# The Evolution of Multicellularity

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Caroline J Rose

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# Abstract

Major evolutionary transitions in Darwinian individuality are central to the emergence of biological complexity. The key to understanding the evolutionary transition to multicellularity is to explain how a collective becomes a single entity capable of self-reproduction – a Darwinian individual. During the transition from single cells to multicellular life, populations of cells acquire the capacity for collective reproduction; however, the selective causes and underlying mechanisms are unclear. This thesis presents long-term evolution experiments using a single-celled model system to address fundamental questions arising during the evolution of multicellularity. Populations of the cooperating bacterium *Pseudomonas fluorescens* were subjected to experimental regimes that directly selected on the capacity for collectives to differentially reproduce – an essential requirement for the evolution of collectives by natural selection.

A crucial stage during an evolutionary transition to multicellularity occurs when the fitness of the multicellular collective becomes 'decoupled' from the fitness of its constituent cells. Before this stage, any differences in collective fitness are due to selection at the cellular level. In the present study, collectives that competed to reproduce via a cooperative propagule cell attained high levels of cooperation and also reached high levels of collective fitness. However, these improvements were shown to be a consequence of selection acting at the cell-level. In contrast, Darwinian individuality emerged in collectives that reproduced via a primitive life cycle that was fueled by conflict between cooperating cells and cheating cells that did not bear the cost

of cooperation. Cheats were analogous to a germ line, acting as propagules to seed new collectives. Enhanced fitness of evolved collectives was attributable to a property selected at the collective-level, namely, the capacity to transition through phases of the life cycle, and was not explained by improvement in individual cell fitness. Indeed, the fitness of individual cells declined.

In addition to providing the first experimental evidence of a major evolutionary transition in individuality, the work presented in this thesis highlights the possibility that the prevalence of complex life cycles among extant multicellular organisms reflects the fact that such cycles, on first emergence, had the greatest propensity to participate in Darwinian evolution.

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# Chapter 1. Introduction

## 1.1. Units of Selection

Charles Darwin's (1859) theory of natural selection is a simple yet often misunderstood mechanism for evolutionary change. For example, one noticeable difference between Darwin's account and modern explanations is that the latter do not refer to a "struggle for life" – competition for resources in short supply is not essential to the process (Lewontin, 1970; Godfrey-Smith, 2009). The standard approach is to provide an abstract summary of the minimal requirements for evolution by natural selection. Biologist Richard Lewontin (1970) outlined the essential features of a population evolving by natural selection: variation between entities within the population, which is heritable and causally associated with their differential reproduction (fitness). It is only possible for an entity within a population to evolve by natural selection if it satisfies these three conditions (variation, heredity and fitness differences). The 'entities' that possess these characteristics, and upon which natural selection acts, are often referred to as 'units of selection' or 'Darwinian individuals' (Hull, 1980; Buss, 1987; Maynard Smith & Szathmáry, 1995; Michod, 1999; Okasha, 2006; Godfrey-Smith, 2009).

## 1.2. Levels of Selection

Biological life exists in a nested hierarchy in which lower level populations have united together to form cohesive units of selection at higher levels (Buss, 1987; Maynard Smith & Szathmáry, 1995). For example, cells, which exist as independent entities, also exist as components of multicellular organisms. Likewise, some species of ants, bees, and shrimps (among other multicellular organisms), whose ancestors were capable of independent living, now exist only as parts of eusocial colonies. The abstract nature of the Darwinian principles of natural selection (Section 1.1) leads to their potential applicability at multiple levels of the biological hierarchy. Natural selection can, in principle, operate at any level of the hierarchy for which these properties hold.

Lewontin's classical approach to natural selection can be contrasted with the 'replicator' perspective. This 'genic' view (Dawkins, 1976, 1982) maintains that genes are the only 'true' unit of selection, and higher levels of the biological hierarchy are merely vehicles on which to carry these replicators. Hull (1980) added that the 'units of selection' debate might be resolved by distinguishing the role of 'interactors' from that of replicators. Replicators are entities of which copies are made (*i.e.* genes), whose differential transmission is due to the interaction as a cohesive whole of interactors with their environment. Hull's interactors are equivalent to the classical units of selection at levels including and higher than that of replicators.

While Hull's replicator/interactor distinction certainly resolved much of the confusion surrounding units of selection, several objections have led to a more widespread acceptance of the hierarchical view in recent years. First, there is now considerable evidence for the existence of evolutionary changes brought about non-genetic inheritance systems rather than the differential transmission of replicators (reviewed in Bonduriansky & Day, 2009). Secondly, the existence of replicators

themselves requires an evolutionary explanation. It makes no sense to presume genes as the cause of their own evolution (Griesemer, 2000). The replicator view mistakes an outcome of Darwinian evolution (genes) for a prerequisite of it (Okasha, 2006).

The genetic, phenotypic and ecological causes and mechanisms of evolution within each level of the biological hierarchy has been, and continues to be, an exciting field of enquiry in which much progress towards an understanding of evolutionary processes has been made. However, while Lewontin's classical approach describes the operation of natural selection at different levels, this abstract summary takes for granted the evolutionary origins of these levels. Evolutionary theory needs to explain how the biological hierarchy itself came into existence (Buss, 1987; Bonner, 1988; Maynard Smith & Szathmáry, 1995; Michod, 1999; Carroll, 2001).

## 1.3. The Major Transitions in Evolution

The evolutionary origin of the biological hierarchy is a perplexing biological problem. During each transition in the hierarchy, the lower level units cease to survive and replicate independently, and have instead come to replicate exclusively as components of the higher level (Maynard Smith & Szathmáry, 1995). Importantly, a new level of selection emerges during a major transition – the collection of lower level units becomes a Darwinian individual. Consequently, the major evolutionary transitions are often referred to as 'transitions in individuality'.

Table 1.1 lists the major levels of biological complexity, and the transitions between them, as presented in Maynard Smith and Szathmáry's (1995) seminal work on the major transitions in evolution. One noticeable omission from this set is cultural evolution (other than in primates), which has since been widely recognised as a level of complexity (*e.g.* Jablonka & Lamb, 2006; Danchin *et al.*, 2011). While human language has traditionally been given a special ranking in discussions of biological complexity, many have argued that non-human and indeed non-primate organisms exist in societies that necessitate complex communication systems (*e.g.* Avital & Jablonka, 2000; Whiten *et al.*, 2011). Jablonka and Lamb (2006) regard the emergence of the nervous system as a major transition because it enabled evolution by a non-genetic inheritance system that is based on the transmission of behaviourally acquired information. While there are clear theoretical parallels between the different transitions, there are important differences. One noticeable difference is the distinction between 'egalitarian' and 'fraternal' transitions (Queller, 1997). Egalitarian transitions unify 'unlike' units, for example, the transition to eukaryotic life involved a union between the ancestors of mitochondria and another, unlike, prokaryotic cell (Emelyanov, 2003). In contrast, fraternal transitions involve alliances between units of the same kind, for example, the

unification of 'like' cells during the transition to multicellularity. The focus of this thesis is the fraternal transition from single cells to multicellularity

**Table 1.1 The major transitions in evolution.**

| | | |
|---|---|---|
| Replicating molecules | —› | Populations of molecules in compartments |
| Independent replicators | —› | Chromosomes |
| RNA as gene and enzyme | —› | DNA + protein (genetic code) |
| Prokaryotes | —› | Eukaryotes |
| Asexual clones | —› | Sexual populations |
| Protists | —› | Animals, plants, fungi (cell differentiation) |
| Solitary individuals | —› | Colonies (non-reproductive castes) |
| Primate societies | —› | Human societies (language) |

From Maynard Smith and Szathmáry (1995). Note that this list does not include the wider spectrum of non-primate cultural evolution.

# 1.4. The Evolution of Multicellularity

Multicellularity has evolved from unicellular ancestors on at least 25 independent occasions (Baldauf, 2003). There is good evidence that multicellular forms of life arose early – not long after the origin of life itself. Multicellular cyanobacteria have been found in the fossil record dating from approximately 3465 million years ago (Schopf, 1993). However, substantial multicellular diversification didn't occur until about 600-700 million years ago, at a time of dramatic increases in atmospheric and oceanic oxygen (Pfeiffer *et al.*, 2001; N King, 2004).

## 1.4.1. Multicellular Semantics

'Multicellularity' is a term that initially appears rather straightforward, yet is surrounded by confusion. On face value, the term 'multicellular' obviously refers to an entity composed of more than one cell, but this definition does not encompass the full meaning usually intended. For example Wolpert and Szathmáry (2002) suggest that "an overall coordination of at least some key function is a necessary and sufficient condition for a colony of cells to qualify as multicellular". Similarly, Kaiser (2001) requires that the activities of cells in a multicellular organism are coordinated and are in physical contact or at least interact strongly. While these descriptions appear to be feasible, their utility remains vague. First, it is uncertain whether a bacterial colony or a biofilm would be considered 'multicellular', because while such assemblages show patterned growth, it is debatable whether they exhibit overall coordination of function. Secondly, such definitions lack any requirement for a group of cells to acquire Darwinian individuality in order to be deemed 'multicellular'. However, the secure placement of 'multicellularity' as a major transition in evolution (Section 1.3) necessitates that a

collection of cells not only displays coordination of function, but also emerges as a higher unit of selection during the transition to multicellularity.

Evidently, there is some confusion regarding the usage of the term 'multicellularity'. This disparity stems from the fact that there exists a large spectrum of marginal to paradigm cases of multicellular assemblages (Godfrey-Smith, 2009). As a result, the general view of evolution with rules and 'necessary and sufficient conditions' is not practical when exercising a diachronic[1] approach to evolution. Maynard Smith and Szathmáry (1995) offer the interesting alternative of interpreting the exceptions to rules as transitions to, or in some cases, from (McShea, 2001), another level of selection.

The shift from a synchronic to a diachronic view of evolution (Okasha, 2005) has contributed to the conception of individuality and organismality as derived characters at each level of biological organisation. Historically, evolution has been considered only at the level of the multicellular organism, and as a consequence the terms 'multicellularity' and 'individuality' are often used interchangeably (*e.g.* Buss, 1987). However, evolutionary transitions from 'groups' to 'Darwinian individuals' can occur between any two levels in the hierarchy and therefore these terms are purely relative designations (Okasha, 2011). Likewise, Okasha's (2011) concept of an 'organism' as "any biological unit with a high degree of functional integration that is capable of reproduction, so has a life cycle, and whose parts work (mainly) for the good of the whole" is 'rank free'. Queller (1997) also challenges the term 'superorganism' as a special designation for eusocial colonies on grounds that the concept of 'organism' has no absolute level in the biological hierarchy.

[1] The 'diachronic' view is concerned with changes in evolutionary processes through time, whereas the more traditional 'synchronic' approach relates to only to one point in time.

In summary, the term ‘multicellular’ denotes a specific level in the biological hierarchy, whereas the specifications ‘cooperative group’, ‘individual’ and ‘organism’ may be applied at any level. For example, ‘multicellular cooperation’, ‘multicellular individual’ and ‘multicellular organism’ refer to three classifications of multi-celled assemblages that occur during the major evolutionary transition from single cells to multicellularity.

## 1.4.2. The Transition to Multicellularity: Three Stages

Given the multiple classifications of ‘multicellularity’ (above), I propose three sequential stages during the transition to multicellularity, or any major evolutionary transition (Table 1.2).

**Table 1.2 The three stages of a major evolutionary transition.**

| Stage | Process | Focal Level of Selection | Resulting Designation |
|---|---|---|---|
| One | The Evolution of Cooperation | Lower | Collective / group |
| Two | The Evolution of Individuality | Lower to Higher | Individual |
| Three | The Evolution of Complexity | Higher | Organism |

During Stage One, lower level entities unite together in a cooperative group to gain an advantage over solitary units. The focus of selection remains at the lower level, albeit in a group-structured context. Stage Two is the true 'transitional stage' of a major evolutionary transition. The focus of selection transitions from the lower to higher level as the cooperating group becomes a unit of selection (a Darwinian individual). Crucially, in order to satisfy the conditions of Darwinian individuality (see Section 1.1), the group itself must become capable of reproduction. The 'overall coordination of function' required by definitions of multicellularity above (Kaiser, 2001; Wolpert & Szathmáry, 2002), must evolve *after* Stage Two, because Darwinian individuality enables the evolution of the groups themselves. Functional organization is presumably an adaptation of groups, *resulting from* selection operating at the higher level (Okasha, 2006). Therefore, complex adaptations of groups occur during the third stage of an evolution transition. Eventually, adaptations at the higher level lead to such complex integration of the lower level entities that they can no longer exist independently, and now only survive and replicate as components of the higher level – the 'organism'.

# 1.5. Stage One – The Evolution of Cooperation

Social interaction among lower level individuals is essential during an evolutionary transition. Social behaviours are those that have fitness consequences for both the actor (the individual performing the behaviour) and another individual, the recipient (West *et al.*, 2007). Hamilton (1964a) first classified social interactions according to whether the fitness consequences for the actor and recipient are beneficial or costly (Table 1.3). If a behaviour increases the fitness of the recipient, it is deemed either 'mutually beneficial' when the actor also gains a fitness advantage, or 'altruistic' if the behaviour is costly to the actor. Alternatively, 'selfish' behaviour increases the fitness of the actor at a cost to the recipient, while 'spiteful' behaviour is costly to both.

**Table 1.3 Classification of social interactions.**

| | | Effect on Recipient | |
|---|---|---|---|
| | | + | - |
| Effect on Actor | + | Mutualism | Selfishness |
| | - | Altruism | Spite |

Adapted from Hamilton (1964a).

A cooperative behaviour is generally described as an investment in resources that benefits an individual (the recipient) other than the actor (Chase, 1980). Two important features of cooperation are:

1) that the behaviour exhibited by the actor is costly relative to a non-actor, and
2) that the behaviour increases the fitness of other individuals, regardless of whether or not they adopt the same behavioural strategy.

Under this definition, both mutualism and altruism (Table 1.3) are considered to be cooperative interactions because they both increase the fitness of the recipient. Velicer

(2003) emphasises that altruism is a subset of cooperation in which there is no direct benefit to the actor, cautioning that semantic confusion arises when the use of the terms 'cooperation' and 'altruism' are used interchangeably. For example, Nowak (2006) defines a cooperator as someone who pays a cost *for* another individual to receive a benefit. Similarly, West *et al.* (2007) assert that a cooperative behaviour is one that "provides a benefit to another individual (recipient), and which is selected for *because* of its beneficial effect on the recipient". The common theme in these descriptions of cooperation is that the cooperative behaviour is *selected to enhance* the fitness of others, and can therefore only be a target for selection if there is a fitness feedback from the recipient to the actor.

This additional criterion for cooperation appears to have stemmed from concerns over the potential misclassification of cooperation in cases of incidental by-products. West *et al.* (2007) illustrate this point with two examples, both involving the excretion of waste products that bestow a fitness benefit upon a) dung beetles, in the case of elephant waste products, and b) waste-utilizing microbes, in the case of bacterial waste products. Under a very broad definition of cooperation, these examples might be considered 'mutually beneficial cooperation', because both the actors and recipients receive a benefit from the waste produced by the actors. To prevent the misclassification of biological phenomena such as by-products, the authors add the criterion that a cooperative behaviour must be selected *because* of its beneficial effect on the recipient. I have two objections to this stipulation. First, under the definition of cooperation described above by Chase (1980), Velicer (2003), and many others, a cooperative behaviour is costly relative to non-actors. The production of waste products by elephants and bacteria is not costly relative to those who do not produce waste; in fact waste disposal is individually beneficial, therefore these are not examples of

cooperative interactions. Secondly, mutually beneficial interactions, by definition, benefit the actor, and therefore may originate and be maintained in a population because of the benefit incurred by actors. While non-actors may arise in the population and receive a benefit, it does not logically follow that the cooperative behaviour was selected *because of* the benefit received by the recipients. In cases of cooperation such as 'synergism', "the situation where a group of individuals gain some benefit together that they could not obtain alone" (Velicer, 2003), the cooperative trait may be selected to enhance the fitness of recipients; however the recipients are also actors, hence in effect it is selected to enhance the fitness of the actor.

Cooperative interactions among lower level units are crucial to an evolutionary transition because the necessary fitness cost associated with cooperation is offset by a group-level benefit. Michod and Roze (1999) even define cooperation in terms of the group-level fitness benefit: "an interaction that possibly decreases the fitness of the individual while increasing the fitness of the group". The concept of emergent fitness is discussed in greater detail in Section 5.1.3.

This thesis focuses on the role of cooperation during an evolutionary transition. The broader definition of cooperation (Chase, 1980) will be utilized, without the need to invoke the additional requirement that a cooperative trait be selected 'to enhance' the fitness of recipients. The defining feature of cooperation, and that of most relevance to evolutionary transitions, is that there is a cost to actors relative to non-actors. Therefore, there are two categories of explanation for the evolution of cooperation (Calcott, 2011). First, there is a need to explain how cooperation generates a benefit, and secondly, there is a need to explain how individually costly behaviours can continue to exist. The next sections will examine both the advantages (Section 1.5.1 Benefits of Multicellular Cooperation) and costs (Section 1.5.2 Cooperation and Conflict) associated with

multicellular cooperation, followed by a discussion of proposed mechanisms for solving these problems (Section 1.5.3. Explanations for Cooperation). The debate and confusion over the various explanations for cooperation leads into an introduction to Multilevel Selection (Section 1.5.4), the theoretical framework upon which the remainder of this thesis is based.

## 1.5.1. Benefits of Multicellular Cooperation

One challenge for understanding the evolution of cooperation is explaining how cooperation generates a benefit (Calcott, 2011). During the evolutionary transition to multicellularity, cooperation between cells resulted from the advantages gained by 'sticking together'. This occurred through two mechanisms: clonality and aggregation (Tarnita *et al.*, 2013). During a transition to clonal multicellularity, cells fail to adequately separate after cell division and ergo remain attached. Consequently, clonal forms of multicellularity, such as plants and animals, develop from a small number of cells – an evolutionary 'bottleneck'. The aggregative mode of multicellularity usually results from motile single cells clustering together to form fruiting bodies for sporulation and dispersal, often in response to environmental starvation (Gross, 1994). Aggregative forms of multicellularity have arisen independently in eubacteria, several cellular slime moulds, and in ciliates (Bonner, 1998). While aggregative forms of multicellularity are numerous and widespread, particularly in terrestrial environments (Bonner, 1998), clonal multicellularity has led to greater diversity and complexity (Fisher *et al.*, 2013), probably as a result of development from a bottleneck (see Section 1.6.2.1 below for a discussion on the importance of bottlenecks in multicellular life cycles).

Multicellular cooperation in many lineages may have originally obtained the advantage of increased size afforded by the ever-present open niche at the top of the

size scale (Bonner, 1988, 2000). One proposed advantage of increased size is that larger assemblages of cells avoid predation by filter feeders (Bell, 1985; Boraas *et al.*, 1998). The argument may be reversed such that increased size enhances feeding efficiency. For example, a low sucrose environment selects for multicellular clumps in experimental populations of the yeast *Saccharomyces cerevisiae* because this nutrient is digested extracellularly by a secreted enzyme (invertase), preventing solitary cells from growing at low cell and sucrose concentrations (Koschwanez *et al.*, 2013). Primitive flagellated or ciliated cells may be able to swim faster as part of a group, enabling the group to catch prey (Bonner, 1998). Colony formation in a species of choanoflagellate (the closest unicellular relatives of animals) is induced by a compound produced by its prey bacterium, possibly to promote more efficient capture of the planktonic bacteria (Alegado *et al.*, 2012). Both the eukaryotic myxomycetes (true slime moulds) and the prokaryotic myxobacteria can feed more effectively by increasing the size of the feeding mass (Bonner, 1998). These species produce extracellular enzymes to digest large food, which they subsequently absorb directly. This form of feeding was originally termed ‘pack’ feeding in the case of myxobacteria (Dworkin, 1972) and is now more commonly referred to as ‘wolf-pack’ feeding. Cooperative feeding may have arisen in heterotrophs because the formation of multicellular groups allowed cells to benefit from the efficiency of aerobic respiration (Pfeiffer *et al.*, 2001). The higher yield of ATP (chemical energy) resulting from cooperative resource consumption during respiration is produced at a lower rate than from fermentation and therefore may be advantageous in the presence of a slowly diffusing food source.

Other advantages of cellular cooperation include benefits associated with both fixed surface attachment and enhanced dispersal. Single cells located in an ideal position for growth may be swept away by currents or wind, whereas an increased

ability to adhere to surfaces by cell clusters might be selectively advantageous (Bonner, 1998). Conversely, primitive clustering aggregates may enhance dispersal of spores (Gross, 1994).

## 1.5.2. Cooperation and Conflict

Cooperation exists in all kingdoms of life, and yet the phenomenon has sparked debate in the field of evolutionary biology for the greater part of the last century (Sewall Wright, 1945; Wynne-Edwards, 1962; Hamilton, 1963; Maynard Smith, 1964; Williams, 1966; Trivers, 1971; DS Wilson, 1975; Dawkins, 1976; Frank, 1998). The challenge is to explain how cooperation can be both costly to an individual and an adaptation. Natural selection favours types that are relatively more fit than others, and therefore the evolution of cooperative behaviours seems paradoxical because they are, by definition, costly to actors relative to non-actors.

The paradox of cooperation is sometimes regarded in the context of multiple levels of selection. The possibility of natural selection acting at different levels of the biological hierarchy was introduced in Section 1.2. However the widespread existence of cooperation draws attention to the idea of selection operating at multiple levels *simultaneously*. One important consequence of this notion is that there is potential for the existence of conflict between higher and lower levels, because selection may operate in opposing directions at each level. Meiotic drive (segregation distortion) and metazoan cancer are examples of conflict between levels of selection. Meiotic drive occurs when one gametic type is favoured during meiosis and is consequently over-represented in the next generation, often at the expense of the higher level (Cosmides & Tooby, 1981; Lyttle, 1993). Cancer cells proliferate in the absence of restraining mechanisms normally in place to serve the interests of the organism, thereby increasing their own (short-term) fitness at the expense of the fitness of the higher level (Merlo *et*

*al.*, 2006). The non-cooperative lower level entities are termed "cheats" if they gain an advantage from their defection (Velicer, 2003).

Conflict between levels of selection has important consequences during an evolutionary transition because a newly emerging higher unit of selection may be compromised by selection continuing to operate at the lower level. During the transition to multicellularity, competition between cells may destroy the integrity of the emerging multicellular organism by favouring cheating cells that fail to cooperate at the expense of the multicellular organism (Michod, 1997). While there are numerous potential advantages of multicellular cooperation (Section 1.5.1), the question of how a cooperative group evolves to become a multicellular organism when confronted with defection remains unanswered (Buss, 1987; Maynard Smith & Szathmáry, 1995; Griesemer, 2000; Michod & Nedelcu, 2003).

## 1.5.3. Explanations for Cooperation

Various methods have been proposed that can, in principle, explain how cooperative behaviours can exist in an evolutionary context. Within a Multilevel Selection framework (see Section 1.5.2) these explanations can be viewed as mechanisms by which the cost of a cooperative behaviour can be offset by benefits gained at different levels of the biological hierarchy. Individually costly behaviours have been explained at the level of selection below the individual (*e.g.* genes *sensu* Dawkins/Hull), at the level of the individual (*e.g.* reciprocal interactions between individuals), and at levels above the individual (*e.g.* interactions between groups). These three categories of explanation for the evolution of cooperation are discussed in the following subsections.

### 1.5.3.1. Shared Genes

Inclusive fitness theory (Hamilton, 1964a, 1964b), kin selection (Maynard Smith, 1964), and selfish gene theory (Dawkins, 1976) are all variations of explanations for cooperation involving shared genes. According to these theories, cooperation can be maintained in a population when one individual benefits another individual with whom it shares genetic material through descent from a common ancestor. By definition, this mechanism can only explain cases of cooperation between members of the same species (Sachs *et al.*, 2004). Inclusive fitness theory posits that if cooperative behaviours are directed towards relatives, the 'inclusive fitness' (a measure of an individual's fitness that includes the reproductive success of its kin) of the actor can increase because other copies of its genes receive the benefit of the cooperative action. In other words, cooperation increases the reproductive success of the actor's genetic material (which encodes the cooperative trait), regardless of the reproductive fate of the actor. This phenomenon was mathematically formalised by Hamilton's inequality, $rb - c > 0$, where $r$ is the coefficient of relatedness between the recipient and the actor, $b$ is the additional reproductive benefit gained by the recipient, and $c$ is the reproductive cost to the actor. Hamilton's rule predicts that cooperation will be favoured by natural selection when the inequality is satisfied, and that cooperation is more likely to be directed towards relatives.

The shared gene framework can be partitioned into the categories of kin fidelity and kin choice, which differ in the mechanism by which they satisfy Hamilton's rule (Sachs *et al.*, 2004). Kin fidelity is contingent on the natural spatial distribution of related individuals (*e.g.* nestmates, microbial colonies). Cooperators do not actively direct cooperative actions towards kin, but towards physically close individuals who will likely be more closely related to the actor than the average member of the

population. Parental care of young birds in nests and coinfection in clonal microbes are examples of cooperative behaviours passively directed towards relatives. Alternatively, if individuals exhibit a distinguishing phenotype that enables recognition of relatives, cooperative actions may be actively directed towards kin, even in unstructured environments. Characteristic plumage and learned vocal cues in birds are examples of mechanisms for kin recognition (EO Wilson, 1975).

Although kin selection is an empirically well-supported mechanism for explaining the evolution of cooperation, it is not sufficient for explaining all cooperation, which has also been observed between unrelated individuals and even between members of different species (Nowak, 2006). Evidence is continually revealing that relatedness is not an essential requirement for the evolution of cooperation and, particularly in the case of kin fidelity, may often be a spurious by-product of cooperation by other mechanisms (Kaushik & Nanjundiah, 2003; Smukalla *et al.*, 2008; Nowak *et al.*, 2010). Even kin choice may be achieved through the 'green beard' effect (Dawkins, 1976), which requires a single genetic locus to encode both the cooperative trait itself and a distinguishing feature (a 'green beard') by which to recognise, and direct cooperative behaviour towards other individuals carrying this locus. The green beard effect is also effective between non-relatives who share a green beard gene(s). For example, flocculation in the yeast *S. cerevisiae* is induced by expression of the green beard gene *FLO1*, which causes cells to preferentially stick to other cells expressing *FLO1* regardless of genetic relatedness across the rest of the genome (Smukalla *et al.*, 2008). To summarise, formal descriptions of the essential features of kin selection reveal that relatedness is optional and the essence of cooperation can be captured by any mechanism that promotes assortment between cooperating types (Godfrey-Smith, 2009).

### 1.5.3.2. Reciprocity

Reciprocal interactions between individuals can offset the cost of cooperation with fitness benefits gained at the same level, *i.e.* the level of the cooperating individuals themselves. Reciprocity, or 'reciprocal altruism' (Trivers, 1971), is classified as a mutually beneficial form of social interaction (Table 1.3) because the both the actor and the recipient benefit from the cooperative interaction. An actor can benefit from the reciprocal cooperative actions of the either the recipient ('directed reciprocity'), or other actors ('indirect reciprocity'). It is often noted that reciprocal altruism is poorly named, because altruism requires a direct fitness cost to the actor (Section 1.5), and therefore 'reciprocal cooperation' might be a more appropriate term (West *et al.*, 2007). While the evolution of a trait that directly benefits the actor may appear to be straightforward selection at the level of the individual, the challenge, as with all cooperation, is to explain how cooperation is maintained amongst the ever-present possibility of cheats.

The paradox of cooperation can be examined within the framework of evolutionary game theory (Lewontin, 1961; Maynard Smith & Price, 1973), and its application to the 'Prisoners Dilemma' game – a game which tests the consequences of cooperation and defection (Trivers, 1971). Directed reciprocity can evolve if there are repeated encounters between the same individuals (in an iterated Prisoner's Dilemma game), and if an individual has the ability to vary its strategy according to its partner's previous actions. One successful strategy for this game is 'tit-for-tat', in which an individual responds towards its partner with the same action the other player performed in the previous round (Axelrod & Hamilton, 1981). Tit-for-tat is an efficient catalyst of cooperation, however it cannot correct mistakes and inadvertent defection leads to a long sequence of retaliation. A more robust strategy for maintaining cooperation is the

win-stay, lose-shift strategy, where an individual repeats its behaviour from the previous round if it was successful, but changes it otherwise (Nowak & Sigmund, 1993). Direct reciprocity can result in the evolution of cooperation only if the probability, *w*, of another encounter between the same two individuals is greater than the cost-to-benefit ratio of the altruistic act, that is, if $w > c/b$ (Nowak, 2006).

Indirect reciprocity evolves when an actor is rewarded for cooperation by individuals other than the recipient, and therefore is highly susceptible to defection unless there is some measure of reputation. Reputation is expressed mathematically by *q*, the probability of knowing another's reputation, and can lead to the evolution of indirect reciprocity if it exceeds the cost-to-benefit ratio, that is, if $q > c/b$ (Nowak & Sigmund, 1998). Only humans and other cognitively complex organisms are likely to engage in cooperation by indirect reciprocity because gauging reputation requires substantial cognitive processes (*e.g.* language) (Nowak, 2006). Direct reciprocity is also unlikely to have played a role in the early stages of the transition to multicellularity, because even if spatial structure allowed for repeated interactions, cells must have already evolved mechanisms for changing their behaviour based on that of their partners. Paradoxically, such mechanisms are social traits, which therefore require previous social interactions to have been selected.

### 1.5.3.3. Group Selection

Individually costly behaviours can be offset by benefits gained at levels above the individual – the level of the group. While cooperative actions may be selectively disadvantageous during competition between lower level entities *within* a group, they can be maintained by enhancing the competitive ability of the group itself during selection *between* groups. Darwin himself recognised that natural selection between

groups might explain the evolution of self-sacrificial behaviour among humans (Darwin, 1871).

The evolution of cooperation as a group-level adaptation has been (and still remains) one of the most debated areas in evolutionary biology, largely due to confusion over different modes of group selection. The debate was sparked in the 1960s when Wynne-Edwards (1962) argued that reproductive constraint evolved "for the good of the group" because groups that do not exhibit restraint go extinct due to over exploitation of resources. The process of differential proliferation and extinction of groups leads to the evolution of behaviours that are adaptations of the group, rather than adaptations of the individual. His arguments were vigorously criticised by the likes of Hamilton (1963, 1964a, 1964b), Maynard Smith (1964, 1976), EO Wilson (1973) and Williams (1966), who insisted that natural selection was intrinsically selfish and could account for the evolution of cooperation at the level of the individual. They viewed individual selection as a much more potent mechanism for evolutionary change because individuals within groups were more variable, numerous, and reproduced more rapidly than groups, and therefore group selection could not restrain invasion by opportunistic cheats.

The 1970s saw both the culmination of the gene-centric view of evolution with Dawkins' popular book *The Selfish Gene* (1976), and the resurgence of the group selection approach with empirical studies (Wade, 1977) and a new theoretical model of interdemic group selection (DS Wilson, 1975). Wade's experimental work on the flour beetle *Triboleum casteneum* demonstrated the efficacy of differential reproduction and extinction of groups. Compared to selection on individual productivity within groups, there was a much higher response to selection when the most prolific group of beetles was chosen to colonize new groups. This result was confounded by the fact that in both

treatments Wade selected on the fitness of individual beetles, however group-selected beetles also evolved a lower rate of cannibalism compared to those in the individual selection treatment, in which cannibalism increased (Wade, 1979). The opposing directions of selection for cannibalism can be interpreted as selection for selfish behaviour in the individual selection treatment, while group selection resulted in evolutionary restraint, a cooperative group-level adaptation.

David Sloan Wilson's 'trait-group' model of group selection explicitly demonstrated that the cost of cooperation within groups could be offset by the differential productivity of groups (DS Wilson, 1975). However, Wilson's model was based on a different conception of the group, leading to a widespread misunderstanding of group selection. The global population repeatedly separates into temporarily interacting 'trait-groups' with varying compositions of the group-beneficial cooperative trait. Despite the individual cost of cooperation, groups with a higher proportion of cooperators contribute more individuals to the total population than groups with a lower proportion of cooperators. Importantly, the differential extinction of groups is not required for this kind of group selection as it treats the group-structured population as part of the environment of the individuals. In the absence of differential extinction and reproduction, trait-groups cannot themselves be units of selection (Section 1.1). As such, the group-structured environment can only equilibrate the changing frequencies of individual characters in the overall population, and does not give rise to group-level adaptations (Okasha, 2006). The differences between this model of group selection, and one that requires differential reproduction of groups (*sensu* Wynne-Edwards and Wade) are illustrated in Figure 1.1.

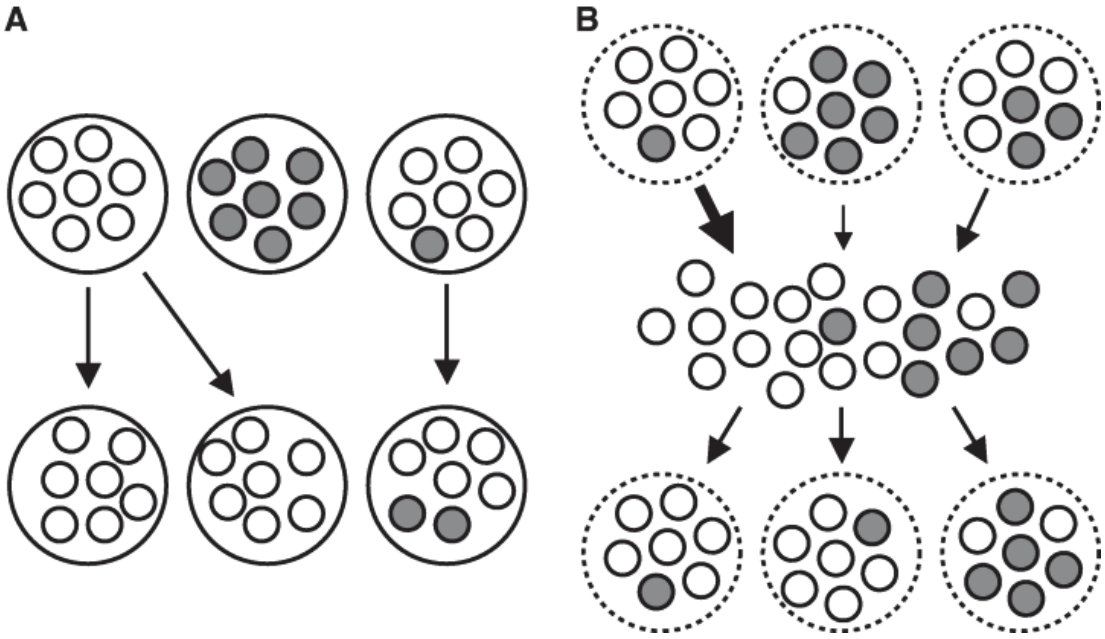


**Figure 1.1 Two models of group selection.** A) 'Old' Wynne-Edwards type of group selection. Selection is between groups via differential extinction and reproduction of groups, with well-defined groups and little gene flow between them (solid line). White circles represent cooperators, whereas grey circles represent selfish types. Selfish types spread within groups, however groups with more cooperators leave more group offspring. B) Trait-group model of group selection with arbitrarily defined groups (dashed lines). Groups with more cooperators make a higher contribution to a shared reproductive pool, from which new groups are formed. From West *et al.* (2007).

The trait-group model of group selection can be alternatively conceptualized as individual selection with fitness-affecting interactions between neighbours - as is the case for kin selection. Group selection can effect the evolution of cooperation when the variation between groups is increased relative to the variation within groups, a situation that can be achieved by increasing the coefficient of relatedness, *r*, between interacting individuals (Frank, 1998). Indeed, it has since been argued that the two models are identical (Wade, 1985; Queller, 1992; Frank, 1998; Nowak *et al.*, 2010), although Traulsen (2010) has demonstrated that this mathematical equivalence is in fact only found in special cases. Similarly, reciprocity can drive assortative interactions between cooperators, decreasing the variation within groups of interacting individuals, and increasing variation (and selection) between groups. Theoretically, groups of any kind

are in fact not essential for the evolution of cooperation if the population is structured so that individuals interact with their neighbours (Maynard Smith, 1976; Godfrey-Smith, 2008). Cooperators can persist in a population if they are more likely to interact with each other than by chance (Godfrey-Smith, 2009). Therefore, the key principle common to the three classes of explanation for the evolution of cooperation is 'correlated interaction' between individuals (Godfrey-Smith, 2009). Preferential interactions among kin, reciprocity, and group structure are all different mechanisms for achieving correlation between traits expressed in a population.

The mathematical and logical similarities between group selection and the other classes of explanation for the evolution of cooperation apply only to trait-group types of group selection, in which groups constitute the environmental structure for selection on individual behaviours. While there are some parallels between 'trait-group' type models of group selection and those featuring differential extinction and reproduction of groups (the 'old' group selection of Wynne-Edwards, 1962), these similarities do not extend to the mathematical equivalence discussed above. Nevertheless, many protagonists of the kin selection approach, while recognising the theoretical possibility of 'old' group selection, dismiss it as a rare occurrence that is unlikely to be of importance in nature (*e.g.* West *et al.*, 2007). However, the very existence of a biological hierarchy in which higher level individuals are composed of groups of lower level individuals demonstrates that groups must have become units of selection during the evolution of the hierarchy itself. I would argue that the frequency of this kind of group selection is of little consequence to its implications – multicellular organisms are known to have evolved just ~25 times in 3.5 billion years (Baldauf, 2003). Progressively, group selection theory has developed into Multilevel Selection theory (Heisler & Damuth, 1987), which successfully distinguishes between the type of group selection in which groups are part

of the environment of individual, and the type in which groups themselves are units of selection.

## 1.5.4. Multilevel Selection Theory

Multilevel Selection (MLS) theory recognizes that natural selection can operate at different levels of the biological hierarchy simultaneously, because entities existing at multiple levels may qualify as units of selection (Heisler & Damuth, 1987; Damuth & Heisler, 1988). Unlike the models of kin selection, reciprocity and group selection above, which bias understanding towards gene-centric, individual, or group-centric views of evolution, MLS theory does not lean toward any particular level of selection, but can be used to assess the strength of selection at different levels on a case-by-case basis. This powerful framework can be used to evaluate the relative contributions of selection at different levels in ambiguous cases such as Wade's (1977) beetle experiments, where the influence of group and individual selection on beetle productivity was unclear. Selection at the level of the individual beetle was evident from the increased beetle productivity in the individual selection treatment, however the response was much stronger in the group selection treatment, indicating that selection for beetle productivity operated simultaneously at multiple levels. MLS theory also acknowledges the possibility of selection driving the evolution of traits in opposing directions at different levels – the potential for conflict between levels of selection (Section 1.5.2). The MLS framework extends group selection to a diachronic view of evolution by recognizing that the multiple levels of the biological hierarchy are simultaneously under selection and are themselves products of evolution (Okasha, 2006).

The two types of group selection discussed in Section 1.5.3.3 are delineated by MLS theory as Multilevel Selection 1, in which the groups are part of the environment

of individual, and Multilevel Selection 2, in which groups themselves are units of selection.

### 1.5.4.1. Multilevel Selection 1

The level of interest in Multilevel Selection 1 (MLS1) is that of the individuals ('particles'[2]) within groups ('collectives'). MLS1 considers how particle fitness is influenced by collective membership (Heisler & Damuth, 1987). Collectives themselves are not differentially successful in terms of their reproduction or extinction, however other differences between collectives can affect the fitness of their constituent particles. Collective fitness in MLS1 ('collective fitness 1') is therefore calculated as the average particle fitness (Okasha, 2006).

Trait-group selection and kin selection are MLS1 models because they are concerned with the evolution of a particle phenotype (*e.g.* cooperation) in a structured population. MLS1 is therefore relevant during Stage One of an evolutionary transition because this stage is characterized by the spread of cooperation among particles.

### 1.5.4.2. Multilevel Selection 2

In Multilevel Selection 2 (MLS2) the interest is the collective itself as an evolving unit of selection. Collectives are themselves capable of reproduction, and therefore their differential viability and fecundity is the measure of collective fitness ('collective fitness 2') in MLS2, regardless of the number and fitness of their constituent particles.

[2] The discussion of group selection literature in the sections above required a relaxation of the usage of terms such as 'individual' as clarified in Section 1.4.1. For example, Wade's manuscripts (1977, 1979) refer to beetles as 'individuals', however the term 'individual' could also be applied to the groups in the group selection treatment according the specifications outlined in Section 1.4.1. The MLS framework acknowledges the existence of individuality at multiple levels, and therefore I will henceforth adopt Okasha's terminology of 'particles' and 'collectives' for lower and higher level entities existing at any abstract level of the biological hierarchy (Okasha, 2006).

The crucial difference between the MLS1 and MLS2 perspectives is that MLS1 concerns the evolution of *particles* organised into collectives, whereas the focus of MLS2 is the evolution of the *collectives* themselves. Table 1.4 outlines the main conceptual differences between the MLS1 and MLS2 approaches to Multilevel Selection.

**Table 1.4 Conceptual differences between MLS1 and MLS2.**

| | **MLS1** | **MLS2** |
|---|---|---|
| **Group Selection** | Effect of group membership on *particle* fitness | Change in the frequencies of different kinds of *collectives* |
| **Fitness** | Property of *particles* | Property of *collectives* |
| **Character** | Value attributed to *particles* | Value attributed to *collectives* |
| **Populations** | Consist of *particles,* organised into collectives | Consist of *collectives,* composed of particles |
| **Inferences** | Can be made only about changing proportions of different kinds of *particles* in the meta-population | Can be made only about changing proportions of different kinds of *collectives* in the population |

Adapted from Damuth and Heisler (1988).

The distinction between the MLS1 and MLS2 approaches to Multilevel Selection is not between mutually exclusive biological processes (Heisler & Damuth, 1987; Damuth & Heisler, 1988), but can instead be interpreted as alternative frameworks appropriate for addressing different scientific questions. For example, MLS2 is clearly the relevant type of selection during Stage Three of an evolutionary transition (the evolution of complexity) because this stage is concerned with the accumulation of collective-level adaptations *after* the collectives have become units of selection. Okasha (2005, 2006) argues that an evolutionary transition is likely to begin as MLS1 and result in an MLS2 process of evolution, shifting from a synchronic to a diachronic perspective. It has already been established that MLS1 is the relevant process during

Stage One of a transition while MLS2 is operational during Stage Three - by which stage the collectives are themselves units of selection, possessing Lewontin's three requirements for Darwinian individuality: variation, reproduction and heredity (Section 1.1, Lewontin, 1970). The question of how the process of selection shifted from MLS1 to MLS2, that is, how collectives became Darwinian individuals, is the subject of the next section.

## 1.6. Stage Two – The Evolution of Individuality

Stage Two is the true 'transitional' phase of a major evolutionary transition because the focus of selection shifts from the lower to higher level, *i.e.* selection transitions from an MLS1 to an MLS2 process. In order to satisfy the conditions of Darwinian individuality (see Section 1.1), the collective itself must become capable of reproduction. A largely unappreciated problem is that the capacity for collective reproduction is both a prerequisite for individuality and a derived trait whose evolution necessitates collective-level individuality. This paradox suggests that collectives must have acquired the capacity for reproduction 'for free' - *without* MLS2-like evolution of collectives.

Conventional hypotheses for the origins of multicellular individuality tend to focus on solving the issue of cheating in cooperating groups (Michod, 1997, 1999; Michod & Roze, 1999; Michod & Nedelcu, 2003). Conflict mediation is generally assumed to be a necessary prerequisite for the emergence of individuality, yet the evolution of such a mechanism is a problem that is strongly related to the paradox of collective reproduction. A mechanism for controlling cheats is a collective-level adaptation that can therefore only evolve after collectives have solved the reproduction problem and are consequently capable of participating in evolution at the collective-level. Michod (2003) defines a conflict mediator as a feature of the emerging multicellular organism, yet Michod and Roze (1999, p.47) assert that

> *"Heritability of fitness and individuality at the new level emerge as a* ***result*** *of the evolution of organismal functions that restrict the opportunity for conflict within and ensure cooperation among cells."*

However, such 'organismal functions' are traits that must evolve at the level of the collective *as a result of* the evolution of multicellular individuality (*i.e.* collective reproduction). Michod's theories require collective-level adaptations (Step 3) to inexplicably emerge *prior to* the evolution of individuality (Step 2).

The following sections discuss the problems associated with collective reproduction (Sections 1.6.1 and 1.6.2) and a hypothesised solution to this problem (Section 1.6.3).

## 1.6.1. Collective Reproduction

Reproduction is at the heart of Darwinian evolution, yet there is some ambiguity surrounding what it means to reproduce. Standard definitions of 'reproduction' of an individual (*e.g.* Griesemer, 2000) require two elements:

1) Material overlap
2) The capacity to multiply is *acquired* during the lifetime of the individual, *i.e.* the individual must develop via a life cycle.

This seemingly inclusive definition may nevertheless be too narrow to include certain instances of reproduction. For example, retroviruses can undergo evolution by natural selection despite offspring receiving no material contribution from their parents (Godfrey-Smith, 2009). There also exist many cases that fit this definition but are actually instances of other biological processes, such as growth of the same individual, and production of waste and other artefacts. For example, many plants produce runners that could be viewed either as new individuals or as growth of the original individual.

The particular question of relevance to major evolutionary transitions is the puzzle of collective reproducers – reproducing units comprised of particles which themselves have the capacity to reproduce. Multicellular organisms, herds of buffalo, and eusocial colonies are all examples of collective reproducers. The challenge is to identify which

are cases of reproduction of collectives, and which are cases of growth of collectives resulting from reproduction and structural organization of their particles. This problem is discussed in depth in Section 5.1 (The Levels of Selection Problem).

A second challenge during Stage Two of a major evolutionary transition is to explain how collectives acquired the fundamental requirement for reproduction – a developmental life cycle.

## 1.6.2. The Origin of Life Cycles

The particular mode by which the earliest multicellular collectives acquired the capacity to reproduce has implications for their ability to transition in individuality and participate in natural selection. Libby and Rainey (2013) outlined a framework describing three distinct pathways that encompass the earliest stages of possible multicellular life cycles. Collectives may be maintained in a population by recurrently arising from single cells, however unless the collectives themselves reproduce they have no possibility of acquiring Darwinian individuality. The other two routes outlined by Libby and Rainey (2013) involve multiplication of primordial collectives either by cycling between single-celled and collective states, or without the need for a unicellular stage (*i.e.* by fragmentation). The majority of clonal multicellular organisms develop from a bottleneck (usually single-celled), which is often a specialized reproductive cell - a germ line. The following sub-sections will discuss the implications of single-celled bottlenecks and germ lines during multicellular development and their significance for major evolutionary transitions.

### 1.6.2.1. Bottlenecks

A unicellular stage in the multicellular life cycle may seem very costly, especially given the multitude of advantages gained by increased size (discussed in Section 1.5.1).

Single cells are exposed to physical and biological hazards that no longer affect multicellular collectives, *e.g.* small organisms tend to get eaten by larger ones (Grosberg & Strathmann, 1998). A unicellular stage also requires that more time be invested in the developmental stage before an organism reaches adult reproductive age. Many organisms invest substantial resources into protecting their offspring during this long developmental period, much of which can be lost due to juvenile mortality (reviewed in Grosberg & Strathmann, 1998). Given the costs of a unicellular bottleneck, why do multicellular organisms not develop from larger propagules?

There may be some ecological advantages of a unicellular bottleneck, for example, a unicellular stage may enhance dispersal (Grosberg & Strathmann, 2007). It has also been suggested that a single-celled stage is necessary for sexual reproduction, however unicellular modes of propagation also persist in asexual organisms (Grosberg & Strathmann, 1998). Aside from these ecological possibilities, there are two main patterns of explanation for the existence of a unicellular stage during the multicellular life cycle. First, the costs of a unicellular bottleneck may be offset by the benefits associated with reduced conflict between levels of selection. A second line of reasoning concerns an evolutionary constraint: a unicellular bottleneck may have been essential during the evolutionary transition to multicellularity.

Historically, the idea of group selection was disputed on the grounds that selection acts more strongly at the lower level due to the greater abundance of variation within, compared to between, collectives (Maynard Smith, 1964; Section 1.5.3.3). However, a unicellular bottleneck acts to reduce the level of variation between cells and therefore may be a mechanism to reduce within-organism conflict. First, reducing variation within an organism eliminates any deleterious mutations that arose in the parent because offspring that develop from deleterious cells will not survive (Grosberg & Strathmann,

1998). Secondly, any non-deleterious variation that has arisen in the parent will be redistributed among offspring, rather than within offspring, in the next generation. This regular redistribution of genetic variation from *within* the parent to *between* its offspring prevents the persistence of selfish cell lineages by allowing selective processes to operate on the variation between the higher level individuals (multicellular organisms) rather than at the lower level (cells) (Michod & Roze, 1999).

Another (related) explanation for the existence of a unicellular bottleneck is the possibility that it was necessary during the primitive *origins* of multicellularity - whereas conflict resolution may be a beneficial consequence that explains its *maintenance*. In experimentally evolved clusters of *Chlamydomonas reinhardtii*, Ratcliff *et al.* (2013) showed that a unicellular bottleneck provided a fitness advantage even in the absence of between-cell conflict. Multicellular organisms must reproduce, and the capacity for reproduction must develop (Griesemer, 2000); therefore a life cycle is a prerequisite for the evolution of multicellular organisms. The presence of a unicellular bottleneck is linked to the evolution of novelty – it rendered primitive life cycles 'evolvable' because development from a smaller stage provides a window of opportunity for organizational change (Dawkins, 1982). A unicellular stage allows a beneficial mutation that arose in the parent to become established in all cells of an offspring (Grosberg & Strathmann, 1998), making this variation available to affect the offspring's basic organization and to be presented for selection. This genetic uniformity is important for the overall coordination of function of cells within a multicellular organism. Reliable large-scale organization requires signalling between cells that obey the same rules and therefore have the same genes – and this constrains development to be from a single cell (Wolpert & Szathmáry, 2002).

### 1.6.2.2. Germ Lines

A unicellular bottleneck stage may not be adequate to cope with the conflict arising from lower level selection in a multicellular organism. While a bottleneck reduces the scope for conflict by reducing variation within an organism, any cell-level competition that *does* occur will be no less consequential; each new multicellular collective will nevertheless be initiated by successively better within-organism competitors (Michod, 1999). Complex multicellular organisms not only have a unicellular bottleneck stage in their life cycle, but this single cell is from a dedicated set of reproductive cells, the germ line. Thus, a typical initiator of a new collective is not only a descendant of a successful competitor in the within-organism context, but also a descendant of a cell in the germ line of a successful collective.

Michod (1996, 1997) demonstrated that levels of cooperation can be low in organisms that do not have a germ line, despite high levels of kinship among cells resulting from a unicellular bottleneck stage. Specialised reproductive cells may therefore be a defence against the accumulation of deleterious mutations in organisms evolving a progressively larger body size (Solari *et al.*, 2013), and the accumulation and transmission of cheating cell lineages across generations (Buss, 1987). Michod's model (1996, 1999) of the transition to multicellularity in *Volvox carteri* demonstrated how reproductive division of labour could prevent the proliferation of cheats. It has also been suggested that there may be reduced variation (and therefore selection) among germ line cells because they often experience lower mutation rates than somatic cell lineages, perhaps due to their lower metabolic rates (Maynard Smith & Szathmáry, 1995; Michod & Roze, 2000; Michod, 2003).

Aside from Michod's theoretical speculations, there is no conceptual or biological rationale for a germ line to reduce within-organism selection compared to a non-

specialised bottleneck. In the case of the *V. carteri* (the model organism upon which Michod's models are based), somatic and germ cells go through approximately the same number of cell divisions, so the evolution of the soma would have no effect on the transmission of mutations that accumulate during development (Koufopanou, 1994). Buss (1987) also argues that the idea of treating an "individual" (a multicellular organism) as genetically uniform is a flawed concept persisting from the "zoological bias" of genetic work during the first part of the 20$^{th}$ century. Early genetic research was dominated by work on the fruit fly, *Drosophila*, which has the 'preformation' mode of development – the germ line is terminally differentiated in early ontogeny. Preformation modes of development are consistent with the idea of conflict reduction mediated by the germ line because early sequestration of germ cells entails that the majority of mutations that arise in a developing organism terminate in the soma, rather than the germ line. However, terminal differentiation of the germ line is limited to a few metazoan species, and is certainly not the most prevalent form of development in multicellular organisms. The fluid boundary between germ and soma in the most basal branches of the Metazoan phylogeny (Porifera (sponges) and Cnidaria (corals, jellyfish and hydra)) suggests that the ancestral mechanism of early germ cell segregation was likely epigenesis – where the germ cell arises later in development as a result of inductive signals from surrounding tissues (Extavour & Akam, 2003). The fact that preformation is not the ancestral mode of development in multicellular organisms suggests that subversion prevention may sometimes be an incidental by-product of a germ/soma distinction that evolved for other reasons.

Trade-offs between cellular functions may have led to the division of reproductive and non-reproductive tasks among cells in primitive multicellular collectives. For example, the need for somatic cells in the large volvocacean algae arose from a peculiar

constraint that prevents simultaneous cell division and locomotion in cells with rigid cell walls. Somatic cells lost mitotic activity in exchange for flagella, because basal bodies (which are involved in both flagellation and mitosis) cannot remain attached to flagella while migrating to the mitotic poles (Koufopanou, 1994). Consequently, the cells cannot divide while they have a functional flagellum. While this solved the problem of conflict among somatic cells, termination of post-embryonic cell division prevented the long-term evolvability of the volvocacean algae (Michod *et al.*, 2003). A similar trade-off between motility and mitosis exists in metazoans. Metazoans, like their protozoan ancestors, have a single microtubule organising centre that can function either in flagellation or mitosis, but not both simultaneously (N King, 2004). Some early metazoans (*e.g.* sponges) solved the motility/mitosis problem by either abandoning dispersal, or moving to a suitable location before developing into an adult sponge (Buss, 1987). Such a strategy is an example of temporal germ/soma differentiation, as opposed to the subsequent spatial germ line differentiation (Mikhailov *et al.*, 2009), and may have limited the evolution of complex developmental pathways in these basal lineages. Gastrulation (reorganization of the single-layered blastula into three layers of specialized cells) solved the problem of simultaneous motility and development in the higher metazoans, leading to greater efficiency of cellular function and greater complexity (Buss, 1987).

An interesting alternative to conventional hypotheses for the evolution of reproductive specialisation is one that involves selection at a third level – that of selfish genetic elements. Johnson (2008) outlined a simple model of selfish element evolution demonstrating that such an element is theoretically capable of creating or strengthening germ-soma differentiation. This model postulates that reproductive division of labour could potentially evolve without any selective benefit for the host. Germ line

sequestration may instead benefit selfish genetic elements, which could evolve to adjust their replication rate in different cell types to reduce their harmful impact on host fitness while maximising their transmission to gametes. Reproduction in somatic cells is of no benefit to the element, and can also reduce host fitness thereby decreasing representation of the selfish element in future generations. Indeed, selfish elements such as transposons often refrain from replication in somatic tissue.

While the mechanisms underlying the evolutionary origins of germ lines are unknown, it is clear that reproductive specialisation has had tremendous consequences: germ lines led to the evolution of multicellular complexity. While some degree of multicellularity has evolved on at least 25 independent occasions (Baldauf, 2003), only three lineages produced particularly complex multicellular organisms: plants, animals, and fungi (Buss, 1987; Maynard Smith & Szathmáry, 1995; Wolpert & Szathmáry, 2002; Niklas, 2014). All species within these three branches have developmental life cycles involving reproductive specialisation, making the germ line one of the key innovations allowing the evolution of integrated multicellular organisms (Grosberg & Strathmann, 2007). Specialised germ cells appear to have been derived at or near the origins of these clades. For example, animal development from a germ cell occurred *prior to* the bilaterians (Blackstone & Jasker, 2003), indicating that the major diversification of this branch of animals may have resulted from the evolution of the germ line.

Within a single clade, the degree of reproductive specialisation appears to correspond to the extent of complexity. Godfrey-Smith (2009) considered this relationship by comparing species of colonial green algae in the *Volvocine* group, which is often viewed as an informative system for studying the evolution of multicellularity (*e.g.* Michod *et al.*, 2003). A spatial representation of several *Volvocine* species (Figure

1.2) demonstrates that the degree of germ line specialisation (*G*) and the level of 'integration' (*I*) of the organism appear to be linked. "*I*" is a measure of complexity that encompasses features such as the extent of non-reproductive division of labour, the mutual dependence (loss of autonomy) of parts, and the maintenance of a boundary surrounding the collective. Levels of *G* and *I* are similar within a single species. For example, *Gonium* colonies, which consist of loosely organized clumps of cells that can all reproduce, are neither well-integrated organisms (low *I*), nor have a germ line (low *G*). Phylogenetic analyses also demonstrate that *Gonium* species have remained simple while germ lines are only present in the more complex *Volvocine* species (Hallmann, 2011). While Figure 1.2 represents extant organisms, the path from lower left to top right is also hypothesized to correspond to the evolutionary path that was actually taken (Kirk, 2005). The relationship between reproductive specialisation and complexity also exists in bee colonies (Godfrey-Smith, 2009).

The emergence of a germ line removed the constraints on the evolution of complexity in plants, animals and fungi. This thesis explores how a germ line may enable selection to act on the developmental life cycle as a unit, rather than on a particular cell state – shifting the level of selection from that of single cells to the multicellular collective.

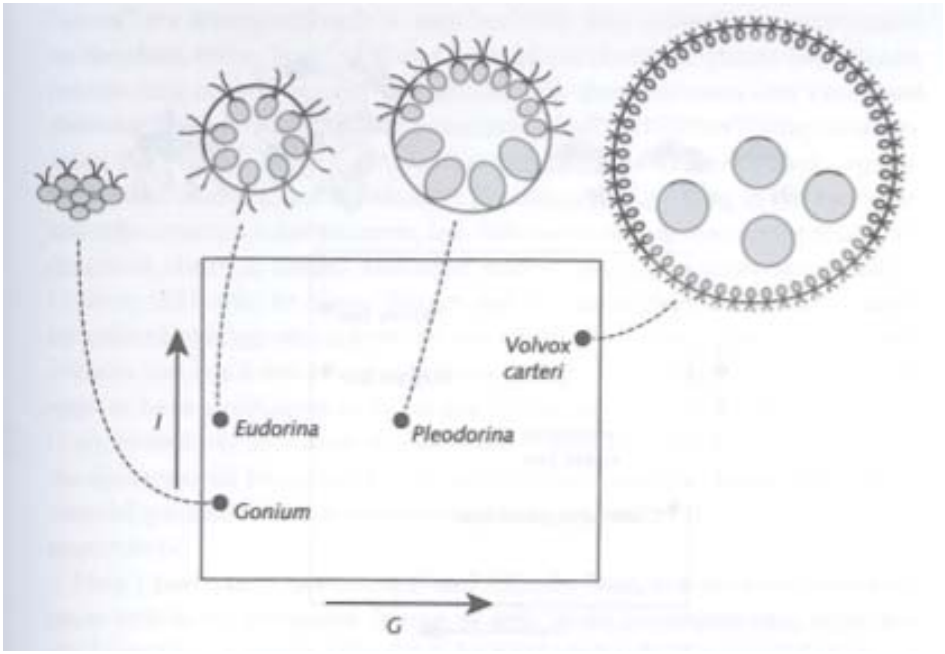


**Figure 1.2 Comparisons of reproductive specialisation (G) and integration (I) in colonial green algae.** From Godfrey-Smith (2009).

## 1.6.3. A Novel Hypothesis for the Evolution of Life Cycles

In a radical reversal of the conventional view of cheats as an obstacle to be overcome during the evolutionary transition to multicellularity, a recent hypothesis embraces the role of cheats in a primitive life cycle (Rainey, 2007; Rainey & Kerr, 2010). According to this hypothesis, a group of cooperating cells is an evolutionary dead end and is therefore equivalent to multicellular soma. Cheats, however, are analogous to a germ line and may act as propagules to seed new collectives. By equating cheats with the germ line, simple collectives are no longer an evolutionary dead end, and the inevitable cycling of cooperation and conflict can be viewed as a 'life cycle' (Figure 1.3). Central to this 'life cycle hypothesis' is the realization of the importance of Multilevel Selection – that selective processes occurring at different levels are intimately linked – so that events at each level can be considered as different stages of the multicellular life cycle. Under this framework, selection at the lower level

and the evolution of cheats is no longer considered an impediment to the evolution of multicellularity. Cheats, while detrimental to the long-term viability of collectives, are essential to their fecundity, and therefore evolvability. The cogency of this hypothesis lies in the fact that the capacity for collective reproduction arises *without* the need for inexplicable MLS2-like evolution of collectives - cheats arise in groups of cooperating cells as a result of within-collective selection. As a result, this proposal simultaneously provides solutions to the problems of defection and collective reproduction, and a mechanism for the origin of germ lines.

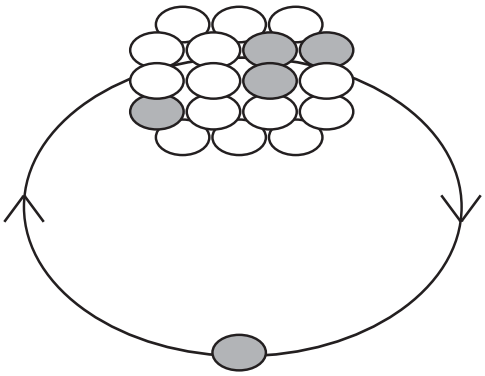

**Figure 1.3 A multicellular life cycle fuelled by within-collective conflict.** White ovals represent cooperating cells (soma) while grey ovals represent cheats (germ cells). Selection within a group of cooperating cells favours the evolution of cheats, which leave the collective to seed offspring collectives.

It is quite feasible that the primitive life cycle outlined in the hypothesis above bridges Stage One, the evolution of cooperation with Stage Two, the evolution of Darwinian individuality. The fundamental requirement for reproduction, that the capacity to reproduce must develop during the lifespan of an individual (Griesemer, 2000), substantiates the idea of a life cycle already being in existence at the beginning of the transition from cooperating cells to individuality. The life cycle hypothesis fulfils this perplexing requirement by embracing cellular differentiation *prior to* the evolution of individuality. This is a strong possibility because cooperation does not prevail as a

steady state – cooperation always exists as oscillations between types rather than in equilibrium (Hauert *et al.*, 2002; Traulsen & Nowak, 2006). Mikhailov *et al.* (2009) also suggest that multicellularity did not trigger cellular differentiation, but emerged *as a result* of integration of different cells types during a transition from temporal to spatial differentiation of cells. This conjecture is supported by morphological and gene expression data, which suggests that Urbilateria (the last common ancestor of the bilaterians) was likely to have had its germ cells scattered throughout its body, only later evolving spatial differentiation (Extavour, 2007). Several theoretical studies have also indicated that cellular differentiation can be driven by within-collective selection, which could then lead to selection between collectives. For example, Gavrilets (2010) demonstrated that germ-soma differentiation can be explained in terms of the immediate selective advantage to individual cells if there are significantly strong trade-offs between somatic and reproductive functions. Additionally, the specialisation of cheats as dispersers could have existed before the transition to multicellular individuality because of the selective advantage gained by individual cells exploiting more niche space by dispersing (Hochberg *et al.*, 2008).

The plausibility of the life cycle hypothesis described above is supported not only by theoretical predictions and phylogenetic data, but by contemporary observations that such 'life cycles' are achieved in simple collectives by the inevitable oscillations between cheats and cooperators. It is commonly suggested that the growth of bacterial biofilms be considered as a developmental cycle (Kolter & Losick, 1998). For example, populations of cooperating *S. cerevisiae* cells can be invaded by mutant cheats that do not produce invertase (an enzyme that hydrolyses glucose), however cooperators can also invade a population of cheats (Gore *et al.*, 2009). Cells of the biofilm-producing social bacterium *Myxococcus xanthus* are able to cheat by achieving disproportionate

representation in the spore pool (the 'germ line'), however, by a single mutation, a superior cooperator can then actively exclude its cheating progenitor (Fiegna *et al.*, 2006). The cycling between cooperators and cheats exists because rare strategies outperform common strategies – a defining feature of the 'snowdrift' game in evolutionary game theory, enabling cooperation to persist at an intermediate frequency (EO Wilson, 1973). This 'negative frequency-dependent selection' also produces oscillations between cooperators and cheats in populations of the bacterium *Pseudomonas fluorescens* (Rainey & Rainey, 2003). Rare cooperative mutant cells invade populations of non-cooperative wildtype cells by exploiting a previously unavailable niche. However cheats then rise again in frequency when rare (Rainey & Travisano, 1998). *P. fluorescens* cooperation and cheating will be discussed in more detail in Section 1.8.1.2.

## 1.7. Stage Three – The Evolution of Complexity

When a collective evolves the capacity to reproduce, it is possible for natural selection to operate on traits that enhance the fitness of the collective. The accumulation of such traits leads to the evolution of progressively higher complexity. Hence, the term 'complexity' does not refer to a specific state reached by a collective, but it is a relative term used to describe a wide spectrum of collective functions. Multicellular complexity is often described by the number of different cell types coexisting in the collective (Bonner, 1988), although epigenetic control of this cellular differentiation is clearly an important innovation resulting from collective-level selection (Buss, 1987; Arnellos *et al.*, 2013). Epigenetic regulation of development itself evolves as increasingly more complex genetic networks. The accumulation of collective-level adaptations may eventually lead to such a degree of integration of parts that the lower level units no longer exist independently – their survival and reproduction depends entirely on the survival of the collective. I suggest that this loss of lower level autonomy be the defining feature of the term 'organism'. In the level above multicellular organisms, eusocial insect colonies are sometimes referred to as 'superorgansims' when the lower level units no longer exist autonomously and instead subsist as sterile workers. There are, however, objections to the use of the term 'superorganism' because organismality can occur at any level (discussed in Section 1.4.1 above) (Queller, 1997).

The evolution of developmental regulation is mechanistically unproblematic because the genetic machinery for coordination of differentiated cell types existed in primitive 'multicellular' prokaryotes (Kaiser, 2001) and close eukaryotic unicellular relatives of metazoans (Sebé-Pedrós *et al.*, 2013; Gombar *et al.*, 2014). Cell-cell signalling in prokaryotes is not restricted to simple autocrine signalling and can generate sophisticated coordination of function. In fact, paracrine signalling (a form of

cell signalling in which the signal-producing cell is immune to, or does not respond to, the signal) has been demonstrated in the differentiated biofilm-forming bacterium *Bacillus subtilis* (Lopez *et al.*, 2009). It is therefore surmised that few mutational steps should be required in a regulatory pathway to produce additional cellular differentiation. Indeed, thousands of differences in gene expression between cell types in multicellular organisms are often controlled by a small set of regulatory proteins (Carroll, 2001). Nevertheless, important metazoan developmental gene families, notably the homeobox genes, are not present in unicellular ancestors (Ruiz-Trillo *et al.*, 2007), indicating that these gene regulatory pathways evolved later as a consequence of collective individuality.

The existence of a 'genetic tool-kit' for substantial cellular differentiation in the unicellular ancestors of multicellular organisms raises questions about the ecological factors that might limit the evolution of complexity. Higher levels of complexity are found in clonal organisms than in aggregate organisms such as cellular slime moulds, which have remained very simple for hundreds of millions of years (Wolpert & Szathmáry, 2002). While it is possible that pre-Metazoans may have been formed from aggregates of differentiated cells (Mikhailov *et al.*, 2009; Olson, 2013), time-lapse microscopy of choanoflagellates (the closest unicellular relatives of Metazoa) has revealed that the transition from single cells to multicellular colonies in the *Salpingoeca rosetta* species occurs not by aggregation, but by cell division with sister cells remaining attached (Fairclough *et al.*, 2010). This may be because aggregation will result in much higher levels of genetic variation than within clonal organisms, increasing the scope for within-organism conflict (Tarnita *et al.*, 2013). Moreover, clonal organisms develop from a unicellular bottleneck, which renders the organism

more evolvable. The crucial role of bottlenecks and germ lines during the evolution of complexity are discussed in Sections 1.6.2.1 and 1.6.2.2 above.

# 1.8. Research Objectives

This aim of this thesis is to experimentally explore each of the three stages of the evolution of multicellularity. The challenge during Stage One is to understand how cooperation can be maintained in multicellular collectives in the face of defection. Here I experimentally test the hypothesis that competition between collectives can maintain high levels of cooperation and reduce the level of cheating within collectives (Section 1.5.3.3). The evolution of collective reproduction via a developmental life cycle and the origin of germ lines are particularly intriguing problems during Stage Two. This thesis experimentally realises the primitive life cycle described above in Section 1.6.3, and tests the hypothesis that a germ line shifts the level of selection from single cells to the multicellular collective – permitting the evolution of complexity (Stage Three).

## 1.8.1. Experimental Approach

The standard method for tackling these issues is to use a 'top-down' approach by exploring the evolution of multicellularity in primitive multicellular organisms such as the volvocacean algae (*e.g.* Michod, 1999) and social amoebae (*e.g.* Strassmann & Queller, 2011). While these studies have been extremely informative, they have two major limitations. First, these organisms have already completed the transition to multicellularity and consequently one can only speculate as to the selective and ecological circumstances that led to the shift in the level of selection. Secondly, focusing on a few model organisms biases the interpretation of observations from these species. These primitive multicellular organisms have remained very simple for hundreds of millions of years – perhaps their evolution was restrained because of an incomplete Stage Two. In the case of the volvocaean algae, termination of post-embryonic cell division has prevented the evolution of complexity (see Section 1.6.2.2

for details). Similarly, slime moulds such as *Dictyostelium discoideum* form multicellular fruiting bodies via aggregation of single cells, thereby supressing the continued evolvability of the collective (for reasons discussed in Section 1.7). The appeal of these model organisms (their simplicity) also limits their usefulness for understanding general principles governing the evolution of multicellularity; their evolutionary history might be more suitably viewed as the 'path not taken'.

An alternative strategy – and the one adopted here – is the 'bottom-up' approach of experimental evolution. Experimental evolution is a field of enquiry that aims to directly test hypotheses of evolutionary theory using controlled experiments. By subjecting extant organisms to different environments we can observe the emergence of adaptations to these varying selective pressures in real-time. Modern molecular tools enable researchers to pinpoint the exact mutations that brought about these adaptations, creating a comprehensive genotype-phenotype-environmental account of evolution. Wade's beetle experiments (1977; Section 1.5.3.3) and Goodnight's (1985) experiments on *Arabidopsis thaliana* are examples of social evolution experiments using animals and plants respectively - both demonstrating the efficacy of group selection in real-time. However, experimenting with unicellular organisms is a 'bottom-up' approach that can facilitate an understanding of conditions necessary at the very origins of multicellularity.

#### 1.8.1.1. Microbial Model Systems

Until recently, much of the empirical evidence for the mode of evolution has focussed on observations of macroorganisms. However, microbial organisms provide unique opportunities to address fundamental evolutionary questions (*e.g.* Lenski, 2011). Microbial model systems are particularly amenable to experimental evolution studies because they quickly reach large population sizes due to their short mean generation

time. Microorganisms usually reproduce asexually, allowing experimenters to create identical laboratory conditions from clonal replicates. This precise control of laboratory conditions allows considerable manipulation, enabling evolution in varying environments to run in parallel. Perhaps one of the greatest advantages of using microorganisms in evolution experiments is their remarkable ability to be frozen, stored and revived for comparison among derived lines, or between derived and ancestral lines.

### 1.8.1.2. The *P. fluorescens* Experimental System

*P. fluorescens* is a gram-negative, rod-shaped bacterium that is particularly suited to evolution experiments because of its ability to colonise numerous environments. Outside of the laboratory, *P. fluorescens* is commonly found in the plant rhizosphere as a free-living motile organism through use of multiple flagella, however it has also been the focus of much experimental interest because of its ability to form biofilms (*e.g.* Rainey & Travisano, 1998). In this thesis I use SBW25, a strain of *P. fluorescens* originally isolated in 1989 from a sugar beet plant at University Farm, Oxfordshire (Rainey & Bailey, 1996). SBW25 has since been the subject of evolution experiments in many laboratories worldwide - providing a rich source of ecological and genetic data, including the availability of the full genome sequence (Silby *et al.*, 2009).

Experimental populations of *P. fluorescens* are ideal for investigating the evolution of multicellularity because the evolution of cooperation has been observed in this species. The wild-type phenotype of SBW25 is termed “smooth” (SM) because of the appearance of its colony morphology when grown on solid agar plates (Figure 1.4a). In spatially structured ‘microcosms’ containing the ancestral SM genotype (Figure 1.4c), collectives arise from single mutant cells that overproduce acetylated cellulose, which acts as a cell-cell glue (Spiers *et al.*, 2003). The failure of the mutant “wrinkly

spreader" (WS) cells (so-called because of their distinctive colony morphology, Figure 1.4b) to separate at cell division leads to the formation of a 'mat' at the air-liquid interface (Figure 1.4d). Natural selection favours WS cells because they gain access to an abundance of oxygen denied to SM cells due to the vertical oxygen gradient that develops rapidly within the medium (Ibelings *et al.*, 2012).

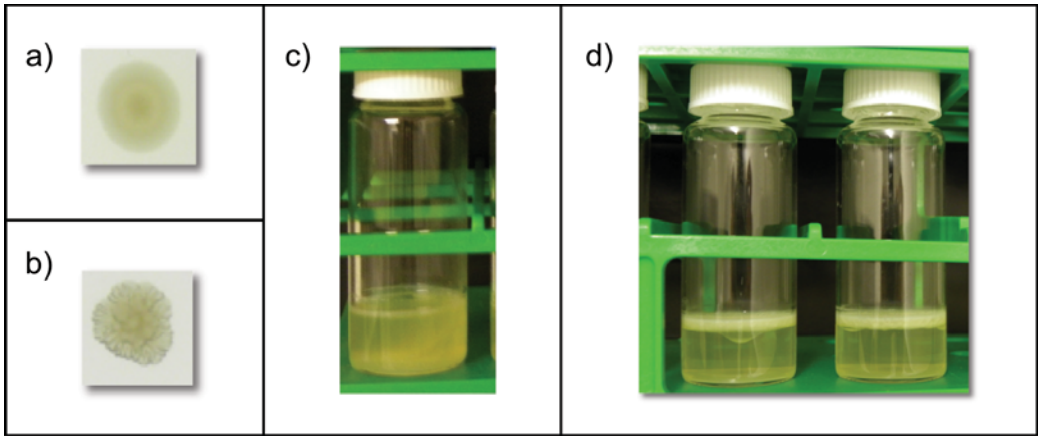


**Figure 1.4 *P. fluorescens* grown on solid and liquid media**. a) SM colony on solid agar b) WS colony on solid agar c) SM cells colonise the broth phase of liquid media in a microcosm d) WS cells form a visible mat at the air-liquid interface in a microcosm. SM and WS phenotypes (a,b) are heritable and correlate with both genotype and niche specificity (c,d) (Rainey & Travisano, 1998; Spiers *et al.*, 2002; Bantinaki *et al.*, 2007).

Crucially, the WS phenotype meets the criteria of a cooperative trait outlined in Section 1.5 (Chase, 1980). First, while WS cooperators invade SM populations by occupying a vacant niche, cellulose production by actors (WS cells) is costly relative to non-actors (SM cells) (Rainey & Rainey, 2003). Secondly, cellulose production increases the fitness of other individuals: SM cells not only increase in frequency in populations founded with WS, but SM are fitter in the presence of WS than in their absence (Figure 1.5; Rainey & Rainey, 2003). Therefore, cooperating WS collectives are inevitably short-lived. Selection continues to act at the level of the individual cell by favouring mutant SM types that cheat by no longer producing cellulose, yet reap the

benefits of cooperation (access to oxygen). If the number of defecting SM cells increases to too high a frequency to maintain the integrity of the mat, this emergent collective-level phenotype will be destroyed, *i.e.* the mat collapses (Figure 1.6).

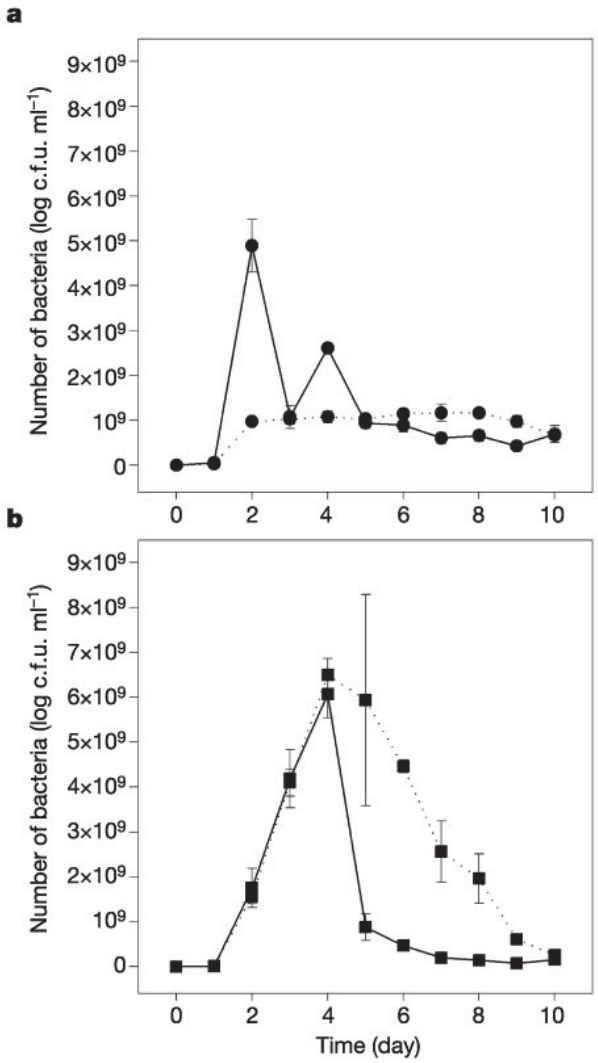


**Figure 1.5 Evidence for cooperation in *P. fluorescens*: population dynamics of WS and SM genotypes in the presence and absence of competition**. a) The effect of WS on the fitness of SM genotypes is the difference between dotted (absence of WS) and solid (presence of competing WS) lines. b) The effect of SM genotypes on the fitness of WS genotypes is the difference between the dotted (absence of competition) and solid (presence of competing SM genotype) lines. From Rainey and Rainey (2003).

**Figure 1.6 Microcosms containing collapsed *P. fluorescens* WS mats.**

Populations of *P. fluorescens* have completed Stage One of an evolutionary transition (the evolution of cooperation), and thus provide an ideal starting position for investigating the evolution of multicellularity. This model system can support Multilevel Selection experiments (Section 1.5.4) by introducing competition between multiple collectives (mats, or 'microcosms') – enabling investigations into how group selection acts as a mechanism to promote cooperation in the face of defection by SM cells. This Multilevel Selection platform is also ideal for testing the predictions of the 'life cycle hypothesis' outlined in Section 1.6.3. A fundamental realization of this proposal is that tension between levels of selection can fuel evolutionary transitions. Through careful manipulation of environmental conditions, this tension can be created in *P. fluorescens* populations by ensuring that selection is operating simultaneously at both the lower (within-collective) and higher (between-collective) hierarchical levels.

## 1.8.2. Thesis Structure and Key Findings

I have conducted long-term evolution experiments using the *P. fluorescens* model system to address fundamental questions arising during the evolution of multicellularity. Chapter 3 tests the hypothesis that group selection can maintain cooperation in the face of defection. In accordance with theoretical predictions (Section 1.5.3.3) competition between *P. fluorescens* mats led to high levels of cooperation

(more WS cells) and less cheating (fewer SM cells). Furthermore, a reduced transition rate between WS and SM cells is indicative of the evolution of a policing mechanism. Chapter 4 describes a test of the 'life cycle hypothesis' for the evolution of multicellularity described in Section 1.6.3. The resulting experiment is presented as a (joint) first-author article submitted for publication in the journal *Nature* (Hammerschmidt *et al.,* 2014). This experiment imposes selection between collectives of *P. fluorescens* sustaining a developmental life cycle involving WS soma and SM propagules (a germ line). Darwinian individuality (Stage Two) emerges in these collectives because the germ line decouples the fitness of the cells from the fitness of the collective. The evolution of traits selected at the level of the collective demonstrates the emergence of the evolution of complexity (Stage Three). Several philosophical problems became evident during the multicellularity experiment. In particular, identifying a true transition in Darwinian individuality is not straightforward because apparent selection at one hierarchical level can be a by-product of natural selection acting at another level. Chapter 5 provides an in depth discussion of these issues with a focus on the evolutionary transition witnessed during the experiment performed in Chapter 4.

# Chapter 2. Materials and Methods

## 2.1. Strains and Medium

*P. fluorescens* SBW25 (Silby *et al.*, 2009) was grown at 28°C in 25-ml static glass vials (with loose caps) containing 6 ml King's Medium B (KB: 10 g/l glycerol; 20 g/l Proteose Peptone No.3; 1.5 g/l $Mg_2SO_4$; 1.5 g/l $K_2HPO_4$ (EO King *et al.*, 1954), and on King's Medium B agar plates for 48 h. For competitive assays strains marked with *lacZ* (Zhang & Rainey, 2007) allowed marked colonies to be distinguished by plating on media containing 60 µg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside dissolved in dimethyl formamide – DMF).

### 2.1.1. Strain Storage

All bacterial strains were grown for 16 h at 28°C in a shaking incubator at 150 rpm in KB medium to be stored at -80℃ in 70% glycerol saline solution (8.5 g NaCl, 300 ml $H_20$, glycerol to 1 litre).

# 2.2. Selection Experiments

## 2.2.1. Cheat-Embracing Regime

The cheat-embracing **(**CE) regime (Figure 4.1, left hand panel) involved a two-phase life cycle (Figure 4.2). The experiment was founded by a single ancestral SBW25 genotype that initiated Phase II. After three days of static incubation (Phase II), $1.5x10^{-7}$ ml was plated and a single wrinkly-spreader (WS) colony of the most abundant morphotype was picked from each plate to inoculate a fresh microcosm (Figure 4.2). This marked the start of Phase I of the first generation. Following six days of static incubation (Phase I), microcosms were visually inspected to check for the presence of an intact mat. Microcosms that had no mat at day 6 were deemed extinct. Microcosms containing intact mats were vortex mixed and $2.5x10^{-8}$ ml plated on solid media. After 48 h incubation plates were inspected. To avoid extinction and successfully passage to Phase II it was necessary for collectives to have produced SM types. All SM colonies were transferred to 200 µl liquid medium and incubated for 24 h under static conditions. These cultures were pooled, mixed, and 6 µl was used to inoculate Phase II microcosms.

## 2.2.2. Cheat-Purging Regime

The cheat-purging (CP) regime (Chapter 3 and Figure 4.1, right hand panel) was identical to the CE regime except that during the life cycle cheats were purged and WS founded both phases of the cycle. Both regimes were conducted in parallel.

## 2.2.3. Between-Collective Selection

Each treatment consisted of 15 replicates of eight competing microcosms, and replicates from both treatments were spread evenly across four experimental blocks. All

microcosms in the CE regime were subjected to the selection regime outlined above, however, following assessment of mat integrity at the end of Phase I, surviving collectives within each replicate were harvested by vortex-mixing and dilutions spread on agar plates (Figure 4.4). Extinct collectives (due to mat collapse / no mat / no SM colony post Phase I, or no WS colony post Phase II) were immediately replaced by randomly chosen surviving collectives taken from the same replicate. On rare occasions all eight microcosms of a replicate were eliminated: in these instances one replicate, chosen at random from the same experimental block, was used to re-found the replicate. The CP treatment was carried out as above, differing only following Phase I when WS colonies were picked instead of SM colonies (Figure 3.1). A smaller volume ($6.25x10^{-9}$ ml) was plated due to the absence of a phenotype-switching requirement for the CP treatment. Both treatment regimes were implemented for ten generations.

## 2.2.4. Selection of Representative WS Genotypes

One single WS genotype to represent each replicate population (set of eight microcosms) from the evolved CE and CP "between-collective selection regimes" was generated after 10 generations as illustrated in Figure 2.1. To obtain the representative set of evolved types under the CE regime SM colonies were collected from the end of Phase I at generation 10 and pooled. The pooled sample was used to found Phase II, at the end of which microcosms were harvested and plated. The single most abundant WS type from the most densely populated plate was selected as representative of that replicate. A second set of representative WS genotypes was obtained from populations evolved under the CP regime. This was as for the CE regime, but instead of pooling SM at the end of Phase I, WS were collected and pooled. A single dominant WS type was chosen from each replicate. To obtain the set of baseline types representing the ancestral state, SBW25 was used to found Phase II. At the end of the three day period a single

WS type representative of each replicate was selected as described for the CE regime. The representative WS colonies were grown in shaken microcosms (16 h) and stored at -80°C for post-selection analyses. This yielded 15 such types each for the ancestral and CP regimes, and 14 types for the CE regime (bacteria from one CE replicate could not be revived from the freezer stock).

A *lacZ*-marked reference strain (W1-*lacZ*) for fitness comparisons in the CP regime (Figure 2.2, right hand panel) was generated the same way from 3-day static microcosms inoculated with a *lacZ*-marked ancestral strain SBW25-*lacZ* (Zhang & Rainey, 2007).

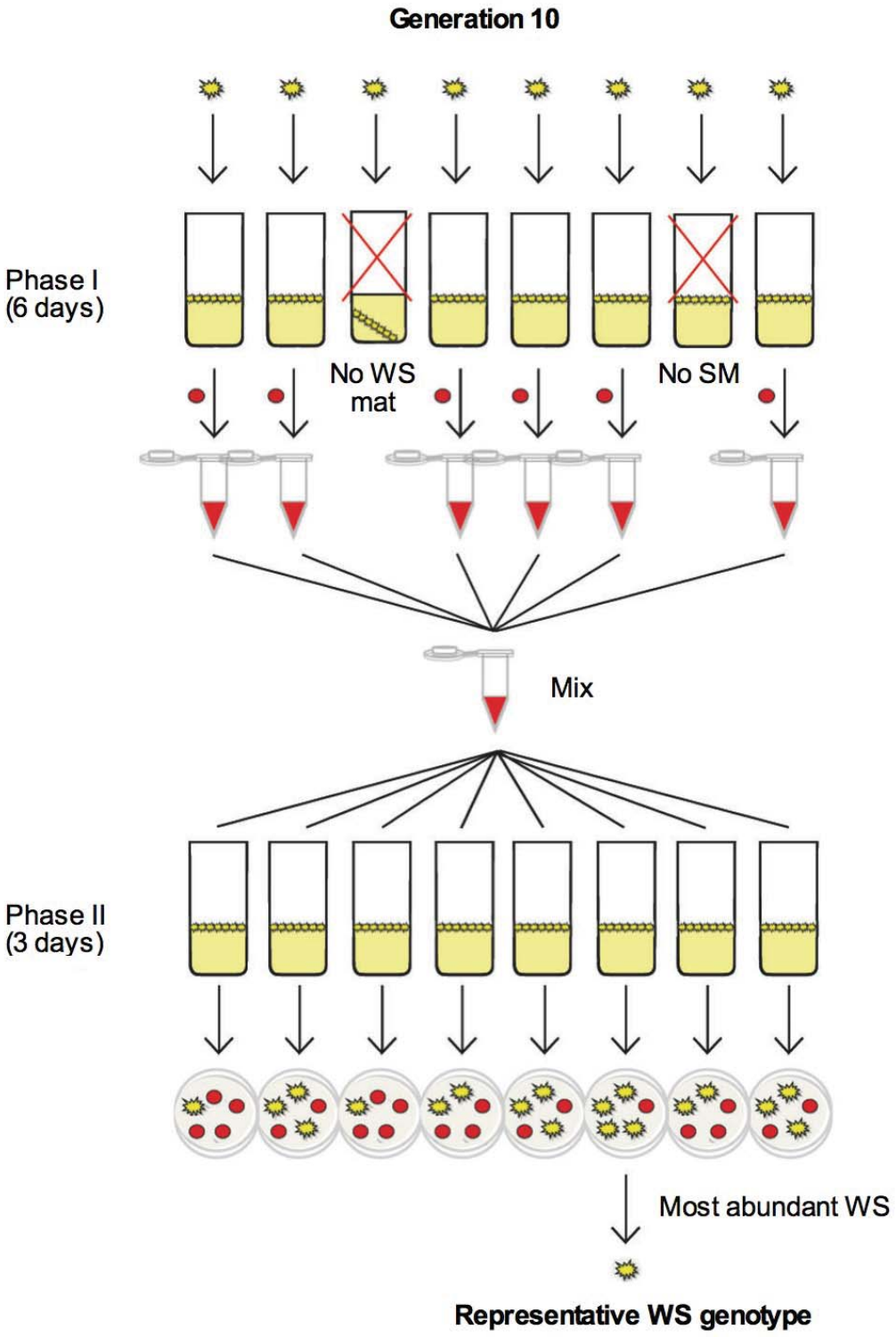


**Figure 2.1 Selection of representative WS genotypes.**

## 2.3. Fitness Assays

Collective and cell level fitness was assessed for all 44 representative ancestral and evolved types (Figure 2.2). The representative types were not directly competed against each other but against a single, neutrally marked reference strain assayed under the appropriate regime: the ancestral and evolved CE types were competed against SBW25-*lacZ*, and the ancestral and evolved CP types were competed against W1-*lacZ*. This yielded independent fitness values for each type (*i.e.* potential interactions between ancestral and evolved types didn't affect the result), and allowed for estimates and comparison of relative performance for all types.

One collective generation was performed for each of three replicate fitness assays for all 44 representative types (as shown in Figure 2.2 for one CE and one CP replicate). For each representative type, eight microcosms were each inoculated with one WS colony in Phase I. After mat assessment surviving microcosms were pooled, and $2x10^{-7}$ ml (CE) or $5x10^{-8}$ ml (CP) of this mixture plated. The total number of colonies (in mats) was recorded as a measure of cell fitness. All SM (CE) and WS (CP) colonies, and colonies of the competitor strains SBW25-*lacZ* and W1-*lacZ* were grown overnight and subsequently pooled as described above (CE regime). The evolved and ancestral types were mixed with the competitor strains in proportion to their performance during Phase I (details displayed in Figure 2.2). 6 µl of this mixture was used to inoculate the eight Phase II microcosms. After three days of static incubation (Phase II), microcosms were plated. The most abundant WS colony morphotype on each plate determined whether the ancestral/evolved representative type or the marked reference type was more successful. The fitness of types representative of each line was calculated for each replicate of eight microcosms as the number of successful offspring of this type as a proportion of the total number of potential offspring (Figure 2.2).

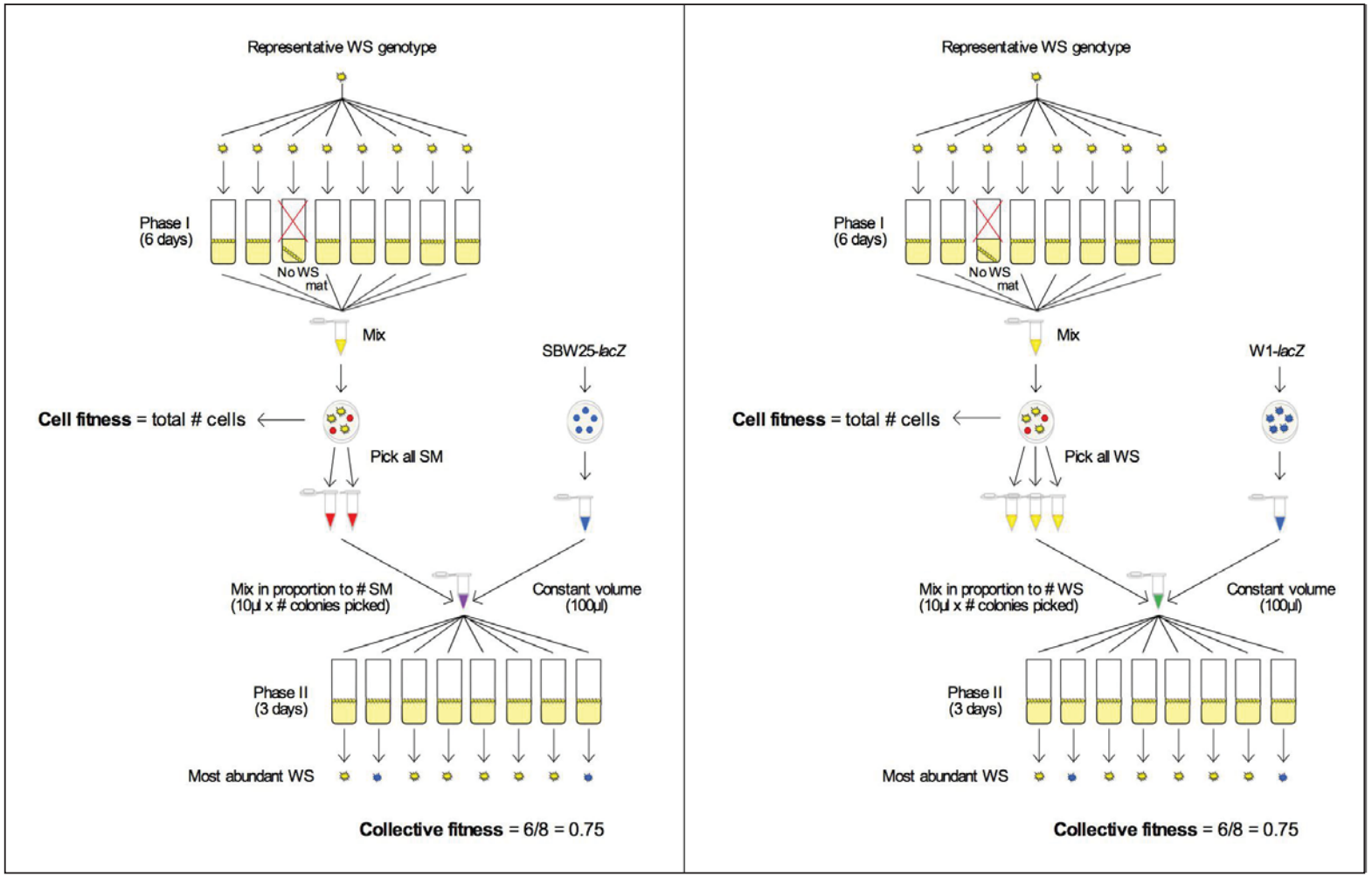


**Figure 2.2 Cell and collective fitness assays for the CE (left) and CP (right) selective regimes.** Microcosms founded by representative WS genotypes (from ancestral and evolved lineages) were competed against a marked (blue colonies) reference strain (SM and WS, for the CE and CP regimes, respectively). In total 2472 microcosms were assayed.

## 2.4. Life History Analysis

Static microcosms (36 per representative genotype) were individually inoculated with single colonies of the representative WS types (1584 microcosms in total). Each day, three replicates were destructively harvested, plated, and the number of SM and WS colony forming units per microcosm was recorded. Phase I was extended from 6 to 12 days, Phase II from 3 to 6 days. At Day 6, propagules were collected for Phase II, and microcosms inoculated (18 per type). Each day, three replicate microcosms per representative type were destructively harvested and number of SM and WS colony forming units recorded.

## 2.5. SM Growth Assay

Three biological replicate SM colonies were derived from each of the representative ancestral and derived CE and CP WS types. Four day static microcosms seeded from a single WS colony were destructively harvested and plated ($5x10^{-8}$ ml). SM colonies were chosen, grown in shaken microcosms (16 h), and stored at -80°C. This procedure was repeated until three biological SM replicate colonies were obtained. For the types where three biological replicate SM colonies couldn't be derived, additional experimental replicates of SM growth rate were assessed. In a small number of instances no SM types were obtained.

SM growth kinetics were determined in 96-well microtitre plates shaken at 28°C, and absorbance ($OD_{600}$) measured in a microplate reader (BioTek®). Each well was inoculated with approximately $10^4$ SM cells in 180 µl KB medium and absorbance measured every 10 m for 24 h. The growth of each biological replicate was determined in three different well locations on independent 96-well plates and on separate days. The maximum growth rate ($V_{max}$) was calculated from the maximum slope of the

absorbance over time. The mean $V_{max}$ for each representative type was calculated from all biological and experimental SM replicates.

## 2.6. Statistical Analysis

Analysis of variance (ANOVA) was used to test for differences in total # cells (cell fitness), #WS/µl, #SM/µl (if present), proportion of SM, and SM growth rate between the different regimes. Explanatory variables were regime and representative type (nested within regime). Post hoc contrasts revealed differences between the ancestral and their respective derived regime.

For detecting differences in collective-level fitness between the ancestral and evolved regimes, a generalized linear model (error structure: binomial; link function: logit) was calculated with the explanatory variables regime and representative type (nested within regime). Contrasts revealed differences in collective-level fitness between regimes.

Generalized linear models (error structure: binomial, link function: logit) were used to test for the difference between regimes in life history parameters during the course of the experiment. The response variable was ‘proportion of microcosms with the new type’. Explanatory variables were regime, representative type (nested within regime), and time. Analysis of variance was performed with the same explanatory variables but for ‘new cell type/µl’, ‘total cells/µl’, ‘proportion of the new cell type within a microcosm’ (all three variables were box cox transformed), and ‘# colony types’. Contrasts revealed differences between the regimes on the individual days.

Relationships between all parameters were tested using the mean per representative type accounting for regime. Pearson, Spearman rank correlations, and regressions (collective-level fitness: generalized linear models with the error structure:

normal, and the link function: identity; cell-level fitness and #SM/µl (if present): general linear models) were performed. SM growth rate, #SM/µl (if present), and SM occurrence were box cox transformed.

Akaike's Information Criterion (AICc, Akaike, 1973) was used to determine the model (linear or quadratic) that was most likely to have generated the data for the relationship between the proportion of SM and collective fitness.

Assumptions of the tests, *i.e.* normality and equal distribution of variances were visually evaluated. Non-significant interactions were removed from the models. All tests were two-tailed. Effects were considered significant at the level of $P < 0.05$. All statistical analyses were performed with JMP® Version 9, SAS Institute Inc., Cary NC, except for Akaike's Information Criterion, which was performed using GraphPad Prism, version 5.0f for Mac OSX, GraphPad Software, San Diego California USA.

# Chapter 3.
# Stage One: The Maintenance of Cooperation

## 3.1. Introduction

The maintenance of cooperation within a population of cells requires that the cost of cooperation be outweighed by the benefits gained through cooperative interactions. Cooperative cells increase the fitness of individuals other than those that cooperate and are therefore susceptible to exploitation by cheating cells that are favoured by natural selection because they do not incur the cost of cooperation (refer to Section 1.5.2 for a discussion on the paradox of cooperation and conflict during the evolution of multicellularity). The challenge during Stage One of the evolution of multicellularity is to understand how cooperation can be maintained in multicellular collectives in the face of defection.

Recently, the experimental evolution of cheater suppression in both eukaryotic and prokaryotic microbial model systems has advanced our understanding of how cooperation and multicellularity can evolve in microbial populations. Khare *et al.* (2009) showed that exposure to cheats can select for resistance to cheats in a randomly mutated population of cooperating *D. discoideum* cells. The *de novo* evolution of a policing mechanism, in which evolved strains actively suppressed cheating cells, has also been demonstrated in populations of a cooperative strain of the bacterium *M.*

*xanthus* that repeatedly encountered a non-evolving developmental cheater (Manhes & Velicer, 2011). Not only did the fitness of the evolving populations improve, but the productivity of the ancestral strain when competing against the cheater also increased with the addition of evolved strains to the culture. Cheater suppression was costly; ancestral strains were more fit than the evolved in the absence of cheats.

Various methods have been proposed that can, in principle, explain how cooperative behaviours might exist in an evolutionary context (see Section 1.5.3 details). Within a Multilevel Selection framework these explanations may be viewed as mechanisms by which the cost of a cooperative behaviour can be offset by benefits gained at different levels of the biological hierarchy. The key principle common to the various classes of explanation for the evolution of cooperation is 'correlated interaction' between cooperating individuals (Godfrey-Smith, 2009). Preferential interactions among kin, reciprocity, spatial heterogeneity in population structure, and Multilevel Selection are all different mechanisms for achieving assortment between cooperating cells (Nowak, 2006; Celiker & Gore, 2013). This chapter explores the Multilevel Selection mechanism because the emerging collective level of selection is highly relevant in the context of cooperation as the first stage in the evolution of multicellularity.

Multilevel Selection can increase the frequency of cooperators in the global population. Chuang *et al.* (2009) established a synthetic microbial system consisting of cooperating and non-cooperating *Escherichia coli* cells, and observed that subpopulations with a higher frequency of cooperators grow to higher densities. Within collectives, the presence of cheats decreases the frequency of cooperators, however the greater productivity of collectives with higher cooperator frequency and periodic

mixing of collectives ensured that the number of cooperators increased in the overall population.

The mode of Multilevel Selection may have a significant effect on the evolutionary outcome of competition between collectives. Section 1.5.4 outlined the fundamental differences between the MLS1 and MLS2 models of group selection. Chuang *et al.* (2009)'s trait-group experiment is an MLS1 model because it concerns the evolution of a particle phenotype (*i.e.* cooperation) in a group-structured population that was part of the environment of the individual cells. In the absence of differential extinction and reproduction, collectives cannot themselves be units of selection (see Section 1.1). While the group-structured environment provided the essential assortment of cooperating cells required for the cooperative phenotype to be maintained in the population, it did not give rise to collective-level adaptations or reduce the level of cheating (Chuang *et al.*, 2009).

The MLS1 selective environment is effective for maintaining cooperation in the global population; however an MLS2 population structure, with differential multiplication of collectives, is clearly essential during Stage Two of an evolutionary transition and the emergence of individuality. Here I experimentally test the hypothesis that competition between collectives via MLS2 death and birth processes can maintain high levels of cooperation within *P. fluorescens* collectives. Collectives are founded by a single WS cooperating cell, allowing SM cheating cells to be regularly purged by new colonization events. In accordance with theoretical predictions (Section 1.5.3.3), between-collective selection leads to high levels of cooperation and less cheating. Furthermore, a reduced transition rate between WS and SM cells hints at the possibility of the evolution of a cheater repression mechanism.

## 3.2. Results

To allow for selection to operate among collectives, *P. fluorescens* collectives founded with a single WS colony were allowed to compete to leave collective offspring via differential extinction and reproduction (Figure 3.1). Collectives that proved unviable (as a consequence of either mat collapse, or failure to complete the life cycle due to invasion of non-cooperating cells) provided opportunity for viable mats to multiply. Following an extinction event, a collective was randomly replaced by a surviving competitor collective. Extinction/replacement of collectives occurred with high frequency (approximately one third were replaced after the first generation) and therefore imposed strong between-collective selection. 15 replicates of eight competing mats were subjected to ten collective generations of between-collective selection.

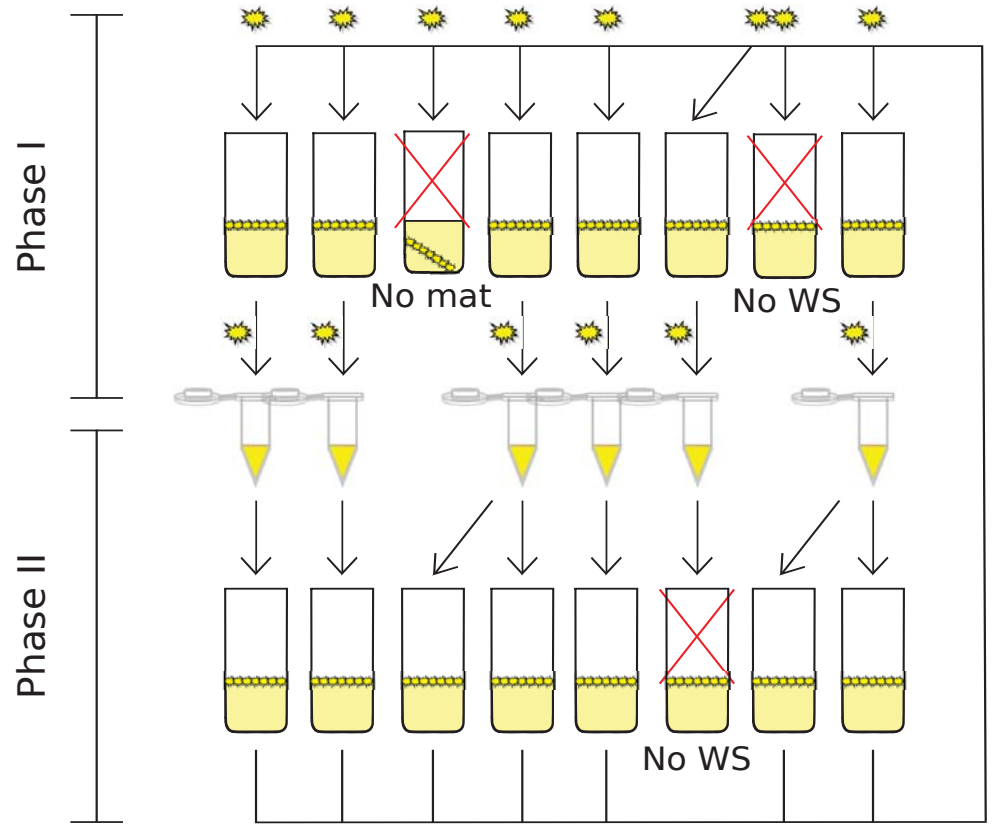


**Figure 3.1 Experimental Regime.** Collectives (N=120) were arranged as 15 replicates of eight (one replicate is depicted). The experiment involved a total of 2400 microcosms. The two phases of the collective life cycle allowed this experimental regime to serve as a control in the experiment described in Chapter 4. Each collective was founded by a single WS genotype. After six days in Phase I cells were harvested, plated and WS colonies pooled to found Phase II. After three days cells were again harvested, plated and a single WS colony of the dominant type founded the next generation. To avoid extinction each mat must be intact at the end of Phase I and WS colonies must be present (*i.e.* the collective has not been overrun by non-cooperating cells). Extinction events provide opportunity for viable collectives to reproduce. Replacements of extinct collectives were chosen at random within the same replicate.

### 3.2.1. Evolution of Increased Cooperation and Decreased Cheating

To examine the changes in collective composition during the selection period, cell densities of the evolved collectives were compared to those of the ancestral collectives. A single WS genotype representative of each population (of eight lines) was taken after ten collective generations (see Methods Section 2.2.4 and Figure 2.1) for further

analyses. In addition, 15 independent WS genotypes were obtained one mutational step from the ancestral SM genotype, thus providing a "baseline" reference for ancestral properties (see Methods Section 2.2.4 and Figure 2.1).

Levels of cooperation significantly improved over the course of the experiment: after 6 days of Phase I growth evolved strains had more WS cooperating cells ($F_1$=25.318, $P$<0.0001; Figure 3.2a; Appendix 8.1) and fewer SM cheating cells, if SM cells were present in the collective ($F_1$=7.516, $P$=0.0109; Figure 3.2b; Appendix 8.1), leading to a substantial reduction in the proportion of SM cheating cells within collectives ($F_1$=21.959, $P$<0.0001; Appendix 8.1).

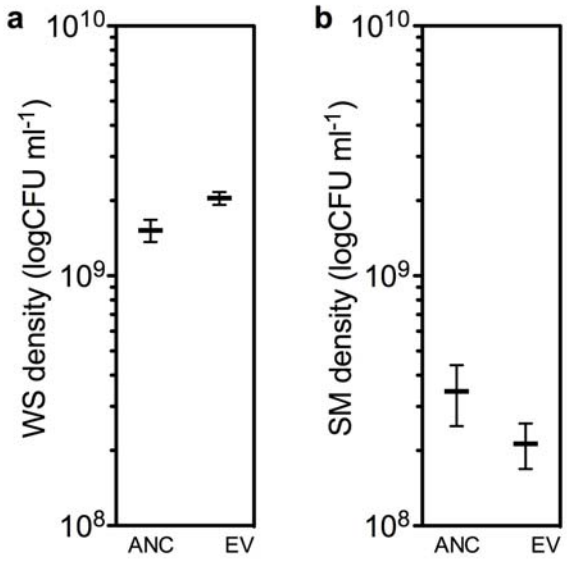


**Figure 3.2 Change in collective composition after ten generations of between-collective selection.** Evolved collectives had significantly more WS cells (a), and fewer SM cells (b) compared with ancestral representative clones. ANC = ancestral, EV = evolved. Error bars are SEM, based on N=11-15.

### 3.2.2. Collective-level Competition Improved Collective Fitness

To investigate whether competition between collectives may have contributed to increased levels of cooperation, collective and cell fitnesses were determined. Experimental microbial evolution studies typically determine relative fitness by directly competing evolved and ancestral cells and calculating the ratio of final to initial population densities (the Malthusian parameter, Lenski *et al.*, 1991). The Multilevel Selection experiment described here warrants an alternative assay that can determine both the competitive ability of collectives and cells within the collective environment. All representative WS genotypes were competed against a *lacZ*-marked reference strain (Zhang & Rainey, 2007) (see Methods Section 2.3 and Figure 2.2, right-hand panel), allowing the competitive performance of all ancestral and evolved strains to be assessed against a single common genotype. Fitness of collectives was defined as the number of WS mat offspring produced relative to the marked competitor (Figure 2.2); cell fitness was assessed as the total number of cells contained within individual mats (irrespective of type) at the end of Phase I. Consistent with an evolutionary response to competition between collectives, the fitness of evolved collectives improved significantly ($\chi^2$=15.737, df=1, $P$<0.0001; Figure 3.3a; Appendix 8.2.1), and was accompanied by a similar increase in cell fitness ($t_{86}$=2.132, $P$=0.036; Figure 3.3b; Appendix 8.2.1).

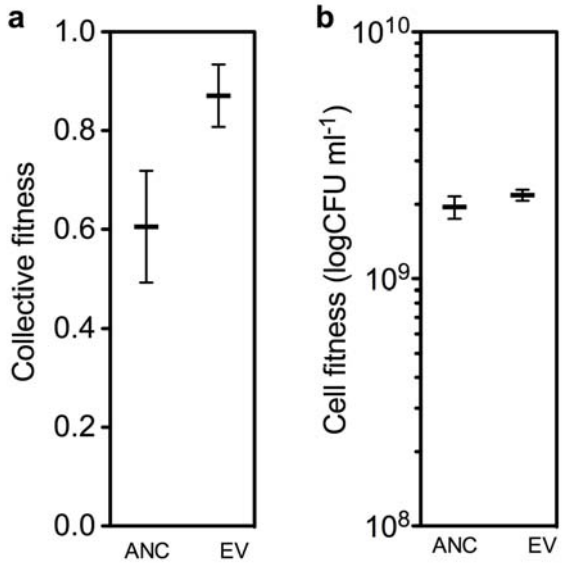


**Figure 3.3 Fitness of evolved and ancestral collectives and their single cell constituents.** Relative to a marked reference strain, both collective fitness (a) and cell fitness (b) improved significantly. ANC = ancestral, EV = evolved. Error bars are SEM, based on N=15.

### 3.2.2.1. Maintenance of Cooperation is Associated With Collective Fitness

Improvements in collective-level fitness were associated with increased levels of cooperation. The number of cooperating WS cells significantly explains collective fitness in both the ancestral ($\chi^2$=6.153, df=1, $P$=0.0131) and evolved strains ($\chi^2$=7.552, df=1, $P$=0.0060; Figure 3.4a). Furthermore, ancestral collective fitness is negatively associated with the density of cheating SM cells ($\chi^2$=17.568, df=1, $P$<0.0001; Figure 3.4b). No statistical relationship exists in the evolved strains because these collectives tended to evolve to near maximum levels of collective fitness and minimal levels of cheating, thereby lessening the pattern of variation (Figure 3.4b).

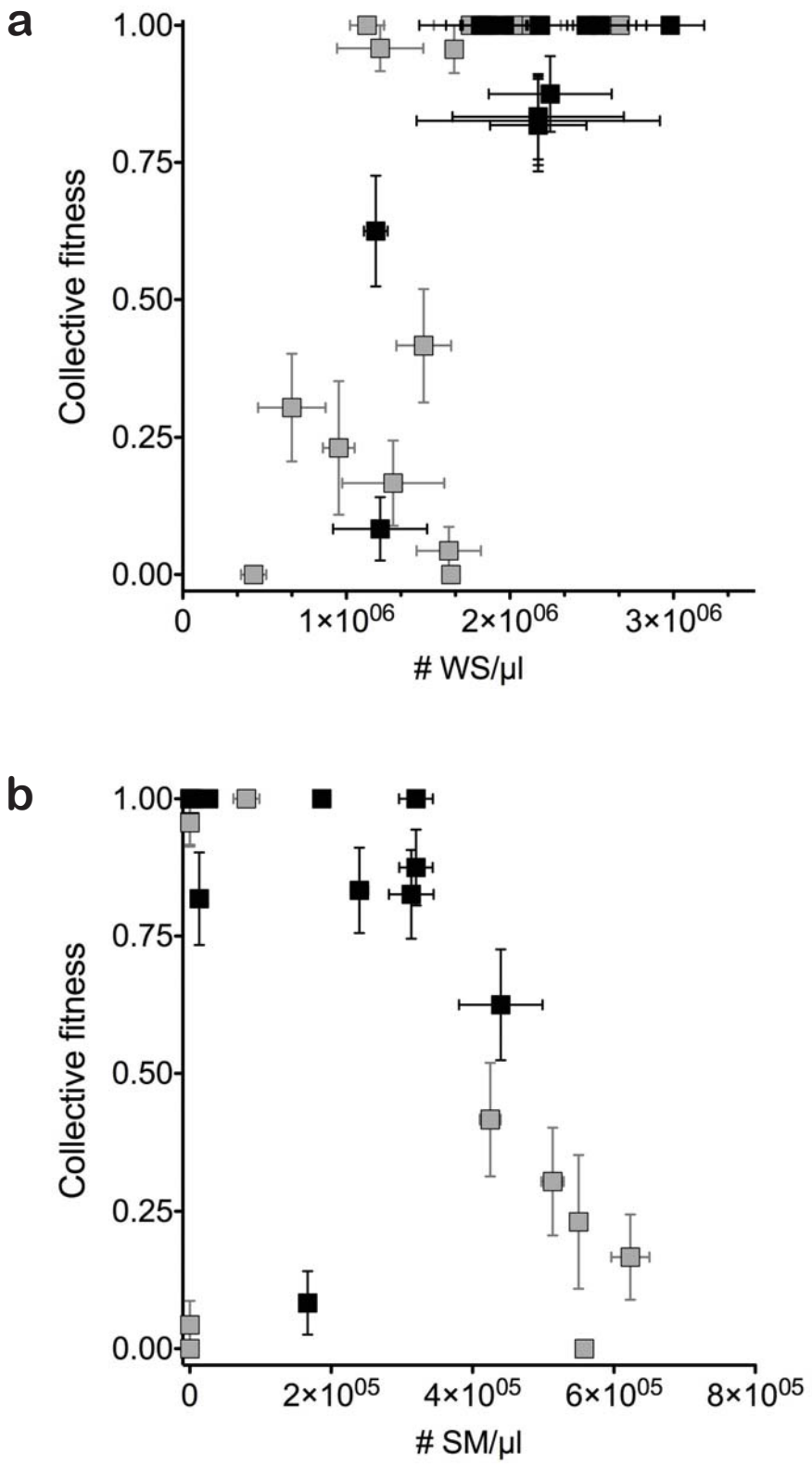


**Figure 3.4 The effect of cooperation (a) and cheating (b) on collective fitness.** Ancestral = grey, evolved = black. Error bars are SEM, based on N≤3.

Competition between collectives therefore provides a selective explanation for the maintenance of cooperation. The following sections investigate the mechanistic basis for the reduction in cheating.

## 3.2.3. Evolution of Reduced Transition Rate

To explore adaptations that contributed to increased cooperation and reduced cheating, life history properties were determined relative to ancestral types. Three replicate microcosms of each representative genotype were destructively sampled each day throughout Phase I and II of the experimental regime, and the frequency of each cell type determined. With interest in the possibility that selection might have optimised life history characteristics to suit the duration of each phase, the number of days of propagation was doubled in both Phase I (12 days) and Phase II (6 days).

A significantly lower proportion of the evolved collectives produced SM cells compared to the ancestral strains ($\chi^2$=8.199, df=1, $P$=0.0042), and any SM types that did arise took longer to do so (Figure 3.5a). Furthermore, a high proportion of cooperating WS cells was maintained in the evolved strains over time, whereas the level of cooperation in the ancestral lines steadily declined (Figure 3.5b), indicating that a mechanism for cheater suppression might have begun to evolve.

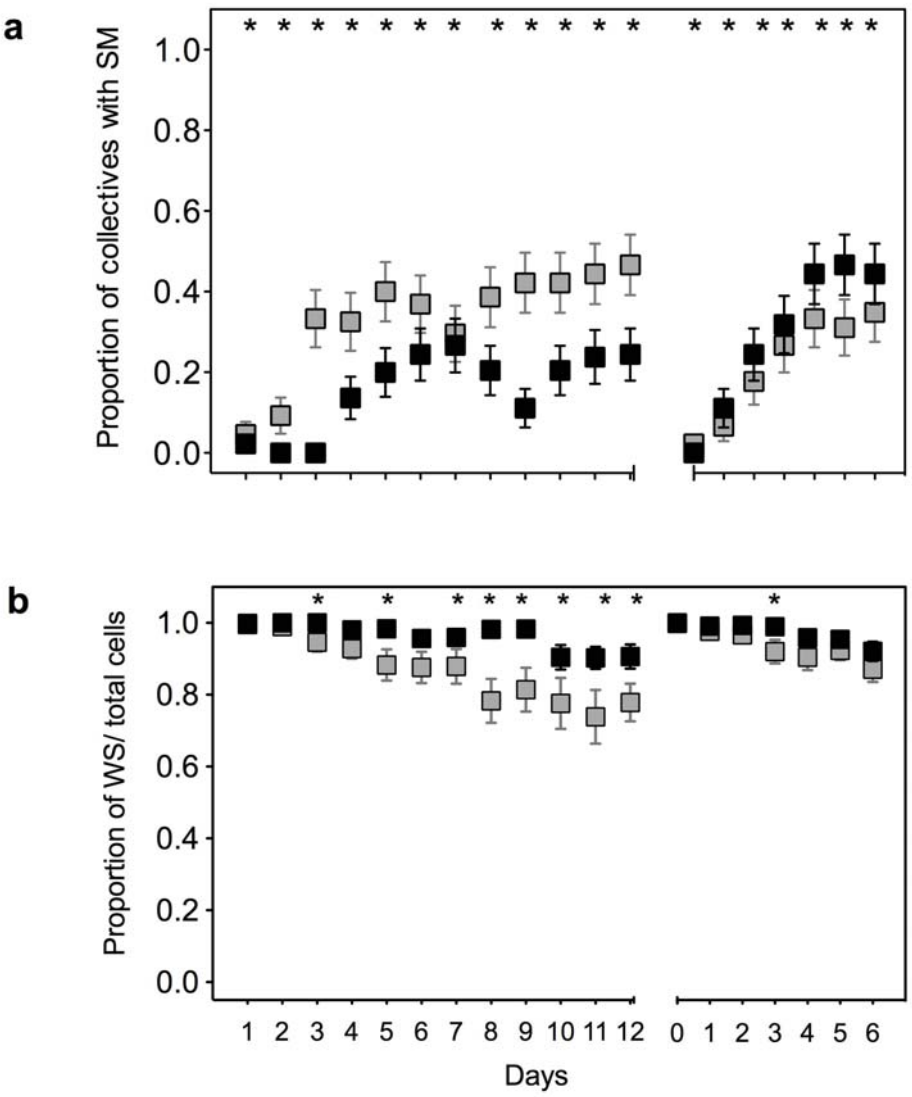


**Figure 3.5 Life history traits** a) Proportion of strains that produced SM b) WS cells as a proportion of total cells. Each circle represents the mean of 45 lines (*i.e.* 3 replicates for each of the 15 representative strains). Ancestral = grey, evolved = black. Error bars are SEM, based on N≤15. * represents a significance level of $P<0.05$.

The rate of SM occurrence is related to collective fitness in the ancestral strains ($\chi^2$=8.710, df=1, N=15, $P$=0.0032). As above (Section 3.2.2.1), a significant relationship between the success of collectives and the rate of SM occurrence does not exist in the evolved strains ($\chi^2$=2.129, df=1, N=15, $P$=0.1445) because these collectives evolved to near maximum levels of collective fitness and minimal levels of cheating.

The reduction in the rate of cheating in the evolved strains, and its effect on the success of collectives is depicted in Figure 3.6.

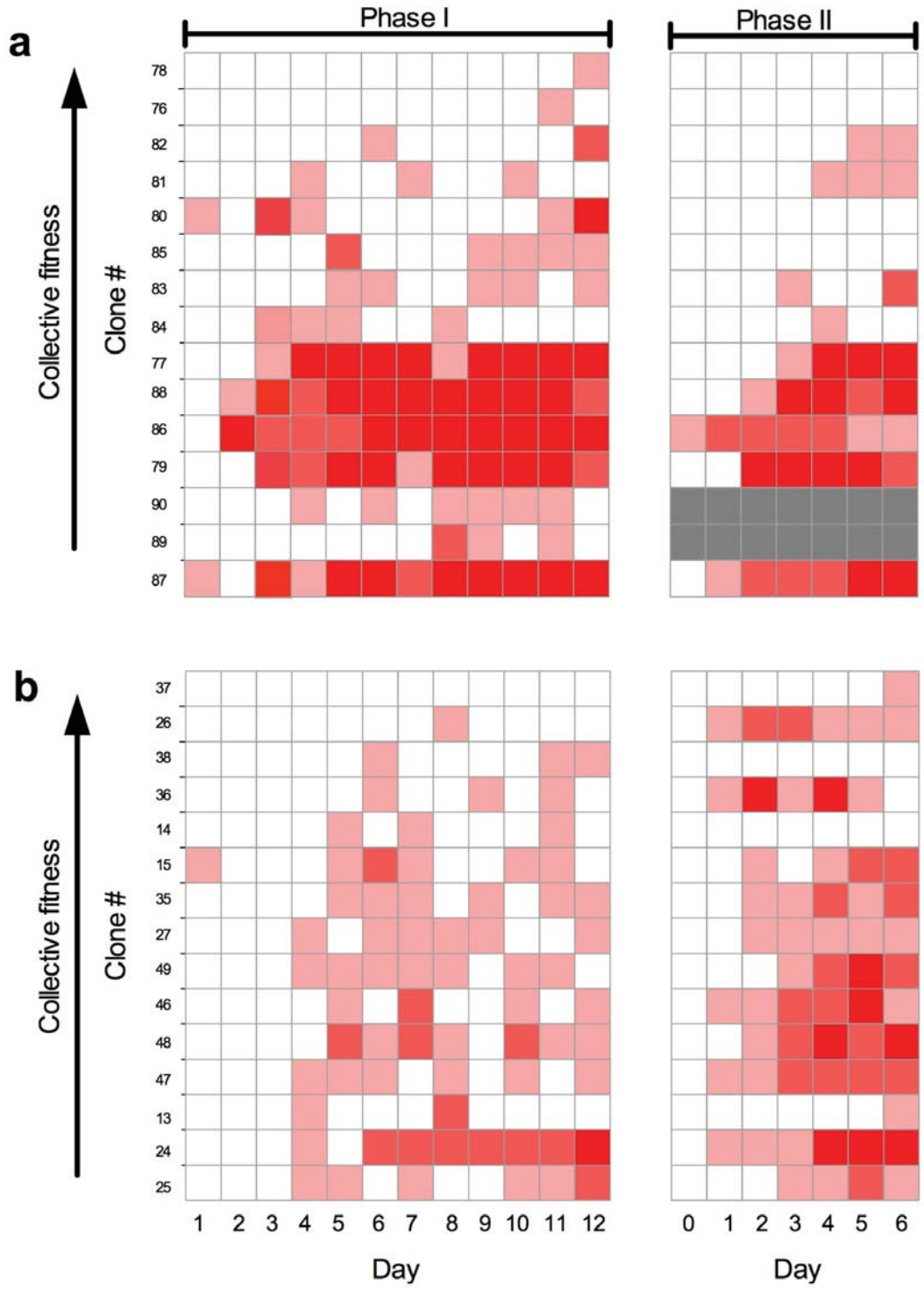


**Figure 3.6 Collective fitness of ancestral (a) and evolved (b) strains and relationship with rate of SM occurrence.** Data are a breakdown of data in Figure 3.5a. Colour intensity represents the proportion of microcosms harbouring SM cells: white = absence of SM; dark red = presence of SM in all three microcosms. Clones are ordered according to their collective fitness. Grey cells indicate loss of lines due to extinction in Phase I.

### 3.2.3.1. Increased SM Growth Rate

A number of possible explanations exist for the decrease in the rate of SM occurrence in the evolved strains. The pattern seen above could result from a reduction in the growth rate of SM cells, a reduced transition rate to SM, or other ecological factors. The maximum growth rate of SM cells was therefore measured in order to elucidate the mechanism for decreased cheating in the evolved collectives. Compared to the growth rate of SM cells harvested from the ancestral lineages, the growth rate of SM cells harvested from the evolved strains significantly increased ($F_1$=42.919, $P$<0.0001; Figure 3.7; Appendix 8.1).

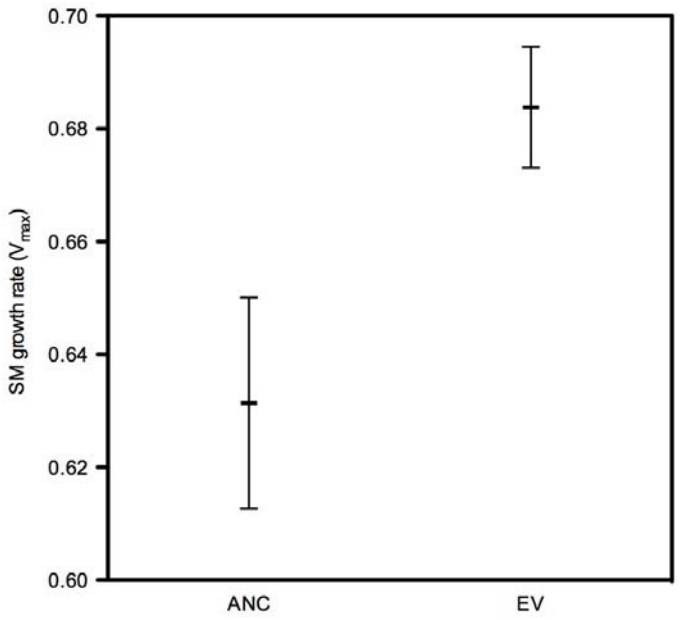


**Figure 3.7 Growth rate of SM. Growth rate of ancestral (ANC) and evolved (EV) SM cells harvested from the representative genotypes.** Error bars are SEM, based on N=12 for ANC and N=11 for EV.

### 3.2.3.2. Evolution of Reduced Mutation Rate?

The increase in SM growth rate reveals that the reduction in likelihood of detecting a SM cell in the evolved collectives must be due to a decreased transition rate

between WS and SM cells. The evolved strains also produced fewer different cell types (including additional WS types) than the ancestral strains ($F_1$=147.893, $P$<0.0001, Figure 3.8, Appendix 8.1) indicating that a reduced overall mutation rate may have caused the lowered transition rate. Further tests of mutation rate are needed to support this conjecture.

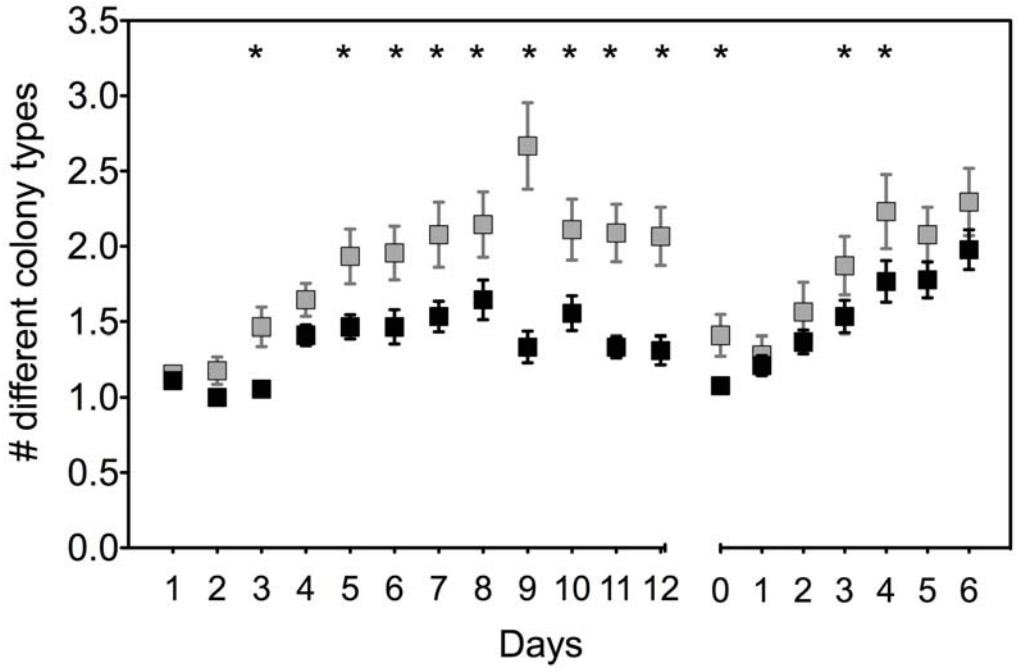


**Figure 3.8 Colony diversity**. Number of different colony types present on plates during each day of the life history analysis. Each circle represents the mean of 45 lines (*i.e.* 3 replicates for each of the 15 representative strains). Derived = black, ancestral = grey. Error bars are SEM, based on N≤15. * represents a significance level of $P$<0.05.

## 3.3. Discussion

The evolution of cooperation within collectives is essential during Stage One of the evolutionary transition from single cells to multicellular organisms. Here, increased levels of cooperation and reduced cheating in collectives of *P. fluorescens* were associated with increased collective fitness, demonstrating that Multilevel Selection is an effective mechanism for the maintenance of cooperation.

Potential mechanisms to control cheating can generally be categorized as either benefit strategies (*e.g.* green beard genes, Section 1.5.3.1), or punishment strategies (*e.g.* toxin-antitoxin "mafia" systems, Nogueira *et al.,* 2009) (Travisano & Velicer, 2004). Other experimental evolution studies (*e.g.* Manhes & Velicer, 2011) have witnessed the *de novo* evolution of cheater suppression when cooperating cells have been exposed to a constant cheater presence. In the current experiment, however, the emergence of new cheats from cooperating cells is suppressed. The reduction in cheating is therefore unlikely to be due to such strategies outlined above; rather the prevention (as opposed to the restraint) of cheating is a consequence of a lowered transition rate between cooperating and cheating cells.

The lowered transition rate may be caused by a reduction in the overall mutation rate. Mutation provides the essential variation required for natural selection to operate. Therefore, an optimal rate of mutation results from the interplay between adaptation and the catastrophic effects of deleterious mutations. Drake (1991) suggested that the minimum mutation rate in microbes is set by the physiological costs of limiting the rate of DNA synthesis in order to maximize the fidelity of replication, and this idea has since received much consideration (*e.g.* Sniegowski *et al.,* 2000). Here, a reduced mutation rate combined with increased rate of cell growth disproves this 'cost-of-fidelity' hypothesis. Alternatively, the lower limit is probably set by drift, which

explains why mutation rate is inversely related to genome size – microbes reach very high effective population sizes (Lynch, 2010). Therefore, the fitness advantage gained by lowering the mutation rate in this Multilevel Selection environment must be greater than the power of drift. Indeed, both collective fitness and cell fitness significantly improved as a consequence of reduced transition rate.

In the long-term evolution experiment with *E. coli* (Lenski *et al.*, 1991) a reduction in mutation rate followed the evolution of hypermutator types. Wielgoss *et al.* (2012) showed that *mutT mutY* double mutants reduced the mutation rate in the *muT* hypermutator background. Loss of function *mutY* mutations on their own also confer a hypermutator phenotype; however in combination with the *mutT* mutations they curtail the effect of the hypermutator. Therefore the genetic basis for this mutation rate reduction is only effective after the mutation rate has been elevated. While the mutation rate in the double mutants was reduced by ~56% and ~36% compared to the *mutT* hypermutator, it still far exceeded the ancestral mutation rate. McDonald *et al.* (2012) also witnessed the evolution of decreased mutation rate in engineered hypermutator strains of *S. cerevisiae*, and even in nonmutator backgrounds (although the latter trend was not significant due to the reduced power of the assay for distinguishing between lower mutation rates). Here, the decreased transition rate in the *P. fluorescens* populations is potentially the first experimental evidence for the evolution of a reduction in wildtype mutation rate.

Selection for high levels of cooperation has led to the evolution of cheater suppression, which is often regarded as an essential first step during the evolution of multicellularity (*e.g.* Michod, 1999; Section 1.6). This has, however, come at the cost of reduced variation; a lower mutation rate limits the scope for innovation and the potential for further evolution of collectives. A more promising path to the evolution of

multicellularity is the evolution of collective adaptations such as conflict mediation *as a result of* first completing Stage Two: the evolution of individuality.

# Chapter 4.

# Stage Two: The Evolution of Individuality

Chapter 4 is based on work that is currently in revision for publication as a full article in the journal *Nature*:

**Katrin Hammerschmidt*, Caroline Rose*, Ben Kerr, Paul B. Rainey (2014)** Cooperation, conflict and the major evolutionary transition to multicellularity. *Nature,* **in revision.**

***These authors contributed equally to this work

All authors contributed to the conception and design of the study. KH and CR performed research, undertook data analysis and prepared figures. All authors wrote the paper.

Alterations have been made for continuity in the context of this thesis.

# 4.1. Abstract

Cooperation is central to the emergence of multicellular life, however the means by which the earliest collectives maintained integrity in the face of destructive cheating types is unclear. One idea posits cheats as a primitive germ line in a life cycle that facilitates collective reproduction. Here we describe an experiment in which simple cooperating populations of bacteria were propagated under a selective regime that rewarded collective-level fecundity. Collectives reproduced via life cycles that either embraced, or purged, cheating types. When embraced, the life cycle alternated between phenotypic states. Selection fostered inception of a developmental programme that underpinned the emergence of collectives whose fitness, during the course of evolution, became decoupled from the fitness of constituent cells. Such development and decoupling did not occur when collectives reproduced via a cheat-purging regime. Our findings capture key events in the evolution of Darwinian individuality during the transition from single cells to multicellularity.

# 4.2. Introduction

Cooperation plays a central role in the evolution of multicellularity (Buss, 1987; Maynard Smith & Szathmáry, 1995; Bonner, 1998; Sachs *et al.*, 2004; Nowak, 2006; Okasha, 2006; Grosberg & Strathmann, 2007; Godfrey-Smith, 2009; Ratcliff *et al.*, 2012). Even under laboratory conditions, simple undifferentiated groups of cooperating cells readily evolve, however, such groups are often short lived: selection typically favours the evolution of cheats (Maynard Smith, 1988; Sober & Wilson, 1998; Strassmann *et al.*, 2000; Velicer *et al.*, 2000; Pfeiffer *et al.*, 2001; Rainey & Rainey, 2003). Cheats are cells that do not contribute toward group integrity, but nonetheless, take advantage of benefit that accrues from being part of a collective (Sachs *et al.*, 2004). In the absence of cheater suppression mechanisms, cheats may prosper to the point where integrity of any newly emerged group is compromised (Wade & Breden, 1980; Nunney, 1985; Michod, 1996; Santorelli *et al.*, 2008). The problem of cheating has led to the suggestion that the evolution of mechanisms for cheater suppression is a critical step in the transition to multicellularity (Frank, 1995; Michod, 1999; Queller, 2000; Santorelli *et al.*, 2008).

The evolution of simple groups of bacteria occurs repeatedly when populations of the bacterium *P. fluorescens* are propagated in spatially structured microcosms (Rainey & Travisano, 1998; Rainey & Rainey, 2003; McDonald *et al.*, 2009). Such collectives – "wrinkly spreader" (WS) mats – constitute a plausible starting position from which to explore mechanisms for cheater suppression in the evolution of simple cooperative groups (Rainey, 2007).

WS mats arise by spontaneous mutations from the ancestral "smooth" (SM) genotype (Bantinaki *et al.*, 2007; McDonald *et al.*, 2009). The mutations cause WS cells to overproduce a cell-cell glue (Spiers *et al.*, 2002; Spiers *et al.*, 2003; Goymer *et al.*,

2006; Bantinaki *et al.*, 2007) that prevents separation following cell division (Tarnita *et al.*, 2013). The net effect is a cellular mat that colonizes the air-broth interface. Although glue production is costly to individual cells, the trait spreads (Trivers, 1971) because the group of mat-forming cells reaps an advantage (access to oxygen) that is denied to individual cells (Rainey & Rainey, 2003).

The life span of WS mats is brief: selection acting on individual cells favours mutant types that cheat. Cheating cells are phenotypically SM and no longer produce adhesive glues (Rainey & Rainey, 2003); nonetheless they take advantage of the benefit that accrues from being part of the mat. In the absence of any mechanism of cheater repression, cheats prosper – ultimately weakening the fabric of the mat to the point where it collapses (Rainey & Rainey, 2003).

While cheats pose a significant problem, the tension between cooperating and cheating cells could fuel evolution (Rainey, 2007). Repression of cheats is not the only way towards greater collective functionality (Rainey & Kerr, 2010; Libby & Rainey, 2013). Consider a newly emergent WS mat. In the absence of a means of collective level reproduction, the mat, like soma, is an evolutionary dead end. The emergence of cheats however is guaranteed. While cheats may destroy the mat, they also stand as a means of mat reproduction, provided they can regenerate the mat. The cycling between WS collectives and SM cells has potential to generate a primitive life cycle (Rainey & Kerr, 2010) (Figure 4.1, lefthand panel) and with this the possibility that WS collectives might participate, even just marginally, as units of selection (Lewontin, 1970) in the process of Darwinian evolution.

# 4.3. Results

## 4.3.1. Embracing Cheats

To test the plausibility of the idea that SM cells might function as the seeds for a new generation of WS mats, we propagated mats under a regime in which SM cells were integral to mat reproduction (Figure 4.1, left hand panel) [this treatment is in stark contrast to the regime discussed in Chapter 3 and below in which cheats are purged (Figure 4.1, right hand panel)]. Each generation was founded by a single WS genotype (Figure 4.2). For collectives to persist WS mats had to remain intact (viable) until the end of Phase I, and be fecund (produce SM types). In addition, during the three-day Phase II period, SM cells were required to transition back to WS. On completion of the cycle a single colony of the most dominant WS type was transferred to a fresh microcosm ensuring new WS mats arise through a bottleneck and are thus re-established free of within-mat conflicts (Wolpert & Szathmáry, 2002; Godfrey-Smith, 2009).

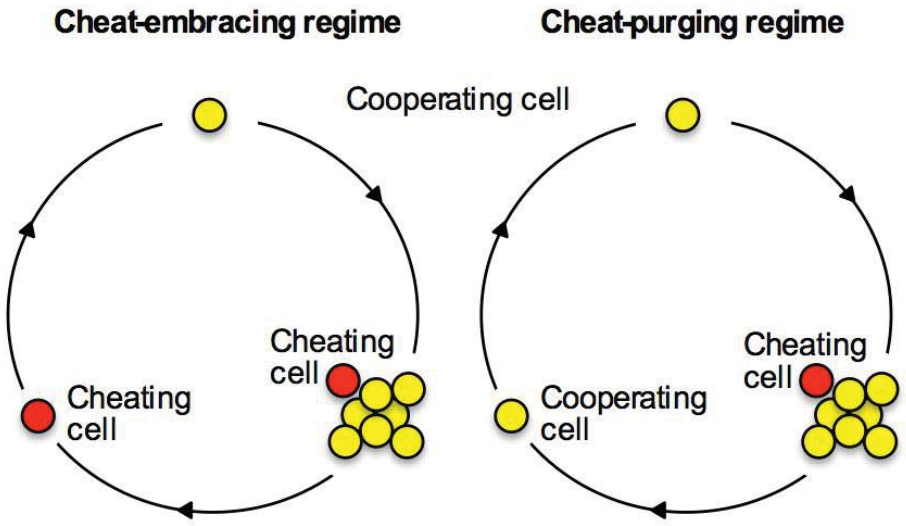


**Figure 4.1 Experimental regimes.**

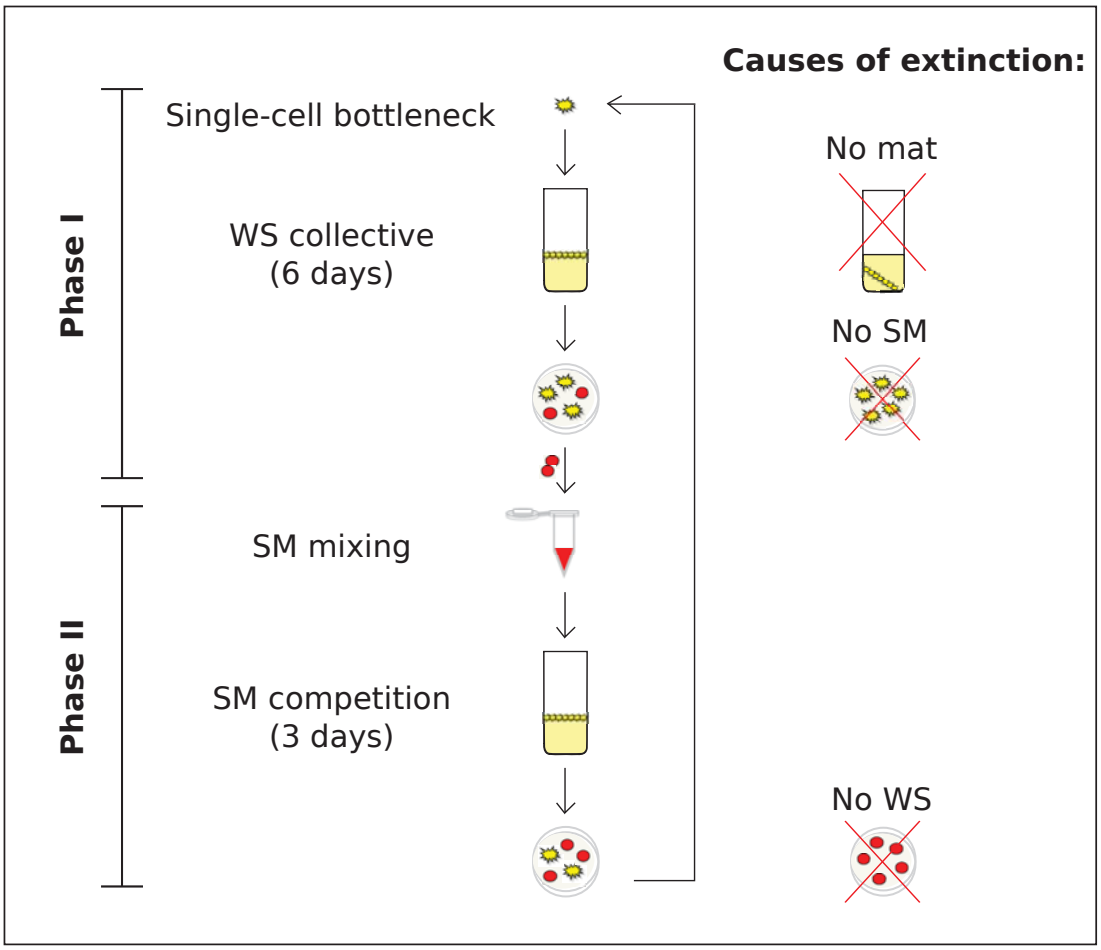


**Figure 4.2 Cheat-embracing experimental regime.** Each collective is founded by a single WS genotype (yellow). After six days in Phase I cells are harvested, plated and screened for SM colonies (red). Pooled SM colonies found Phase II. After three days cells are harvested, plated and screened for WS colonies. A single WS colony of the dominant type founds the next generation. To avoid extinction the WS mat of each line must be intact at the end of Phase I and WS types must have produced SM cells; by the end of Phase II, SM types must have produced WS cells.

Transitioning between collective and single cell phases relies initially upon mutation and poses significant challenges. In an initial experiment in which 120 collectives were required to repeatedly transition between WS and SM states, all collectives went extinct by the sixth life cycle generation. Extinction was primarily due to insufficient SM cell production before death of the mat at day six (Figure 4.3).

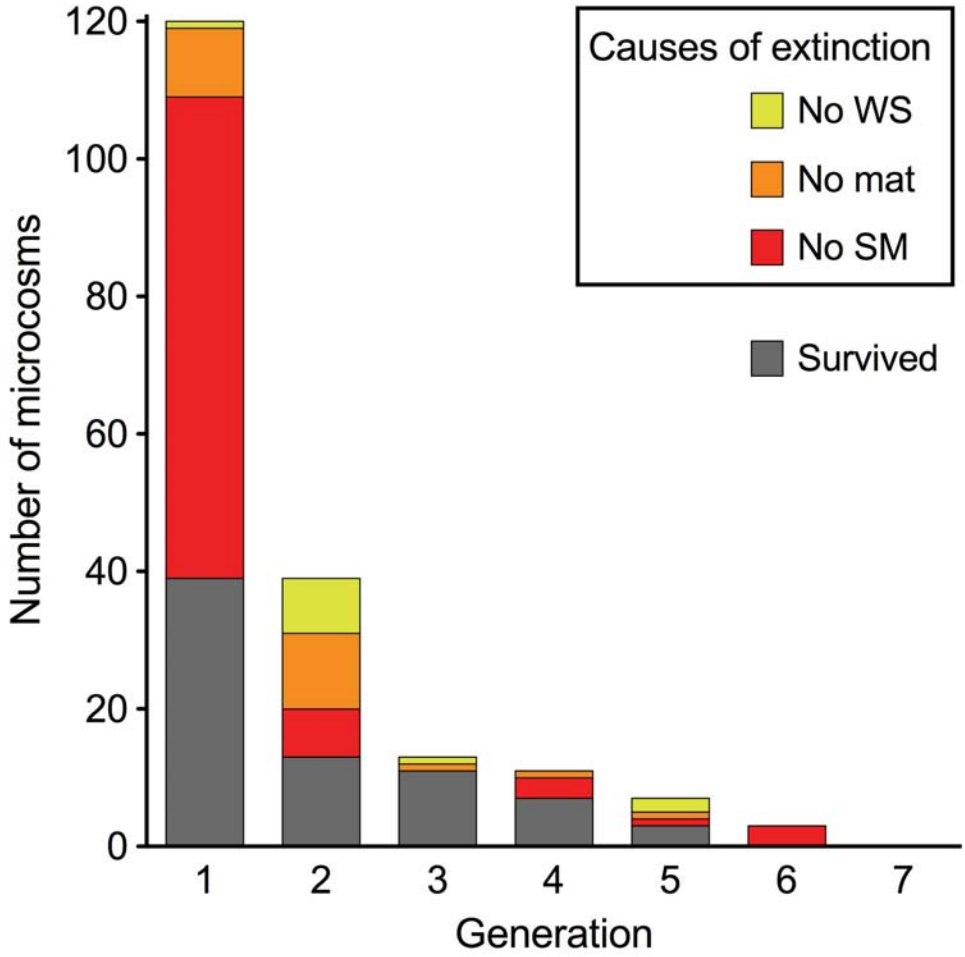


**Figure 4.3 Persistence of collectives and extinction dynamics.**

Given the prominent role of cheats and the requirement for mutation to transition each phase of the life cycle, the persistence of collectives through six generations is surprising: it indicates a capacity for innovation. Such a capacity might, under different circumstances, provide opportunity for evolutionary refinement to the point where cycling through phases could come under developmental control.

Most non-neutral mutations are deleterious and thus prone to eventually disrupt the life cycle. Persistence is therefore likely to depend on viable collectives having opportunity to export their success to a new microcosm by division of the lineage. Were the splitting of viable collectives to operate concomitantly with the elimination of

unsuccessful collectives then selection among lineages might allow the possibility that life cycle-enhancing mutations, which are beneficial in the long run, outrun life cycle-disrupting mutations (Leigh, 1977; Slatkin & Wade, 1978; Craig, 1982; Nunney, 1985, 1999).

## 4.3.2. Adaptive Evolution and Fitness Decoupling

To allow for selection to operate among collectives, we took 120 microcosms, each containing a single WS mat, and divided these into 15 populations, each comprised of eight collectives (Figure 4.4). Collectives that proved unviable (either due to mat collapse, or failure to complete the life cycle) provided opportunity for viable collectives to export their success to a new microcosm. Upon demise of a particular collective, a viable collective within the same replicate was chosen at random and allowed to replace the extinct type (Figure 4.4). Extinction occurred at high frequency resulting in ~5 replacements per generation (per replicate). After ten life cycle generations each population housed viable lines. Selection on collective viability – and concomitantly fecundity – was thus central to persistence.

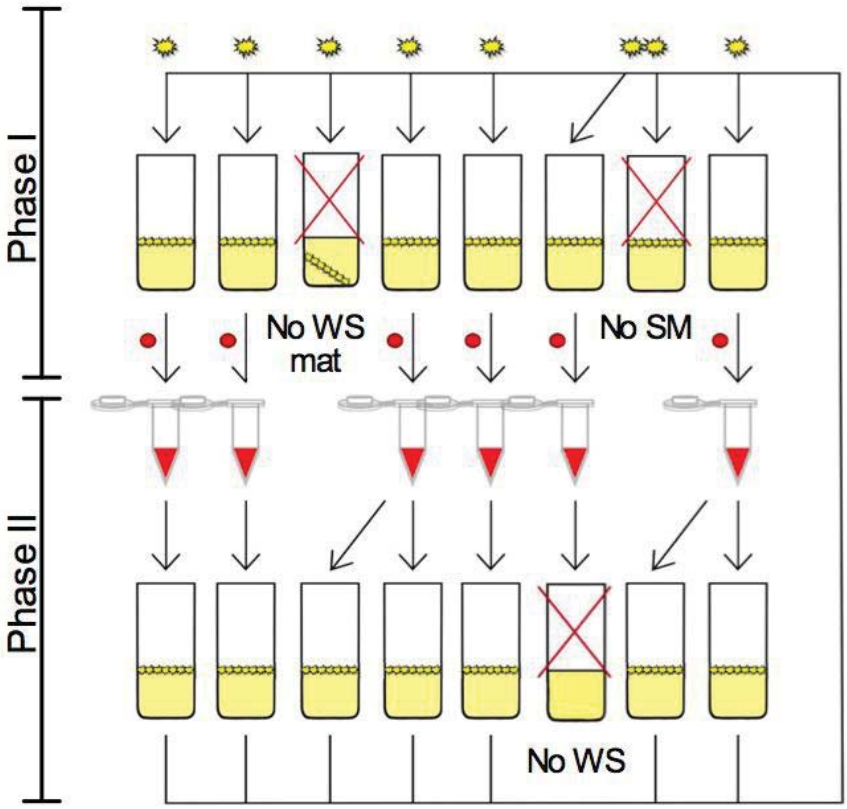


**Figure 4.4 Between-collective cheat-embracing experimental regime.** Collectives (N=120) are arranged as 15 replicate populations of eight collectives each (one replicate is depicted). The experiment involved a total of 2,400 microcosms and 140 days of selection. Extinction events provide opportunity for viable collectives to reproduce. Reproduction allows export of a viable collective to a new microcosm. Collectives marked for reproduction were chosen at random and replacements took place within the same replicate.

To determine whether evolving collectives were seen by selection the fitness of evolved collectives was determined relative to ancestral types. A single WS genotype representative of each population (of eight collectives) was taken at the end of the selection period (see Methods 2.2.4 and Figure 2.1). In addition, 15 independent WS genotypes were obtained one mutational step from the ancestral SM genotype, thus providing a "baseline" reference for ancestral fitness (see Methods 2.2.4 and Figure 2.1). All representative WS genotypes were competed against a *lacZ*-marked reference strain (Zhang & Rainey, 2007) (see Methods 2.3 and Figure 2.2, lefthand panel),

allowing the competitive performance of all ancestral and evolved types to be assessed against a single common genotype (Queller, 2000). Because our interest is in the outcome of selection at the level of both cycling collectives and individual cells (Damuth & Heisler, 1988), a measure of fitness at the two levels was obtained. Fitness of collectives was defined as the number of WS mat offspring left relative to the marked competitor (Figure 2.2, lefthand panel); cell fitness was assessed as the total number of cells contained within individual mats (irrespective of type) at the end of the Phase I period. Additional measures of cell-level performance are described below.

Fitness of evolved collectives – as determined by ability to leave mat offspring relative to ancestral types – improved significantly ($\chi^2$=4.262, df=1, *P*=0.039; Figure 4.5). This is consistent with an evolutionary response to selection and shows that when SM cells are integral to the reproduction of WS mats, collectives not only persist, but their ecological performance improves as a consequence of selection among collectives.

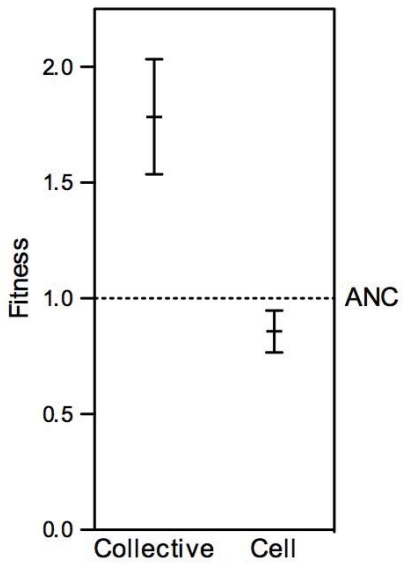


**Figure 4.5 Fitness of evolved collectives and their single cell constituents relative to ancestral (ANC) types.** Collective fitness increased significantly, whereas cell-level fitness decreased. Error bars are SEM, based on N=14.

While fitness of evolved collectives improved, cell fitness significantly decreased ($t_{79}$=3.092, $P$=0.0027; Figure 4.5). Success at the level of evolving collectives has come at a cost to the individual cells of which the collectives are comprised. This is a striking result and represents an important step in understanding how selection can transition to higher levels of individuality. The fact that fitness of the evolved collectives is no longer explicable in terms of the fitness of individual cells indicates that collective fitness has become decoupled from individual cell fitness. This is consistent with theoretical predictions that during major evolutionary transitions selection shifts from the lower (cell) to the higher (collective) level (Maynard Smith, 1988; Michod, 1999; Michod & Roze, 1999; Michod & Nedelcu, 2003; Okasha, 2006; Godfrey-Smith, 2009). With such a shift arises a new kind of biological individual whose emergence is likely to curtail the independent evolution of lower level entities (Godfrey-Smith, 2009).

## 4.3.3. Traits Underpinning Collective Improvement

Traits inherent in the individual cells must explain the improved reproductive capacity of collectives (Damuth & Heisler, 1988). To explore adaptations that contributed to increased fitness of collectives, life history properties were determined relative to ancestral types. Three replicate microcosms of each representative genotype were destructively sampled each day throughout phase I and II of the experimental regime and the frequency of each type determined. With interest in the possibility that selection might have optimised life history characteristics to suit the duration of each phase, the number of days of propagation was doubled in both Phase I (12 days) and Phase II (6 days).

Evolved collectives increased their capacity to generate the phenotype required for the next stage of the life cycle. This was evident in both phases: the new type was

produced earlier and more reliably compared to the ancestral baseline lineages ($\chi^2$=5.442, df=1, *P*=0.0197; Figure 4.6a; Appendix 8.2.2). Moreover it was maximal at day six suggesting optimisation to the periodicity of the selection regime. Enhanced capacity to generate each stage of the life cycle was not explained by an increase in total cell density (density of the ancestral collectives was greater than that of the evolved types ($F_1$=51.521, *P*<0.0001; Figure 4.6b; Appendix 8.2.2), the number of cells of the next stage in the life cycle ($F_1$=0.589, *P*=0.4431; Figure 4.6c; Appendix 8.2.2), nor by the proportion of cells of the next stage in the life cycle ($F_1$=1.701, *P*=0.1926; Figure 4.6d; Appendix 8.2.2). In fact the proportion of cells (and number of cells) marking the next stage of the life cycle was often higher in ancestral lineages (Figure 4.6c and Figure 4.6d).

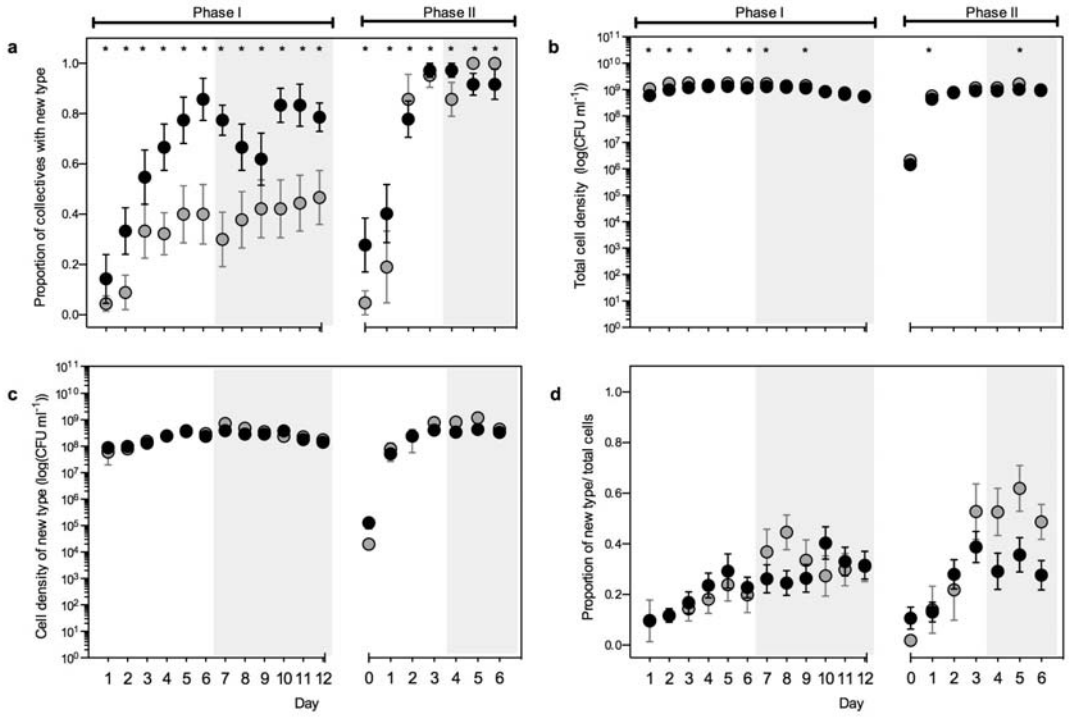


**Figure 4.6 Life history traits under the cheat-embracing regime.** (a) Proportion of collectives producing the morphotype required for the next phase of the life cycle (*i.e.* SM from WS during Phase I and WS from SM during Phase II). (b) Total cell density. (c) Cell density of the new type. (d) Proportion of the new cell type/total cells. Each circle represents the mean of 42-45 collectives (*i.e.* 3 replicates for each of the 15 ancestral and 14 evolved replicates), however, for c and d lines that failed to produce the required type were excluded. Ancestral = grey, evolved = black. Error bars are SEM, based on N≤15. * represents a significance level of P<0.05.

The level of SM occurrence in Phase I, and WS occurrence in Phase II, is strongly related to collective fitness (Figure 4.7a and Figure 4.7b) in both the evolved and ancestral types (evolved: $\chi^2$=12.324, df=1, N=14, *P*=0.0004; ancestral: $\chi^2$=22.801, df=1, N=15, *P*<0.0001; Appendix 8.2.3). Interestingly, five ancestral collectives with high fitness had a tendency toward early production of the next life cycle phase (Figure 4.7a). After selection, this trait was present in the majority of collectives (Figure 4.7b) despite there being no change in the proportion, or number of the respective alternative cell type (Figure 4.6c and Figure 4.6d).

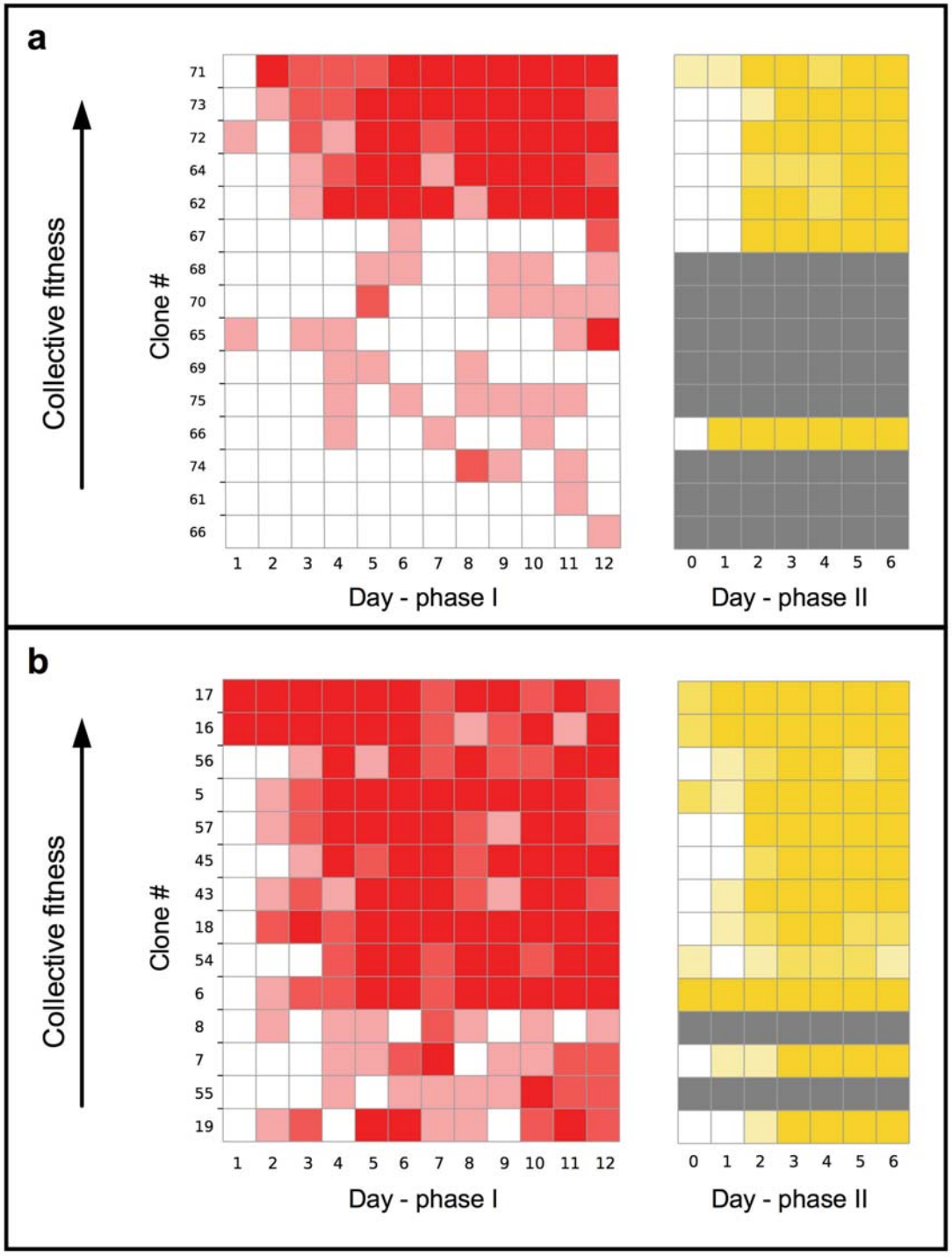


**Figure 4.7 Collective fitness and life cycle perpetuation.** Fitness of ancestral (a) and evolved (b) collectives and relationship with capacity to perpetuate the two phase life cycle. Data are a breakdown of data in Figure 4.6a. Colour intensity represents the proportion of microcosms harbouring the new type (white = absence of new type; dark red (yellow) = presence of SM (WS) in all microcosms. Collectives are ordered according to their fitness. Grey cells indicate loss of collectives due to extinction.

The increase in the frequency of occurrence (rather than the number) of the alternative cell type suggests that the transition rate between stages of the life cycle – a form of development – was the focus of selection. Such a finding is consistent with a simple mathematical model which shows that the possibilities for producing cells of the opposing type via evolution of increased rates of switching readily out paces any such possibilities arising from improvement in cell growth rate (Libby & Rainey, 2013). Nonetheless, to see whether growth rate had changed we determined the maximum growth rate of SM cells.

Compared to the growth rate of SM cells harvested from the ancestral lineages, the growth rate of SM cells harvested from the evolved cheat-embracing collectives did not increase ($t_{74}$=1.527, $P$=0.1315; Figure 4.8). This leaves increase in the rate of transition as the only possible explanation for the increased likelihood of detecting the new type in evolved collectives.

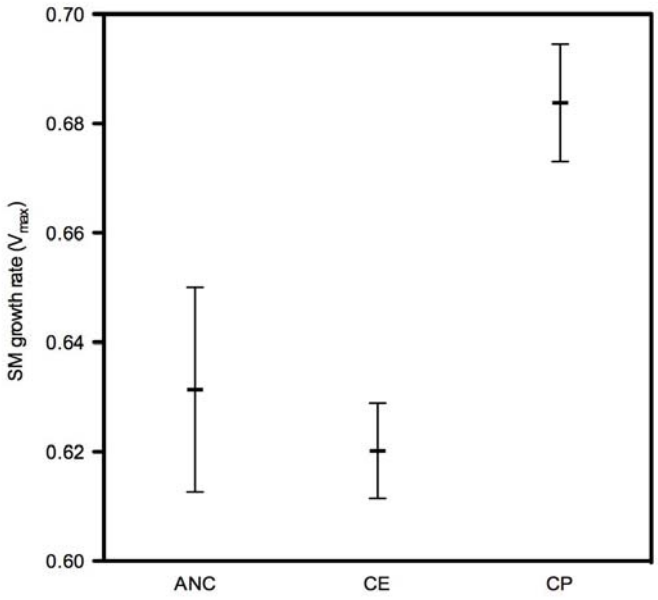


**Figure 4.8 Growth rate of SM.** Growth rate of the ancestral (ANC) and evolved SM types from the cheat-embracing (CE) and cheat-purging (CP – see below) regimes obtained from the representative genotypes. (ANC: N=81; CE: N=95; CP=81). Error bars are SEM, based on N≤15.

We examined the capacity for the fittest collectives to transition through phases of the life cycle. Three replicate populations of the two ancestral and evolved collectives with the highest fitness: 71, 73 and 16, 17, respectively, were founded by the representative WS type and plated to check for SM types. Whereas ancestral WS took 48 hours to generate detectable levels of SM, the two evolved WS collectives contained a mixture of WS and SM colonies, such that even at the time of initial inoculation, both types were present, with SM cells making up ~5% of the collective. SM colonies were then used to found collectives that were plated to check for WS types. Examination of the resulting plates showed a mixture of types. Significantly, in lines 16 and 17 (evolved collectives), the WS morphs from successive generations were phenotypically indistinguishable from the founding WS types (Figure 4.9). The high rate at which these lines transitioned between phases of the life cycle, combined with the phenotypic similarity between the states of successive generations, is indicative of an epigenetic switch (Beaumont *et al.*, 2009; Gallie *et al.*, 2014) (Figure 4.10). Such a switch would be highly advantageous because it would free the life cycle from reliance on mutation and mark a critical first step in the evolution of development (Buss, 1987; Libby & Rainey, 2013).

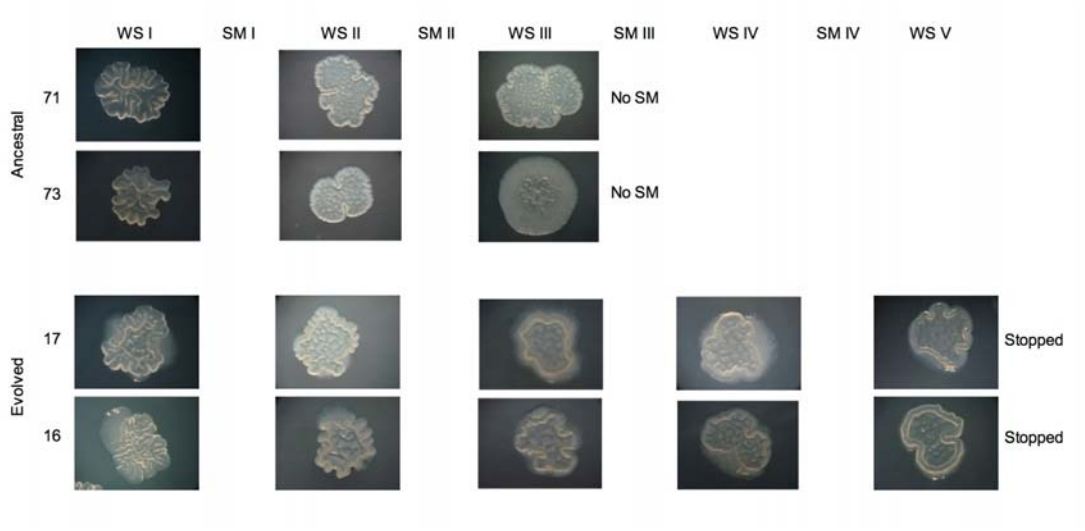


**Figure 4.9 Cycling through phases.** Ancestral and evolved collectives differ in their capacity to transition between phase of the life cycle. Ancestral lineages 71 and 73 completed two cycles before extinction through failure to produce SM, whereas evolved lines 16 and 17 completed five cycles before termination of the experiment. Colonies were photographed after 48 h growth. Notable in the evolved lines is the phenotypic similarity of WS colonies over time and the visible presence of zones of SM cells surrounding the central WS colony.

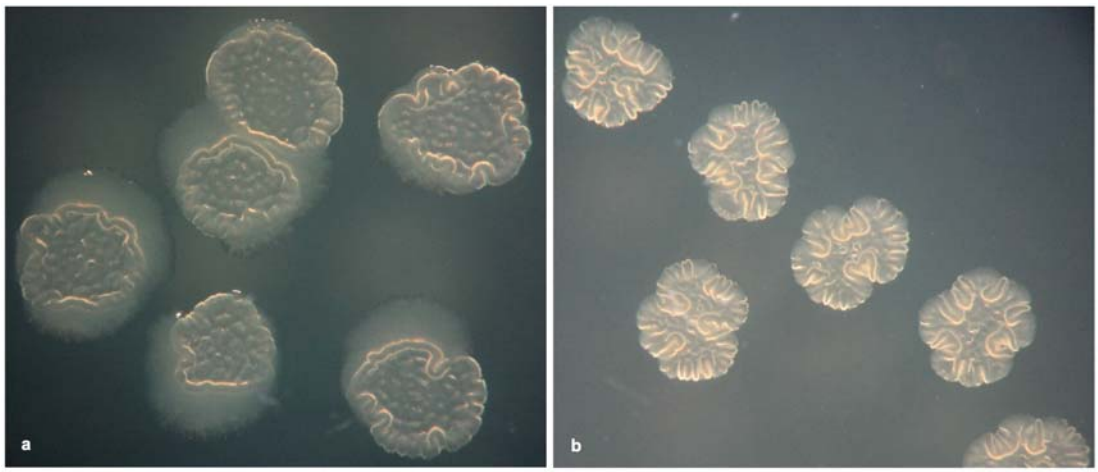


**Figure 4.10 Dimorphic colonies.** Photos of the fittest evolved (a) and ancestral (b) colonies (lines 17 and 71, respectively) from the CE regime. Colonies of the evolved type have a WS centre, but SM types at the periphery. No evidence of this dimorphism is evident in the ancestral WS colonies.

Regression and correlation analyses summarising the relationship between the traits of collectives and the single cells of which they are comprised are shown in Figure 4.11a Figure 4.11b (also see Appendix 8.2.3). Collective fitness is explained by

increased capacity to transition from WS to SM and not by improvement in the competitive performance of single cells. Increased collective fitness is therefore a product of selection at the level of the evolving collective.

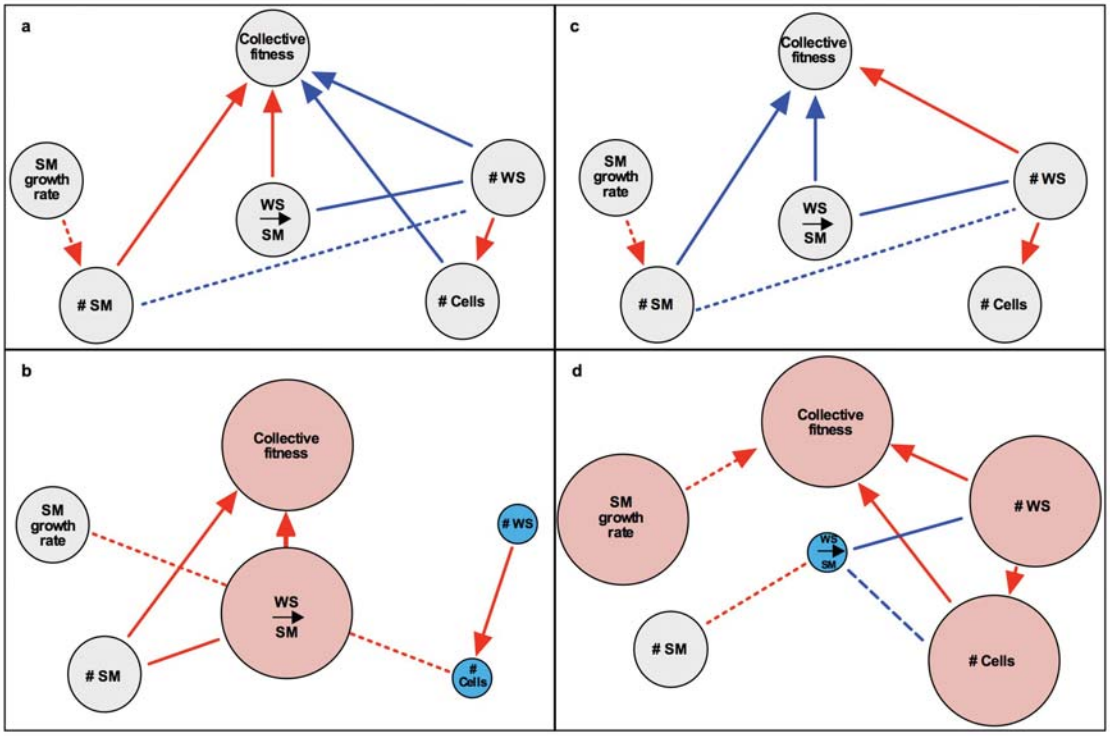


**Figure 4.11 Summary.** Summary of parameters describing collective and cellular properties and the relationship among these parameters in ancestral (a) and evolved (b) CE and ancestral (c) and evolved (d) CP populations. Traits in the evolved regimes (b, d) are depicted relative to their respective ancestral states (a, c): significant increase (large red circle), significant decrease (small blue circle), and no significant change (grey circle). WS→SM=SM occurrence. Arrows indicate significant regressions, and lines significant correlations between traits, dashed lines indicate trends ($0.05<P<0.09$). The colour represents the direction of the relationship: red: positive, blue: negative. The significance level is $P<0.05$. Individual cell properties displayed in (a) and (c) are identical for the ancestral state in both CE and CP regimes, but measures of collective fitness are regime-specific. Parameters that relate positively to collective fitness in the CE regime, negatively affect collective fitness in the CP regime, and visa versa (a vs. c). For example, in the CE regime, the number of SM cells and the rate at which WS cells give rise to SM cells positively regresses on collective fitness (red arrows (a)), whereas only the number of WS cells shows a positive regression with collective fitness in the CP regime (red arrows (c)). After 10 generations of between-collective selection the relationships between cell and collective-level parameters significantly altered in both CE and CP regimes (b and d). In the CE regime enhanced collective fitness is explained by a significant increase in the capacity to transition from WS to SM and is not explained by enhanced performance of single cells: the fitness of single cells either remained unaltered or declined. Increased collective fitness is therefore a product of selection at the higher (collective) level. In marked contrast is the CP regime (see below) where improved collective fitness is readily explained by changes in traits that improve the competitive ability of individual cells. Enhanced line fitness in the CP regime is thus likely a by-product of selection at the lower (cell) level.

## 4.3.4. Purging cheats

Reproduction of mats via the cheat-embracing (CE) regime, in conjunction with selection among collectives, decoupled fitness, effected a shift in selection to the higher level, and fostered initial steps toward the emergence of development. The causative factor is of central interest. While it is possible that a life cycle of two phases is key, reproduction via a bottleneck phase, combined with between-collective selection, may be sufficient. For example, it is conceivable that the fitness of WS mats propagated via a bottleneck without requirement to pass through an alternate (SM) phase could improve as a consequence of the production of more substantive adhesive polymers that come at the expense of individual cell performance. To test for such a possibility we performed a control experiment in which WS mats were propagated under a "cheat-purging" (CP) regime. The sole difference between the CP and CE regimes is the cell that passes through the bottleneck: under the CP regime the cell passing through the bottleneck is a WS cell (Figure 4.1, righthand panel). Reproduction via the CP regime is analogous to fragmentation.

Conventional wisdom predicts the CP regime to have greatest evolutionary potential. Indeed, after 10 generations of lineage selection (Figure 3.1) fitness of evolved collectives improved significantly ($\chi^2$=15.737, df=1, $P$<0.0001; Figure 4.12; Appendix 8.2.1). However, this was accompanied by a similar improvement in the fitness of single cells ($t_{86}$=2.132, $P$=0.036; Figure 4.12; Appendix 8.2.1). Improvement in the fitness of evolved collectives is thus explained by improvement in individual cell performance. The striking response observed under the CE regime can therefore be attributed to a life cycle of alternating phases.

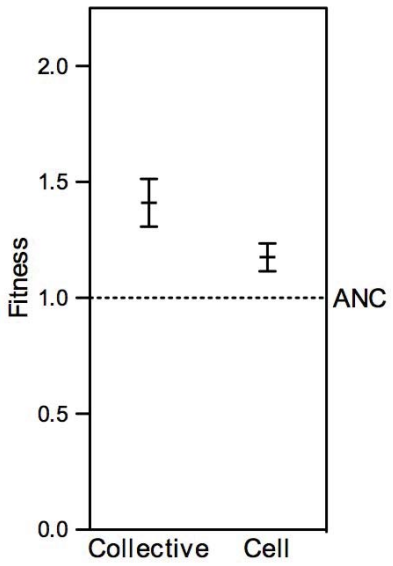


**Figure 4.12 Fitness of evolved CP collectives and their single cell constituents relative to ancestral types.** Both collective and cell-level fitness increased significantly. Error bars are SEM, based on N=15.

To explore adaptations of the CP regime that contributed to increased collective performance, life history properties were determined relative to ancestral types, as for the CE regime. No improvement was seen in the capacity for WS types to transition to SM. A significantly lower proportion of the evolved collectives produced SM ($\chi^2$=8.199, df=1, *P*=0.0042): SM types took longer to arise (Figure 4.13a) hinting at the possibility that cheater suppression might have begun to evolve under this regime (Figure 4.13b-d).

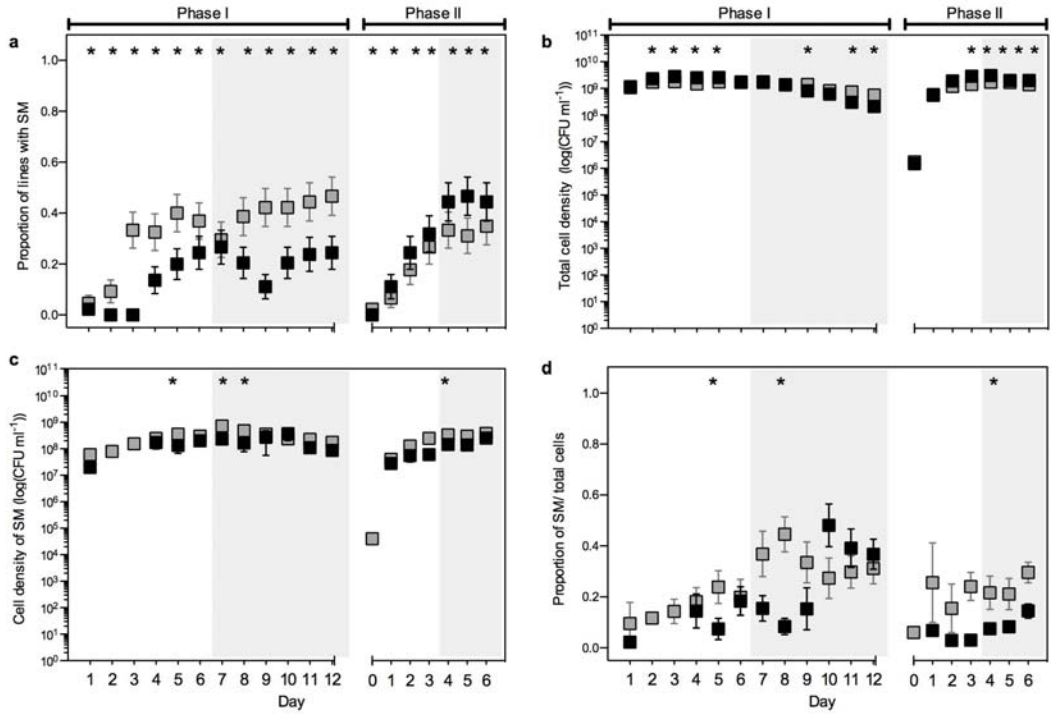


**Figure 4.13 Life history traits under the CP regime**. (a) Proportion of collectives producing SM (note that this data is identical to panel (a) of Figure 3.5) (b) Total cell density. (c) Cell density of the new type. (d) Proportion of SM cell types/total cells. Each circle represents the mean of 45 collectives (*i.e.* 3 replicates for each of the 15 collectives), however, for c and d collectives that failed to produce SM were excluded. Ancestral = grey, evolved = black. Error bars are SEM, based on N≤15. * represents a significance level of P<0.05.

Regression and correlation analyses summarising the relationship between traits of collectives evolved under the CP regime and the single cells of which they are comprised are shown in Figure 4.11c and d (Appendix 8.2.3). Under the CP regime enhanced collective fitness is explained by changes in traits that improve the competitive ability of individual cells. Enhanced collective fitness under the CP regime is thus most likely a by-product of selection at the lower (cell) level.

## 4.4. Perspective

Multicellular organisms are descendants of once free-living cells (Buss, 1987; Maynard Smith & Szathmáry, 1995; Bonner, 1998; Grosberg & Strathmann, 2007). By virtue of their capacity for differential reproduction, ancestral free-living cells were units of selection (Lewontin, 1970). As such they evolved by Darwinian processes (Okasha, 2006; Godfrey-Smith, 2009). During the transition to multicellularity, collectives of cells emerged that came to participate in Darwinian processes in their own right (Maynard Smith, 1988; Michod, 1999; Okasha, 2006; Godfrey-Smith, 2009). The essential ingredient was a means of collective reproduction (Maynard Smith, 1988; Godfrey-Smith, 2009). This most seminal of Darwinian properties emerges afresh at each transition and requires evolutionary explanation (Griesemer, 2000). Here we have shown that cheating cells – those types seemingly most detrimental to the persistence of newly formed cooperative entities – can function as a germ line to facilitate reproduction of collectives. In assuming the role of germ line, cheats not only solve the very problem they pose, but underpin the emergence of a new level of biological organisation – a level comprised of collectives with properties sufficient to participate in Darwinian evolution (Lewontin, 1970; Michod, 1999; Okasha, 2006; Godfrey-Smith, 2009).

While emphasising distinctive roles for cooperating and cheating cells, similar two-phase life cycles could conceivably emerge from a variety of simple founding states in which the nascent multicellular organism comprises both group-forming and individually-acting cell types (Libby & Rainey, 2013). Such founding states have implications for understanding the origins of complex life cycles, including those that involve development of a new organism from a single cell (Godfrey-Smith, 2009; Libby

& Rainey, 2013, 2013), and where the single cell is derived from a dedicated germ line that is distinct from soma (Buss, 1987).

Mode of reproduction also has profound implications for how selection sees nascent multicellular organisms (Stearns, 2007; Libby & Rainey, 2013). When collectives reproduce via a two-phase life cycle, success depends on the existence of both collective and individual cell states. Each state is in effect a different attribute of a single and altogether new kind of organism whose evolution stands to be unified through a developmental programme (Wolpert, 1990). Selection acting at the higher level favours those collectives most able to perpetuate the life cycle (Libby & Rainey, 2013). Because the properties that engender success at the higher level are not equivalent to properties yielding successful individual cells, improvement of the collective comes at a cost to the cell. Decoupling of fitness between levels is a signature of this conflict. When reproduction of collectives is via fragmentation (a single phase life cycle), the traits that yield success at the higher level are largely those that determine success at the lower level. The focus of selection remains, at both collective and single cell phases, the individual cooperating cell. This offers limited opportunity for the emergence of new kinds of biological individuality because properties of higher and lower levels remain aligned. It is possible that the prevalence of complex life cycles among extant multicellular organisms reflects the fact that such cycles, on first emergence, delivered to selection collectives with greatest propensity to participate in Darwinian evolution.

# Chapter 5.
# Stage Three: The Evolution of Complexity

A ‘Darwinian individual’ is an entity upon which natural selection can act, and must therefore satisfy the three conditions of variation, heredity and associated fitness differences (Section 1.1; Lewontin, 1970). Chapter 4 described an experiment in which populations of the bacterium *P. fluorescens* acquired Darwinian individuality in a long-term evolution experiment that implemented competition between groups of cells. Collectives reproduced via a single-celled bottleneck in two selection regimes, however in one regime this bottleneck was an alternative cell type. Crucially, populations in both regimes exhibited the classic features of individuals (heritable variation between groups of cells resulting in their differential reproduction), however only populations with a germ line acquired true Darwinian individuality. This experiment exposes the difficulties encountered with such a simplistic view of evolutionary transitions. Recognition of a true transition in individuality calls for careful consideration of selective processes occurring at both levels – a multilevel selection approach.

An evolutionary transition in individuality requires natural selection to operate at the higher level – the level of the collective. Selection at the higher level is dependent on the emergence of characters that are causally associated with fitness at that level (De Monte & Rainey, 2014). Identification of such a process is problematic because apparent selection at one hierarchical level may in fact be a by-product of selection

occurring at another level. This 'levels of selection problem' is discussed in Section 5.1. A critique of the literature reveals that other experimentalists have not adequately addressed the levels of selection problem, leaving the field vulnerable to misleading claims. Various approaches to the levels of selection problem are explored in Sections 5.1.1-5.1.4 and applied to the experiment in Chapter 4 where appropriate.

Section 5.2 seeks to identify factors that led to a transition in individuality in the *P. fluorescens* populations. Collective-level competition was imposed in both regimes, yet the levels of selection problem was only solved for populations with a germ line because the fitness of the collectives became decoupled from the fitness of the cells. Section 5.2.2 describes the commonly held notion that the *coupling* (rather than *decoupling*) of the lower and higher level fitnesses controls cheating, which, according to this viewpoint, is an essential prerequisite for the evolution of individuality. An alternative hypothesis – and one that is substantiated by the results from Chapter 4 – is that selection operating at the higher level de-Darwinizes the lower level (Section 5.2.3). A third possibility is that the growth pattern of WS and SM cells within a mat inadvertently resulted in a lower carrying capacity when the transition rate increased (Section 5.2.4).

Fitness decoupling, and the resulting transition in individuality, was observed only in the *P. fluorescens* populations with a germ line. Is the association between the germ line and fitness decoupling specific to this experiment or a feature of all life cycles with germ lines? Decoupled fitness resulted from a germ line that was brought about by transitioning between two cell states. This transitioning itself is due to negative frequency-dependent selection, in which selection favours rare cell types. The cheating (germ line) cells in the *P. fluorescens* populations rise in frequency due to frequency-dependent selection, so it follows that defection must be embraced to decouple fitness

and transition individuality. Section 5.3 explores whether the remarkable results from Chapter 4 can be generalized to devise broad rules for transitions in individuality.

# 5.1. The Levels of Selection Problem

At any level of the biological hierarchy where reproduction is found there is the possibility of a Darwinian process. Collective reproduction is necessary but not sufficient to ensure natural selection at the level of the collective. The essence of natural selection at a particular level is that the character under selection must covary with fitness at that level. However, covariance is a statistical concept and not a causal one. There *may* be a causal (selective) influence of a given trait on the fitness of a collective if the trait covaries with collective fitness, or this covariance may be spurious (Okasha, 2012). Therefore, considering the possibility of natural selection independently at each level is inadequate because an apparent Darwinian process at one level can be a side effect of selection operating at another level – a 'cross-level by-product' (Sober & Lewontin, 1982; Okasha, 2006; Shelton & Michod, 2014). Okasha (2006, p.78-79) frames the levels of selection problem as follows:

> *"When is a character-fitness covariance indicative of direct selection at the level in question, and when is it a by-product of direct selection at another hierarchical level?"*

The levels of selection problem is related to G.C. Williams' (1966) distinction between 'group adaptation' and 'fortuitous group benefit'. In his well-known example of this point, Williams contrasts herds of deer that run at different speeds. Herds made up of faster deer grow and 'reproduce' (split into new herds) more quickly because slower deer are more susceptible to predation. Consequently, selection for fast deer within herds leads to increasingly faster herds of deer – a fortuitous group benefit. The fitness of the herds (the rate at which they reproduce) covaries with average running

speed of the herd, however this covariance is merely a cross-level by-product of within-herd selection for faster deer - not a result of group adaptation.

The cross-level by-product is easily detected in the hypothetical example above, however the levels of selection problem is not often addressed in experimental evolution literature. A recent experimental paper claims to have evolved 'multicellular organisms' from the unicellular yeast *S. cerevisiae* (Ratcliff *et al.*, 2012). Selection for rapid settling in liquid media resulted in the evolution of a clustering 'snowflake' phenotype. These collectives of cells reproduce via multicellular propagules that separate from the parent snowflake by apoptosis (cell death) of a single cell at the break point. The selective regime favoured larger snowflakes and higher rates of apoptosis – resulting in higher rates of collective reproduction. If snowflakes vary in traits that affect their fitness, and if this variation is heritable, then it is possible that these traits have evolved in response to between-snowflake selection. However, to claim that collective-level selection – and a transition in individuality - has occurred, it is necessary to show that the observed adaptations not only improve the fitness of the snowflake, but that this improvement is not a by-product of selection acting at the cell-level – a fortuitous group benefit. In this experiment, cell fitness also increased and was correlated with rate of apoptosis. By forming snowflakes that produce a higher proportion of altruistic cell types, recipient cells can grow faster than their non-cooperative ancestors. While it is possible that collective-level selection may have contributed to the increased rate of apoptosis, Ratcliff *et al*. did not partition the evolutionary change due to selection at the level of the snowflake and change due to selection at the level of the cell. Furthermore, this article is an example the lack of semantic continuity between philosophers, theorists and experimental biologists working in the field of evolutionary transitions. In Section 1.4.1 I propose clear

distinctions between the terms ‘cooperation’, ‘individuality’ and ‘organism’ and their application to any level of the biological hierarchy, including multicellularity. According to this framework it is clear that the evolution of snowflake clusters in *S. cerevisiae* is a remarkable example of the evolution of multicellular cooperation, not ‘multicellular organisms’.

Identifying an evolutionary transition in individuality requires some caution because a character-fitness covariance at a particular hierarchical level is not necessarily indicative of selection operating at that level. Various methods have been proposed for determining the hierarchical level(s) at which the association between a trait and fitness is causal, rather than merely statistical. The following sections (Sections 5.1.1-5.1.4) describe several approaches to tackling the levels of selection problem with a focus on the *P. fluorescens* populations described in Chapter 4.

## 5.1.1. Supervenience

The supervenience argument maintains that genuine collective-level selection is impossible because all properties of collectives depend on those of their constituent particles. The underlying particle characters ultimately determine both the fitness and character of collectives; the whole ‘supervenes’ on its parts (summarised in Okasha, 2012). The argument then follows that there can never be a causal link between a collective’s character and its fitness because *all* causality is at the particle level (Figure 5.1). The supervenience argument against collective-level selection renders the levels of selection problem irrelevant because it makes cross-level by-products ubiquitous.

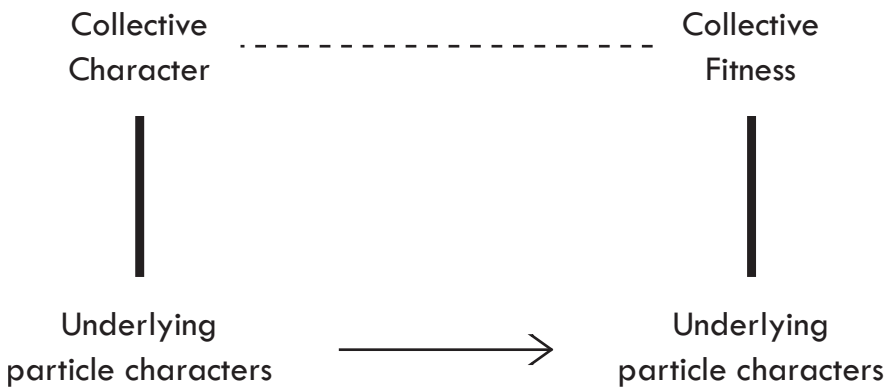


**Figure 5.1 The supervenience argument against collective-level selection.** The solid arrow represents a causal link, whereas the dotted line indicates a spurious correlation. Thick vertical lines represent the 'supervenience' of the higher property on the lower. Any apparent causal relationship between collective character and fitness is reducible to causal processes acting at the particle level. Adapted from Okasha (2012).

The supervenience argument does not provide an account of how the biological hierarchy itself came into existence. The very existence of a biological hierarchy is evidence of transitions from collectives to integrated units of selection. It follows then that at some point during the evolutionary history of the hierarchy, selection (or fitness) must have been transferred up the hierarchy in order to transform collectives into units of selection. Even the reductionist views of Dawkins and others, which maintain that genic selection can account for all evolution (see Section 1.2), are not congruent with the supervenience argument because genes themselves are collectives of lower level molecules whose evolution requires an explanation. Collective-level selection must be considered in order to understand the origin of genes.

It is clear that the supervenience argument against collective-level selection is not a practical framework for investigating evolutionary transitions. Okasha (2006, 2012) reasons that the argument only shows that a character-fitness covariance at the higher level must be a side effect of "*some lower level causal processes or other*, but not necessarily lower level *selection*". Certainly everything in existence can be explained by its constituent particles, however reducibility to lower level *selection* is what is

important to evolutionary biologists. The underlying particle characters on which collective fitness supervenes do not necessarily include particle *fitness*, so it does not follow that collective fitness always has a lower level *selective* explanation. Therefore, Okasha suggests that the concept of a cross-level by-product should be restricted to a character-fitness covariance at one level that results from *selection* at another level.

I agree with Okasha's statement that the levels of selection problem is exactly that – a question of at what level selection is operating, rather than at what level any explanation for a collective character exists. However, I would extend this notion by suggesting that if the level of *selection* is what matters, then characters or traits need not be assigned to any particular level. The distinction between collective character and particle character is unnecessary and can lead to debates that detract from the real issue of the selective cause of the character in question. It makes no sense (and in fact invokes circular logic) to utilize the terms 'collective character' or 'particle character' prior to determining the level at which this character was selected. If a given character is not assigned to a particular hierarchical level, supervenience discussions over whether it is reducible to particle characters are irrelevant. The relevant factor in determining the level of selection is the level at which a given *undesignated* character is causally associated with fitness.

In the absence of level classifications for characters, the relationships depicted in Figure 5.1 can be modified to those in Figure 5.2. When there is a correlation (and perhaps a causal relationship) between particle fitness and collective fitness, it is difficult to determine whether any statistical link between a character and either level of fitness is causal. A character-fitness covariance at the level of the collective may - or may not – supervene on a character-fitness covariance at the particle level. The statistical link between a character and collective fitness may merely be a spurious

association caused 'from below' by selection acting on this character at the particle level, *i.e.* the character is selected because of the causal effect it has on particle fitness, not collective fitness.

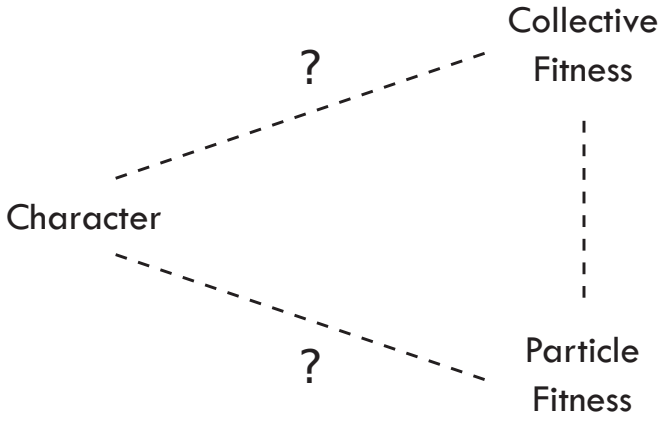


**Figure 5.2 Correlation versus causal links between character and collective and particle fitness.** The level of selection for a given character can be difficult to determine when a positive association exists between collective and particle fitness.

## 5.1.2. The Godfrey-Smith Framework

The essentialist search for 'necessary and sufficient' conditions to categorise evolutionary phenomena is often futile because of the dynamic nature of the biological world, particularly evolutionary transitions. Peter Godfrey-Smith (2009) (hereafter PGS) provides a highly intuitive method for visualising possible cases of Darwinian individuality as a gradient. This conceptualisation of the field eliminates the need to quibble over whether any given biological example meets a certain threshold for individuality.

### 5.1.2.1. Godfrey-Smith on Individuality

PGS defines a Darwinian individual as a member of a Darwinian population: a collection of entities that undergo evolution by natural selection. A transition in individuality therefore involves the appearance a new kind of Darwinian population. These populations do not suddenly appear as unequivocal collections of individuals, but instead exist within a wide spectrum of possible Darwinian populations. Populations residing in the large 'grey area' of the spectrum are perhaps those populations currently undergoing an evolutionary transition – they are 'mid-transition' and have yet to complete Stage Two and solve the levels of selection problem.

The gradient of populations ranges from marginal, to minimal, to paradigm Darwinian populations. The very permissive 'minimal concept' of a Darwinian population features three elements that closely resemble Lewontin's (1970) classic requirements for individuality (Section 1.1): (1) variation in individual character, (2) which affects reproductive output, and (3) which is heritable. The category of 'paradigm Darwinian populations' is the subset of the minimal concept area of the spectrum that can produce novel and complex organisms. If only paradigm Darwinian populations can evolve complexity (Stage Three), then according to the framework outlined in Section 1.4.2 it is only this subset of populations that can be said to have completed the transition to individuality (Stage Two). 'Marginal Darwinian populations' reside at the opposite end of the spectrum and outside the minimal concept boundary because they do not clearly satisfy all three minimal requirements. These marginal populations have a partially Darwinian character but cannot complete the evolutionary transition to individuality.

PGS introduces a range of parameters that can be applied to real biological populations in order to determine their position in the spectrum of Darwinian

populations. The extent to which reproductive differences of members of a population depend on their intrinsic character is captured by the parameter *'S'* (for 'supervenience'). Intrinsic features of an individual are those that are not altered by other individuals in the population. By this definition, the transition rate in the *P. fluorescens* collectives is an intrinsic character (upon which collective fitness highly depends) because it is not affected by the existence or arrangement of the other collectives in the higher-level population. An evolving population exhibits high *'C'* ('continuity') when small changes in an individual's character lead to small changes in fitness. The *C* parameter is related to Sewall Wright's famous 'fitness landscape' (S Wright, 1932). High values of *C* correspond to a smooth landscape in which similar properties are associated with similar fitnesses, as opposed to a rugged landscape that results from similar features associated with very different fitness values (low *C*). The heritability parameter *'H'* distinguishes between reliable and unreliable inheritance of characters, while *'V'* represents the abundance of variation present in a population. In the experiment outlined in Chapter 4, the mixed regimes exhibit much less variation between collectives compared to the non-mixed regimes. Inclusion of the *V* parameter in the spectrum of Darwinian populations makes it apparent that the higher-level populations in the mixed regimes are less 'Darwinian' than those in the non-mixed. Finally, a measure of the strength of competition, $\alpha$, encapsulates the extent to which the reproductive success of one individual affects that of another. PGS suggests that paradigm Darwinian populations have high values of $\alpha$. The *P. fluorescens* populations exhibit strong competitive interaction with respect to reproduction ($\alpha$): the death of one collective has a direct causal effect on another's reproduction. This is in contrast to the within-collective control regime imposed in Chapter 4 in which there was no

replacement of failed collectives: $\alpha$ had a value of 0 and the population of collectives was ultimately extinguished.

PGS introduces a spatial tool that enables us to easily visualise the spectrum of possible Darwinian populations in a 3D 'Darwinian Space' displaying the relationships between these evolutionarily important parameters (Figure 5.3). High values of *H*, *C* and *S* correspond to paradigm Darwinian populations. The list of parameters described above is incomplete. Population size, population structure, strength of selection, sex and niche construction are also important variables in an evolving population. However, in this discussion we are interested in discovering which of these factors make a tangible difference to the Darwinian nature of a population – what are the 'difference-makers'? (Calcott, 2011). PGS suggests that by assuming that all the other parameters have high values on various unseen dimensions, we can focus on the difference-making role of a few key elements. For example, low *S* indicates that reproductive differences are uncoupled from differences in intrinsic character and selection is instead dependent on extrinsic factors. This is the case for populations of human cells: a character-fitness covariance barely exists at the lower (cell) level, whereas the higher-level populations (of humans themselves) - are paradigm Darwinian populations that exhibit strong character-fitness covariance (high *S*). If populations with low *S* also exhibit low *C* then selection is essentially random – the ultra-rugged landscape can lead to the fixation of characters that are not necessarily associated with high fitness – the population evolves by drift rather than by natural selection. In populations with low *H*, paradigmatic Darwinian evolution cannot occur because adaptations cannot accumulate before they disappear again due to mistaken transmission. The low *H* corner of Darwinian space is perhaps the most difficult to overcome during an evolutionary transition in individuality because of the collective reproduction problem introduced in Section 1.6.1. A certain

amount of biological complexity is required before an inheritance system can be reasonably reliable, however paradoxically this complexity is composed of adaptations that must evolve by paradigmatic natural selection. The life cycle experiment described in Chapter 4 attempted to overcome this 'error catastrophe' by providing a mechanism for reproduction of nascent multicellular collectives.

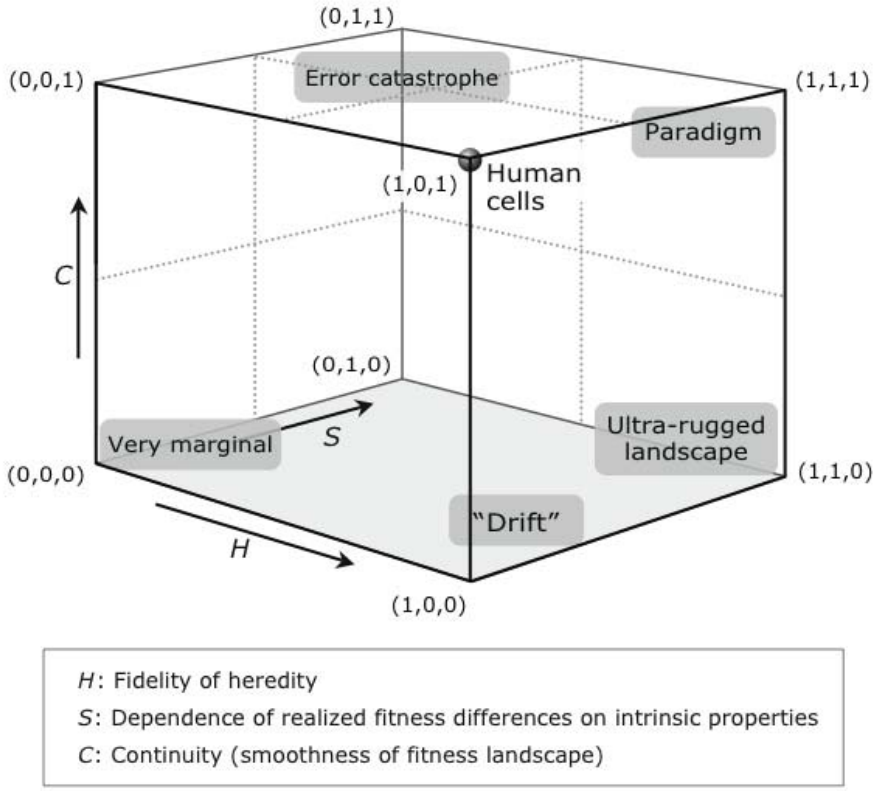


**Figure 5.3 A Darwinian space.** From (Godfrey-Smith, 2009).

## 5.1.2.2. Godfrey-Smith on Collective Reproduction

The numerous problems presented by the notion of collective reproduction have been discussed in Section 1.6.1. The particular challenge of relevance to major evolutionary transitions is to identify which are cases of reproduction of collectives, and which are cases of growth of collectives resulting from reproduction and structural

organization of their particles. PGS recognizes that there are more than these two distinct alternatives and treats the possibility of collective reproduction itself as a gradient of paradigm to marginal forms. A Darwinian population may be a marginal one if the individuals that make up the population are located at the marginal end of the reproduction spectrum. During a transition in individuality, a marginal Darwinian population may initially be composed of collective entities that can hardly reproduce themselves reliably, but are nevertheless able to evolve within Darwinian space towards a more paradigm population.

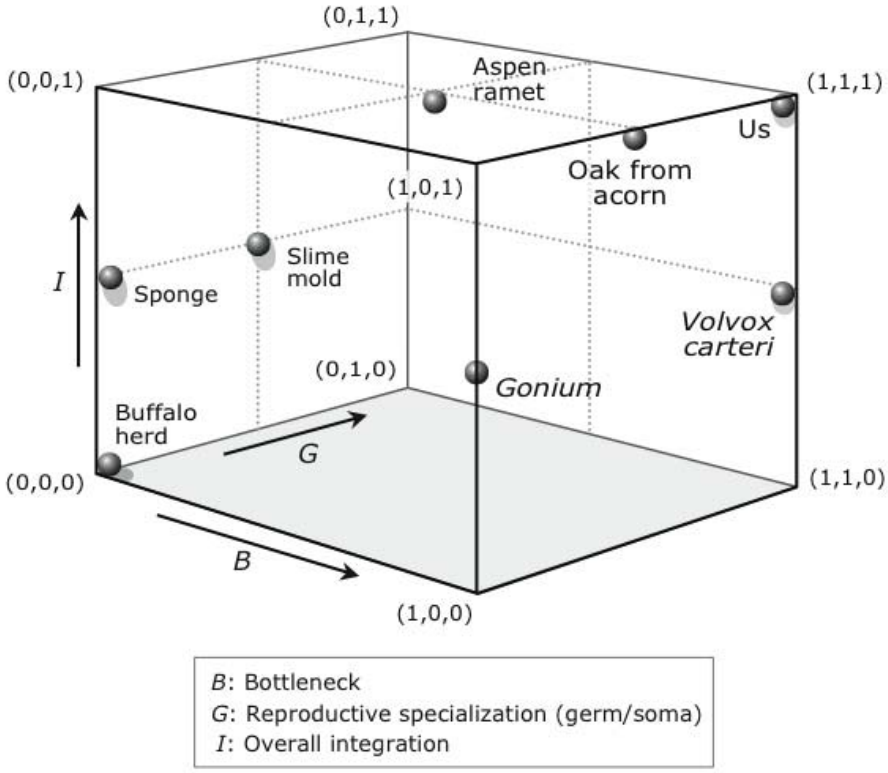


**Figure 5.4 Cases of collective reproduction.** From (Godfrey-Smith, 2009).

A spatial illustration of the reproduction spectrum (Figure 5.4) displays three parameters that characterise paradigm examples of collective reproduction. *'B'* stands for 'bottleneck', and represents the extent of narrowing of a collective during each

generation – distinguishing reproduction from growth. The 'germ line' parameter *'G'* conveys the degree of reproductive specialisation of parts. See Section 1.6.2 for a discussion on the importance of bottlenecks and germ lines during multicellular reproduction. The *P. fluorescens* collectives in the experiments described in Chapter 4 have the highest possible value for *B* (a single-celled bottleneck). However, only the collectives subjected to the cheat-embracing selective regime in have high values of *G*, while those in the cheat-purging regime have no reproductive specialisation: *G*=0. Conversely, the so-called 'multicellular organisms' that emerged in Ratcliff *et al.*'s (2012) yeast settling experiment have evolved a curious mechanism for reproduction of collectives with high *G* and low *B* (described above in Section 5.1). Godfrey-Smith (2011) contends that such cases are rather implausible because there is no advantage to maintaining a germ line when any within-organism genetic conflict would nonetheless be inherited from previous generations via a multicellular propagule.

The 'integration' of the collective is summarised in an overall sense by the *'I'* parameter, which encompasses features such as the extent of non-reproductive division of labour, the mutual dependence (loss of autonomy) of parts, and the maintenance of a boundary surrounding the collective. Both the cheat-embracing and cheating-purging collectives in Chapter 4 have fairly low values of *I*. The cooperation required to form WS mats is a kind of integration in the form of mutual dependence of parts - though the parts that are dependent upon each other (WS cells) are identical. However, the selective advantage generated by the mat is a within-collective advantage (access to oxygen denied to those cells outside of the mat) and therefore the mat does not contribute to the overall integration of a collective. On the other hand, the glass walls of the microcosm contribute to collective integration by artificially imposing a surrounding boundary. A mat was required to be present on Day 6 of both selective regimes, so in

this sense a between-collective selective advantage resulted from this basic level of collective integration (*I*).

Subsequent to between-collective selection, some *P. fluorescens* collectives may have reached a higher level of integration (*I*) due to the evolution of developmental regulation. There is some evidence for this in the cheat-embracing lines in the form of an elevated transition rate that is unlikely to be caused by an increased mutation rate[3], and no indication of any such regulation in the cheat-purging lines. It is possible for non-reproductive division of labour to evolve in the *P. fluorescens* experimental system. In an evolution experiment that favoured WS cooperation by selecting purely on mat strength, two types of WS cells evolved to be mutually dependent on each other in order to form the strongest mats (McDonald, 2009). It is unknown whether any non-reproductive division of labour has evolved in the current experiments, thus I will conservatively assume that *I* is low in both regimes, but may have increased in the cheat-embracing regime due to the evolution of developmental regulation (Figure 5.5).

[3] Preliminary genetic analyses suggest that the accumulation of mutations during the long-term evolution experiment is comparable between the cheat-purging and cheat-embracing lines. This result indicates that the elevated transition rate in the cheat-embracing lines is not due to an increased mutation rate. Further investigations are planned to corroborate this finding.

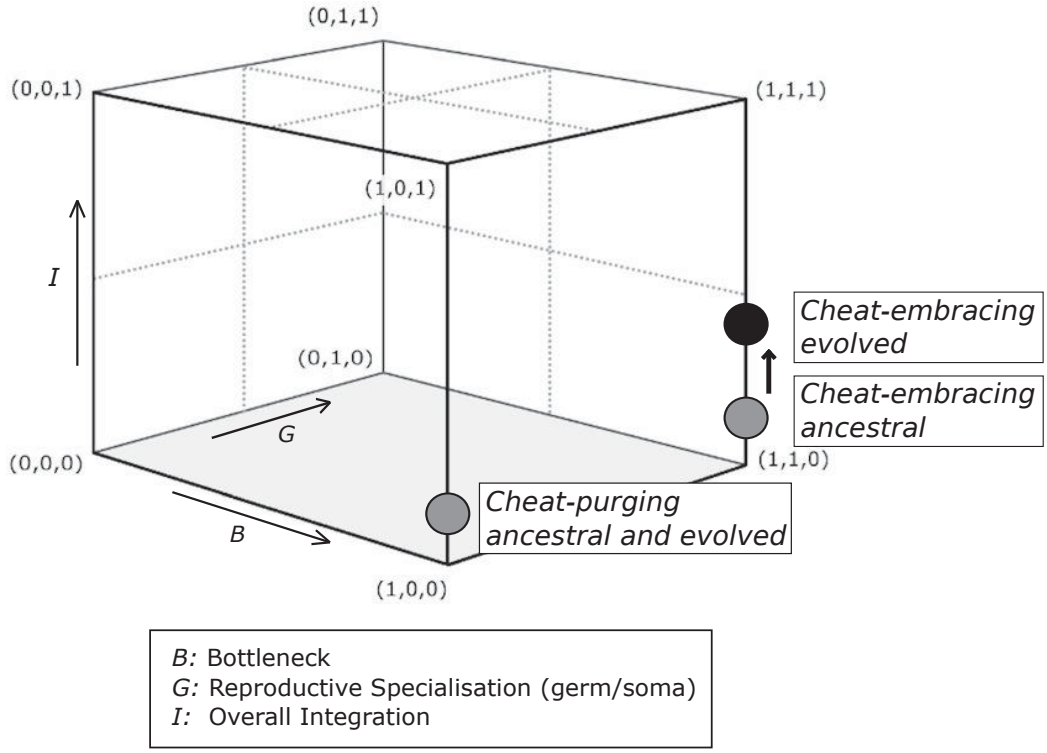


**Figure 5.5 Collective reproduction in P. fluorescens collectives.** Collectives in the cheat-purging and cheat-embracing regimes both reproduce via a single-celled bottleneck, however this is a dedicated germ line only in the latter. Both initially have a similar low level of integration (I) due to the collective benefit gained from mat formation, but the cheat-embracing collectives may have become more integrated with the evolution of developmental regulation. The cheat-embracing collectives are closer to 'paradigm' (1,1,1) forms of reproduction than the collectives in the cheat-purging regime.

### 5.1.2.3. Godfrey-Smith and the Levels of Selection Problem

PGS's approach to the levels of selection problem is to ask separately, at each level, how well the members of the population at that level meet the criteria set out in Section 5.1.2.1 for being a Darwinian population. This strategy may nevertheless mask any cross-level by-products because the parameters depicted in Figure 5.3 may occupy the paradigm corner as a result of selection operating at another level. The character-fitness covariance requirement for natural selection is related to PGS's *S* parameter – the extent to which differences in reproductive output in a population depend on the intrinsic features of entities within the population. The levels of selection problem asks

whether a high value of *S* is actually due to selection *at that particular level*. Perhaps contrary to intuition, a high value of *S* does not necessarily mean that the intrinsic character with which fitness covaries is itself under direct selection.

A 'strength of selection' parameter could be added to the framework (as suggested by PGS), which refers only to the strength of selection at the level in question. While the *P. fluorescens* collectives in Chapter 4 are paradigm Darwinian populations according to the three criteria depicted in Figure 5.3, this designation may change if a 'strength of selection' parameter is considered. For example, if the increased collective fitness was due entirely to selection acting at the cell-level, the strength of selection parameter in the collective-level Darwinian space would be zero. This approach is appealing, however it necessitates a prior knowledge of the strength of selection - and therefore a prior knowledge of the existence of cross-level by-products.

Some clarification is gained when we consider PGS's spatial representation of collective reproduction. Via *G* and *I*, collective-level reproduction is more clearly distinguished from growth and organisation of the lower level. It is therefore not surprising that the increased collective fitness in the cheat-purging regime, which had zero *G* and low *I*, was 'caused from below'. The structure imposed by the formation of mats was merely organisation of the environment in which cell-level selection was operating. In contrast, the high *G* in the cheat-embracing regime facilitated selection at the level of the collective – the *G* parameter was the 'difference-maker' (Calcott, 2011).

PGS's spatial tools highlight the difference-making role of the *G* parameter only because prior knowledge of the level of selection was gained from detection of a cross-level by-product in the cheat-purging regime that was not present in the cheat-embracing regime (Section 5.1.3). Perhaps it is possible to predict the level of selection

from these parameters alone, but a more robust strategy for solving the levels of selection problem is needed.

### 5.1.3. Emergence

One approach to the levels of selection problem is to distinguish between an *emergent* character – a property of a collective that cannot be qualitatively or quantitatively reduced to the sum of its parts (Corning, 2002), and *non-emergent* (or 'aggregate') characters. How such a distinction is made is not always straightforward because all properties of a collective can ultimately be explained by the underlying particles (discussed above in Section 5.1.1). Nevertheless, the 'emergent character requirement' (Vrba & Eldredge, 1984; Vrba, 1989) posits that genuine collective-level selection, that is not reducible to selection at the particle level, can only operate on emergent characters. Okasha (2006) objects to this rule because an emergent character may be a *result* of selection operating at the collective-level, rather than a pre-condition of it. The capacity for collective reproduction, which is necessary for collective-level selection, is itself a derived, and arguably emergent, character (Griesemer, 2000; Rainey & Kerr, 2010). Likewise, cheat-supressing mechanisms are often touted as a prerequisite for the evolution of individuality (*e.g.* Michod, 1997), yet these are emergent characters whose evolution necessitates collective-level selection. It is also possible for an emergent character to increase in frequency in the absence of collective-level selection if it is associated with characters that increase particle fitness, *i.e.* an emergent character may inadvertently result from particle-level selection. It is therefore generally accepted that the emergent/aggregate distinction is not important for solving the levels of selection problem (Damuth & Heisler, 1988; Okasha, 2006; Godfrey-Smith, 2009; Okasha, 2012).

Stephen Jay Gould argued that while a given character need not be emergent to evolve at the level of the collective, emergent *fitness* (in the MLS2 sense) is what matters for collective-level selection (Lloyd & Gould, 1993; Gould, 2002). It is true that collectives must acquire the capacity for reproduction to allow the possibility of collective-level selection and a measure of collective fitness. However, this potential for collective-level selection does not rule out cross-level by-products. Collective fitness emerged (increased) in both the cheat-purging and cheat-embracing selective regimes in Chapter 4 (Figures 4.11c and 4.11d), however selection operated at the collective-level only in the cheat-embracing regime.

Heisler and Damuth (1987) suggest that the emergence of the *relation* between a character and collective-level fitness is of fundamental importance for collective-level selection. A character-fitness relation at the higher level is emergent if it cannot be accounted for by a character-fitness relation at a lower level. Okasha's (2006) formulation of the levels of selection problem (Section 5.1) essentially recapitulates the emergent relation requirement. It is only possible to identify an emergent relation when there is no positive association between particle and collective fitness (Figure 5.6).

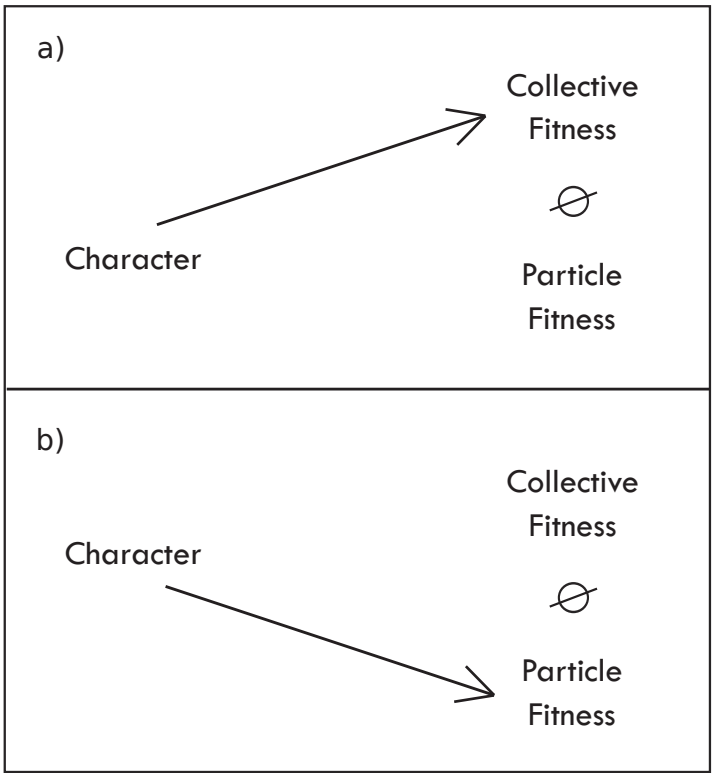


**Figure 5.6 Emergent Relations.** When there is no positive correlation between particle and collective fitness, a character-fitness relation at the collective-level (a) is emergent; it is not a by-product of a character-fitness relation at the particle-level (b).

A relationship exists between particle fitness and collective fitness in the evolved cheat-purging *P. fluorescens* populations from the life cycle experiment in Chapter 4 (Figure 4.11d). This (probably causal) link presents no difficulty in determining the level of selection, however, because the character in question (transition rate) correlates only with parameters of cell fitness, not collective fitness. It is clear therefore, that the transition rate significantly decreased because of selection acting at the cell level, and the increased collective fitness can be attributed to the correlation between collective and cell fitness parameters – collective fitness supervenes on particle fitness. No correlation exists between cell and collective fitness in the evolved populations from the cheat-embracing regime (Figure 4.11c) thus the positive relationship between the increased transition rate and collective fitness is causal - an emergent relation.

### 5.1.3.1. Contextual Analysis

Heisler and Damuth (1987) devised a method for detecting emergent relations by analysing character-fitness covariance from phenotypic data. Contextual Analysis is a generalisation of the selection analysis developed by Lande and Arnold (1983), which in turn is a specific application of Pearson's (1903) multiple regression analysis. This approach treats the levels of selection problem as a special case of selection on correlated characters, where the dependence of fitness on correlated characters is captured with a standard linear regression model. The covariance of collective fitness (*Y*) with a 'collective' character (*Z*) could be non-zero (*i.e. Cov(Y,Z)* $\neq$ 0) in the absence of a causal link between *Z* and *Y* if *Z* correlates with another character that *does* affect fitness. There is a cross-level by-product if *Y* is causally determined by the average fitness of its constituent particles, *W*. The character-collective fitness covariance can be partitioned into two components: direct collective-level selection on a character, $\beta_1 Var(Z)$, and the by-product of particle-level selection, $\beta_2 Cov(W,Z)$:

$$Cov(Y,Z) = \beta_1 Var(Z) + \beta_2 Cov(W,Z) \quad (1)$$

where $\beta_1$ is a measure of the direct effect of *Z* on *Y*, controlling for *W*, and $\beta_2$ measures the effect of *W* on *Y*, controlling for *Z*.

No relationship exists between transition rate and cell fitness in the cheat-embracing *P. fluorescens* populations (Figure 4.11b and Appendix 8.2.3). Contextual analysis is therefore not necessary to partition the change in transition rate due to particle- and collective-level selection, because *Cov(W,Z)* = 0. The strength of collective-level selection for increased transition rate is equal to the covariance between collective fitness and transition rate:

$$Cov(Y,Z) = \beta_1 Var(Z) + 0 \quad (2)$$

$$Cov(Y,Z) = \beta_1 Var(Z) \quad (3)$$

As no relationship exists between transition rate and collective fitness in the evolved populations from the cheat-purging regime (Figure 4.11d and Appendix 8.2.3), there is no need to discriminate between levels of selection – decreased transition rate was selected entirely at the lower level:

$$Cov(Y,Z) = 0 \quad (4)$$

These results corroborate the idea that a trait need not be assigned to a particular hierarchical level. One might regard 'transition rate' as a property of collectives, cells, or even genes. This character evolved to be higher in the cheat-embracing selective regime as a result of its influence on collective fitness, and lower in the cheat-purging regime because of selection at the cell-level. Does it then follow that transition rate is a collective-level character in one selective regime and a particle-level character in another? To reiterate, the level of *selection* is the focus during an evolutionary transition in individuality. An *a priori* designation of transition rate as either a 'collective character' or a 'particle character' may have masked the true level of selection, or worse, led to false conclusions.

## 5.1.4. Additivity

Lloyd (1989) contributed the 'additivity criterion' to the levels of selection debate - a notion that was first presented by Wimsatt (1980) in the philosophical literature disputing the 'gene's eye view' of evolution that was dominant among evolutionary

biologists at the time. The additivity criterion posits that true collective-level selection can only occur when the variance in collective fitness does not relate additively (linearly) to the composition of their particles. When there is a linear relationship between collective fitness and particle composition, then differences in collective fitness can be fully explained by selection at the particle level. A shift in the level of selection occurs when there is a departure from additivity, *i.e.* there is a non-linear relationship between the particle composition of collectives and their fitness. The additivity criterion for collective-level selection is similar to the criterion of emergent relations: a 'new' non-linear relation between character and fitness emerges at the level of the collective.

The main objection to this approach stems from the well-known example of the evolution of altruism, which reduces a particle's relative fitness while increasing the fitness of the collective to which it belongs (Sober & Wilson, 1994). Specifically, the proportion of altruistic particles in a collective can have a linear (additive) relationship with collective fitness, yet altruism must, according to this argument, be selected at the collective level. This is a valid objection in the case of MLS1 because collective fitness is the average particle fitness. Therefore the proportion of altruists will, by definition, always covary with collective fitness linearly, as average particle fitness is derived from the proportion of altruists. However, the collective fitness in MLS2 is the number of offspring collectives produced, which may or may not be affected additively by the proportion of altruists. For example, collective fitness may increase additively with the proportion of altruistic particles only until a certain threshold is reached, above which the relationship between particle and collective fitness 'levels off'. In this scenario, the proportion of altruists would be optimised at this threshold by collective- and particle-level selection acting in opposing directions.

A non-linear relationship clearly exists between collective fitness and particle composition in the *P. fluorescens* populations from the cheat-embracing regime in Chapter 4. SM 'germ line' cell types are required at a high enough frequency to be consistently detected on Day 6 of the regime, however a WS mat must also be present to avoid extinction. Therefore, collective fitness will be highest at intermediate frequencies of SM cells, and zero if the proportion of SM cells is either 0% or 100%. This non-additive relationship between cell composition and collective fitness is not pronounced in the experimental data because the wildtype transition rate is so low that the hypothetical 'high SM/low collective fitness' collectives do not exist in the ancestral populations, and were not selected during the experiment due to their low collective fitness (Figure 5.7a). Nevertheless, a comparison of the fit of linear and quadratic models to the data confirms the non-additive relationship between the proportion of SM cells within a collective and its fitness. Akaike's Information Criterion (Akaike, 1973), which compares the quality of statistical models by giving a relative estimate of the information lost by overfitting a given model, indicated that the quadratic model was 99.99% and 99.88% more likely to explain the data than the linear model in the ancestral and evolved collectives respectively (Appendix 8.3.1). This non-additive, and therefore emergent, relationship between the proportion of SM cells and collective fitness is indicative of selection operating at the level of the collective.

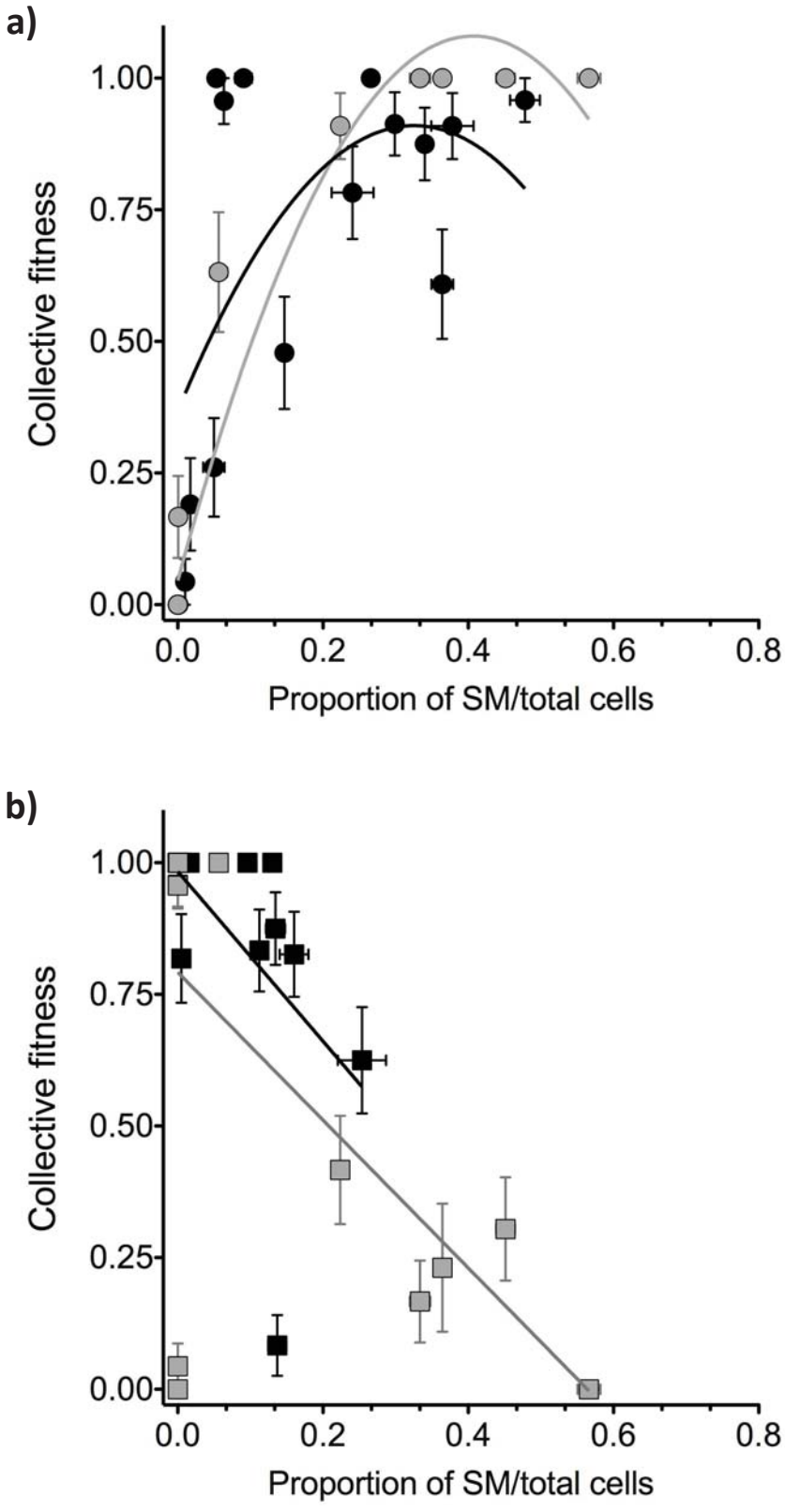


**Figure 5.7 Relationship between particle composition and collective fitness in the cheat-embracing (a) and cheat-purging (b) regimes.** Ancestral = grey, evolved = black. Error bars are SEM, based on N=3.

In contrast, there appears to be an additive relationship between the composition of collectives and their fitness in the cheat-purging selective regime; compared to the quadratic model, the linear model has a 70.54% and 69.94% probability of correctly fitting the ancestral and evolved data respectively (Appendix 8.3.1). Ancestral collectives with a fewer SM cells tend to have higher fitness ($\chi^2$=26.801, df=1, *P*<0.0001). The two ancestral clones that had both low collective fitness and low proportion of SM cells did not exhibit visible mats on Day 6 because of the invasion of a third cell type, the "fuzzy spreader" (described in Ferguson *et al.*, 2013). These collectives were therefore extinguished after one generation and the proportion of SM alone became a reliable predictor of collective fitness. Collective fitness increased after ten generations of evolution under the cheat-purging selective regime ($\chi^2$=15.737, df=1, *P*<0.0001; Figure 4.12), a significantly lower proportion of the evolved collectives produced SM ($\chi^2$=8.199, df=1, *P*=0.0042), and all evolved collectives had a proportion of SM cells lower than 0.3. In contrast to the non-linear relationship between the fitness of a collective and its composition in the cheat-embracing regime, the additive dependence of a collective's fitness on its composition reveals lower-level causality in the cheat-purging regime. Non-additivity in the cheat-embracing life cycle solved the levels of selection problem and led to a transition in individuality that was not possible for collectives in the cheat-purging regime, whose fitnesses depended entirely on particle composition.

## 5.2. Fitness Decoupling

Both the non-additivity and the emergent relations criteria for collective-level selection are strongly related to the notion of fitness decoupling during an evolutionary transition. Michod and Roze (1999) suggest that a crucial stage during the transition to multicellularity occurred when the fitness of the multicellular collective became 'decoupled' from the fitness of its constituent cells. Before this stage, any differences in collective fitness were due to selection at the cellular level: a cross-level by-product.

The experiment reported in Chapter 4 imposed selection between *P. fluorescens* collectives sustaining life cycles that either purged or embraced cheating SM cells. Only those collectives subjected to the cheat-embracing selective regime evolved Darwinian individuality (Stage Two) because the fitnesses of the collectives and their cells became decoupled, solving the levels of selection problem. It is logically inevitable that transitions in individuality are only possible for collectives whose fitnesses are decoupled from the fitnesses of their particles. If the fitnesses of the higher and lower levels are independent of one another, then improvements in higher-level fitness cannot be a cross-level by-product of selection at the lower level, thus transitioning individuality. This argument is somewhat circular: if increased higher-level fitness is not a cross-level by-product, then the fitnesses of the higher and lower levels must be decoupled. Regardless of the direction of causality, it is clear that fitness decoupling corresponds to solving the levels of selection problem and transitions in individuality.

Despite theoretical predictions of fitness decoupling, witnessing such a transformation in real-time in a single-celled organism is quite extraordinary. Although strong competition was imposed between collectives undergoing the cheat-embracing life cycle, it is nonetheless surprising that simultaneous within-collective competition did not select for higher cell fitness. We might still expect the most abundant SM cell

type to be the first to produce WS cells, the fastest growing of which would in turn colonize the vacant niche at the air-liquid interface and subsequently be transferred to the next collective generation. To the contrary, cell fitness significantly decreased ($t_{79}$=3.092, $P$=0.0027; Figure 4.5).

Understanding the mechanistic underpinnings of fitness decoupling in these *P. fluorescens* populations will shed light on whether this remarkable outcome can realistically be generalised to other biological systems including the evolutionary transition to multicellularity from cyanobacteria ~3.5 billion years ago.

## 5.2.1. Stochastic Events

It is conceivable that cell fitness was lowered during the course of the long-term selection experiment either by drift, or by the accumulation of slightly deleterious mutations that are no longer presented to selection because of the unique experimental setup. These two possibilities are unlikely, however, because the reduction in cell fitness (carrying capacity) was not due to lowered growth rate. Unfortunately it was impossible to monitor the growth rate of WS cells in a mat in a static environment because these attached cells construct their own niche that cannot be disturbed for measurement. Monitoring WS growth rate in a shaken environment would require cells to experience an environment in which they did not arise, and in which they are very unfit (Rainey & Travisano, 1998), and therefore growth could not be distinguished from selection during the assay. However, the growth rate of SM cells was measured, and was not significantly lower than the growth rate of SM cells harvested from the ancestral lineages ($t_{74}$=1.527, $P$=0.1315; Figure 4.8). It is highly likely that SM growth rate is correlated with WS growth rate, which would explain the increase in SM growth rate during the cheat-purging regime (in which the number of WS cells increased). While growth rate may affect the time to reach carrying capacity (usually 2-3 days), cell

fitness (# cells) was measured after 6 days. Therefore, regardless of whether SM growth rate corresponds to WS growth rate, the reduction in cell fitness requires an alternative explanation.

## 5.2.2. Conflict Mediation

The idea that fitness decoupling can solve the levels of selection problem is at odds with the conventional notion that conflict mediation must evolve before individuality transitions to a new level (Section 1.6). This is because conflict mediation is equated with, and indeed even defined as, "the process by which the fitnesses of lower level units are aligned with the fitness of the group" (Michod & Roze, 1999). Buss (1987) envisaged that the emergence of multicellular organisms occurred when cells enhanced their own replication rate by restraining or directing the growth of neighbouring cells, incidentally favouring the collective harbouring them, *i.e.* when the interests of the cell and the collective were *coupled*. Likewise, Grosberg and Strathmann (2007) argue that the alignment of the fitness interests of multicellular organisms and their cells is the most common way for multicellular organisms to develop. The two levels of fitness may indeed be coupled in common, simple multicellular organisms such as the slime mould *D. discoideum*, however the aggregative mode of development and propagation in these organisms supresses their continued evolvability (for reasons discussed in Section 1.7). Perhaps it is the uncommon, difficult transitions with decoupled fitness that ultimately give rise to multicellular complexity. For example, the Metazoan kingdom arose only once, yet this major evolutionary transition has since given rise to the panoply of diverse and complex multicellular animals.

Michod & Roze's (1999) claim that conflict mediation (via fitness alignment) is a prerequisite for the evolution of individuality contradicts their assertion that

individuality emerges when the two levels of fitness are decoupled. The evolution of conflict mediation was realised during the experiment described in Chapter 3, in the form of reduced transition rate from cooperative WS cells to cheating SM cells. This is an impressive outcome of between-collective selection, however the levels of selection problem was not solved because the increase in collective-level fitness was fully attributable to the improvements in cell fitness. Fitness alignment was indeed associated with the evolution of conflict mediation, however individuality did not emerge at the level of the collective.

The discrepancies outlined above result from the notion that conflict mediation must correspond to fitness alignment. A broader view allows for the possibility that the decoupled fitness in the cheat-embracing *P. fluorescens* collectives in Chapter 4 may be a form of conflict resolution in an unconventional sense. Consistent with mathematical predictions (Libby & Rainey, 2013), an increase in the transition rate between WS and SM cells outperformed any lines that improved cell growth rate during the cheat-embracing selective regime. While an increase in cell fitness could potentially maximise the potential for a collective to leave collective offspring, SM growth rate may have been curtailed in order to protect collectives from extinction by mat collapse. Despite the higher transition rate to SM ('cheating') cell types, the number and proportion of SM cells did not increase, and the derived collectives did not suffer from a greater propensity for mat collapse. In fact, the derived lines had slightly fewer mat collapses (3.8%) at Day 6 than the ancestral lines (4.7%). While prevailing theory focuses on the role of conflict mediation in reducing within-collective competition (cheating) (*e.g.* Michod & Roze, 1999), the conflict resolution proposed here acts to limit collective extinction in the face of an increased rate of 'cheating'. However, mat collapse only accounted for a small subset of collective extinctions (Figure 4.3), therefore this

selective pressure was unlikely to have contributed significantly to the fitness decoupling.

### 5.2.3. De-Darwinization

An alternative view is that individuality emerges at the higher level when competition at the lower level is suppressed (Okasha, 2006; Godfrey-Smith, 2009), however this does not require the fitnesses of the two levels to be aligned. It is possible for competition to be reduced at the lower level in the absence of conflict mediation – the two levels may continue to have opposing directions of selection, however the strength of selection is greater at the higher level. Godfrey-Smith (2009) suggests that when collectives form paradigm Darwinian populations at a new level, their constituent particles move away from paradigm status, which "de-Darwinizes" the lower level. Human cells illustrate the suppression, or de-Darwinization, of the lower level during the transition to multicellularity. Mutation repair mechanisms are evidence of active suppression of variation (*V*), and therefore competition, among cells.

Another factor contributing to the de-Darwinization of our cells is the repression of Godfrey-Smith's *S* parameter. Much of what determines the relative rate of reproduction of human cells depends upon their arrangement in the body rather than differences in their intrinsic character (*S*). The enormous reproductive difference, in the long term, between a successful germ cell and a somatic cell within the same person (collective) is not due to differences in intrinsic character, *S*, but is primarily a matter of location (Godfrey-Smith, 2009). *S* is greatly affected by the extent of reproductive division of labour, *G*. When there is high *G* at the collective level, there is reduced *S* at the lower level. Therefore, as high *S* (character-fitness covariance) is associated with paradigm cases of Darwinian populations (Figure 5.3), the presence of a germ line in a collective contributes to the de-Darwinization of the population below.

The WS (somatic) cells and the SM (germ) cells within each collective in cheat-embracing *P. fluorescens* collectives in Chapter 4 have the same (or very similar) intrinsic character (transition rate), rendering the determination of the successful SM germ line cells a stochastic event. At the level of the cell, transition rate therefore has little effect on cell fitness, *i.e. S* is low. In fact, there is no significant correlation between cell fitness and transition rate in either the ancestral (r=-0.378, *P*=0.1645; Appendix 8.2.3), or the evolved (r=0.326, *P*=0.2558; Appendix 8.2.3) collectives. At the level of the collective, however, transition rate is strongly related to collective fitness (ancestral: $\chi^2$=22.801, df=1, *P*<0.0001; evolved $\chi^2$=12.324, df=1, *P*=0.0004; Figures 4.11a and 4.11c) – *S* is high. Over the course of the cheat-purging selective regime, however, transition rate ceased to impact on collective fitness ($\chi^2$=2.129, *P*=0.1445; Appendix 8.2.3) while cell fitness became associated with transition rate (r=-0.473, *P*=0.075; Appendix 8.2.3). In contrast to the cheat-embracing lines, cheat-purging led to decreased *S* at the collective level, and increased *S* at the level of the cell – the populations of cells became *more* Darwinian.

Our bodies are made up of Darwinian populations, but they are not paradigmatically Darwinian, in part because the cells have low *S* - an evolutionary product of collective-level selection. Analogously, the *P. fluorescens* cheat-embracing selective regime has relegated the cell level from paradigm Darwinian status to the 'low *S*', minimal region of Darwinian space (Figure 5.3). These populations may have decoupled fitness and transitioned to a higher level of individuality because selection at the level of the collective has de-Darwinized their constituent cells.

## 5.2.4. Ecological Constraint

It is likely that the growth pattern of the cells within the mat may determine its carrying capacity. The evolution of a higher transition rate between WS and SM cells

must have generated mats with a more mosaic structure. This is congruent with morphological and gene expression data that suggests that early multicellular organisms probably had germ cells scattered throughout their bodies, rather than clustered together in one region (Extavour, 2007). It is possible that the mosaic mat structure results in mats that are less dense because the WS cells can no longer grow in a tightly bound, compact formation due to the profusion of SM patches. In addition, the thickness of the mat is probably constrained by the limiting resource (oxygen) present only within a few millimetres of the air-liquid interface (Ibelings *et al.*, 2012). Presumably the selective advantage of colonization at the air-liquid interface is no longer present on the underside of the mat once it reaches a certain thickness, hindering further growth. Therefore, the reduced carrying capacity – and resulting fitness decoupling – may be due to the combined effect of limited mat thickness and reduced mat density caused by the increased transition rate.

## 5.3. Germ Lines Revisited

This chapter has explored the basis for the transition in individuality in the cheat-embracing *P. fluorescens* populations in the experiment described in Chapter 4. It was established that these populations solved the levels of selection problem because the presence of a germ line decoupled the fitnesses of the collectives from their constituent cells. Fitness decoupling will emerge whenever there is a trade-off between the presence of a germ line and cell fitness. In the *P. fluorescens* populations, the presence of the germ line established a trade-off between cell and collective fitness (Sections 5.2.1-5.2.4), which led to fitness decoupling when selection was applied at the level of the collective. Interestingly, the relationships between parameters in the ancestral lines predicted their evolutionary trajectory in each regime, including the fitness decoupling observed in cheat-embracing lines (Figures 4.11a and 4.11b) Selection was imposed on collective reproduction, resulting in increased collective fitness in both regimes, and indirectly selected for parameters that were linked to collective fitness in their respective ancestral baselines. In the cheat-purging treatment, this entailed selection for increased cell fitness parameters and decreased transition rate, while the cheat-embracing regime selected for increased transition rate and decreased cell fitness.

### 5.3.1. Conflict and Individuality

Conventional hypotheses (*e.g.* Michod, 1996) stipulate that the germ-soma distinction evolved as a mechanism for resolving within-organism conflict in multicellular organisms (see Section 1.6.2.2, however the results from Chapter 4 suggest that a germ line can emerge when conflict is embraced. The oscillation between cheating (germ line) and cooperating (somatic) cell types is due to negative frequency-dependent selection, which allows the alternative cell type to increase in frequency

when rare (described in Section 1.6.3). Presumably frequency-dependent oscillations between cooperators and defectors are a common occurrence in the microbial world, however environmental conditions usually ensure that selection continues to operate strongly at the cell-level. Transitions in individuality occur only when the levels of selection problem is solved (by the fitness decoupling attributable to frequency-dependence), AND selection is imposed at the higher level. As the presence of the germ line is essential for solving the levels of selection problem, it thus follows that defection (*i.e.* frequency-dependent selection) must be embraced to decouple fitness and transition individuality.

# Chapter 6. Concluding Discussion

## 6.1. Summary of Main Results

Major evolutionary transitions from single cells to multicellular organisms involve cells overcoming the cost of cooperation, a shift in the level of selection and the emergence of complex traits, ultimately leading to a loss of autonomy of the single cells of which the organisms are composed. The framework presented in this thesis arranges the multitude of features required for this transition into three sequential stages (Section 1.4.2). I have conducted long-term evolution experiments using the single-celled *P. fluorescens* model system to address fundamental questions arising during each of the three stages of the evolution of multicellularity.

### 6.1.1. Stage One: The Evolution and Maintenance of Cooperation

During Stage One, cells unite together in a cooperative group to gain an advantage over solitary cells. The focus of selection remains at the lower level, albeit in a group-structured context. The aim of Stage One research was to understand how cooperation can be maintained in multicellular collectives in the face of defection. Chapter 3 tested the hypothesis that competition between collectives can maintain high levels of cooperation and reduce the level of cheating within collectives. In accordance with theoretical predictions (Section 1.5.3.3) ten collective generations of competition between *P. fluorescens* mats led to high levels of cooperation (more WS cells) and less cheating (fewer SM cells). Both the increase in cooperation and the decrease in

defection were associated with improved collective fitness, indicating that the group-structured environment provided the selective basis for these changes. Life history properties of the evolved collectives were measured relative to ancestral types to determine the mechanistic basis for increased cooperation and reduced cheating. The success of the collectives was associated with a reduced transition rate between WS and SM cells, suggesting the evolution of a cheater suppression mechanism. Finally, a higher SM growth rate and a reduction in diversity evolved over the course of the experiment, suggesting that the lower transition rate may be due to an overall reduction in mutation rate. If so, this would be the first evidence of a reduction in wildtype mutation rate, which already sits at the theoretical 'lower bound' (Sniegowski *et al.*, 2000; Lynch, 2010). Greater certainty about this conclusion will require specific mutation rate measurements.

The evolution of reduced cheating and increased cooperation allowed cooperation to be maintained for ten collective-level generations. While the evolution and maintenance of cooperation is an important first step during the evolution of multicellularity, it is unlikely that these collectives could continue to the second and third stages of the transition. The lowered mutation rate and resulting loss of diversity prevents these collectives from producing the complex adaptations required to complete the transition.

## 6.1.2. Stage Two: A Transition in Individuality

Selection on cooperation (a property of individual cells) is alone insufficient to induce a shift in the level of selection (Stage Two). An emergent multicellular collective constitutes a unit of selection only once collective functionality, rather than size, becomes the main determinant of fitness (Okasha, 2006). The experiments described in Chapter 4 demonstrated that selection on the cycling of a two-phase life cycle allowed

collectives to transition to a higher level of individuality. Collectives that reproduced via an alternative cell type (a SM 'germ' cell) evolved increased collective fitness and decreased cell fitness; the fitness of the higher and lower levels became decoupled – a hallmark of a transition in individuality. The increased capacity for collective reproduction did not result from any other cell-level property: the fitness of the SM "propagules" (Figure 4.8), and the total carrying capacity (Figure 4.5) were both reduced. Rather, the increased collective fitness was due to a shift in the composition of the collective: the proportion of germ (SM) to soma (WS) - a property arising from an increased transition rate between the two cell types.

### 6.1.3. Stage Three: The Evolution of Complexity

The evolution of traits selected at the level of the collective demonstrates the emergence of the evolution of complexity (Stage Three). However, apparent selection (*i.e.* a covariance between a character and fitness) at one hierarchical level can be a by-product of natural selection acting at another level (Okasha, 2006). Chapter 5 provided an in depth discussion of the various approaches to addressing this 'levels of selection' problem. Reductionist arguments that *all* causal explanations lie at the lower level were discussed and the conclusion drawn that this approach need not rule out the possibility of *selection* at the higher level. Application of the Godfrey-Smith framework (Godfrey-Smith, 2009), which views Darwinian individuality as a spectrum of possible cases, illustrated that the cheat-embracing collectives evolved towards the paradigm region of 'Darwinian Space', whereas the cheat-purging collectives did not – despite their increased collective fitness (Figure 5.5). A discussion of the concept of emergence concluded that the evolution of 'emergent characters' (Vrba & Eldredge, 1984; Vrba, 1989) and 'emergent fitness' (Lloyd & Gould, 1993; Gould, 2002) are not good criteria for identifying higher level selection because they do not sufficiently rule out cross-

level by-products. Instead, the emergence of a causal relation between collective character and collective fitness is of fundamental importance for collective-level selection (Heisler & Damuth, 1987). A character-fitness relation at the higher level is emergent if it cannot be accounted for by a character-fitness relation at a lower level. Contextual analysis of the character-fitness covariance present at each level confirmed that the decreased transition rate in the cheat-purging collectives resulted from selection acting at the level of the cell and not at the level of the collective. In contrast, the increased transition rate in the cheat-embracing collectives resulted from selection acting at the level of the collective and not at the level of the cell (Section 5.1.3.1). Finally, while the composition of the evolved cheat-purging collectives relates linearly to their fitness, the non-additive relationship between the composition of the evolved cheat-embracing collectives and their fitness confirmed that these collectives satisfy the 'additivity criterion' (Lloyd, 1989) for true collective-level selection (Section 5.1.4). Therefore, collectives that reproduced via a single 'germ' cell solved the levels of selection problem, allowing individuality to emerge and establishing the conditions necessary for the evolution of traits beneficial at the higher level – the evolution of complexity.

A crucial stage during an evolutionary transition to multicellularity occurs when the fitness of the multicellular collective becomes 'decoupled' from the fitness of its constituent cells. Before this stage, any differences in collective fitness are due to selection at the cellular level. The final sections of Chapter 5 explored possible explanations for the fitness decoupling and resulting emergence of individuality in the evolved cheat-embracing *P. fluorescens* collectives. The presence of the germ line established a trade-off between cell and collective fitness that was most likely due to a combination of ecological constraint and the 'de-Darwinization' (Godfrey-Smith, 2009)

of the cell-level. The escalation of cell fitness typical in microbial experimental evolution studies (*e.g.* Lenski *et al.*, 1991; Ratcliff *et al.*, 2012) was inhibited here by environmental conditions that favoured collectives while restricting the access of large collectives to a limited resource (oxygen). The de-Darwinization of the cell-level resulted from the suppression of Godfrey-Smith's (2009) *S* parameter – the dependence of fitness on the intrinsic character of a cell. During Phase I of the cheat-embracing life cycle, SM cells had the highest fitness because they were selected to found Phase II. However, WS cells were favoured during Phase II. Throughout the duration of the two-week cheat-embracing life cycle, neither WS nor SM cells were ultimately more fit – long term evolutionary success depended on the cycling between these two cell states. Cell fitness therefore became less dependent on intrinsic character. The repression of character-fitness covariance de-Darwinized the cell-level and resulted in decoupled fitness and an evolutionary transition in individuality.

## 6.2. The Evolution of Multicellularity in *P. fluorescens*

Table 1.2 lists features that emerge during the three stages of the evolution of multicellular organisms. Prior to the experiments described in this thesis, Rainey and Rainey (2003) showed that collectives of *P. fluorescens* growing in static microcosms evolve a cooperative phenotype (Section 1.8.1.2). WS cooperators gain an advantage over wildtype cells by occupying a vacant niche, however the cooperative trait is costly relative to non-actors and is therefore inevitably short-lived. In the present study, competition between collectives maintained cooperation for ten collective level generations (20 weeks) in both the cheat-purging and cheat-embracing experimental regimes.

**Table 6.1 Properties contributing to the evolution of multicellularity in P. fluorescens collectives**

| Stages of the Transition to Multicellular Organisms | Cheat-embracing regime | Cheat-purging regime |
|---|---|---|
| **One: The Evolution of Cooperation** | ✔ | ✔ |
| Cost of cooperation | ✔ | ✔ |
| Benefit of cooperation | ✔ | ✔ |
| Long-term maintenance | ✔ | ✔ |
| **Two: The Evolution of Individuality** | ✔ | |
| Collective Reproduction | ✔ | ✔ |
| Bottleneck (development) | ✔ | ✔ |
| Germ line | ✔ | |
| Decoupled fitness | ✔ | |
| **Three: The Evolution of Complexity** | | |
| Trait(s) selected at the collective-level | ✔ | |
| Conflict resolution | ✔ | ✔ |
| Developmental regulation | ✔**?** | |
| Loss of cellular autonomy | | |

Collectives in both regimes reproduced via a single-celled bottleneck, which is hypothesised to be a crucial factor during the evolution of individuality (Grosberg & Strathmann, 1998; Michod & Roze, 1999; Griesemer, 2000; Wolpert & Szathmáry, 2002). First, a single-celled bottleneck reduces the variation within collectives and may therefore be a mechanism for reducing within-collective conflict. Indeed, the bottleneck regularly removed cheats from collectives in the cheat-purging regime, resulting in reduced levels of conflict. Secondly, the presence of a unicellular bottleneck is linked to the evolution of novelty – it rendered primordial life cycles 'evolvable' because development from a smaller stage provides a window of opportunity for organizational

change (Dawkins, 1982). Collectives subjected to evolution under the cheat-purging regime in the present experiment suffered from reduced diversity, possibly as a result of a reduced mutation rate. The regular redistribution of genetic variation from *within* the parent to *between* its offspring theoretically allows selective processes to operate on variation at the higher level rather than at the lower cell level (Michod & Roze, 1999), however the focus of selection during the cheat-purging regime remained at the level of the cell.

The presence of a germ line has also been suggested as a defence against the proliferation of cheats (Buss, 1987; Michod, 1996, 1999). In a radical reversal of the common perception of the role of cheats, which sees cheats as a major impediment to the evolution of multicellularity, results from the experiments presented in Chapter 4 suggest that cheats might be instrumental in fuelling the transition. This idea was realised by a selective regime that embraced cheats as the 'germ line', allowing collectives to reproduce via a two-phase life cycle. This resulted in the decoupling of the lower and higher levels of fitness and collectives emerged as a higher unit of selection as evidenced by selection acting at the collective-level for a higher transition rate between WS and SM cells (discussed above in Section 6.1.3).

Both the cheat-embracing and the cheat-purging regimes fostered the evolution of conflict resolution. The reduction in the level of cheating in the cheat-purging regime was due to a reduced transition rate. This trait evolved to be lower because of its influence on the fitness of cells within the collective – not as a result of selection acting at the collective-level. In contrast, a form of conflict resolution exhibited by the collectives evolving under the cheat-embracing regime may have emerged as a *result* of the transition in individuality. SM growth rate may have been restricted in order to protect collectives from being overrun by cheats. Despite the higher rate of transition to

SM cell types, which 'guaranteed' collective reproduction via a SM propagule, the number and proportion of SM 'cheating' cells did not increase.

The transition between WS and SM cells in some of the evolved cheat-embracing collectives may have evolved developmental regulation. Life history analysis found that the rate of transition was maximal at day six suggesting optimisation to the periodicity of the selection regime (Figure 4.6a). Preliminary genetic analysis has revealed mutations in the mismatch repair locus, MutS, in evolved lines #16 and #17, conferring hypermutator phenotypes. This heritable increase in mutation rate explains the very high transition rate (Figure 4.7) and, consequently, the high collective fitness in these two lines. The increase in consistency of SM detection in the remaining evolved lines suggests that the increased transition rate in these lines is also heritable. However, unlike lines #16 and #17, this may not be due to a general increase in mutation rate – SM and WS cells are not detected until after Day 1 in the life history analysis (Figure 4.7b) implying that the transition between WS and SM cells in some of the evolved lines may be developmentally regulated.

The results from the experiments presented in this thesis demonstrate that a major evolutionary transition in individuality is possible when collectives compete to reproduce via a two-phase life cycle – a germ line. Conflict resolution evolved in collectives that reproduced without a germ line, however these collectives did not complete Stage Two – they did not evolve individuality (Table 1.2). Reproduction via a germ line allowed collectives in the cheat-embracing regime to complete Stage Two by decoupling fitness and emerging as units of selection. This enabled the evolution of complex traits by selection acting at the collective-level. Primitive collectives reproducing via a germ line might eventually accumulate higher level adaptations that lead to such complex integration of their comprising cells that they can no longer exist

independently, surviving and replicating only as components of the collective – the 'organism'.

## 6.3. Future Directions

The experiments presented in this thesis provide the first experimental evidence of a major evolutionary transition in individuality. This has opened many exciting opportunities for continued investigation. An advantage of the experimental evolution approach using a microbial model system is that throughout this long-term study *P. fluorescens* cells from every replicate of every generation have been stored at -80℃ and can be revived for further analyses.

The speculation that the lowered transition rate and resulting conflict resolution in the cheat-purging collectives is caused by the evolution of a reduced mutation rate needs to be experimentally verified. If this hypothesis proves to be correct, it may be the first evidence for a decrease in wildtype mutation rate to below the theoretical minimal rate (Drake, 1991; Sniegowski *et al.*, 2000; Lynch, 2010). Determining the genetic basis for the reduced mutation rate will improve our understanding of mutation rate evolution (the evolution of 'evolvability'), and factors that set the lower bound.

Preliminary genetic analyses have revealed that the two collectives in the cheat-embracing regime with the highest transition rate are probably hypermutators (see Section 6.2). The remaining 12 clones also exhibit increased transition rates, however both the delay and the consistency in SM/WS production suggest that the transition between the two cell states in these replicates may not be due to mutation, but an epigenetic mechanism. Determining the mechanistic basis for the increased transition rate will reveal the extent of complexity these collectives have evolved.

The environmental conditions that favour the evolution of multicellularity may have a huge impact on the progression of the evolutionary transition. Mathematical models predict the evolution of different developmental strategies of collectives depending on the availability of new niches (Rainey & Kerr, 2010). If new niches are

commonly available, then propagule production will be maximised (high fecundity), while if new niches are rarely made available, then the rate of propagule production will be lower. Given that the high rate of SM production was directly related to fitness decoupling in the cheat-embracing *P. fluorescens* collectives, then fitness decoupling and the resulting transition in individuality may be more likely to occur in environments in which new niches are commonly made available. In addition, such an environment would promote high rates of death and birth of collectives, thus elevating the level of competition, and therefore selection, between collectives. Experimental data from this thesis could be used to build on this model by combining the influence of niche availability with the likelihood of fitness decoupling. Furthermore, the availability of new niches could be manipulated in the laboratory to experimentally test these predictions. If this hypothesis is correct, it is likely that historical transitions in individuality occurred in turbulent environments.

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# Chapter 8. Appendices

## 8.1. Chapter 3 Statistics

Results from general linear models are shown.
Regime: Ancestral vs evolved
Rep. type: representative WS genotype
#SM: number of SM at 6 day within a mat (SM=0 were excluded)
Bold denotes significance at the level of $P<0.05$.

| Parameters | N | df | *F* | *P* | $R^2$ |
|---|---|---|---|---|---|
| **#WS** | | | | | |
| Full model | 88 | 29 | 4.562 | **<0.0001** | 0.695 |
| Regime | | 1 | 25.318 | **<0.0001** | |
| Rep. type (Regime) | | 28 | 3.763 | **<0.0001** | |
| **#SM** | | | | | |
| Full model | 44 | 17 | 4.481 | **=0.0003** | 0.746 |
| Regime | | 1 | 7.516 | **=0.0109** | |
| Rep. type (Regime) | | 16 | 3.906 | **=0.0010** | |
| **Prop SM** | | | | | |
| Full model | 44 | 17 | 8.126 | **<0.0001** | 0.842 |
| Regime | | 1 | 21.959 | **<0.0001** | |
| Rep. type (Regime) | | 16 | 6.129 | **<0.0001** | |
| **SM growth rate** | | | | | |
| Full model | 63 | 22 | 8.450 | **<0.0001** | 0.823 |
| Regime | | 1 | 42.919 | **<0.0001** | |
| Rep. type (Regime) | | 21 | 7.027 | **<0.0001** | |
| **# colony types** | | | | | |
| Full model | 1608 | 47 | 18.145 | **<0.0001** | 0.353 |
| Regime | | 1 | 147.893 | **<0.0001** | |
| Rep. type (Regime) | | 28 | 14.737 | **<0.0001** | |
| Time | | 18 | 16.278 | **<0.0001** | |

# 8.2. Chapter 4 Statistics

## 8.2.1. Differences in Collective Fitness, Cell Fitness, and Life History Traits

Results from generalized and general linear models are shown. Values for ancestral lines are the same for all parameters measured during Phase I, whereas line fitness differs between ancestral CE and CP regimes.
Rep. type: representative WS genotype
#SMX: number of SM at 6 day within a mat (SM=0 were excluded); X: box cox transformation
WS➔SM: proportion of lines producing SM during Phase I
Bold denotes significance at the level of P<0.05.

| **Parameters** | **N** | **df** | ***F*/ $\chi^2$** | ***P*** | $R^2$ |
|---|---|---|---|---|---|
| **Collective fitness** | | | $\chi^2$ | | |
| Full model | 1392 | 59 | 1160.211 | **<0.0001** | |
| Regime | | 3 | 61.429 | **<0.0001** | |
| Rep. type (Regime) | | 56 | 1090.351 | **<0.0001** | |
| **Cell fitness** | | | *F* | | |
| Full model | 126 | 43 | 4.404 | **<0.0001** | 0.698 |
| Regime | | 2 | 13.524 | **<0.0001** | |
| Rep. type (Regime) | | 41 | 3.916 | **<0.0001** | |
| **#WS** | | | *F* | | |
| Full model | 126 | 43 | 5.508 | **<0.0001** | 0.743 |
| Regime | | 2 | 28.282 | **<0.0001** | |
| Rep. type (Regime) | | 41 | 4.339 | **<0.0001** | |
| **#SM X** | | | *F* | | |
| Full model | 80 | 31 | 5.148 | **<0.0001** | 0.769 |
| Regime | | 2 | 2.358 | =0.1055 | |
| Rep. type (Regime) | | 29 | 5.190 | **<0.0001** | |
| **SM growth rate** | | | *F* | | |
| Full model | 104 | 36 | 6.450 | **<0.0001** | 0.777 |
| Regime | | 2 | 32.806 | **<0.0001** | |
| Rep. type (Regime) | | 34 | 5.101 | **<0.0001** | |
| **WS ➔ SM** | | | $\chi^2$ | | |
| Full model | 1557 | 43 | 565.727 | **<0.0001** | |
| Regime | | 2 | 289.108 | **<0.0001** | |
| Rep. type (Regime) | | 41 | 467.060 | **<0.0001** | |

## 8.2.2. Differences in Life History Traits Between Ancestral and Evolved CE Regimes Over Time

Results from generalized and general linear models are shown.
All three parameters for the general linear models were Box Cox transformed (X).
Rep. type: representative WS genotype
Bold denotes significance at the level of P<0.05.

| **Parameters** | **N** | **df** | ***F*/ $\chi^2$** | ***P*** | ***$R^2$*** |
|---|---|---|---|---|---|
| **Proportion of replicates with new type** | | | $\chi^2$ | | |
| Full model | 1357 | 64 | 690.216 | **<0.0001** | |
| Regime | | 1 | 5.442 | **=0.0197** | |
| Rep. type (Regime) | | 27 | 457.619 | **<0.0001** | |
| Time | | 18 | 385.403 | **<0.0001** | |
| Regime x Time | | 18 | 62.994 | **<0.0001** | |
| **Total # cells/µl X** | | | *F* | | |
| Full model | 1355 | 46 | 21.697 | **<0.0001** | 0.433 |
| Regime | | 1 | 51.521 | **<0.0001** | |
| Rep. type (Regime) | | 27 | 8.144 | **<0.0001** | |
| Time | | 18 | 39.887 | **<0.0001** | |
| Regime x Time | | | | n.s. | |
| **# new type/µl X** | | | *F* | | |
| Full model | 740 | 46 | 8.823 | **<0.0001** | 0.369 |
| Regime | | 1 | 0.589 | =0.4431 | |
| Rep. type (Regime) | | 27 | 5.620 | **<0.0001** | |
| Time | | 18 | 13.615 | **<0.0001** | |
| Regime x Time | | | | n.s. | |
| **Proportion new cell type X** | | | *F* | | |
| Full model | 740 | 46 | 6.447 | **<0.0001** | 0.300 |
| Regime | | 1 | 1.701 | =0.1926 | |
| Rep. type (Regime) | | 27 | 6.648 | **<0.0001** | |
| Time | | 18 | 6.229 | **<0.0001** | |
| Regime x Time | | | | n.s. | |

## 8.2.3. Relationships Between Fitness and Life History Parameters in (a) CE and (b) CP regimes

X denotes parameters that were Box Cox transformed to meet requirements of normality and equal variance.
WSèSM: proportion of lines producing SM during Phase I
Above the diagonal are tests for the ancestral (grey), below for the evolved regimes (black). Bold denotes significance at the level of P<0.05.

**a**

| | SM growth X | #SM/µl X | #WS | WSèSM X | Collective fitness | Cell fitness |
|---|---|---|---|---|---|---|
| SM growth X | - | $R^2$=0.449, F=4.893, P=0.0690, N=8 | r=-0.372, P=0.2335, N=12 | r=0.222, P=0.4882, N=12 | $\chi^2$=0.711, df=1, P=0.3992, N=12 | r=-0.218, P=0.4964, N=12 |
| #SM/µl X | $R^2$=0.007, F=0.087, P=0.773, N=14 | - | $r_S$=-0.667, P=0.0710, N=8 | $r_S$=0.619, P=0.1017, N=8 | **$\chi^2$=26.801, df=1, P<0.0001, N=8** | $R^2$=0.380 $F_{1,8}$=3.671, P=0.1038 |
| #WS | r=0.381, P=0.1796, N=14 | $r_S$ =0.007, P=0.9822, N=14 | - | **r=-0.689, P=0.0045, N=15** | **$\chi^2$=11.150, df=1, P=0.0008, N=15** | **$R^2$=0.339, $F_{1,15}$=6.673, P=0.0227** |
| WS➔SM X | r=0.063, P=0.8297, N=14 | **$r_S$=0.587, P=0.0274, N=14** | r=0.114, P=0.6987, N=14 | - | **$\chi^2$=22.801, df=1, P<0.0001, N=15** | r=-0.378, P=0.1645, N=15 |
| Collective fitness | $\chi^2$=0.061, df=1, P=0.8046, N=14 | **$\chi^2$=9.305, df=1, P=0.0023, N=14** | $\chi^2$=1.008, df=1, P=0.3154, N=14 | **$\chi^2$=12.324, df=1, P=0.0004, N=14** | - | **$\chi^2$=4.246, df=1, P=0.0393, N=15** |
| Cell fitness | r=0.486, P=0.0783, N=14 | $R^2$=0.009, $F_{1,14}$=0.113, P=0.7428 | **$R^2$=0.890, $F_{1,14}$=97.359, P<0.0001** | r=0.326, P=0.2558, N=14 | $\chi^2$=2.041, df=1, P=0.1531, N=14 | - |

**b**

| | SM growth X | #SM/µl X | #WS | WS➔SM X | Collective fitness | Cell fitness |
|---|---|---|---|---|---|---|
| SM growth X | - | $R^2$=0.449, F=4.893, P=0.0690, N=8 | r=-0.372, P=0.2335, N=12 | r=0.222, P=0.4882, N=12 | $\chi^2$=0.705, df=1, P=0.4010, N=12 | r=-0.218, P=0.4964, N=12 |
| #SM/µl X | $R^2$=0.011, F=0.0656, P=0.8064, N=8 | - | $r_S$=-0.667, P=0.0710, N=8 | $r_S$=0.619, P=0.1017, N=8 | **$\chi^2$=17.568, df=1, P<0.0001, N=8** | $R^2$=0.380 $F_{1,8}$=3.671 , P=0.1038 |
| #WS | r=0.215, P=0.5265, N=11 | $r_S$=0.222, P=0.5372, N=10 | - | **r=-0.689, P=0.0045, N=15** | **$\chi^2$=6.153, df=1, P=0.0131, N=15** | **$R^2$=0.339, $F_{1,15}$=6.673, P=0.0227** |
| WS➔SM X | r=0.221, P=0.4129, N=11 | $r_S$=0.616, P=0.0580, N=10 | **r=-0.669, P=0.0064, N=15** | - | **$\chi^2$=8.710, df=1, P=0.0032, N=15** | r=-0.378, P=0.1645, N=15 |
| Collective fitness | **$\chi^2$=5.567, df=1, P=0.0183, N=11** | $\chi^2$=0.312, df=1, P=0.5762, N=10 | **$\chi^2$=7.552, df=1, P=0.0060, N=15** | $\chi^2$=2.129, df=1, P=0.1445, N=15 | - | $\chi^2$=0.746, df=1, P=0.3878, N=15 |
| Cell fitness | r=0.290, P=0.3874, N=11 | $R^2$=0.074, $F_{1,10}$=0.640, P=0.4470 | **$R^2$=0.887, $F_{1,15}$=101.583, P<0.0001** | r=-0.473, P=0.0750, N=15 | **$\chi^2$=5.339, df=1, P=0.0208, N=15** | - |

# 8.3. Chapter 5 Statistics

## 8.3.1. Model Comparisons

Comparison of the fit of linear and quadratic models of the relationship between the proportion of SM cells and collective fitness in the CE and CP regimes using the Akaike's Information Criterion.
Prob correct: Probability that this model is the most likely to have generated the data.

| **Parameters** | **Difference in AICc** | **N** | **Prob Correct** | **df** | $R^2$ |
|---|---|---|---|---|---|
| **CE ancestral** | 151.5 | 360 | | | |
| Linear model | | | **<0.01%** | 358 | 0.712 |
| Quadratic model | | | **>99.99%** | 357 | 0.812 |
| **CE evolved** | 13.41 | 336 | | | |
| Linear model | | | **0.12%** | 334 | 0.128 |
| Quadratic model | | | **99.88%** | 333 | 0.167 |
| **CP ancestral** | -1.747 | 360 | | | |
| Linear model | | | **70.54%** | 358 | 0.291 |
| Quadratic model | | | **29.46%** | 357 | 0.292 |
| **CP evolved** | -1.689 | 360 | | | |
| Linear model | | | **69.94%** | 358 | 0.141 |
| Quadratic model | | | **30.06%** | 357 | 0.142 |