of the death of SM cells in the broth phase by anoxia caused due to the maturation of the mat (a reason similar to what was observed in case of WS derived from ancestral SM SBW25). On later days (day 3 to 6), WS has no effect on SM. These results are different to what was seen in ancestral SBW25 (section 3.2.1 (a)), where there was a positive effect of WS from day 3 to 6, when WS was 50% in the starting population.

b) Effect of SM on WS

Case 1: populations are founded with a 1:1000 ratio of SM and WS, so SM is 0.1% of the initial inoculum. SM has no impact on the growth of WS (Figure 3.3(d)).

Case 2: populations are founded with a 1:1 ratio of SM and WS, so SM is 50% of the initial inoculum.SM has no effect on WS (Figure 3.3(e)).

Case 3: populations are founded with a 1000:1 ratio of SM and WS, so SM is 99.9% of the initial inoculum. SM has no effect on WS, even at high proportions (Figure 3.3(f)). Overall, SM has no effect on the growth of WS. Also, the effect of SM on WS for Line 71 is different from SBW25, where SM had a strong negative effect on WS (section 3.1.1).

3.2.2.2 Frequency dependent interactions in baseline lineage – Line 70

a) Effect of WS on SM

Case 1: populations are founded with a 1000:1 ratio of SM and WS, so WS is 0.1% of the initial inoculum.WS has no effect on SM at low frequency (WS is 0.1%) (Figure 3.4(a)).

Case 2: populations are founded with a 1:1 ratio of SM and WS, so WS is 50% of the initial inoculum. The effect of WS is predominantly negative that persisted throughout the 6 day period. This can be seen in the data depicted in Figure 3.4(b).

Case 3: populations are founded with a 1:1000 ratio of SM and WS, so WS is 99.9% of the initial inoculum. Again, the effect of WS is predominantly negative that persisted throughout the 6 day period. This can be seen in the data depicted in Figure 3.4(c).

Overall, WS has no effect on SM at very low frequency but the effect turns negative at higher frequencies of WS. The effect of WS on SM is therefore, frequency dependent. Also, the effect of WS on SM is not positive at 50% WS frequency as expected from the interaction in WS derived from ancestral SM SBW25, rather, the effect is negative at higher frequency.

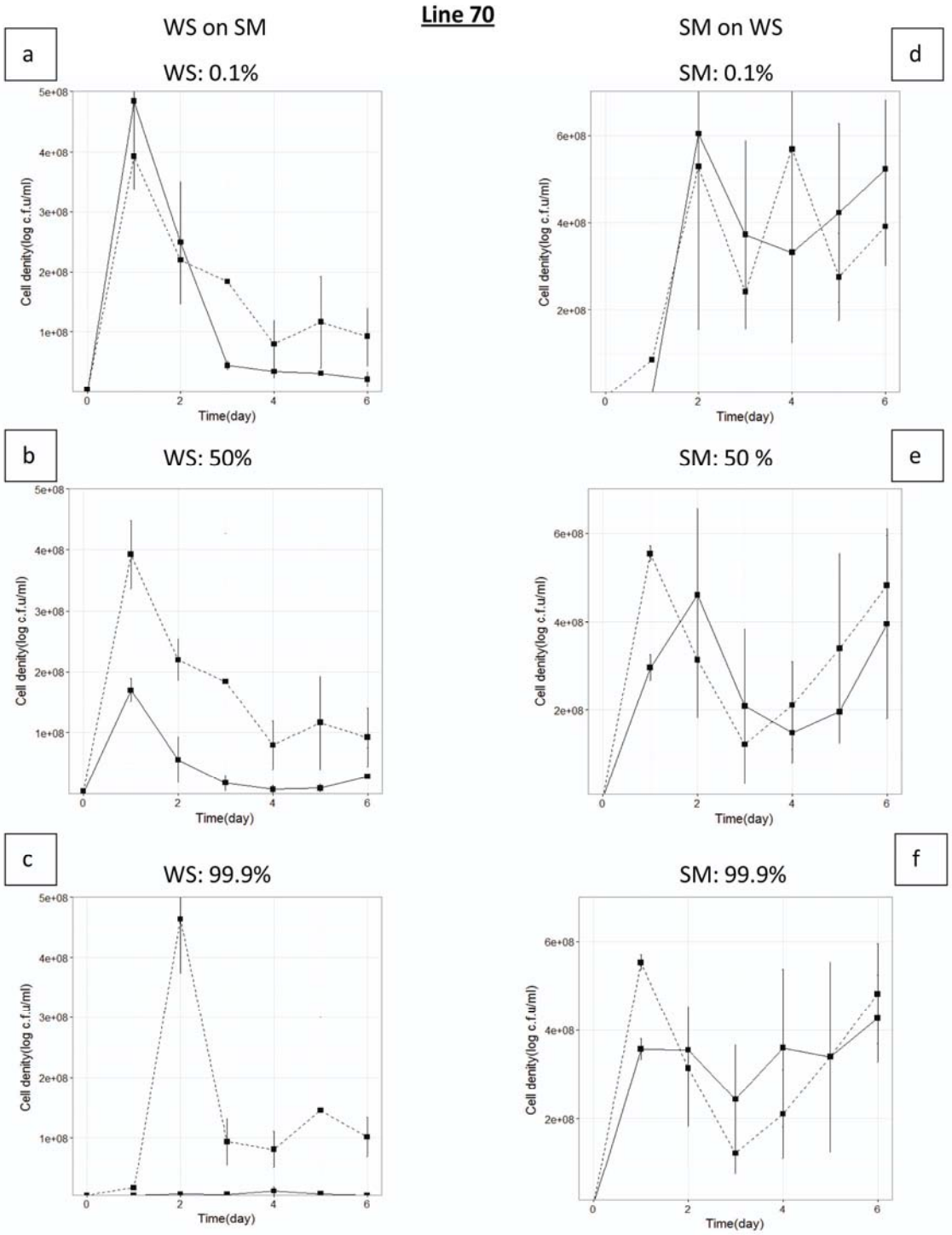

Figure 3.4 Frequency dependent interactions in Line 70. Left panel shows the effect of WS on SM at varying founding frequencies of WS (a) WS is 0.1 % (b) WS is 50% (c) WS is 99.9%. While the right panel shows the effect of SM on WS at varying founding frequencies of SM (a) SM is 0.1 %(b) SM is 50% (c) SM is 99.9%. Each plot shows comparison between two growth profiles: 1. a given type starting from 100% frequency (dotted line) and 2. a given type in the presence of the alternate type (solid line). The difference between the solid and the dotted lines gives the degree of interaction. Values are means ± s.e.m. (n = 3).

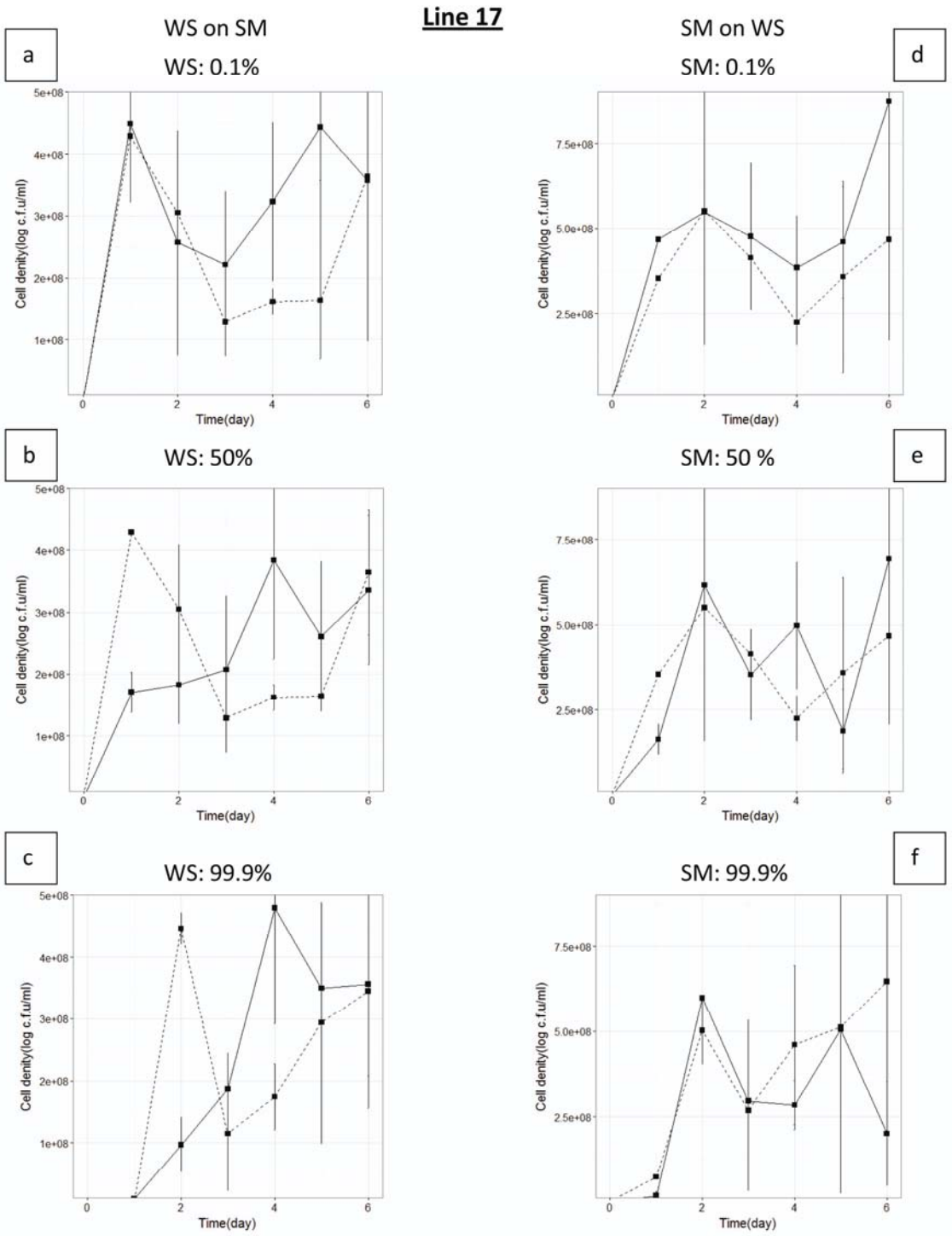

Figure 3.5 Frequency dependent interactions in Line 17. Left panel shows the effect of WS on SM at varying founding frequencies of WS (a) WS is 0.1 % (b) WS is 50% (c) WS is 99.9% .While the right panel shows the effect of SM on WS at varying founding frequencies of SM (a) SM is 0.1 %(b) SM is 50% (c) SM is 99.9%. Each plot shows comparison between two growth profiles: 1. a given type starting from 100% frequency (dotted line) and 2. a given type in the presence of the alternate type (solid line). The difference between the solid and the dotted lines gives the degree of interaction. Values are mean ± s.e.m. (n = 3).

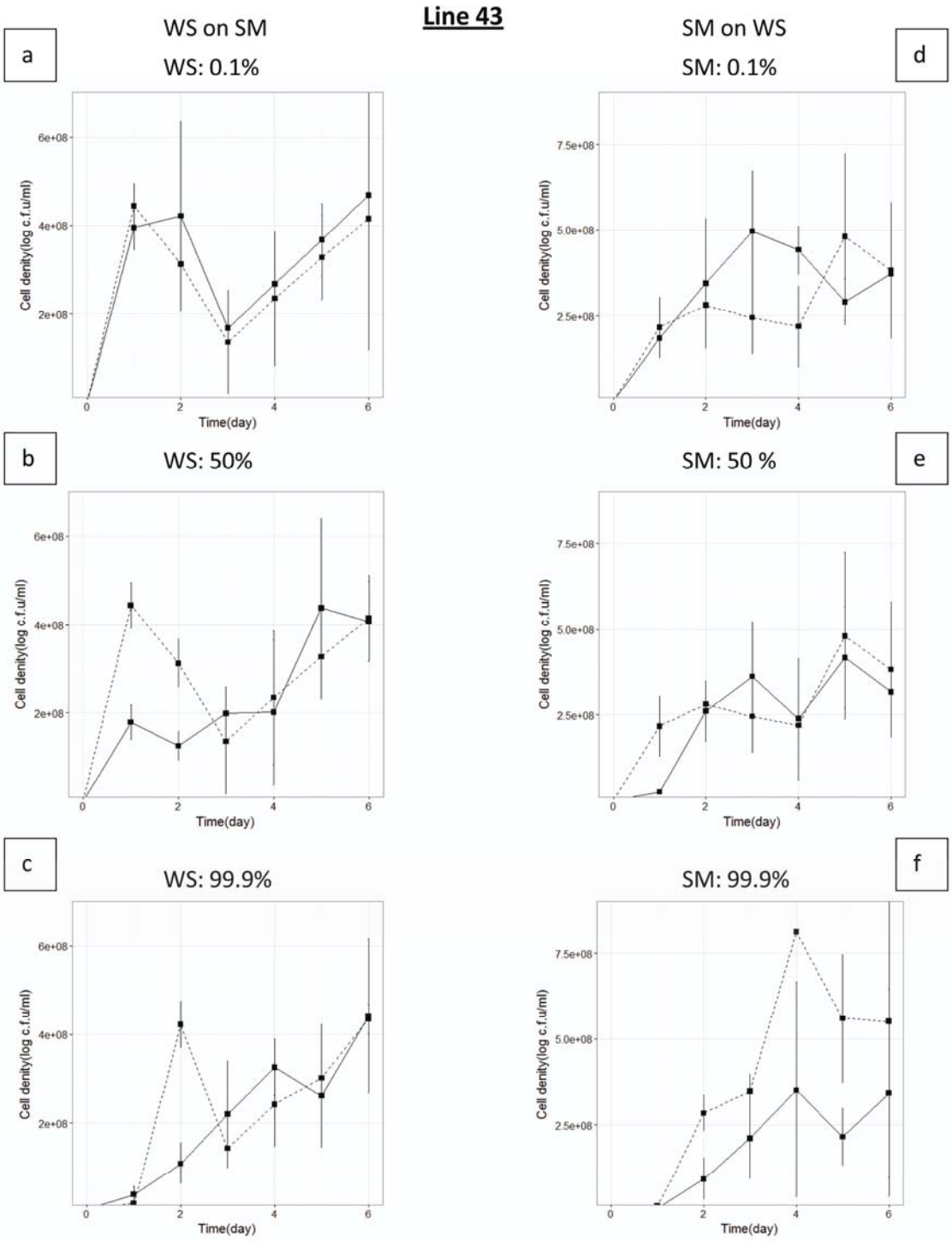

Figure 3.6 Frequency dependent interactions in Line 43. Left panel shows the effect of WS on SM at varying founding frequencies of WS (a) WS-0.1 % (b) WS is 50% (c) WS is 99.9% .While the right panel shows the effect of SM on WS at varying founding frequencies of SM (a) SM is 0.1 %(b) SM is 50% (c) SM is 99.9%. Each plot shows comparison between two growth profiles: 1. a given type starting from 100% frequency (dotted line) and 2. a given type in the presence of the alternate type (solid line). The difference between the solid and the dotted lines gives the degree of interaction. Values are mean ± s.e.m. (n = 3).

b) Effect of SM on WS

The two growth profiles of WS alone and in the presence of SM did not show significant difference at all the three SM frequencies – 0.1%, 50% and 99.9% (Figure 3.4(d), (e) and (f), respectively). Even the increasing proportion of SM – i.e. SM, 50% and 99.9% – didn't't impact upon the growth of WS.

3.2.3 Frequency dependent interactions in evolved lineages

Evolved lineages were derived from the ancestral SBW25 after ten and a half generations of selection. Selection was on perpetuation of the life cycle and the need for WS to produce SM and vice versa. There was an increase in fitness of evolved lineages (Hammerschmidt *et al*., 2014) . The increased group fitness was found to be positively correlated with an overall increased transition rate (the ability of WS and SM to switch to the other type). Thus, evolved lineages had an overall higher transition rate compared to ancestral lines (Hammerschmidt *et al*., 2014). Among the evolved lineages, Line 17 had the highest transition rate while Line 43 had a reduced transition rate.

The expectation is that looking at the evolutionary dynamics (frequency dependent interactions) in evolved lineages would shed light upon the strategies that have enabled the evolved lineages to progress through the life cycle.

3.2.3.1 Frequency dependent interactions in evolved lineage – Line 17

a) Effect of WS on SM

Case 1: populations are founded with a 1000:1 ratio of SM and WS, so WS is 0.1% of the initial inoculum. The data depicted in the Figure 3.5(a) shows that WS has no effect on SM for first two days (day 1 and 2) and a positive effect from day 3 to 6.

Case 2: populations are founded with a 1:1 ratio of SM and WS, so WS is 50% of the initial inoculum. (Figure 3.5(b)). WS has a negative effect on SM for the first 2 days and a positive effect afterwards (day 3-6).

Case 3: populations are founded with 1:1000 ratio of SM and WS, so 99.9% of the initial inoculum. (Figure 3.5(c)). Similar to case 2, WS has a negative effect on SM for the first 2 days and positive afterwards (day 3-6).

b) Effect of SM on WS

There is no significant difference between the two fitness profiles for each of the three frequencies of SM (Figure 3.5(d), (e) and (f)). Even an increasing proportion of SM (Figure 3.5(e) and (f)) didn't impact upon the growth of WS.

3.2.3.2 Frequency dependent interactions in evolved lineage – Line 43

a) Effect of WS on SM

Case 1: populations are founded with a 1000:1 ratio of SM and WS, so WS is 0.1% of the initial inoculum: WS has no effect on SM (Figure 3.6(a)).

Case 2: populations are founded with a 1:1 ratio of SM and WS, so WS is 50% of the initial inoculum. With increasing proportion of WS, the trend appeared to change, the interaction is negative for first 3 days and no interaction from day 3 onwards (Figure 3.6(b)).

Case 3: populations are founded with a 1:1000 ratio of SM and WS, so WS is 99.9% of the initial inoculum. The interaction is still negative and is the same as in case 2 – i.e. negative for first 3 days and no interaction from day 3 onwards (Figure 3.6(c)).

Overall, WS has no effect on SM.

b) Effect of SM on WS

SM has no effect on WS when it is rare (0.1%) and intermediate (50%) (Figure 3.6(d) and e)), the effect only becomes negative at a very high frequency (99.9%) (Figure 3.6(f)).

3.3 Discussion

3.3.1 Summary of the frequency dependent interactions lineage-wise

a) Ancestral SBW25

The results for the interactions in SBW25, from this study show that WS has a positive effect on SM, when WS is 50% (Figure 3.2(b)); however, the effect is less pronounced compared to the data from Rainey and Rainey (2003) (Figure 3.1(a)). While, for other combinations: WS is 0.1% and WS is 99.9%, the effect is not as anticipated (a positive effect of WS on SM). WS has no effect on SM when WS is 0.1 % (no difference in the two trajectories) and a negative effect when WS is 99.9%. Thus, WS seem to have a frequency dependent negative effect on SM.

SM also has a negative effect on WS that increases with an increase in the frequency of SM. Therefore, SM seems to have a frequency dependent negative effect on WS.

b) Baseline lineage – Line 71

WS seem to have no effect on SM and vice versa. This is probably because the high transition capacity in both directions, from WS to SM and from SM to WS of Line 71 may have nullified any initial advantage in numbers arising either, because of difference in initial frequency, or due to the difference in the growth rates of the two types. This means that growth of a given type is similar in the presence of the alternate type and when grown alone, resulting in no growth difference and therefore no interaction.

c) Baseline lineage – Line 70

WS has a negative effect on SM, the effect is counterintuitive, and differs from the conventional role of WS which is facilitative. The negative effect of WS is possibly because of the nature of the WS of Line 70 that produces less glue (Appendix, Figure 8.2), and therefore produces poor mats (Appendix, Figure 8.3). The Line 70 WS, despite forming a poor mat, is still able to outcompete SM. The invasion is possible because the Line 70 WS, unlike other WS types (that are mat dwellers) (Appendix, Figure 8.1) is both a mat and broth dweller that competes with SM for space in the broth, for all six days (Appendix, Figure 8.3). Thus, in competition with SM, WS has an overall dominant effect.

SM on the other hand, had no effect on WS. This is possibly because the Line 70 WS type formed either no mat or a very poor mat. This gave no advantage to SM in its interaction with WS — it appeared not to be able to hitchhike with the mat as ancestral SM did in the case of its interaction with WS. The nature of Line 70 WS led to the development of a unique environment that restricted exploitation of WS by SM. Poor mat formation by Line 70 represents an example of a resource that restricts cheaters from taking advantage of the mat unless a threshold amount of the resource is released. For instance, cellulose secretion by WS is such a resource whose benefits can only be realized if there occurs an event of mat formation. This in turn would depend on whether there is a threshold number of WS cells available, sufficient to form a mat.

d) Evolved lineage – Line 17

WS has a positive effect on SM in Line 17. The effect is similar to what is seen in WS derived from ancestral SM SBW25. However, the reason for the positive effect of Line 17 WS on SM could be different. For Line 17 WS, the positive effect could be a

result of selection on WS to produce SM towards the end of day 6 of phase I of the life cycle experiment. Having higher proportions of SM towards the end of phase I help the lineage to progress successfully to the next phase of the life cycle, thus increasing the chances of completing the life cycle and therefore survival over the long term.

e) Evolved lineage – Line 43

WS has no effect on SM. This could be because of the intermediate transition rate of Line 43 that doesn't facilitate growth of SM. This suggests one possible way of maintaining coexistence: where the cooperator (WS) has neither a positive nor a negative effect on the SM type.

Table 3.1: Summary of the frequency dependent interactions

	SM Baselines		**SM Evolved lines**	
WS	70	71	17	43
0.1%	-	-	+	=
50%	-	=	+	=
99.9%	=	=	+	=
	WS Baselines		**WS Evolved lines**	
SM	70	71	17	43
0.1%	=	=	=	=
50%	=	=	=	=
99.9%	=	=	=	-

The top panel shows the effect of WS on SM while the bottom panel shows the effect of SM on WS in baseline and evolved lineages at different founding frequencies of WS and SM (shown in the first column), respectively. Symbol, '=' means no interaction while, '+' means facilitative interaction and '-'means negative interaction.

Similarly SM has no effect on WS, when SM is 0.1% and 50% in the initial population. The ability to sustain the interaction at a high level of SM invasion (0.1% and 50% SM) indicates a strategy on the part of Line 43 to coexist (i.e. to maintain both the population of cheater and cooperator) through no effect of SM on WS for a large window of the frequency range. However, the balance changes when the frequency becomes exceedingly high (99.9%). This indicates that Line 43 has evolved a resistance to withstand change in interaction for a very high range of frequencies but that resistance is compromised when SM is at high frequency.

3.3.2 Comparison of frequency dependent interactions between baseline and evolved lineages

Table 3.1 summarizes the interactions. The top panel denotes the effect of WS on SM, whereas the bottom panel denotes the effect of SM on WS in populations founded at different frequencies – low, medium and high; each for baseline, evolved and ancestral SBW25 lineage.

Comparison of the effect of WS on SM between baseline and evolved lineages shows an overall shift. The effect changed from being an overall negative in baselines to an overall positive in evolved lineages for the three WS frequencies (0.1%, 50%, and 99.9%) (Table 3.1, top panel). The positive shift in the effect of WS on SM from baseline to evolved lineages is likely due to an increase in the transition rate of evolved lineages during the life cycle experiment.

On the other hand, comparison of the effect of SM on WS between baseline and evolved lineages shows no change. This suggests no effect of SM on WS in both the baselines and evolved lineages at three SM frequencies (0.1%, 50%, and 99.9%) (Table 3.1, bottom panel).

3.3.3 Interactions seem to be density dependent

From the frequency dependent nature of the interaction between WS and SM in SBW25, I learned that increasing the relative frequency of WS cells in the population doesn't necessarily result in an equivalent positive effect of WS on SM. On the contrary, I saw that the high number of WS cells (99.9%) negatively affected the growth of SM. A possible suggestion for the negative effect of WS at high frequency is that at higher density of WS cells there is an increase in competition with SM cells for the air-liquid interface (section 3.2.1(a), case 3). The negative effect of WS on SM at higher WS frequency, specifically in the case of ancestral SBW25 lineage, motivated me to study density dependent interactions between WS and SM.

Chapter 4. Density dependent interactions between WS and SM

4.1 Introduction

Upon recognizing the possible involvement of density dependent factors, I was motivated to explore this in detail by comparing the growth profiles of the given type alone and in the presence of the alternate type (in a 1:1 mix), at varying densities – high (10^8 cells/ml), medium (10^5 cells/ml) and low (10^2 cells/ml); each for ancestral SBW25, baseline lineages – 70, 71 and the evolved lineages – 17, 43. Later, the interactions are compared between the baseline and evolved lineages.

4.2 Results

4.2.1 Density dependent interactions in ancestral SBW25

a) Effect of WS on SM

The data depicted in Figure 4.1(a) shows that WS has no significant effect on SM at high density; however, the effect is positive from day 3 to 6 that increases with the decrease in density, such that it is more at low than at medium density.

b) Effect of SM on WS

SM has no effect on WS, for populations founded at high density, shown in Figure 4.1(d). However, the data depicted in Figure 4.1(e) and (f) shows that SM has a significant negative effect on WS between day 3 and 6, for populations founded at medium and low density. The effect is comparable between the two densities (medium and low), except on day 4, as shown in Figure 4.1(e) and (f).

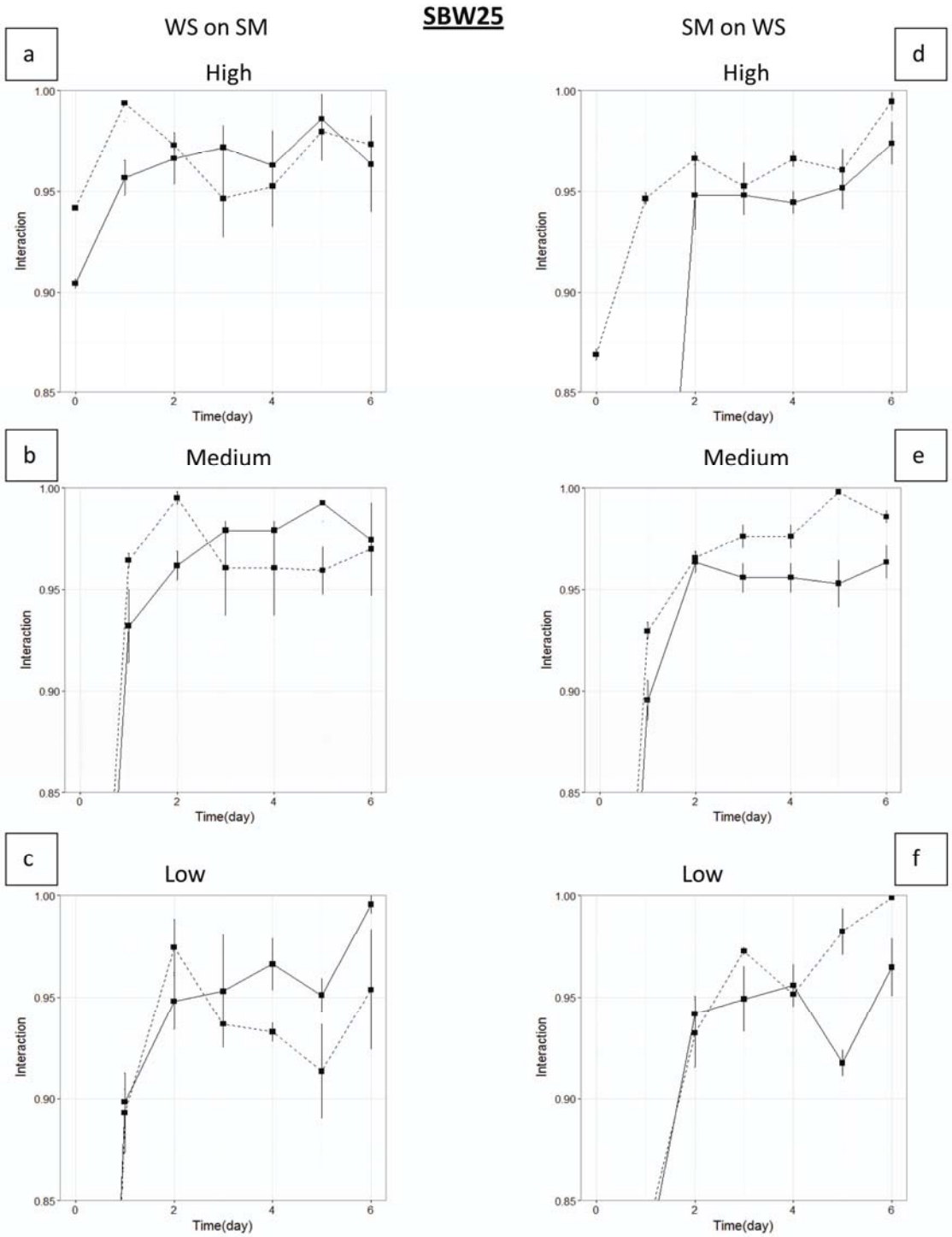

Figure 4.1 Density dependent interactions in Line SBW25. Left panel shows the effect of WS on SM at varying founding density for 1:1 mix population of WS and SM (a) High density-10^8 cfu/ml (b) Medium density-10^5 cfu/ml (c) Low density-10^2 cfu/ml, while the right panel shows the effect of SM on WS at varying founding density for the 1:1 mix. For each plot, Y axis shows the interaction (normalized log of c.f.u/ml), over a period of six days (time in days - X axis). The interaction is inferred by comparison between the two growth profiles: 1. a given type starting from 100% frequency (dotted line) and 2. a given type in the presence of the alternate type (solid line), when alternate type is at 50% initial frequency. The difference between the solid and the dotted lines gives the degree of interaction. Values are mean ± s.e.m. (n = 3).

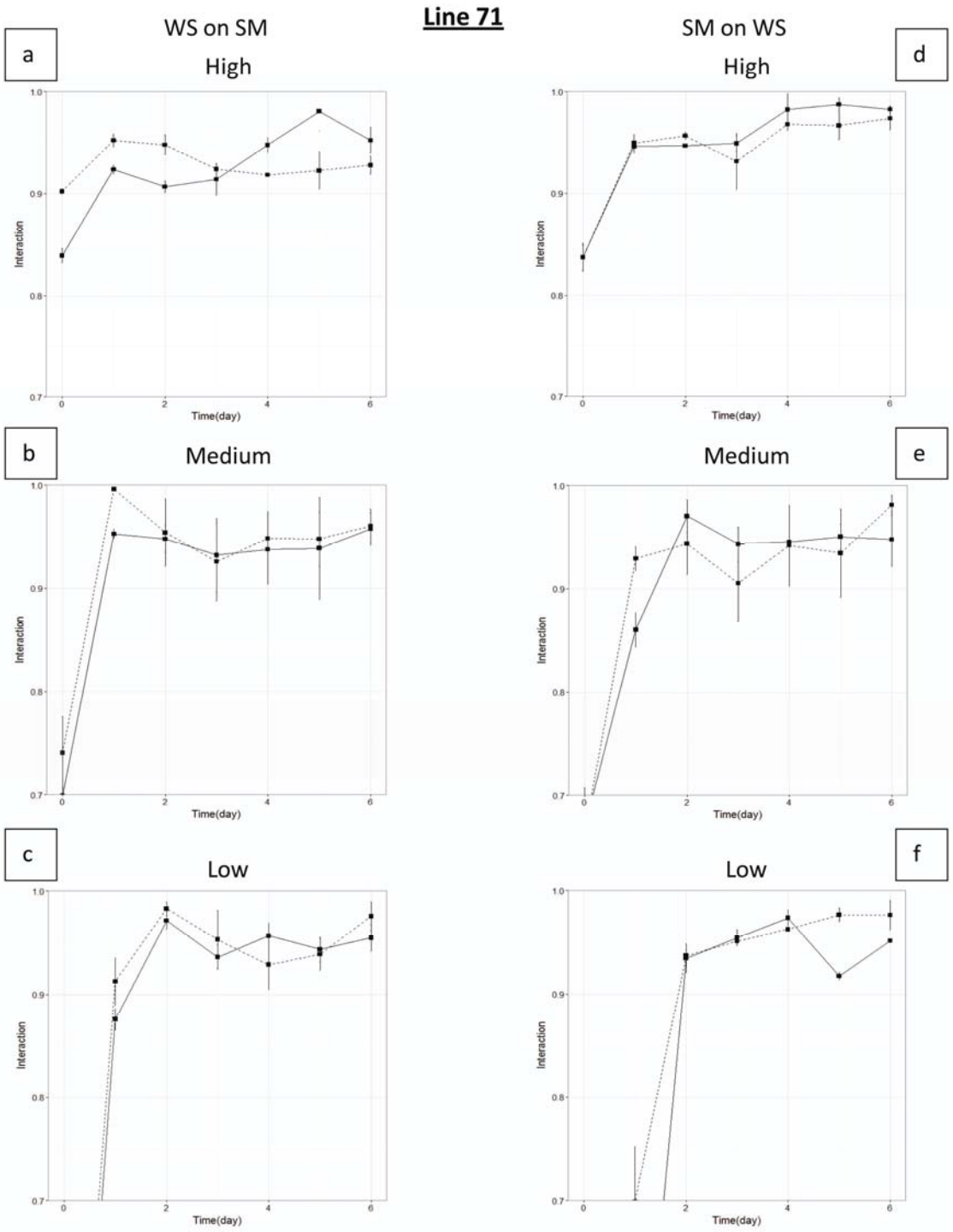

Figure 4.2 Density dependent interactions in Line 71. Left panel shows the effect of WS on SM at varying founding density for 1:1 mix population of WS and SM (a) High density-10^8 cfu/ml (b) Medium density-10^5 cfu/ml (c) Low density-10^2 cfu/ml, while the right panel shows the effect of SM on WS at varying founding density for the 1:1 mix. For each plot, Y axis shows the interaction (normalized log of c.f.u/ml), over a period of six days (time in days -X axis). The interaction is inferred by comparison between the two growth profiles: 1. a given type starting from 100% frequency (dotted line) and 2. a given type in the presence of the alternate type (solid line), when alternate type is at 50% initial frequency. The difference between the solid and the dotted lines gives the degree of interaction. Values are mean ± s.e.m. (n = 3).

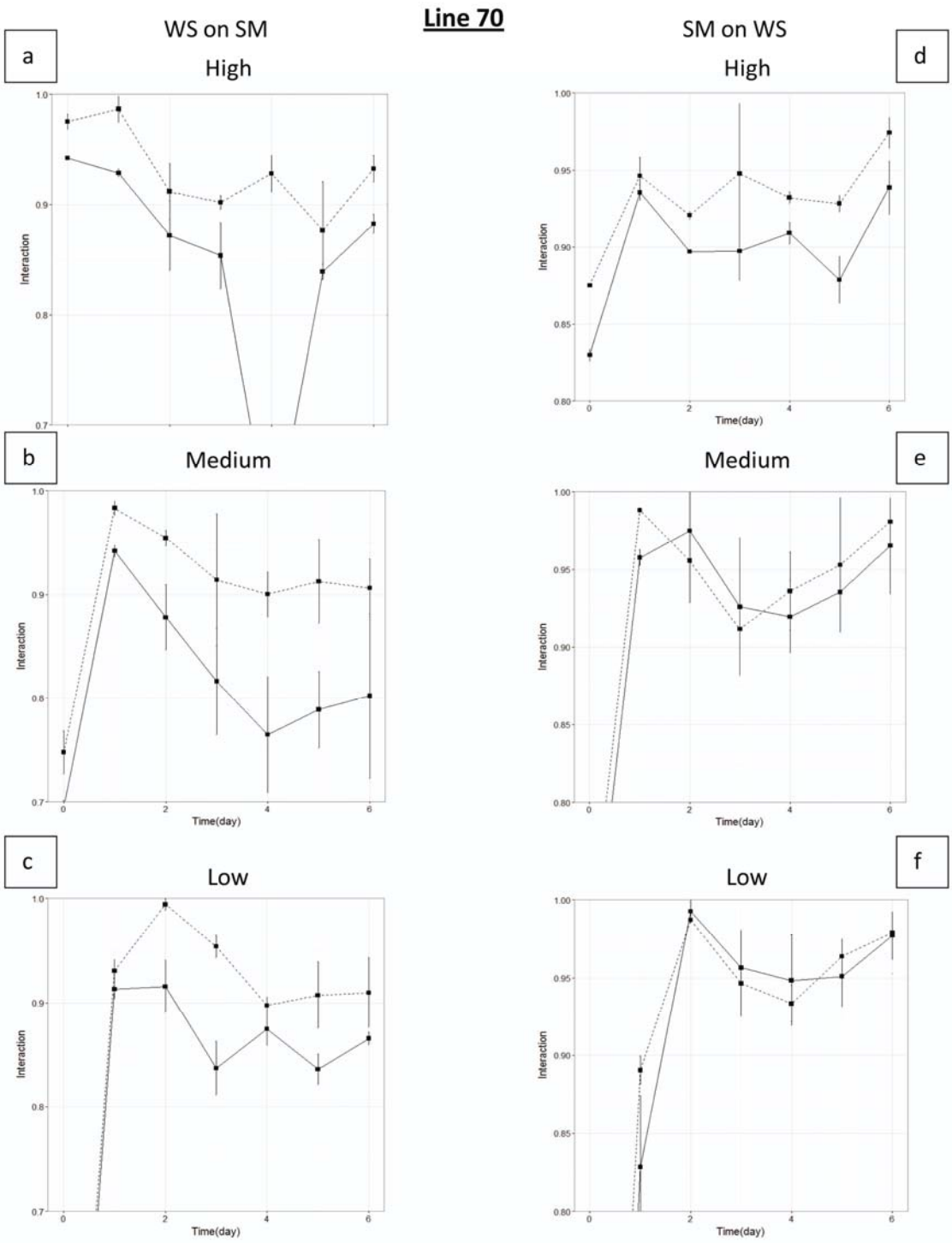

Figure 4.3 Density dependent interactions in Line 70. Left panel shows the effect of WS on SM at varying founding density for 1:1 mix population of WS and SM (a) High density-10^8 cfu/ml (b) Medium density-10^5 cfu/ml (c) Low density-10^2 cfu/ml, while the right panel shows the effect of SM on WS at varying founding density for the 1:1 mix. For each plot, Y axis shows the interaction (normalized log of cfu/ml), over a period of six days (time in days -X axis). The interaction is inferred by comparison between the two growth profiles: 1. a given type starting from 100% frequency (dotted line) and 2. a given type in the presence of the alternate type (solid line), when alternate type is at 50% initial frequency. The difference between the solid and the dotted lines gives the degree of interaction. Values are mean ± s.e.m. (n = 3).

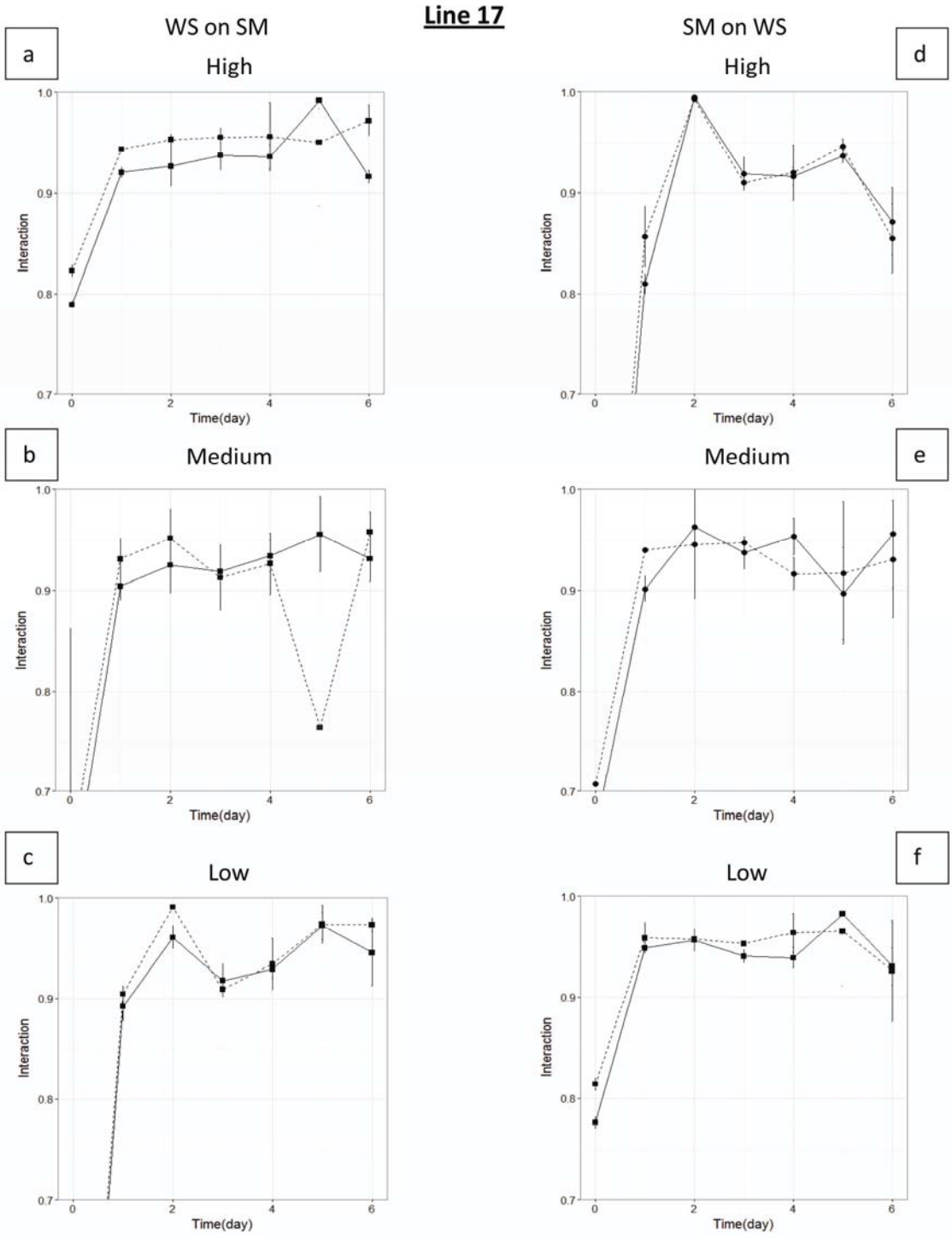

Figure 4.4 Density dependent interactions in Line 17. Left panel shows the effect of WS on SM at varying founding density for 1:1 mix population of WS and SM (a) High density-10^8 cfu/ml (b) Medium density-10^5 cfu/ml (c) Low density-10^2 cfu/ml, while the right panel shows the effect of SM on WS at varying founding density for the 1:1 mix. For each plot, Y axis shows the interaction (normalized log of c.f.u/ml), over a period of six days (time in days -X axis). The interaction is inferred by comparison between the two growth profiles: 1. a given type starting from 100% frequency (dotted line) and 2. a given type in the presence of the alternate type (solid line), when alternate type is at 50% initial frequency. The difference between the solid and the dotted lines gives the degree of interaction. Values are mean ± s.e.m. (n = 3).

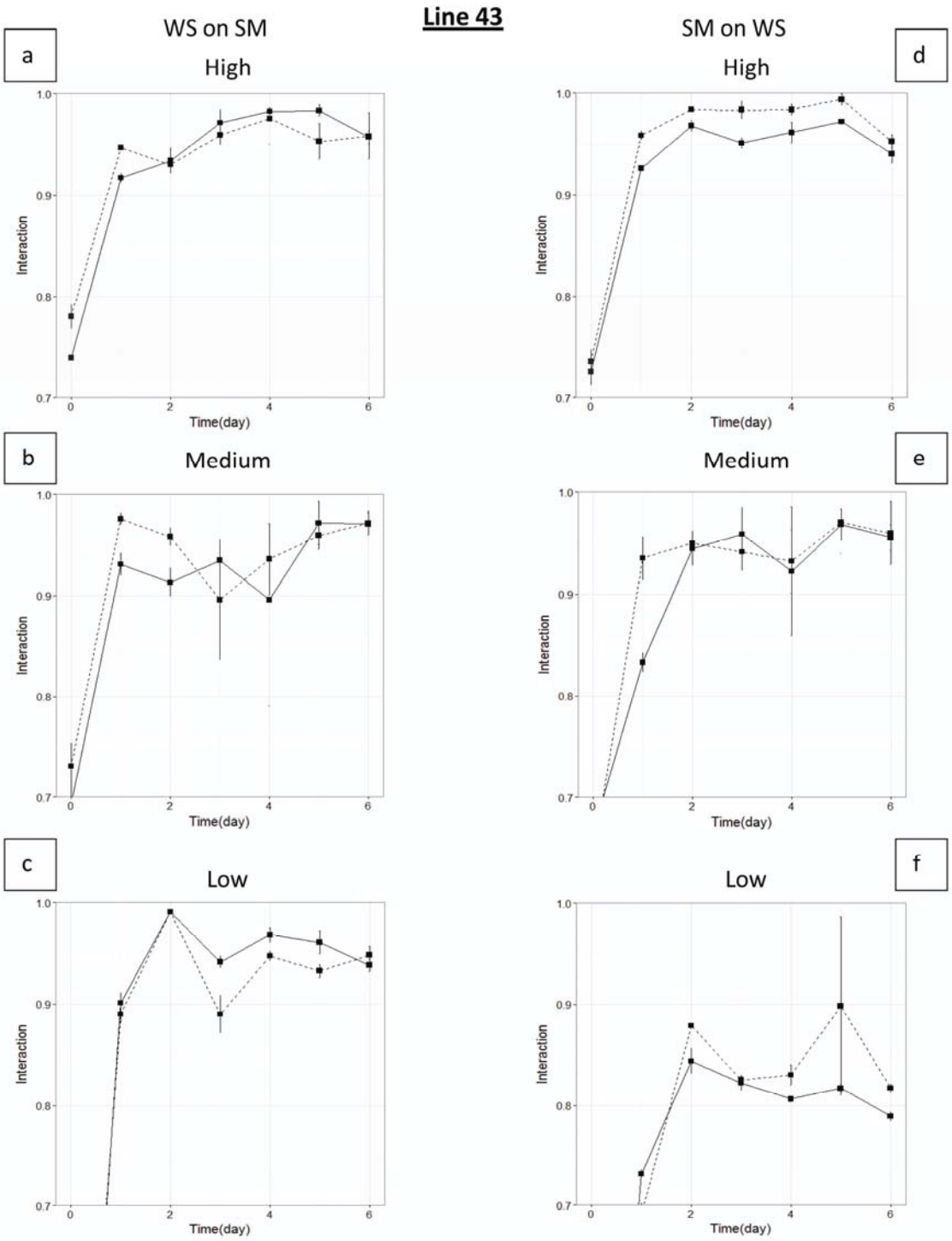

Figure 4.5 Density dependent interactions in Line 43. Left panel shows the effect of WS on SM at varying founding density for 1:1 mix population of WS and SM (a) High density-10^8 cfu/ml (b) Medium density-10^5 cfu/ml (c) Low density-10^2 cfu/ml, while the right panel shows the effect of SM on WS at varying founding density for the 1:1 mix. For each plot, Y axis shows the interaction (normalized log of c.f.u/ml), over a period of six days (time in days -X axis). The interaction is inferred by comparison between the two growth profiles: 1. a given type starting from 100% frequency (dotted line) and 2. a given type in the presence of the alternate type (solid line), when alternate type is at 50% initial frequency. The difference between the solid and the dotted lines gives the degree of interaction. Values are mean ± s.e.m. (n = 3).

4.2.2 Density dependent interactions in baseline lineage – Line 71

a) Effect of WS on SM

The data depicted in Figure 4.2(a) shows that at high density, WS seems to have a negative effect on SM up to 3 days after which it is positive. The result is different to what is seen in the case of WS derived from ancestral SBW25, where it had no effect at high density and a positive effect at lower densities (section 4.2(a)).

For other cases of Line 71, i.e. for the populations founded at medium and low density, the Figure 4.2(b) and (c) depicts that, with decreasing density, the interaction also reduces, with only negative interaction present on day 1 and no interaction present thereafter.

b) Effect of SM on WS

As can be seen in the data depicted in the Figure 4.2(d) and (e), SM has no effect on WS, for populations founded at high and intermediate density. However, Figure 4.2(f) shows that SM has a negative effect on WS from day 3 to 6, at low density.

4.2.3 Density dependent interactions in baseline lineage – Line 70

a) Effect of WS on SM

WS has a negative effect on SM at all the founding densities – high, intermediate and low – during the 6 day period (Figure 4.3(a), (b) and (c), respectively).

b) Effect of SM on WS

For the population founded at intermediate and low density SM has no effect on WS. However, for the populations founded at high density SM has a negative on WS (Figure 4.3(d)). This observation suggest that SM doesn't affect WS in a negative

manner at low and intermediate founding densities (Figure 4.3(e) and (f), respectively), but only effects negatively at very high founding densities (Figure 4.3(d)).

4.2.4 Density dependent interactions in evolved lineage – Line 17

a) Effect of WS on SM

For the populations founded at three initial densities – high, medium and low – WS has no effect on SM (Figure 4.4(a), (b) and (c), respectively).

b) Effect of SM on WS

Similarly, for the populations founded at three initial densities – high, medium and low, SM has no effect on WS (Figure 4.4(d), (e) and (f), respectively).

4.2.5 Density dependent interactions in evolved lineage – Line 43

a) Effect of WS on SM

For populations founded at high density, WS has a slight positive effect on SM (from day 3 to 5). This reduces to being no effect on day 6 (Figure 4.5(a)). For populations founded at medium density, WS has a negative effect on SM, on day 1 and day 2, while there is no effect afterwards (Figure 4.5(b)). Again, at low founding density, WS has a positive effect on SM, from day (3 to 6) (Figure 4.5(c)).

b) Effect of SM on WS

For populations founded at high (Figure 4.5(d)) and low (Figure 4.5(f)) densities, SM has a negative effect on WS. While for the populations founded at intermediate density, SM has no effect (Figure 4.5(e)).

4.3 Discussion

4.3.1 Summary of the density dependent interactions lineage-wise

a) Ancestral SBW25

WS has a positive effect on SM which is more at low than at medium density. The positive effect of WS on SM at medium density is similar to the result of the effect of WS (derived from ancestral SM SBW25) on ancestral SM, when WS (derived from ancestral SM SBW25) is 50% (section 3.2.1(a)).

The positive effect at low density could be because there is a low number of absolute WS present in the starting population (10^2 cells/ml), because of which mat formation will be delayed until the time a threshold of WS is reached. The time taken by WS to increase in number sufficient to form a mat, would then give an opportunity for SM to divide and increase in number. Thus, giving SM an added growth advantage and therefore resulting in a positive effect of WS on SM. Also, WS has no effect on SM at high densities. This could be because of two reasons:

- The competition offered by WS to SM at high densities, a reason similar to what is observed in the case of a negative effect of WS on SM at higher frequencies, for ancestral SBW25 (Chapter 3, section 3.2.1 (a)).
- The low capacity of Line SBW25 to switch from WS to SM.

Both of these factors could be jointly responsible for the missing positive effect of WS on SM at high density.

Overall, the effect of WS on SM is not constant; WS has no effect at high density while a positive effect that increases with decreasing density of the founding population(from medium to low). Therefore, the effect of WS on SM seems to depend negatively on density.

On the other hand, for the effect of SM on WS, the observation indicates that there is a negative effect of SM on WS. Moreover, since there is no interaction in populations founded at high density, while there is a negative interaction at low and medium density. This indicates that the interaction is density dependent.

b) Baseline lineage – Line 71

WS has no effect on SM. The observation is suggestive of the high transition rate of Line 71 that neutralizes the change in WS and SM arising due to the change in initial density or frequency.

Similarly, SM has no effect on WS at high and intermediate densities. Again, the probable reason for no interaction could be the high transition rate of Line 71. However, SM has a negative effect on WS at low density. This could be because of two reasons:

- Firstly, the growth advantage that SM (in general) has at lower densities, similar to the case of ancestral SM SBW25 (Chapter 3, section 3.2.1(b)).
- Secondly, the high transition capacity of Line 71 – from WS to SM – that would additionally support the advantage to SM at lower density.

c) Baseline lineage – Line 70

WS has a negative effect on SM. The probable reason for why WS could have a negative effect on SM is because:

- The WS of Line 70 form a poor mat that leads to improper differentiation between broth and mat niche. As a result of which the WS of Line 70 behaves as a generalist and occupies both broth and mat niche for all six days, and thus remains dominant throughout. A reason similar to what was suggested in the frequency dependent interactions in Line 70 for the effect of WS on SM (Chapter 3, section 3.2.2.2(a)).

- The low transition capacity of Line 70 from WS to SM that doesn't support SM production.

SM has a negative effect on WS at high density. The reason could be that at high density there is a competition for resources between Line 70 WS and SM.

d) Evolved lineage – Line 17

WS has no effect on SM and vice versa. This is probably because of the high evolved transition rate of the line that moderates the differences that arise in the frequency of the WS and SM types, due to the differences in initial population density.

e) Evolved lineage – Line 43

Line 43 shows a positive effect of WS on SM either at very low or high founding densities while it didn't show any interaction at medium founding density. Medium density are the ones at which the lab populations are generally cultured. Also, during the life cycle experiment, populations were grown at medium density. As a result, Line 43 as one of the evolved lineages would have evolved the capacity to have a constant interaction at medium density while any deviation from usual density (growth of the line at high and low founding densities) would have led to a change in interaction (a positive effect of WS on SM). Probably this is the reason why a positive effect of WS on SM is observed at high and low densities.

Again, in case of the effect of SM on WS, Line 43 shows no effect at medium density while a negative effect at low and high founding densities. The probable reason for no effect of SM on WS at medium density could be due to the property of Line 43 to show constant interaction at densities at which the lab populations are generally evolved – i.e. at medium founding density. In such situations, a deviation from the usual

founding density (growth of the line at high and low founding densities) would then lead to a change in interaction because the line is not evolved at extreme densities. Probably, this is the reason why Line 43 shows a negative effect of SM on WS, at high and low founding densities and not at medium density.

4.3.2 Comparison of density dependent interactions between baseline and evolved lineages.

Table 4.1 summarizes the interactions. The top panel denotes the effect of WS on SM, whereas the bottom panel denotes the effect of SM on WS in populations founded at three initial densities (low, medium and high); each for baseline, evolved and ancestral SBW25 lineage.

Table 4.1 Summary of the desnity dependent interactions

	SM Baselines		**SM Evolved lines**	
WS	70	71	17	43
High	-	+	=	+
Medium	-	=	=	=
Low	-	=	=	+
	WS Baselines		**WS Evolved lines**	
SM	70	71	17	43
High	-	=	=	-
Medium	=	=	=	=
Low	=	-	=	-

The top panel shows the effect of WS on SM while the bottom panel shows the effect of SM on WS in baselines and evolved lines at different starting densities (shown in the first column). Symbol, '=' means no interaction while, '+' means facilitative interaction and '-'means negative interaction.

4.3.2.1 Effect of WS on SM

a) Baseline lineages

Table 4.1, top panel shows that baseline lineages have their individual pattern of density dependence. WS has a negative effect on SM, for Line 70 (shown by '-' sign). On the other hand, for Line 71 WS has no effect on SM at medium and low density – but a positive effect at high density.

b) Evolved lineages

Also, evolved lineages have their own individual pattern of density dependence, as seen from the Table 4.1 – top panel. For Line 17, there is no effect (shown by '=' sign) of WS on SM at the three founding densities (Table 4.1, top panel, evolved lineage- Line 17, at densities – high, medium and low). On the other hand, the evolved lineage, Line 43 has no effect at medium density, but a positive effect of WS on SM at low and high densities (Table 4.1, top panel, evolved Line 43, at densities – high, medium and low).

4.3.2.2 Effect of SM on WS

a) Baseline lineages

For baseline lineages, SM has no effect on WS at intermediate densities but a negative effect of SM on WS at very high and low densities. For instance, Line 71 and 70 SM has no effect on WS at intermediate density while a negative effect at low (for Line 71) and high density (for Line 70) (Table 4.1, bottom panel).

b) Evolved lineages

A similar observation is seen with evolved lineages where Line 43 has no interaction at intermediate density and a negative interaction at extreme densities. On

the other hand, Line 17 always maintained a condition of no effect of SM on WS, at all the three initial densities (Table 4.1, bottom panel).

Overall, no distinct pattern of density dependence is observed between the baseline and evolved lineages on both the aspects of the interaction i.e. from WS to SM and vice versa.

Chapter 5. Eco-space diagrams

5.1 Introduction

In the two previous chapters where, I considered firstly frequency and secondly, density dependent factors, the data appeared to suggest that the change in initial frequency and initial density both affected the interaction. Since the interactions are seen to be dependent on both the components – frequency and density – this motivated me to interrogate the joint influence of both these factors on the interaction between WS and SM.

To study the effect of both the factors I plotted, frequency and density data as a function of the other. These plots are called as eco-space diagrams (Sanchez & Gore, 2013). The diagrams measure the response of population density (Y-axis) against the changing frequency (X-axis) for each line's population over the 6 day period. Keeping track of frequency represents measurement of evolutionary change while keeping track of population density represents measurement of ecological change.

It is hypothesized that the frequency (evolutionary) and population density (ecological) changes are involved in an eco-evo feedback during the life cycle experiment and this feedback would lead to an eco-space trajectory that is conserved for evolved lineages.

The results from the eco-space diagrams are presented in the following manner: (i) lineage-wise: eco-space trajectories are presented on a single plot for populations starting at different initial WS frequencies at medium density (section 5.2.1). (ii) combination-wise: eco-space trajectories are presented on a single plot for all the lineages starting at a particular combination of frequency and density (section 5.2.2).

Plotting frequency versus density data lineage-wise, made it possible to visualize the equilibrium (in terms of frequency and density) that each lineage attains for variable populations (starting at different initial frequencies). On the other hand, plotting data combination-wise for all the lineages together, allowed comparisons to be made between the lineages (between baselines and evolved lineages).

The section below (section 5.2.1) presents the results of the eco-space diagrams, lineage-wise.

5.2 Results

5.2.1 Lineage-wise eco-space trajectories

Consider a bacterial strain consisting of two behavioral types A and B, when mixed together in competition, can give rise to different strategies like dominance (one type dominates over the other irrespective of initial frequency), coexistence (both types exist irrespective of initial frequency), or bi-stability (founding frequency decides which type dominates) (Chapter 1, section 1.4(a)). Similarly, the data depicted from the eco-space diagrams shows that WS and SM types (like the two behavioral types A and B mentioned above) exhibit different strategies.

In this study, a strategy for a lineage is termed as: (i) *dominance*, when either a WS or SM type belonging to the respective lineage has more than 50% frequency in the population at day 6, for all initial WS frequencies (ii) *coexistence*, when both types exist at 50% frequency in the population, for all the initial WS frequencies (iii) *bistability*, when the population divides into two frequencies at day 6, depending upon the initial WS frequency in the population.

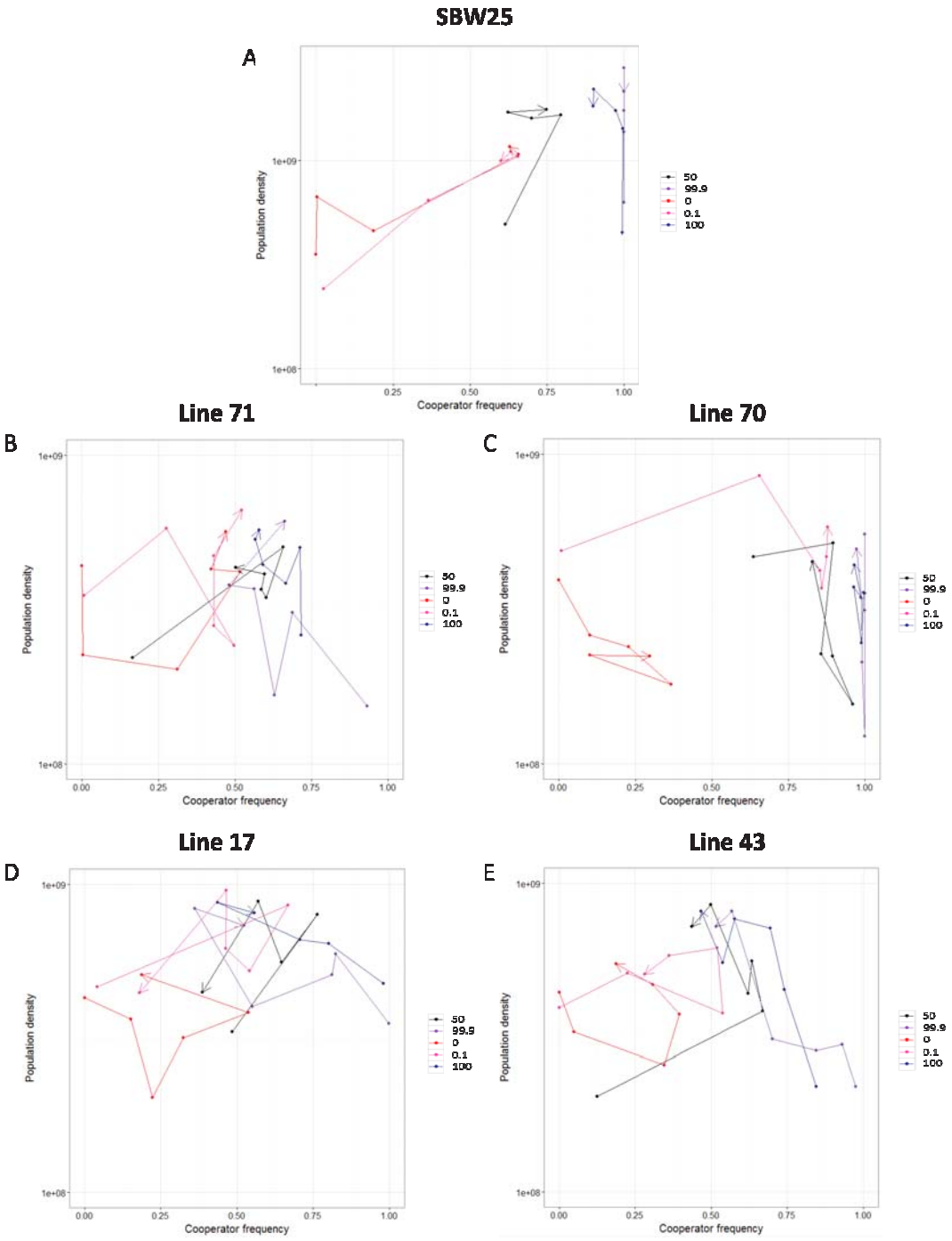

Figure 5.1 Lineage-wise plots of population density versus frequency. Lineage-wise plots for different initial conditions of frequency are given as follows. (A) SBW25 (B) 71 (C) 70 (D) 17 and (E) 43. The plot represents individual strategies employed by each line to progress through the life cycle. Strategies employed are *dominance* – SBW25 (more than 50% WS final frequency for all starting frequencies of WS), *coexistence* – 71 (50% WS final frequency for all starting WS frequency) and *bistability* – 70, 17 and 43 (two stable states for different initial conditions of WS frequency). The colour represents different initial frequency, black – 50% WS, purple – 0.1% WS, red – 0% WS, pink – 99.9% WS and blue – 100% WS. The Y-axis represents the population density while the X-axis represents the WS frequency. Each point on the graph represents the mean of population density and WS frequency on each day and the arrow denotes the end of the trajectory at day 6.

For each eco-space plot, lineage-wise, the Y-axis represents the total population density that varies from 1e+08 to 1e+09 cfu/ml, for all the plots except for ancestor SBW25 for which it varies from 1e+08 to 5e+09 cfu/ml. For each eco-space plot, combination-wise, the Y-axis represents the total population density that varies from 1e+08 to 5e+09 cfu/ml for all the combinations. The X-axis represents the WS frequency, given by the formula:

$$n\,(WS)/(n\,(WS) + n\,(SM))$$

Where,

n (WS)	:	number of WS cells in the population, measured in cfu/ml
n (SM)	:	number of SM cells in the population, measured in cfu/ml
n (WS)+n (SM)	:	number of total cells in the population, measured in cfu/ml

The X-axis varies from 0 to 1, where '0' means 0% WS or 100% SM frequency and '1' means 100% WS frequency or 0% SM frequency.

a) Ancestral lineage – SBW25

- Cases where the populations are founded with a 1:1000 ratio of SM: WS, so WS is 99.9% (Figure 5.1(A), purple trajectory), and for pure WS population, where WS is 100% (Figure 5.1(A), blue trajectory), the final mean frequency of the populations remain 100 % WS at day 6.
- Cases where the populations are founded with a 1000:1 ratio of SM: WS, so SM is 99.9% (Figure 5.1(A), pink trajectory), and for pure SM population, where SM is 100% (Figure 5.1(A), red trajectory), the mean frequency of the populations remain 64% and 60% WS, respectively, at day 6.
- Cases where the populations are founded with a 1:1 ratio of SM: WS, so SM and WS both are 50% (Figure 5.1(A), black trajectory), the mean frequency of the populations remain 75% WS at day 6.

Overall, for different WS frequencies in the population, the lineage settles at more than 50% WS frequency on day 6. This indicates a strategy of WS *dominance* for ancestral Line SBW25.

b) Baseline lineage – Line 71

- Cases where populations are founded with a 1:1000 ratio of SM: WS, so WS is 99.9% (Figure 5.1(B), purple trajectory), and for pure WS population, where WS is 100% (Figure 5.1(B), blue trajectory), the final mean frequency of populations remain 58% and 66% WS, respectively, at day 6.
- Cases where the populations are founded with a 1000:1 ratio of SM: WS, so SM is 99.9% (Figure 5.1(B), pink trajectory), and for pure SM population, where SM is 100% (Figure 5.1(B), red trajectory), the mean frequency of the populations remain 52% and 47% WS, respectively, at day 6.
- Cases where the populations are founded with a 1:1 ratio of SM: WS, so SM and WS both are 50% (Figure 5.1(B), black trajectory), the mean frequency of the populations remain 50 % WS at day 6.

Overall, for different WS frequencies in the population, the lineage settles around 50% WS frequency on day 6. Therefore, Line 71 indicates a strategy of *coexistence*.

c) Baseline lineage – Line 70

- Cases where populations are founded with a 1:1000 ratio of SM: WS, so WS is 99.9% (Figure 5.1(C), purple trajectory), and for pure WS population, where WS is 100% (Figure 5.1(C), blue trajectory), the final mean frequency of the populations remain 97% and 96% WS, respectively, at day 6.

- Cases where the populations are founded with a 1000:1 ratio of SM: WS, so SM is 99.9% (Figure 5.1(C), pink trajectory), and for pure SM population, where SM is 100% (Figure 5.1(C), red trajectory), the mean frequency of the populations remain 88% and 30% WS, respectively, at day 6.
- Cases where the populations are founded with a 1:1 ratio of SM: WS, so SM and WS both are 50% (Figure 5.1(C), black trajectory), the mean frequency of the population remain 83% WS at day 6.

Overall, for different WS frequencies in the population, the lineage settles at more than 50% WS frequency, except for pure SM population that settles at equilibrium mean frequency of 30% WS, on day 6. Thus, the presence of two equilibrium frequencies on day 6 that depend upon the initial WS frequency in the founding population indicates a strategy of bistability.

d) Evolved lineage – Line 17

- Cases where populations are founded with a 1:1000 ratio of SM: WS, so WS is 99.9% (Figure 5.1(D), purple trajectory), and for pure WS population, where WS is 100% (Figure 5.1(D), blue trajectory), the final mean frequency of the populations remain 63% and 52% WS, respectively, at day 6.
- Cases where the populations are founded with a 1000:1 ratio of SM: WS, so SM is 99.9% (Figure 5.1(D), pink trajectory), and for pure SM population, where SM is 100% (Figure 5.1(D), red trajectory), the mean frequency of the populations remain 28% and 19% WS, respectively, at day 6.
- Cases where the populations are founded with a 1:1 ratio of SM: WS, so SM and WS both are 50% (Figure 5.1(D), black trajectory), the mean frequency of the populations remain 59 % WS at day 6.

Line 17 shows two equilibrium states depending upon different WS frequencies in the initial inoculum. For WS dominated population (WS is 99.9% and 100%) and mixed population (WS is 50%), lineage settles at an equilibrium frequency of about 50% WS (Figure 5.1(D). Whereas, for SM dominated population (SM is 99.9% and 100%), lineage settles at an equilibrium frequency of less than 30% WS. The presence of two equilibrium states at day 6 indicates bistability (Figure 5.1(D)).

e) Evolved lineage – Line 43

- Cases where populations are founded with a 1:1000 ratio of SM: WS, so WS is 99.9% (Figure 5.1(E), purple trajectory), and for pure WS population, where WS is 100% (Figure 5.1(E), blue trajectory), the final mean frequency of the populations remain 53% and 56 % WS, respectively, at day 6.
- Cases where the populations are founded with a 1000:1 ratio of SM: WS, so SM is 99.9% (Figure 5.1(E), pink trajectory), and for pure SM population, where SM is 100% (Figure 5.1(E), red trajectory), the mean frequency of the populations remain 28% and 19% WS, respectively, at day 6.
- Cases where the populations are founded with a 1:1 ratio of SM: WS, so SM and WS both are 50% (Figure 5.1(E), black trajectory), the mean frequency of the populations remain 44% WS at day 6.

Line 43 shows two equilibrium states depending upon different WS frequencies in the initial inoculum. For WS dominated population (WS is 99.9% and 100%) and mixed population (WS is 50%), lineage settles at an equilibrium frequency of about 50% WS (Figure 5.1(E)). Whereas, for SM dominated population(SM is 99.9% and 100%), lineage settles at an equilibrium frequency of less than 30% WS. The presence of two equilibrium states at day 6 indicates bistability (Figure 5.1(E)).

Table 5.1 Transition capacity (TC)

A.

	Strain name	Proportion of microcosms with alternate type for 6 days*
WS to SM	71 WS	
	17 WS	
	70 WS	
	43 WS	
	WS¶	

B.

SM to WS	71 SM	
	17 SM	
	70 SM	
	43 SM	
	SBW25 SM[¥]	

SM: smooth cell type, WS: wrinkly spreader. BL: baseline lineage, EV: evolved lineage, ANC: ancestor.*White: absent, yellow: the proportion WS type, red: the proportion of SM type, extinction. Colour intensity shows the proportion of microcosms containing a new type; the more the intensity the higher the proportion. [¥] The strain used in the Rainey and Rainey (2003). ¶ The WS derived from ancestral SM SBW25.

Panel A shows the transition capacity from WS to SM while panel B shows the transition capacity from SM to WS.

From the results of the lineage-wise eco-space diagrams, I now move onto combination-wise eco-space diagrams.

5.2.2 Combination-wise eco-space diagrams: for populations founded with different combinations of frequency and density.

Before proceeding with the results of this section, it is important to understand the role of transition capacity of the individual lines in affecting the eco-space trajectory. Transition capacity (TC) gives an indirect measure of the switching rate of the lines (Chapter 2, section 2.1.1). Given in Table 5.1 are the TC of all the lineages used in this study. Panel A shows the TC from WS to SM while panel B shows the TC from SM to WS. The TC for each lineage is represented by a band consisting of six divisions, where each division has a colour intensity that gives the measure of the TC on that day. The more the colour, the higher the TC of the lineage.

In case of ancestral and baseline lineages, the eco-space trajectory is predicted to be a function of its respective TC whereas for evolved lineages the eco-space trajectory is hypothesized to be a function of both its TC and eco-evo feedback (Chapter 2, section 2.4.4). Hence, whenever, the evolved lineage exhibits a deviation in trajectory to its respective TC in the eco-space diagram, it suggests the presence of an eco-evo feedback.

Given below are the eco-space diagrams which forms the following combinations:

1. Pure WS population initiated at high density.
2. Pure WS population initiated at medium density.
3. Pure WS population initiated at low density.
4. Mixed (1:1 ratio of WS: SM) population initiated at high density.
5. Mixed (1:1 ratio of WS: SM) population initiated at medium density.
6. Mixed (1:1 ratio of WS: SM) population initiated at low density.
7. Pure SM population initiated at high density.
8. Pure SM population initiated at medium density.
9. Pure SM population initiated at low density.

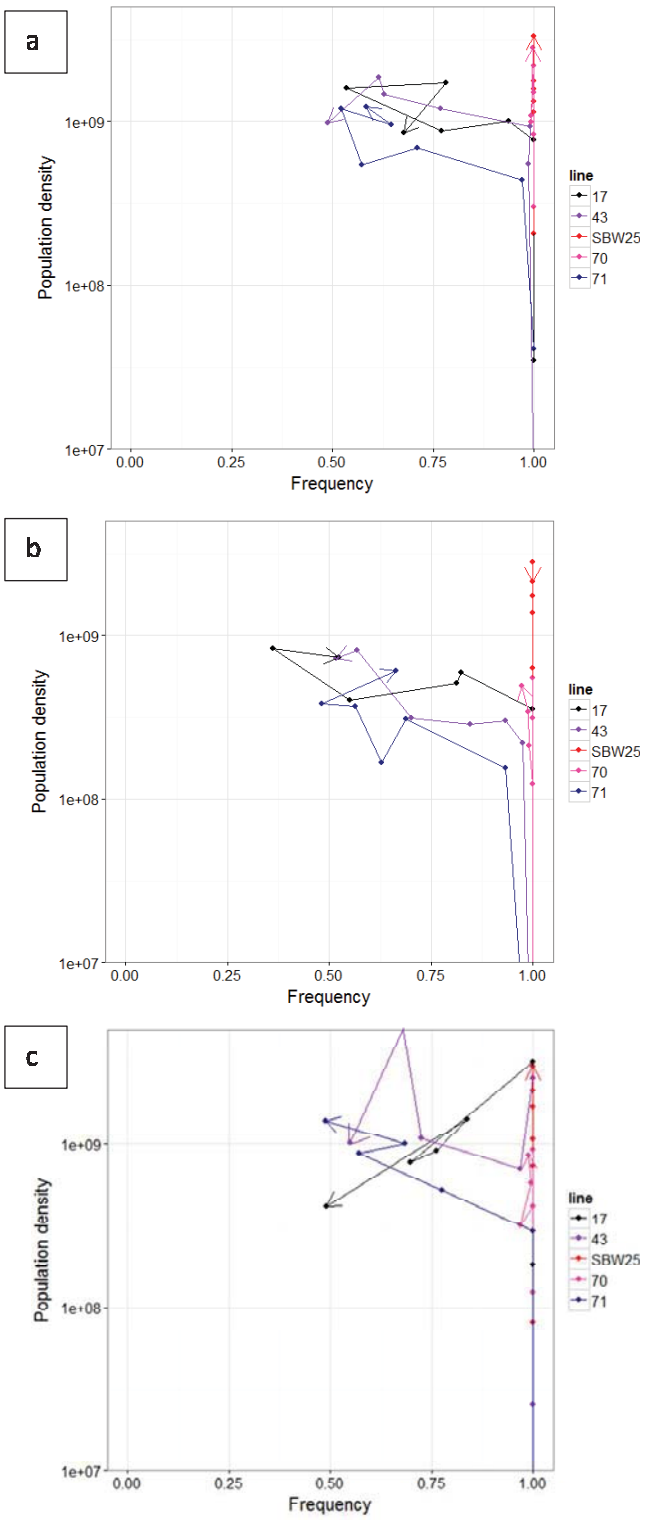

Figure 5.2 Eco-space trajectories of pure WS populations. Figure shows the population density versus frequency trajectories for different lines for a pure WS population at different initial densities: (a) high (b) medium (c) low, from top to bottom, respectively. Each point on the graph represents the mean of population density and WS frequency on each day and the arrow denotes the end of the trajectory at day 6. Colours are representative of lineages, black: 17 (evolved), purple: 43 (evolved), red: SBW25 (ancestral), pink: 70 (baseline), blue: 71 (baseline).

It is found that in most cases there is no difference in the trajectory with the change in initial density and hence the explanation for such cases is combined.

5.2.2.1 Eco-space diagram for pure WS population founded at different densities

The data depicted in Figure 5.2 shows the eco-space diagram for pure WS populations (SM-0%) of different lineages (represented by different colors on the diagram), founded at three initial densities: (a) high, (b) medium (c) and low. The expectation is that in the absence of an eco-evo feedback, line's eco-space trajectory for pure WS population would be according to its transition capacity (panel A, Table 5.1, shows the TC for each individual lineage). While in the presence of the feedback, the trajectory would show deviation from its respective TC profile. Given below is the explanation for the eco-space trajectory for all the lineages, case-wise. For each case, WS trajectory founded at medium density is discussed first and later it is compared to the WS trajectory founded at high and low density.

Case 1: Eco-space trajectory for pure population of WS derived from ancestral SM SBW25.

If the eco-space trajectory of WS derived from ancestral SM SBW25 is according to the TC profile of the lineage given in panel A ,Table 5.1, then WS will show no transition to SM for first 5 days (day 1-5) and there will be a low TC value on day 6. The trajectory of WS derived from ancestral SM SBW25 shown in red color in Figure 5.2(b), behaves in the same manner. It is seen that the WS didn't produce SM in 6 days (100% WS frequency). Also, Figure 5.2(a), (b) and (c) shows that the trajectory didn't get affected by the change in the initial density.

Case 2: Eco-space trajectory for pure population of WS of Line 70.

If the eco-space trajectory of Line 70 is according to the TC profile of the lineage given in panel A, Table 5.1, then WS will show no transition to SM for all the 6 days, except on day 4, where it will have a slight (shown in light red color in panel A, Table 5.1) transition to SM. The trajectory of Line 70 WS shown in pink line in Figure 5.2(b) depicts that WS didn't produce SM i.e. it didn't deviate much from 100% WS frequency on all the 6 days, except for a slight deviation on day 6 (99.9%).Thus, the observation is in near accordance to the WS TC profile of Line 70. Also, the data depicted in Figure 5.2(a) shows that the populations founded at high density settles at a comparatively higher density (2.81e+09 cfu/ml) to the populations founded at medium (4.95e+08 cfu/ml) and low density (8.53e+08 cfu/ml) (depicted in Figure 5.2(b) and (c), respectively).

Case 3: Eco-space trajectory for pure population of WS of Line 71.

If the eco-space trajectory of Line 71 is according to the TC profile of the lineage given in panel A, Table 5.1, then WS will show transition to SM on all 6 days. Moreover, it will have a high frequency of SM on each day. Accordingly, the trajectory of Line 71 WS, depicted in Figure 5.2(b) by blue line, increases in SM frequency at each day and settles at a final frequency of 65% WS on day 6. Also, the data depicted in the Figure 5.2(a), (b) and (c) shows that the trajectory didn't get affected by the change in the initial density.

Case 4: Eco-space trajectory for pure population of WS of Line 17

If the eco-space trajectory of Line 17 is according to the TC profile of the lineage given in panel A Table 5.1, then WS will show transition to SM on all 6 days. Accordingly, it is observed with the trajectory of Line 17 depicted by black line in

Figure 5.2(b), that the SM frequency increases each day and settles at an equilibrium frequency of 52% WS, for Line 17 Also, the equilibrium frequency (frequency on day 6) of Line 17 did not change with the change in the initial density, as can be seen in the Figure 5.2 (a), (b) and (c).

Case 5: Eco-space trajectory for pure population of WS of Line 43.

If the eco-space trajectory of Line 43 is according to the TC profile of the lineage given in panel A, Table 3.3, then WS will show transition to SM on all 6 days. Accordingly, the trajectory of Line 43 depicted by purple line, in Figure 5.2(b), shows that the SM frequency increases each day and settles at an equilibrium frequency of 52% WS. Also, the equilibrium frequency (frequency on day 6) of Line 43 did not change with the change in the initial density, as can be seen in the Figure 5.2(a), (b) and (c).

Overall, the observation of eco-space trajectory for pure WS population shows that:

- The TC's of Line 17, 43 and 71 are similar, so are there eco-space trajectories (compared to Line 70 and ancestral SBW25). This indicates that lines with similar TC's display similar eco-space trajectories.
- The lineage's eco-space trajectories are in accordance to their respective TC profile. This indicates an absence of an eco-evo feedback in evolved lineages.

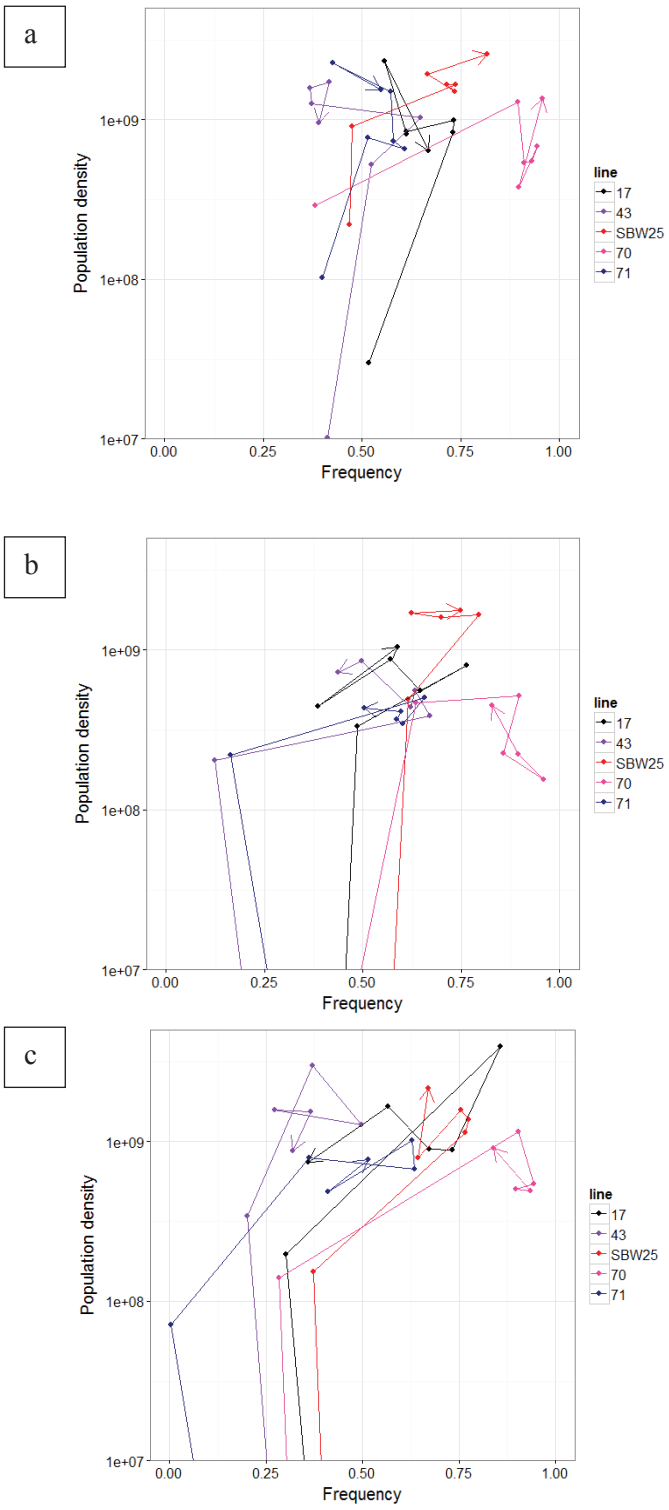

Figure 5.3 Eco-space trajectories of mixed populations. Figure shows the population density versus frequency trajectories for different lines for a mixed (1:1 ratio of WS: SM) population at different initial densities: (a) high (b) medium (c) low, from top to bottom, respectively. Each point on the graph represents the mean of population density and WS frequency on each day and the arrow denotes the end of the trajectory at day 6. Colours are representative of lineages, black: 17 (evolved), purple: 43 (evolved), red: SBW25 (ancestral) , pink: 70 (baseline), blue: 71 (baseline).

5.2.2.2 Eco-space diagram for mixed (1:1 WS: SM) population founded at different densities

The data depicted in Figure 5.3 shows the eco-space diagram for populations founded at mixed frequency (1:1 WS: SM) and three initial densities – high, medium and low – for different lineages (represented by different colors on the diagram). Again, the expectation is that in the absence of an eco-evo feedback, line's eco-space trajectory for mixed population would be according to its TC such that the trajectory would move towards an increase in SM or WS frequency depending upon which is greater: -TC from SM to WS or from WS to SM (Table 5.1, panel A shows the TC from WS to SM and panel B, shows TC from SM to WS). While in the presence of the feedback, the trajectory would show deviation from the expected pattern.

Case 1: Eco-space trajectory for mixed (1:1 ratio of WS: SM) population of WS derived from ancestral SM SBW25 and SM of ancestral SBW25.

For ancestral line, the TC from SM to WS is greater than from WS to SM (panel B and A, respectively, Table 5.1, ancestral SBW25). The expectation is that the unequal transition capacities would shift the equilibrium towards more WS than SM frequency in the population. Accordingly, Figure 5.3(b) shows that the WS frequency on day 1 is 61% WS that increases from day 2 to 77%, and remain the same thereof (from day 3 to 6). This leaves the population with more frequency of WS than SM. Also, the equilibrium frequency (frequency on day 6) of SBW25 did not change with the change in the initial density as can be seen in the Figure 5.3(a), (b) and (c).

Case 2: Eco-space trajectory for mixed (1:1 ratio of WS: SM) population of WS and SM of Line 70.

Like in the case of ancestral SBW25, the TC of Line 70, from SM to WS is greater than from WS to SM, (panel B and panel A, respectively, Table 5.1, Line 70), which again shifts the equilibrium towards more WS. Probably, this is the reason why Line 70 frequency shoots up to 90% WS on day 2 and remains same thereof until day 6 (87% WS). This can be seen from the trajectory depicted in the Figure 3.13(b). Again, the equilibrium frequency (frequency on day 6) of Line 70 WS did not change with the change in the initial density as can be seen in the Figure 5.3(a), (b) and (c).

Case 3: Eco-space trajectory for mixed (1:1 ratio of WS: SM) population of WS and SM of Line 71.

Line 71 has similar TC's in both directions i.e. from SM to WS and from WS to SM as can be seen in panel B and panel A, respectively, Table 5.1, Line 71.Therefore, it is expected that the trajectory would not diverge much from 50% WS frequency. Accordingly, the trajectory in Figure 5.3(b) shows that from day 2 onwards, line reaches 54% WS frequency and remains the same thereof until day 6 (52% WS). Thus, the line behaves in accordance to its TC profile. Also, Figure 5.3(a), (b) and (c) show that the trajectory did not change with the change in the initial density.

Case 4: Eco-space trajectory for mixed (1:1 ratio of WS: SM) population of WS and SM of Line 17.

Line 17 has similar TC's in both directions i.e. from SM to WS and from WS to SM as can be seen in the panel B and panel A, respectively, Table 5.1, Line 17 .Therefore, it is expected that the trajectory would not diverge much from 50% WS

frequency. Accordingly, trajectory depicted in the Figure 5.3(b) shows that on day 2, the line reaches 66% WS frequency and remains the same thereof until day 6 (54% WS). Thus, the line behaves in accordance to its TC profile. Also, Figure 5.3(a), (b) and (c) show that the trajectory did not change with the change in the initial density.

Case 5: Eco-space trajectory for mixed (1:1 ratio of WS: SM) population of WS and SM of Line 43.

Line 43, has similar TC's in both directions i.e. from SM to WS and from WS to SM as can be seen in the panel B and panel A, respectively, Table 5.1, Line 43. Therefore, it is expected that the trajectory would not diverge much from 50% WS frequency. Accordingly, the trajectory depicted in the Figure 5.3(b) shows that on day 2, the line reaches 56% WS frequency and remains the same thereof until day 6 (40% WS). Thus, the line behaves in accordance to its TC profile. Also, the Figure 5.3 (a), (b) and (c) shows that the trajectory did not change with the change in the initial density.

Overall, the observation of eco-space trajectory for mixed population shows that:

- The TC for SM to WS for Line 70 is high like SBW25 but unlike SBW25, transition from SM to WS starts from day 1 itself compared to SBW25 ,where the transition starts from day 3 onwards. The difference in their TC is also reflected in their WS frequencies, where line 70 has a mean average of 90% WS from day 2-6 compared to SBW25 which has a mean average of 72 % WS.
- The lineage's eco-space trajectories are in accordance to their respective TC profile. This indicates an absence of an eco-evo feedback in evolved lineages.
- Also, the equilibrium frequency (frequency on day 6) in all the cases did not change with the change in initial density.

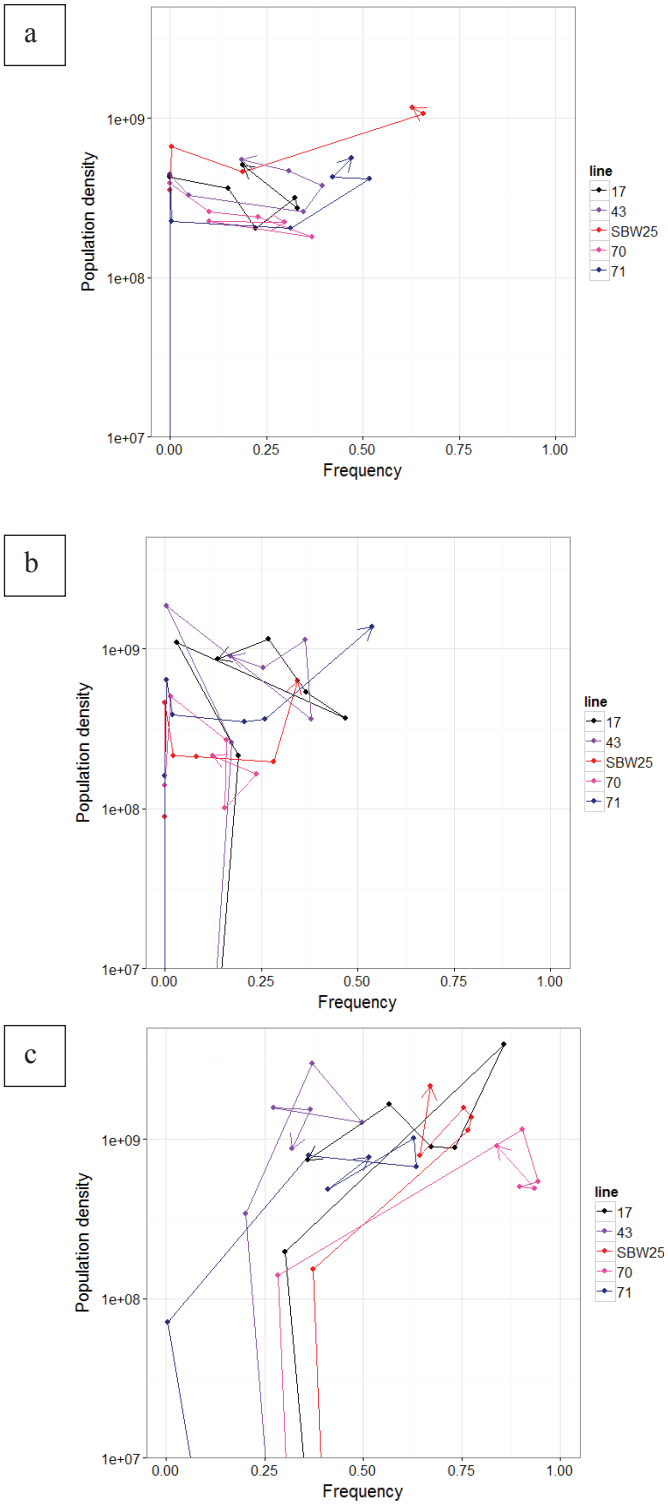

Figure 5.4 Eco-space trajectories of pure SM population. Figure shows the population density versus frequency trajectories for different lines for a pure SM population at different initial densities: (a) high (b) medium (c) low, from top to bottom, respectively. Each point on the graph represents the mean of population density and WS frequency on each day and the arrow denotes the end of the trajectory at day 6. Colours are representative of lineages, black: 17 (evolved), purple: 43 (evolved), red: SBW25 (ancestral), pink: 70 (baseline), blue: 71 (baseline).

5.2.2.3 Eco-space diagram for pure SM population founded at different densities

The data depicted in Figure 5.4 shows the eco-space diagram for pure SM populations (WS-0%) of different lineages (represented by different colors on the diagram), founded at three different densities: high, medium and low. The expectation is that in the absence of an eco-evo feedback, line's eco-space trajectory for pure SM population, given in panel B Table 5.1, would be according to its TC from SM to WS. While in the presence of the feedback the trajectory would show deviation from its respective TC profile. Given below is the explanation for the eco-space trajectory for all the lineages, case-wise.

Case 1: Eco-space trajectory for pure population of SM of ancestral SBW25.

If the eco-space trajectory of SM of ancestral SBW25 is according to the TC profile of the lineage given in panel B, Table 5.1, then SM that has a high TC, will transition to WS on each day, from day 2 onwards. Trajectory of ancestral SM SBW25 shows the same result. Figure 5.4(b) shows that the WS frequency increases almost linearly from day 2 onwards and reaches an equilibrium frequency of 52%. Also, the equilibrium frequency and density is lower in pure SM population founded at low density (34%, 6.43e+08 cfu/ml) (Figure 5.4(c)), compared to the population founded at medium (63%, 1.17e+09 cfu/ml) and high density (60%, 1.23e+09 cfu/ml), seen in Figure 5.4(b) and (a), respectively.

Case 2: Eco-space trajectory for pure population of SM of Line 70.

If the eco-space trajectory of Line 70 is according to the TC profile of the lineage given in panel B, Table 5.1, then SM will show transition to WS on all 6 days, except on day 1. Moreover, the frequency of WS produced will be high in frequency.

Although, the trajectory depicted in Figure 5.4(b) shows that there is WS production from SM from day 2-6; however, the frequency of WS produced from SM on these days is low (21% WS). This observation is seen for all the initial densities (Figure 5.4(a), (b) and (c)). Thus, the trajectory observed is not in accordance to the TC for Line 70.

The WS of Line 70 couldn't invade the population when the initial frequency of WS is 0% while it can invade at all other WS founding frequencies, so much so, that it dominates the population (Figure 5.1(c)).The inability of Line 70 WS to invade pure SM population when WS is 0%, is probably because there is not enough threshold of WS cells at 0% WS frequency to form a mat at the air-liquid interface. This limits the growth of WS and so decreases its frequency in the overall population.

According to our hypothesis, if a pattern differs from its TC, the pattern is attributed to the eco-evo feedback. In case of Line 70, even though the trajectory differs from what is expected from its TC, yet this difference cannot be attributed to an eco-evo feedback, because the pattern is possibly a result of the unique ecology of line 70 WS, than due to the feedback.

Case 3: Eco-space trajectory for pure population of SM of Line 71.

Line 71 has a high TC for SM to WS, on each day. If the eco-space trajectory of Line 71 is according to the TC profile of the lineage given in panel B, Table 5.1, then WS will increase in frequency on each day. Accordingly, the trajectory depicted in Figure 5.4(b) shows that the WS increases progressively in frequency to reach an equilibrium frequency of 55% WS on day 6.

Case 4: Eco-space trajectory for pure population of SM of Line 17.

Line 17, has a comparable TC to line 71(panel B, Table 5.1, Line 17 and Line 71), and the expectation is that Line 17 will have a trajectory very similar to Line 71, i.e. the WS frequency would increase every day and would reach an equilibrium frequency of about 50% on day 6. However, Line 17 shows an unusual trajectory. The trajectory depicted in Figure 5.4(b) shows that on day 1, the frequency of WS stays 0% and then it increases gradually to reach a maximum frequency at around day 3 or day 4. The populations founded at low density (47% WS) reaches a maximum on day 3 while populations founded at high (48% WS) and medium density (32% WS), reaches a maximum on day 4. The maximum frequency attained varies between 30-50%, after which the frequency starts to decline to reach an equilibrium frequency of less than 20% WS (15% WS frequency on day 6). Thus, the trajectory observed is not in accordance to the TC for Line 17.

Case 5: Eco-space trajectory for pure population of SM of Line 43.

Line 43, although having intermediate TC among evolved lineages, has a comparable TC to baseline lineage Line 71 (panel B, Table 5.1, Line 43 and Line 71), and the expectation is that Line 43 would also have a trajectory very similar to Line 71. Line 43 comparable TC with Line 71 mean that the WS frequency of Line 43 would increase every day and would reach an equilibrium frequency of about 50% on day 6. However, Line 43 like Line 17 also shows an unusual trajectory. The trajectory depicted in Figure 5.4(b) shows that on day 1, the frequency of WS stays 0% and then it increases gradually to reach a maximum frequency on day 3 or day 4. Populations founded at medium density (40% WS) reaches a maximum on day 4, while populations founded at high (28% WS) and low density (39% WS), reaches a maximum on day 3. The maximum frequency attained varies between 25-40%, after which the frequency

starts to decline to reach an equilibrium frequency of less than 25% WS. Thus, the trajectory observed is not in accordance to the TC for Line 43.

Overall, the trajectory shows that:

- Line 70 shows deviation from its respective TC. The deviation in this case is due to the specific nature of the WS that forms poor or no mat and not due to the presence of the feedback.
- The ancestor and the baseline lineage 71 behave in accordance to their respective TC.
- The two evolved lineages, 17 and 43 show deviation from their TC profiles. This can be seen in their eco-space trajectories that are depicted in Figure 5.4. The uniqueness of the pattern to the evolved lineages and not the baseline lineages suggest that this might be an evolved pattern that has emerged during the life cycle experiment.
- This indicates the presence of an eco-evo feedback in evolved lineages.

5.3 Discussion

5.3.1 Summary and comparison of strategies between baseline and evolved lineages

The baseline lineages show different strategies: (i) d*ominance* in ancestral SBW25 (ii) *bistability* in Line 70 and (iii) *coexistence* in Line 71. The strategy displayed by each lineage is found to be unique. This is because each lineage's WS-SM pair differs from each other in their genotypic constitution and therefore its interaction with the environment, thus giving rise to different strategies.

Moreover, I believe that a given strategy is optimized for each lineage. For instance, it is seen that Line 71 ranked highest in line fitness among the baseline lineages. There is a possibility that the high line fitness of Line 71 is a result of the strategy exhibited by Line 71. This is because the *coexistence* strategy followed by Line 71 allows for equal proportion of WS and SM in the population on day 6. Having almost equal proportion of WS and SM maximizes the requirement of the life cycle for survival (intact WS mat) and reproduction (production of propagules by WS mat) of the lineage (Chapter 1, section 1.6). Therefore, it is likely that the *coexistence* strategy of Line 71 (Figure 5.1(B)) helps the lineage to have highest line fitness

The most important finding that became apparent with the comparison of strategies between baseline and evolved lineages is that , while the ancestral SBW25 and baseline lineages – 71 and 70, exhibited different strategies, evolved lineages – 17 and 43, shows a common strategy of bistability. Bistability as a strategy in biological systems have been known to arise where there is a potential for the development of a relationship between ecology and evolution. Therefore, the response of bistability is likely indicative of an emergence of an eco-evo feedback in evolved lineages.

5.3.2 Eco-evo feedback indicates evolution on the part of the cheater

Observation from the eco-space trajectories of pure SM populations of evolved lineages indicates that there is selection on the pattern of emergence of WS from SM during the life cycle experiment rather than the other way round. This is because the pattern of emergence of WS from pure SM population deviates from its TC profile compared to the pattern of emergence of SM from WS, that didn't deviate much from its respective TC.

The nature of the deviation is found to be a reduction in the equilibrium frequency (WS frequency on day 6) in Line 17 and 43. The reduced WS frequency is not expected from Line 17 and 43, given the high TC of both the lines. This deviation indicates that there is a selection on the SM for less production of WS. The reduced capacity of SM to produce WS indicates a strategy on the part of SM (cheat) to produce less WS (cooperator). Producing less WS indirectly increases the SM proportion in the population which help maintains a higher SM proportion at the end of phase I (in the life cycle experiment). Higher proportions of SM is what that determines the success of the lineage to pass onto the succeeding phase (phase II), and determines the lineage's fitness.

Therefore I think that reduced transition rate of SM from WS is one of the tactics of evolved lineages that have helped them progress through the phases of the life cycle, and eventually increase in fitness compared to the baseline lineages.

Chapter 6. Conclusion

Evolution of multicellularity from unicellular organisms is an evolutionary transition (Grosberg & Strathmann, 2007) that is hypothesized to have progressed through three seminal steps: evolution of cooperation, evolution of groups and evolution of complexity (Rose, 2015). Among these, the evolution of cooperation is the primary step. Therefore, understanding the evolution of cooperation is essential before gaining insight into further stages. Understanding the evolution of cooperative behaviour has been a challenge. This is because natural selection rewards selfish behaviour (Nowak & Sigmund, 2004). Yet, in nature we see numerous examples where cooperation exists (Clutton-Brock, 2009). Here arises a conflict between the interests of individuals and those of collectives and thus raises a question as to the evolution of cooperative behaviour (Dawkins, 2006).

Several ideas have been put forward to explain the cooperative behaviour. These include theories based on direct and indirect fitness benefits. Recent work has identified assortment as the fundamental principle behind all the theories and thus encompasses mechanism based on both (Fletcher & Doebeli, 2006, 2009. However, these theories assume the environment to be stable and fail to acknowledge the self-referential property of evolution, i.e. – that evolutionary outcomes are capable of affecting the evolutionary process itself (Watson *et al*., 2015). In other words, this means that the prevailing theories underestimate the role of interactions and in particular interactions that are density and frequency dependent (the two components of environment) that are capable of generating co-evolutionary interactions (Heininger, 2015). One such mechanism that addresses the role of environment, is eco-evo feedback, in which the interplay between density – representing ecological change – and frequency –

representing evolutionary change – maintains cooperation (Tudge *et al*., 2013; Gore *et al*., 2009; Sanchez & Gore, 2013).

Many empirical studies use microorganisms to answer questions pertaining to the eco-evo feedback (Ribeck & Lenski, 2015). This is because microorganisms have short generation times that allow overlapping of ecological and evolutionary timescales, which have the potential to generate feedbacks. Recently, a long term evolution experiment was conducted using bacterial lineages of *Pseudomonas fluorescens* (Hammerschmidt *et al*., 2014). The *P. fluorescens* cheater-cooperator model system consists of a cellulose-producing, group-living cooperator type termed WS and a solitary, free-living cheater type termed SM. In this experiment, *Pseudomonas* populations were repeatedly transitioned between phases of the life cycle – from WS to SM and from SM to WS. At the end of the experiment, the fitness of derived lineages was compared to the ancestral types. The result depicted that, the overall fitness of derived lineages increased and was found to be positively correlated with increased transition rate of the lineages.

It was predicted that ecological-evolutionary (eco-evo) feedback had likely occurred both on the WS-SM interaction and their transition rate. In principle, the existence of the feedback can be identified by comparing the evolutionary dynamics, population dynamics and joint evolutionary and population dynamics of the ancestor and evolved lineages. And so this study aimed to determine: frequency dependent interactions (an investigation of evolutionary dynamics), density dependent interactions (an investigation of population dynamics) and to represent the combined effect as a series of eco-space diagrams, which provides understanding of the joint influence of evolutionary and population dynamics.

6.1 Frequency dependent interactions

The nature of interaction between WS and SM is seen to be frequency dependent for most lineages. Also the effect differed between lineages. For instance, the interaction in ancestral lineage SBW25 is seen to be frequency dependent. The WS (WS derived from ancestral SM SBW25) has a negative effect on SM for populations that began with higher WS frequency, while WS has a positive effect for populations that began with intermediate frequencies. The result of negative effect of WS on SM at high WS frequency is contrary to the expectation that asserted the effect of WS on SM to remain positive even at high WS frequency. This expectation is formed due to two reasons: firstly, the result from Rainey and Rainey (2003) that determined the effect of WS on SM as positive, at intermediate initial WS frequency; and secondly, the evidence from previous studies that increasing the relative proportion of cooperator (WS) in the population leads to an increased positive effect on cheater (SM) (Ross-Gillespie *et al*., 2007).

Contrary to expectation, I observed a negative effect of WS on SM when WS was at high frequency. This may be related to mat formation by WS cells. Mat formation requires a process of development that takes place from a limiting number of cells. So adding more cells, other than required for mat formation, is likely to increase the relative frequency of WS cells and this would impose stringent competition on SM cells for resources, thus resulting in a negative effect of WS on SM when WS is initially present at high frequency.

Another example where the effect of WS on SM is found to be frequency dependent is for baseline lineage 70, where WS has a negative effect on SM – which increases with the increase in initial WS frequency.

There are also examples of lineages wherein the interaction of WS on SM stayed the same and therefore is found to be frequency independent. Examples of such lineages include the two evolved lineages – Line 17 and Line 43 – where WS always has a positive effect on SM in case of Line 17, and no effect in case of Line 43.

6.1.1 Increased transition rate from WS to SM

The result of the overall comparison between the evolved and baseline lineages for the frequency dependent nature of interactions between WS and SM revealed a positive shift in the capacity of WS to facilitate the growth of SM in the evolved lineages. A similar observation was seen in Hammerschimdt *et al.* (2014), where there was found to be an overall increased transition capacity from WS to SM in evolved lineages. This led to the believe that the positive shift, seen in this study, is likely to be a consequence of the overall increase in transition capacity of the evolved lineages.

6.2 Density dependent interactions

After investigation of frequency dependent nature of interactions, I then examined the extent to which interactions between SM and WS types were influenced by density dependent factors. I found that the effect of WS on SM is density dependent. Also, the interactions are different for different lineages. For instance in ancestral lineage SBW25, it was observed that at high initial density of the population, WS (derived from ancestral SM SBW25) has no effect on SM while at low initial density WS has a positive effect on SM. No positive effect of WS on SM at high initial density indicates resilience to SM invasion. The possible suggestion for why this occur, could to do with the spatial structure of mat in SBW25 at high density that minimises the interaction between WS and SM; thus, decreasing the chances of direct exploitation of WS by SM (Hilbe, 2011).

Another example, where the effect of WS on SM was found to be density dependent is in the case of baseline lineage 71. Contrary to the result found with the ancestral lineage SBW25, the effect of WS (Line 71) on SM is positive at high density while no effect was observed at low density. The possible reason for a positive effect of WS on SM at high density could be that Line 17, which has a high transition capacity, would give rise to a spatially mixed population of WS and SM, where the members are in close physical contact. This might increase the chances of direct exploitation of WS by SM; thus leading to a positive effect of WS on SM at high density.

The result from density dependent study indicates that there are some lineages whose interaction did not change with the change in initial density, and hence were found to be density independent. For example, in Line 70 the interaction (effect of WS on SM) remained the same, i.e. negative throughout, at all the three densities. The negative interaction is because of WS dominance in Line 70 at all the three densities, and represents an example of an ecological community whose function resides in its "driver species". Driver species, like WS of Line 70, are species that significantly structure the ecosystems in which they persist. They may be ecological engineers which physically structure the community – like WS; thus, they hold an ecological dominance and stability, in all the fluctuating environments (changes in density) (Peterson *et al.*,1998).

Another finding that came from density dependent study is that the variation in interaction between SM and WS is greatest at low and high population densities, and is diminished at population densities that mirrored standard laboratory levels. For example, in Line 43 interactions were detected at both high and low densities that were absent at standard intermediate densities. A similar observation was seen in the

interactions between X and Y in the cooperative bacterium *Myxococcus xanthus*, which shows variation in the tendency to cooperate at very low and high densities but not at intermediate population densities (Kadam, 2006). The reason for this observation could be relegated to the evolutionary history of the lineages.

So far I have seen that interactions between SM and WS are often both frequency and density dependent. This motivated me to interrogate the joint influence of both these factors on the WS-SM interaction. For this purpose, eco-space diagrams were used in which population density and frequency is represented on a single graph.

6.3 Joint influence of frequency and density reveal presence of an eco-evo feedback

6.3.1 Common strategy in evolved lineages

The results of the lineage-wise plotting shows that each lineage displayed a strategy reminiscent of the outcome of game theoretic studies where two players interact and their interaction results in different responses (Doebeli & Hauert, 2005).The ancestral and the baseline lineages exhibit different strategies, for example (i) *dominance,* in case of ancestral SBW25, (ii) *bistability*, for baseline lineage – Line 70 and (iii) *coexistence,* for baseline lineage – Line 71. This is possibly due to the presence of different environmental conditions that generate different optimal strategies (Dekel & Alon, 2005).

Evolved lineages – Line 17 and 43, on the other hand, both show interactions that have the hallmarks of bistability. Bistability in biological systems has been known to arise where there is a potential for the development of a relationship between ecological and evolutionary factors (Tiwari *et al*., 2011). In fact, models suggest that for

fluctuating environments of the kind that result in the evolution of evolvability, bistability and phenotypic diversity arises as a by-product (Kuwahara & Soyer, 2012). I think that during the life cycle, evolved lineages have faced similar fluctuating environments over long time periods giving rise to positive feedbacks. Such feedbacks in turn are capable of generating graded responses like bistability. Therefore, the response of bistability shown by the evolved lineages is likely indicative of an emergence of an eco-evo feedback.

6.3.2 Reduced transition rate of SM of evolved lineages

Comparison of eco-evo trajectories between baseline lineages and evolved lineages (Line 17 and Line 43) indicates differences in their capacity to produce WS and SM. It is found that for evolved lines, capacity of SM to produce WS is reduced compared to what is seen in the baseline lineages. This means that during evolution, the tendency of SM to produce WS could be the target of selection.

The tendency to produce fewer WS cells seemed beneficial for the evolved lineages in the life cycle that required sufficient number of SM cells for the lineage to progress to the next phase of the life cycle. Therefore, producing less WS may have indirectly helped the lineage to increase the relative number of SM cells in the population.

The next question that arises is whether having a reduced capacity of SM to produce WS is adaptive. The transition of SM to WS is a 'gain of function' phenotype, although often underpinned by a loss of function mutation (Bantinaki *et al.*, 2007; McDonald *et al.*, 2009; Lind *et al.*, 2015) that renders a genotype the ability to over-produce cellulose. Production of cellulose is metabolically expensive and comes at a fitness cost to the lineage that produces it. So, reduced transition capacity from SM to

WS, indicates a strategy on the part of the SM of evolved lineages (of Line 17 and 43) to save the cost of production of cellulose (by WS) and to trade-off this cost with an increase in the fitness of the lineages.

Thus, the high fitness of evolved lineages in the life cycle experiment is possibly due to the presence of an eco-evo feedback, signature of which is seen in the tendency of the evolved lineages to produce fewer WS cells from SM types.

6.4 FUTURE WORK

1. Strategy for other evolved lineages

For other evolved lineages the strategy to maximise their fitness during the life cycle could be different, and therefore the signature of an eco-evo feedback may differ. For future work, the eco-evo feedback pattern for other lineages would be a profitable line of investigation.

2. Temperature affecting the nature of interactions

Temperature has been identified as a strong abiotic factor that has the capacity to change ecological interactions (Callaway *et al*., 2002). One of two possible ways by which it affects the nature of interactions is by changing the phenological responses of the coevolving species and thus rendering unique properties to the interaction (Hegland *et al*., 2008). The other way the temperature can affect an interaction is by exercising control over the two factors of density and frequency; the results from this study suggest that changes in density and frequency alter the nature of the interaction. Thus, the role of temperature in affecting the phenological properties of the coevolving species and the relationship of temperature with population density and frequency could be explored in future studies.

3. Modelling eco-evo feedback

This study has indicated the presence of an ecological evolutionary feedback in two of the lineages that were evolved in a long term evolutionary experiment. The results obtained highlight eco-evo feedback as one potential factor influencing evolution of the lineages. Similarly, there is a possibility for other evolved lineages too, to have been shaped by the feedback between ecology and evolution (in comparison to the ancestral lineages). Also, other studies working on similar lines have demonstrated eco-evo feedback as a characteristic feature of coevolving systems (Reznick, 2013; Travis *et al*., 2013, Harrington & Sanchez, 2014). Therefore, it becomes imperative to delve deep into the knowledge of the general mathematical principles underlying the mechanism. Theoretical studies have already started thinking to incorporate the property of eco-evo feedback (central to the coevolving systems) in the existing models of cooperation, by borrowing a concept from a pre-existing idea in mathematical sciences called "reinforcement learning" (Erev & Roth, 1998; Aunger & Curtis, 2014). Like in eco-evo feedback, in reinforcement learning too, the evolutionary parameters of the system coevolve with the environment (Watson *et al*., 2015). Thus, there are tools available to model eco-evo feedback and in future work the "reinforcement learning" principle could be applied to study coevolving systems like the one dealt in this study.

4. Assortment and eco-evo feedback

As discussed before in the previous section (Chapter 1, section 1.3.2 (f)), assortment is a general mechanism on which existing theories on the evolution of cooperation are based. However, there still remains a gap in understanding the relationship between assortment and eco-evo feedback. It is not understood how these two mechanisms relate with each other to support cooperation, especially in populations that have already been known to show assortment; for instance, *P. fluorescens* bacterial

populations are known to show high degree of assortment due to the property of WS cells that over-produce cellulose. The relationship between assortment and eco-evo feedback is unclear in this system. Therefore, future studies might consider the interplay between these two mechanisms – assortment and eco-evo feedback – that could be explored using the evolved lineages (from the life cycle experiment) of the *P. fluorescens* model system.

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Chapter 8. Appendix

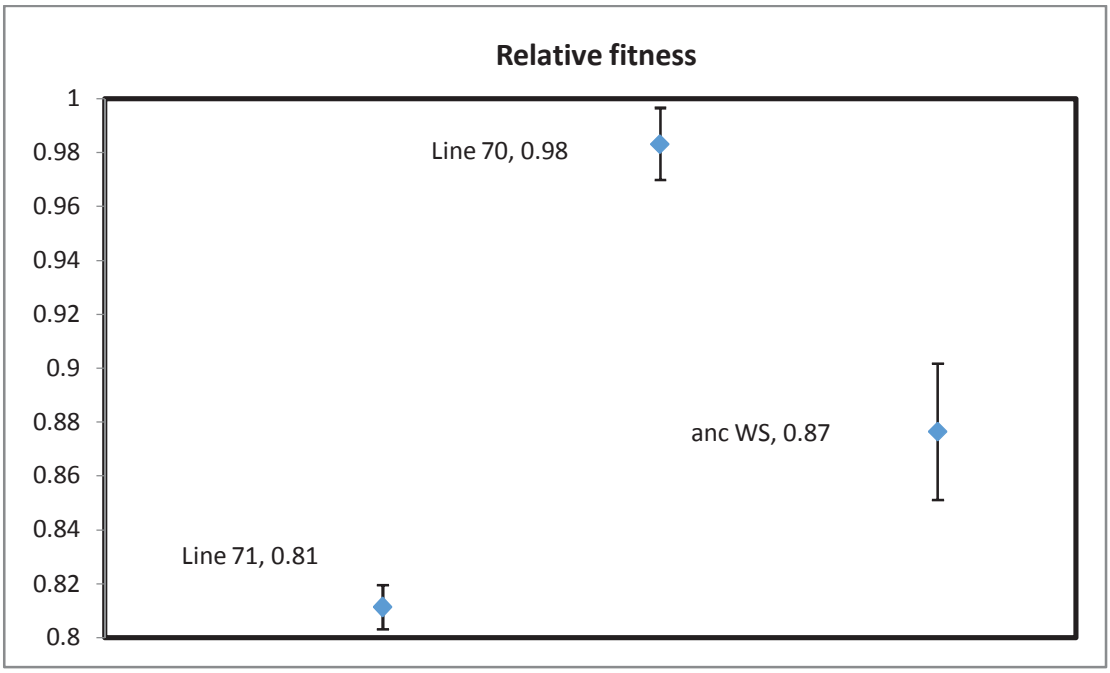

Figure 8.1 Comparison of relative fitness of Line 70 WS with WS of other ancestral lineages. The figure shows the comparison of the relative fitness of WS of Line 70, 71 and anc WS (WS derived from ancestral SBW25), w.r.t SM of ancestral SBW25, in shaken environment. Relative fitness is calculated by obtaining a ratio of Malthusian parameter of WS by SM over a time period of 24 hours. The relative fitness of 1 indicate the fitness of the ancestral SM. The figure shows that Line 70 WS is the fit in the shaken environment compared to the WS of other ancestral lineages and its fitness being comparable to the fitness of ancestral SM. Values are mean ± s.e.m (n=3).

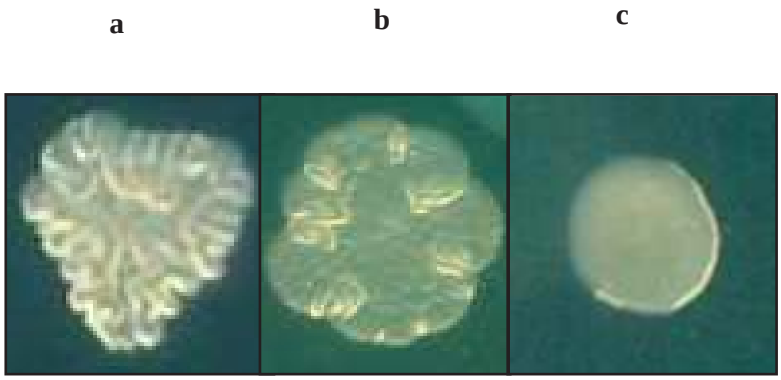

Figure 8.2 WS morphology. The figure shows the WS morphology of (a) Line 71 (b) anc WS (WS derived from ancestral SBW25) (c) Line 70. Note that the WS of Line 70 lacks the typical wrinkly appearance compared to WS of Line 71 and anc WS.

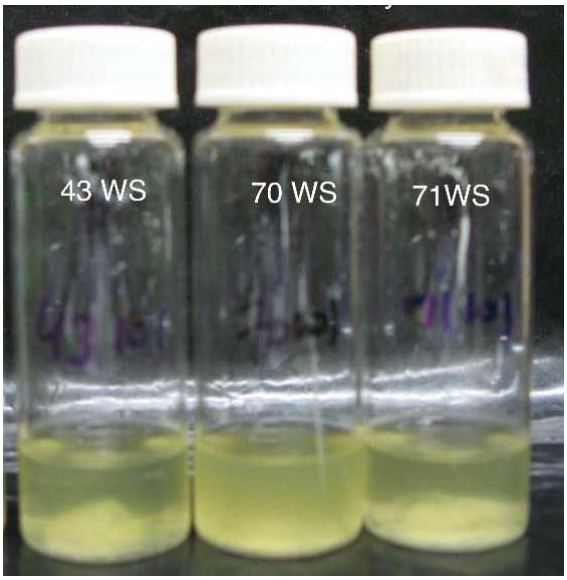

Figure 8.3 The growth in static on day 6 for Line 43, 70 and 71, when they begin with pure WS population. From the figure we can see that Line 70 broth phase appears to be hazy indicating growth compared to the broth phase for Line 17 and 43 that appears to be clear indicating no/less growth. Also, there is mat formation by line 43 and 71 WS (indicated by collapsed mat in the figure), while there was no apparent mat formation for Line 70 WS.