The ecology and evolution of diversity and cooperation in bacterial public-goods

Submitted by

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To the University of Exeter as a thesis for the degree of

Doctor of Philosophy in Biological Sciences

September 2017

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University

Signature:.....

Acknowledgements

The work presented in this thesis would not have been possible without the excellent supervision of Angus. His work has been an inspiration since I first read a paper of his to over a decade ago, and it will continue to be so for the rest of my life.

I cannot understate the value of the encouragement, support, and company of my friends and colleagues in getting this work completed. So, I thank Chris Lowe, Pawel Sierocinski, Uli Klümer, Florian Bayer, Lena Bayer-Wilfert, Daniela Farina, Siobhan O'Brien, Daniel Padfield, Edze Westra, Christina Van Houte, Helen Leggett, Elze Hesse, Sean Meaden, Adela Luján, Pedro Gómez, Andrea Dowling, Suzanne Kay, Martin Grunnill, Lewis Campbell, and Ben Makin. Special thanks to Andy Gardner for modelling our ideas regarding resource availability in chapter 4.

I thank the BBSRC and the hard work of the supervisors who secured the grant providing me with my studentship, and allowing me the freedom to explore my interests.

I thank my parents and my sister, for their consistent support over the years. I thank Jack and Tom for their true friendship. And lastly, Mariann for being a truly wonderful partner.

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Foreword

The maintenance of cooperation is the central topic linking the two themes of this thesis. Chapters 2 and 3 deal with how cheats interacting with tag-based cooperative public-goods systems effect diversity. Chapters 4 and 5 deal with ecological aspects of cooperation; how patterns of resource availability simultaneously alter both the cost of cooperation and the population dynamics. Chapter 1 will introduce to the reader the fundamental concepts of social evolution before providing a broader general background to each chapter.

Abstract

Explaining why cooperation exists despite the persistent advantage of cheats has been the focus of much theoretical and empirical attention in biology. Using the bacterium *Pseudomonas aeruginosa* as a model system for the evolution of cooperation, I investigate two distinct phenomena which may develop our understanding of how cooperation is maintained; 1) tag-based cooperation and diversity; and 2) environmental heterogeneity.

The first investigates how diversity in cooperative systems may be a response to the selective pressure exerted by cheating, and how cheats may then regulate communities to maintain diversity: I demonstrate that in competition, tag-based cooperation is able to evade parasitism, provided the public-good is only accessible to producer strains, i.e., the cheat possesses the "wrong" tag. I also demonstrate that cheats can have a marked influence on diversity: In a community of two producer strains with different tags, if a third cheater strain is introduced, it will drive both its own producer and itself extinct. I do not find that the presence of cheats maintains diversity in either structured or unstructured environments, and discuss the possible causes of this.

In the second topic of this thesis, I investigate the effect of environmental heterogeneity in resource availability, through space and time, on the evolution of cooperation. Environmental heterogeneity is a ubiquitous feature of natural landscapes, yet its effect on the evolution of cooperation

has not been extensively studied. I demonstrate that resource availability heterogeneity, in both time and space, acts to maintain cooperation at higher levels than homogeneous environments of the same total resource value. This effect is due to the covariance between productivity and the cost of cooperation: high resource availability periods and spaces are highly productive, and also incur a relatively lower cost of cooperation.

1 Introduction

1.1 Social evolution theory

When organisms interact, and fitness consequences ensue, social evolution theory provides a paradigm with which to explore the biological causes and consequences of these interactions. In the simplest consideration, an interaction between two organisms - an actor and a recipient - there are four possible fitness consequences; 1) mutualism; a fitness benefit for both actor and recipient, 2) altruism; a fitness cost for the actor and benefit for the recipient, 3) selfishness/parasitism; a fitness benefit for the actor and cost for the recipient, 4) spite; a fitness cost for both actor and recipient.

Conceptually, behaviours resulting in fitness benefit for the actor are easy to understand in the light of evolution; individuals performing such behaviours are simply increasing their own replication frequency. Altruism and spite, however, being costly to the actor, require a different appreciation of the evolutionary maximand. For altruism to evolve, this maximand cannot be individual fitness, as by definition altruism is costly to the individual. Hamilton (1964) proposed that rather than simply maximizing personal fitness, evolution would favour genes maximizing their total frequency regardless of the organism in which they were found; this is the "inclusive fitness" approach to understanding adaptation.

Kin selection is the most easily illustrated example of inclusive fitness, as cooperation between family group members is perhaps the most readily observable altruistic behaviour. Kin groups share genes, by definition, so it is no surprise that social behaviours arise here. The evolutionary biologist J.B.S. Haldane, is said to have first outlined the notion verbally:

"I would gladly lay down my life for two brothers or eight cousins"

But the work of Hamilton in 1964 is where greatest recognition of the idea is bestowed. We now refer to "Hamilton's rule", which states that the cost an actor is willing to incur in performing a social action will be weighed against the benefits it brings to the recipient and the probability of their shared genes. Expressed algebraically, a behaviour will be selectively favoured if $rb - c > 0$, where r is the relatedness, c is the costs incurred to the actor, and b is the benefit to the recipient.

1.2 The greenbeard effect

Inclusive fitness requires that actors are somehow able to direct their cooperative behaviour towards individuals sharing their genes. One way this can be achieved is if dispersal is limited: Provided social interactions are locally limited, interacting individuals then necessarily share genes by descent (Hamilton, 1964; Lehmann & Keller, 2006). Other solutions to the problem of shared-gene discrimination rely on some form of recognition

mechanism. Individuals may learn to recognise a location that predictably contains kin, or a phenotype matched to known kin (Blaustein, 1983). Alternatively, the recognition may be based on a genetic system, of which there are two, both dubbed by Dawkins: The first is based on self-referent phenotypic matching (Mateo & Johnston, 2000), called "the armpit effect" (Dawkins, 1982), in which an individual evaluates relatedness by comparing their own phenotype to that of a conspecific (i.e., by assessing the degree of similarity in each other's armpit odour), and then directing behaviour according to the degree of similarity. The second is a distilled form of this idea, originally proposed by Hamilton (1964), in which tags are used as a form of genetic identification. Known as the "greenbeard effect" (Dawkins, 1976), an individual gene, or tightly linked cluster of genes, encodes three functions: 1) a phenotype (e.g., a green beard); 2) recognition of that phenotype in other organisms and; 3) directing of cooperative behaviour towards those organisms, thus increasing the frequency of the gene. Chapters 2 and 3 explore greenbeard-type public-goods cooperation. Specifically, the diversity that such systems display, and the causes and consequences of this diversity.

Hamilton's tag-based cooperation concept (the greenbeard effect) was introduced as just that – a hypothetical concept – not thought necessarily to exist. Alleged barriers to the real-world existence of greenbeard genes included the burden of complexity required of one gene encoding three

functions; collapse of the system through the evolution of non-cooperative "false beards" – individuals possessing the phenotype, receiving the cooperative behaviour, but not themselves behaving cooperatively; and simply that the gene would be so successful to render it fixed within a population and thus unnoticeable (Dawkins, 1982; Blaustein, 1983).

Naturally then, greenbeard genes were discovered. The first being in the imported red fire ant, *Solenopsis invicta*, by Keller & Ross (1998). In multiple queen colonies, it was noticed that all egg-laying queens were *Bb* heterozygous at the Gp-9 locus. Homozygous *bb* queens were intrinsically inviable, whilst *BB* homozygous queens were killed, predominantly by heterozygous, *Bb,* workers, who used an olfactory que to identify the Gp-9-linked phenotype. Gp-9^b was acting according to the greenbeard effect. In the language of social evolution, inclusive fitness is maximised through spite as an indirect fitness benefit is garnered through increased resource availability for Gp-9^b queens.

Other real-world examples of the greenbeard effect illustrate different ways in which greenbeards can operate. These can be broadly broken-down into helping and harming greenbeards (Gardner & West, 2010). In the case of the red fire ant, the greenbeard is harming, as the inclusive fitness benefits are secured by executing a competitor.

Harming greenbeards get their inclusive fitness benefits through elimination of competition, with harm conferred exclusively to non-bearers. An example from the microbial world; bacteriocin production is a harming greenbeard: These anti-competitor toxins, produced by many microorganisms, are secreted into the environment, encountering bearers and non-bearers alike. Encoded in tight linkage with the toxin is a gene that deactivates the it, rendering bearers immune (Riley & Wertz, 2002).

Helping greenbeards include expression of the csA cell adhesion gene in the social amoeba *Dictyostelium discoideum* (Queller et al., 2003): A slime-mould that propagates via the formation of cell aggregations known as "fruiting bodies", which require the expression of an adhesion protein, coded by the gene *csA*. In this example, individuals with *csA* adhere to each other more effectively than genetic knock-outs, which are left behind as the fruiting body develops.

Cell-to-cell adhesion is also the mechanism behind which the yeast *Saccharomyces cerevisiae* appears to benefit from the greenbeard gene, *FLO1* (Smukalla et al., 2008). Here the greenbeard gene is involved in flocculation, a behaviour in which cells aggregate when under conditions of stress. The number of tandem repeats within the *FLO1* gene correlates with the strength of flocculation, and exhibits a wide degree of variation (Smukalla et al., 2008). This may be the result of varying selective pressure in different

environments but could also be a consequence of social cheating. Foregoing the cost of expressing *FLO1* with a high number of tandem repeats, cheats may yet retain the benefit of adhesion provided by conspecifics, making them social "green-bearded cheaters" (Brown & Buckling, 2008), also referred to as "false beards".

The ability of greenbeard systems to potentially generate and maintain diversity has been explored in a theoretical treatment of a helping greenbeard system: That of siderophore production in the bacterium *Pseudomonas aeruginosa* (Lee *et al.*, 2012). As with most forms of life, bacteria require iron to grow (Andrews *et al.*, 2003). When limited, bacteria secrete siderophores into their environments. Having a strong affinity for iron, these molecules are able to bind the nutrient from otherwise biologically unavailable sources, after which they are taken-up via a cognate receptor. In the case of *P. aeruginosa,* the siderophore is called "pyoverdine" and is structured of a chromoflurophore, which gives the molecule its characteristic fluorescent green colour and iron binding property, and an attached peptide chain (Wendenbaum *et al.*, 1983; Demange *et al.*, 1990). The function of the peptide chain is to mediate up-take via binding to the receptor, *fpvA*, which is encoded adjacent to the pyoverdine cluster (Merriman *et al.*, 1995). There are three main classes of pyoverdine, designated types -I, -II, and -III, with uptake fairly restricted to type (Meyer, 2000), though as with *FLO1*, the pyoverdine region exhibits a wide degree

of variation within and between types (Smith *et al.*, 2005; Bodilis *et al.*, 2009).

Phylogenetic studies have shown that the pyoverdine locus is under diversifying selection; the gene is present in all strains, but multiple alleles are maintained (Smith *et al.*, 2005). The evolutionary reasons for this may be environmental variation, for example, environmental viscosity has been shown to correlate with siderophore diffusibility (Kümmerli *et al.*, 2014). Alternatively, receptor diversity could be a resistance mechanism as, for example, the pyoverdine receptor, fpvAII, is an entry site for pyocin S3 (a bacteriocin) (Baysse *et al.*, 1999).

As with *FLO1,* an intriguing alternative explanation is whether the diversity exhibited at this locus results from the selective pressure exerted by false-beard cheats. After the breakdown of greenbeard cooperation brought about by a false-beard cheat invasion, a novel beard colour would then be able to invade but would itself be susceptible to novel cheats. Hamilton recognised that this succession of events could lead to the continual generation of tag diversity (Hamilton, 1964). "Beard chromodynamics" has emerged as a field studying the interplay between different greenbeard systems and their cheats. Simulations have shown that rather than the continual generation of diversity, maintenance of cooperation is possible

even with small numbers of beard colours (Jansen & van Baalen, 2006; Lee *et al.*, 2012).

Such maintenance of cooperation is possible in patch-based landscapes as the fitness of each beard-colour/social strategy combination is negatively related to its frequency: High frequency of one beard colour means a large available niche for that same coloured "false beard"; whilst a high frequency of any one false-beard colour means a large available niche for an alternative coloured greenbeard (Lee *et al.*, 2012).

However, the maintenance of siderophore based cooperation is further complicated by the ability of siderophores in mixed populations to act simultaneously as helping and spiteful greenbeards: Iron bound in complex with one siderophore is unable to be dissociated by another, and in this way siderophore production can be used as a competitive trait (Niehus *et al.*, 2017). For example, in a competition between two *P. aeruginosa* strains producing two different pyoverdine types, one strain engaging in substantial overproduction of pyoverdine could, even in lieu of growth, rapidly saturate the environment with its own pyoverdine, rending it uninhabitable to a competitor.

Specific hypothesis testing using empirical investigation of public-goods based greenbeard systems, particularly with respect to the maintenance of

cooperation and the generation of diversity discussed above, has been scant until recently (Inglis *et al.*, 2016; Butaitė *et al.*, 2017; Leinweber *et al.*, 2017). Chapters 2 and 3 aim to expand our understanding of the real-world dynamics of tag-based cooperation by testing specific hypotheses regarding the interactions of two strains of *P. aeruginosa* producing two different pyoverdines and their corresponding, non-pyoverdine producing mutant cheats.

1.3 Spatial and temporal heterogeneity

Much of the work on cooperation, both empirical and theoretical, has focussed on the "*rb*" components of Hamilton's inequality, i.e., the conditions under which relatedness favours cooperation, the fitness benefits it brings, and the interplay between the two. In experimental systems at least, the effects of variation in the cost of cooperation are an often overlooked, yet crucial factor in developing our understanding of social evolution. Chapters 4 and 5 explore how different environments incur different costs upon cooperative behaviour. Specifically, we explore how cooperation responds to environments with spatial (chapter 4) and temporal (chapter 5) heterogeneity in resource availability, which will alter both the cost of cooperation and the population productivity.

Heterogeneity is a ubiquitous feature of natural landscapes which has been shown to influence population dynamics and community structure

(Waxman & Peck, 1999; Chesson, 2000; Maestre & Reynolds, 2006; Oliver *et al.*, 2010; Blanquart & Gandon, 2011; Eilts *et al.*, 2011; Chisholm *et al.*, 2014). The "geographic mosaic theory of coevolution" provides a useful framework to conceptualise how heterogeneity can effect populations (Thompson, 1999, 2005). This concept considers an environmental landscape as a mosaic, made up of patches, within which species interact and between which species migrate. Studies of coevolving hosts and parasites have shown that variation in productivity in spatially heterogeneous landscapes leads to "hotspots" of coevolution (Forde *et al.*, 2004; Vogwill *et al.*, 2009): Whilst productivity within patches may alter the velocity, or possibly even trajectory of coevolution, migration between patches of different productivity can provide an asymmetry, homogenising the metapopulation in favour of the dynamic of the highest productivity patches.

This asymmetry depends entirely upon the mode of migration. If a fixed fraction of each patch within the mosaic is allowed to migrate, then indeed the metapopulation will become over-represented by individuals from the patches of highest productivity. This is a form of "hard selection" (Christiansen, 1975; Wallace, 1975): The number of migrants from each patch that form the next generation is a function of the number of individuals within the patch. This is opposed to "soft selection", in which an equal number of migrants from each patch would form the next

generation. Where an effect of selection covaries with productivity, under hard selection the makeup of the metapopulation is skewed towards the selective effect in highly productive patches.

Resource availability drives population productivity. Resource availability can also affect other aspects of biology, from evolutionary diversification (Hall & Colegrave, 2007), to host/parasite interactions (Westra *et al.*, 2015), the position of partners in the mutualism parasitism spectrum (Boza & Scheuring, 2004; Bull & Harcombe, 2009; Hom & Murray, 2014; Hoek *et al.*, 2016), and patterns of species diversity (Eadie & Keast, 1984; Zhou *et al.*, 2002; Eilts *et al.*, 2011).

Cooperative behaviour is also affected by the level of resource availability (Brockhurst *et al.*, 2008, 2010; Xavier *et al.*, 2011). The reason for this relationship is likely due to the pay-out from the metabolic trade-off between investment in growth and investment in cooperation, providing diminishing returns on growth and favouring cooperation as resource availability increases. Chapter 4 aims to study the link between productivity and investment in cooperation in populations experiencing heterogeneous resource availability. Specifically, we transfer populations of *P. aeruginosa* over evolutionary time-scales (~40 two-day transfers) through either spatially homogeneous or heterogeneous environments with respect to

resource availability, measuring the per capita investment in cooperation along the way.

Heterogeneity can also vary with respect to time. The majority of previous research in this area has focussed again on the relationship between heterogeneity and species diversity, with not much attention on the effect of temporal heterogeneity on the evolution of cooperation. Studies that have looked at cooperation to date, have found that temporal heterogeneity correlates with increased levels of cooperation. For example, a greater number of bird species are cooperative breeders in heterogeneous environments (Rubenstein & Lovette, 2007; Jetz & Rubenstein, 2011). The mechanism underlying this pattern is unknown. Rubenstein & Lovette (2007) posit that individuals reared in favourable periods behave cooperatively in periods of low resource availability, as non-cooperative breeding in harsh periods is likely to fail. Cockburn & Russell (2011) identify that this may be a population dynamic effect, due to the additional accumulation of offspring during favourable conditions.

Further contributing to the over-representation of cooperation in variable environments, alternating harsh and favourable conditions can create bottlenecks in populations, increasing relatedness, which, referring back to Hamilton's rule, also favours cooperation (Brockhurst, 2007).

An extension of the work on spatial heterogeneity, the effect of temporal variation on the evolution of cooperation is the subject of chapter 5. Specifically, we transfer populations of *P.aeruginosa* through environments that vary with respect to resource availability through time, following the fitness of wild-type cooperators relative to pyoverdine knock-out cheats along the way.

1.4 The model system *Pseudomonas aeruginosa*

P. aeruginosa is a rod-shaped gram-negative member of the class gammaproteobacteria. Having an unusually large number of genes in its genome, *P. aeruginosa* is a versatile species, able to grow in a wide variety of environments (Woolverton *et al.*, 2016). *P. aeruginosa* is a species of interest as opportunistic human pathogen, infecting burns victims, immunocompromised patients, and cystic fibrosis sufferers, in which chronic infection of the lung is of particular concern (Bodey *et al.*, 1983).

Iron is crucial for the survival of almost all life (Andrews *et al.*, 2003). In iron-limited environments, microbes secrete siderophores; iron-scavenging molecules that diffuse into the environment, bind otherwise biologically unavailable iron, and are subsequently taken-up by any bacteria with the cognate receptor. *P.aeruginosa,* like other members of its genus, producers a green-yellow fluorescent siderophore called "pyoverdine". As described in

the previous section, a fluorophore at the centre of the molecule gives pyoverdine the characteristic pigmentation and iron binding property, whilst an attached peptide chain is involved in receptor binding and uptake (Wendenbaum *et al.*, 1983; Demange *et al.*, 1990).

As a secreted siderophore will diffuse away from the individual producing it, a direct benefit from production cannot be guaranteed. Pyoverdine production has been shown to incur a metabolic cost, which becomes a relative fitness cost when producers are grown in competition with mutants, who forego pyoverdine production but retain the receptor (West & Buckling, 2003). See figure 1.

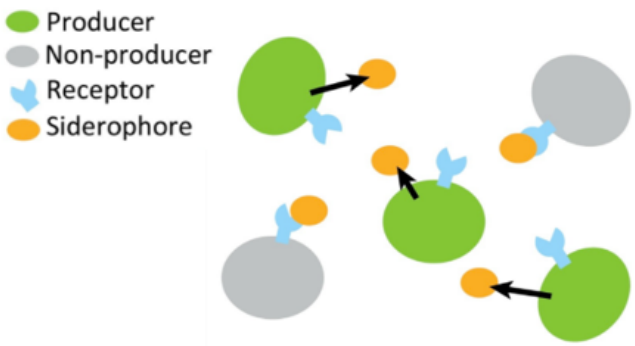

Figure 1. Secreted siderophores (yellow) can be taken up by any cell with the cognate receptor (blue). Non-producers (grey) incur no cost of production and will outgrow producers (green). (Reproduced from Ghoul et al., (2017)).

Pyoverdine production can therefore be considered a social trait, and as such has been extensively and successfully employed as a model organism

in study of the evolution of public-goods based cooperation (West & Buckling, 2003; Harrison *et al.*, 2006; Kümmerli *et al.*, 2009, 2014; Lee *et al.*, 2012; Zhang & Rainey, 2013; Inglis *et al.*, 2014; Butaitė *et al.*, 2017; Leinweber *et al.*, 2017).

Understanding the ecology and evolution of public-goods based cooperation using the *P.aeruginosa* model is not only fascinating in its own right, but potentially useful for developing novel therapy (Foster, 2005), as pyoverdine production is a known virulence factor (Meyer *et al.*, 1996; Takase *et al.*, 2000; Harrison *et al.*, 2006)

2 Cheat-mediated evolution of siderophore diversity in Pseudomonas aeruginosa

2.1 Abstract

Cooperation can be maintained if cooperative behaviours are preferentially directed towards other cooperative individuals. Tag-based cooperation (greenbeards) – where cooperation benefits individuals with the same tag as the actor - is one way to achieve this. Tag-based cooperation can be exploited by individuals who maintain the specific tag but don't cooperate, and selection to escape this exploitation can result in the evolution of tag diversity. We tested key predictions crucial for the evolution of cheat-mediated tag diversity using the production of iron-scavenging pyoverdine by the opportunistic pathogen, *Pseduomonas aeruginosa* as a model system. Using two strains that produce different pyoverdine and their respective cheats, we show that cheats outcompete their homologous pyoverdine producer, but are outcompeted by the heterologous producer. Moreover, co-inoculating two types of pyoverdine producer and one type of pyoverdine cheat resulted in the pyoverdine type whose cheat was not present having a large fitness advantage, and often reaching fixation. However, when all pyoverdine producers and cheats were co-inoculated, both types of pyoverdine producers were outcompeted. These data are

consistent with theory suggesting that cheats can maintain tag diversity under metapopulation dynamics, where not all cooperating and cheating tag combinations are present in all patches simultaneously, but that tag-based cooperation will be lost in well-mixed populations, regardless of tag diversity.

Keywords: cooperation; cheating; public-goods; Greenbeard effect

2.2 Introduction

Cooperation, where an individual pays a cost to benefit others, can be evolutionary unstable because non-cooperative organisms can exploit cooperators. Cooperation can however be maintained if the benefits of cooperation can be directed towards other co-operators, and this can occur in two ways. First, when cooperative interactions occur more frequently between genealogical kin, as a result of either limited dispersal and/or kin recognition (Hamilton, 1964; Lehmann & Keller, 2006; West *et al.*, 2007). Second, when a single gene complex encodes a tag and a behaviour to help individuals with that tag ("greenbeard genes") (Hamilton 1964, Dawkins, 1976; Biernaskie *et al.*, 2011, 2013); or more generally if helping and interaction with individuals with same helping genes are encoded together (Gardner & West 2010). While originally thought to be very rare, recent years have uncovered more and more examples of both helping and harming greenbeard genes (the latter encoding harming behaviour towards

individuals without the greenbeard (Gardner & West 2010; Biernaskie et al 2013)), in organisms including lizards, fire ants, bacteria, yeast and amoeba (Keller & Ross, 1998; Queller *et al.*, 2003; Sinervo *et al.*, 2006; Inglis *et al.*, 2009; Smukalla *et al.*, 2008).

There is also growing evidence that in some organisms multiple "beard colours", or tags, exist (Smukalla 2008; Bodilis *et al.*, 2009). Such diversity can theoretically be explained by the evolution of "falsebeards" - cheats that are able receive the benefits of directed cooperation without paying the cost (Jansen & van Baalen, 2006; Rousset & Roze, 2007; Lee *et al.*, 2012) - in spatially structured populations. This is because a tag type that is not exploited will out-compete both the tag types that are exploited and their associated cheats within a patch; and population structure allows the combination of tag-types and their cheats that interact to vary in space and time. As such, different tag types will win out at different localities. Crucially, a tag-type is less likely to encounter its own cheat if that tag-type is rare in the population, and it is this negative frequency dependence that can then result in stable coexistence of multiple tag-types (Lee et al 2012). Note that if populations are well mixed, such that all tag-types and their respective cheats are present in all patches, the maintenance of tag-based cooperation is unlikely (Rousset & Roze 2007). This is because the advantage of being a rare tag-type with few cheats in a patch is offset by the

advantage of being a common tag type that necessarily receives more helping behaviour.

Here we experimentally determine whether the presence of cheats can explain the maintenance of tag diversity in a potential multi-beard colour system: pyoverdine production in the bacterium *Pseudomonas aeruginosa*. Pyoverdine is costly to make (Griffin et al 2004) and pyoverdine-iron complexes can be used by any cells with an appropriate receptor (Hohnadel & Meyer, 1988; Cornelis *et al.*, 1989; Smith *et al.*, 2005), hence pyoverdine can be a cooperative trait and producers can be invaded by cheats (West & Buckling, 2003; Griffin *et al.*, 2004; Harrison *et al.*, 2006; Buckling *et al.*, 2007; Harrison & Buckling, 2009). Three types of pyoverdine and receptor pairs have been described to date (Cornelis *et al.*, 1989; Meyer *et al.*, 1997; De Vos *et al.*, 2001; Bodilis *et al.*, 2009), and cross-feeding, binding, and uptake assays suggest that pyoverdine-mediated iron transport is relatively specific to the pyoverdine-receptor combination (Hohnadel & Meyer, 1988; Cornelis *et al.*, 1989), although a generalist receptor can also be present. We determine whether the within-patch competitive outcomes required for cheat-mediated maintenance of tag diversity hold for pyoverdine diversity, namely: 1) Tag-based cooperators are outcompeted by their tag-specific cheats, but outcompete cheats associated with a different tag; and 2) A specific cooperator tag type without cheats will outcompete other cooperator tag types and their associated cheats. Finally, we test the

prediction (3) that tag-based cooperation will be selected against when each tag cooperator type has its cheat present, regardless of tag diversity.

2.3 Materials and methods

2.3.1 Bacterial strains

Pseudomonas aeruginosa strains PA01 and 59.20 produce siderophore types I and III respectively, each expressing a surface receptor cognate with the siderophore type produced (*fpvAI* in the case of PA01 and *fpvAIII* in the case of 59.20) (De Chial *et al.*, 2003). After 10-20 two-day transfers in iron-limited casamino acid media (CAA) (see below), one non-siderophore producing mutant colony from each strain was picked based on loss of yellow-green pigmentation. As pyoverdine is a green, fluorescent siderophore, non-producing mutants (cheats) are demarcated from pyoverdine producing strains (cooperators) due to their colonies' lack of pigmentation when grown on KB agar. Picked colonies were then grown in CAA, and from each 1mL was mixed with glycerol and frozen at -80˚C. The chrome azurol sulphate S (CAS) assay was used, as described in (Harrison & Buckling, 2007), to derive *per capita* iron chelator activates of pyoverdine producers and non-producers. The activity of each cheat was compared with the cooperator from which it was derived with a two-sample t-test.

To unambiguously distinguish strains in pairwise competitions, both the PA01 cheat and 59.20 cooperator were transformed with the *lacZ* operon (following (Choi *et al.*, 2006)). Briefly, to render bacteria electro-competent, populations were grown overnight in 6mL Luria-Bertani (LB) medium, harvested by centrifugation (2 min at 14,000 rpm), washed twice with room temperature 300mM sucrose, and resuspended in 100μL sucrose. For electroporation, 500ng of purified plasmid DNA was mixed with 100μL electrocompetant cells and transferred to a 2mm gap width electroporation cuvette (BioRad). A pulse of 2.5kV was applied to the cells, after which 1mL of LB was used to wash the cells from the cuvette. This wash was then incubated for 1 hour at 37˚C, shaken at 180 r.p.m. 100μL of this final culture was plated onto an LB+Gm (30μg/mL) +X-gal (20μg/mL) plate. Colonies with the LacZ phenotype were blue on x-gal (20μg/mL final concentration) plates, and colony from each genetic background was selected for the experiment. Note that there was no detectable growth rate cost associated with insertion of the *lacZ* operon under the culture conditions described below.

We used colony PCR in in conjunction with the LacZ phenotype to distinguish strains in 3- and 4-way competition experiments. Populations were plated onto KB agar containing 30μg/mL X-Gal and incubated overnight at 37˚C. Colonies from these plates were picked into 75μL Milli-Q H_20. 1μL of this was used as template for a PCR: GoTaq Green Master

Mix (Fisher) containing 0.25µM of each forward and reverse primers for PA01 and 59.20 ferripyoverdine genes FpvAI and FpvAIII (De Chial *et al.*, 2003), respectively. The thermocycler was run with the following parameters: 96˚C for 10 minutes, followed by 35 cycles of 95˚C for 30 seconds, 55˚C for 30 seconds, and 72˚C for 30 seconds, with a final extension of 7 minutes at 72˚C. Two distinct bands, corresponding to each strain, were observable following electrophoresis (1.2% agarose in TAE, 35 minutes at 120V).

2.3.2 Culture conditions

Six replicate cultures were established from a single colony of each of the four strains. These starting cultures were grown overnight at 37˚C in 30mL glass universal tubes containing 6mL (KB) on a 180 r.pm. orbital shaker. 1mL of each culture was centrifuged at 13,000 r.p.m. for 5 minutes at room temperature, then suspended in 1mL of M9 salts (70 g Na_2HPO_4•$7H_2O$, 30 g KH_2PO_4, 5 g NaCl, 10 g NH_4Cl). Suspensions were diluted in M9 salts to OD 600nm 0.2 (~10^8 cfu.mL^{-1}). Strains were then combined as appropriate for the specific experiment and treatment, described below and 60µL from each mix was inoculated into 6mL of casamino acids (CAA) media (5g casamino acids, 1.18g K2HPO4•3H2O, 0.25g MgSO4•7H2O, 1L H2O), supplemented with 100µg/mL human apotransferrin (sigma, Gillingham, UK), an iron chelator, and 20mM NaHCO3 (sodium

bicarbonate), required for iron chelator activity (Meyer et al., 1996). Cultures were plated immediately after inoculation and incubated at 37˚C in static, 30mL glass universal tubes. Every 24 hours, cultures were plated and 1% transferred into new iron-limited CAA media, for 6 transfers, or until a strain was no longer detectable.

2.3.3 1. Monoculture growth.

Each strain was grown as a monoculture, and Malthusian parameters ((m = ln(final density/starting density) (Lenski et al., 1991)) of cooperators and corresponding cheats compared using t-tests.

2.3.4 2.. Cheat specificity.

To investigate the performance of cheats in the background of either the strain from which they were derived (their homologous strain), or the alternative siderophore-type producer (their heterologous strain), four two-way competition treatments were setup competing each cooperator strain against each cheat. The relative fitness (*W*) of the focal competitor in each competition was calculated from the ratio of each strain's 24 hour Malthusian growth parameter (*m*) averaged through time. Fitness differences between competitors were determined by carrying out 1 sample t-tests.

2.3.5 3. Three-way competitions.

To determine if being cheat free allows one co-operator strain to outcompete both the other cooperator strain and its associated cheat, we competed both cooperators with one or other of the cheats. The relative fitness *(W)* of the non-exploited cooperator was determined with respect to growth of the total competitor population, and analysed as above.

2.3.6 4. Four-way competitions

To test whether the presence of cheats affected the diversity of cooperator types when competing directly, we determined the frequency of each type when all four strains were competing and when only the two producing strains were competing. This experiment was replicated in two blocks because of high-within treatment variation. We estimated diversity as the proportion of the rarest strain background (i.e. summing the density PAO1 and its cheats and 59:20 and its cheats in the 4-way competition experiments) which was used as the response variable in a linear mixed model, with treatment (with or without cheats present), time, experimental block, and the treatment by time interaction fitted as explanatory variables, and replicate as a random factor nested within treatment.

2.4 Results

2.4.1 Growth cost of cheats in monoculture

Both cooperators had a greater growth rate that their respective cheats (PAO1: $t_{10} = 7.089$, $p < 0.0001$; 59.20: $t_{10} = 2.457$, $p = 0.0378$) as monocultures, demonstrating a growth rate cost of the non-pyoverdine producing mutants under iron-limited conditions.

2.4.2 Per capita iron chelator activity of cheats and cooperators

Significant differences in iron chelator activity were found between PA01 cooperator and PA01 cheat ($t_{10}=14.038$, $p<0.0001$), and between 59.20 cooperator and 59.20 cheat ($t_{10}=10.664$, $p<0.0001$)).

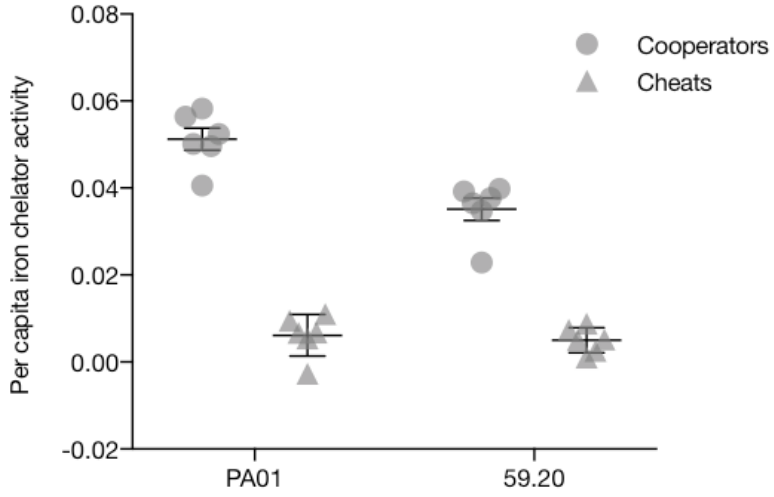

Figure 1. Results of CAS assay showing per capita iron chelator activities of PA01 WT (cooperator), PA01 cheat, 59.20 WT (cooperator), and 59.20 cheat. Error bars show mean ±SEM.

2.4.3 Growth rates with and without lacZ operon

There was no detectable growth rate cost associated with insertion of the *lacZ* operon under the culture conditions described below (59.20, with and without *LacZ*, T_{10} = 1.314 p = 0.218; PA01, with and without *LacZ*, T_{10} = 1.153, p = 0.276,

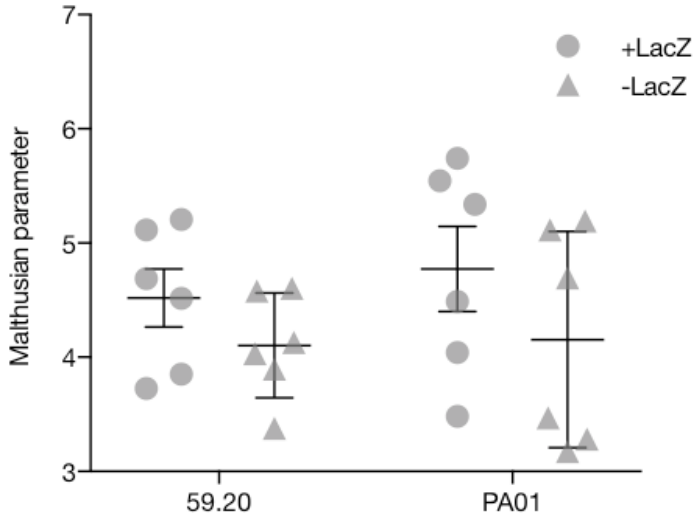

Figure 2. Malthusian parameters of strains 59.20 and PA01 with and without the *LacZ* marker inserted. Error bars show mean ±SEM.

2.4.4 Cheat specificity

In two-way competition, the PA01 cheat had a higher fitness than PAO1 cooperator (Figure 1; t_5 =74.538, $p < 0.001$) and a lower fitness than the 59.20 cooperator (t_5 = -12.86, $p < 0.001$). Similarly, the 59.20 cheat had a higher fitness than 59.20 cooperator (t_5 = 9.676, $p < 0.001$) and a lower fitness than PAO1 cooperator (t_5 = -8.419, $p < 0.001$). At the final time

point, cooperators in all six replicate populations from both homologous competitions had been eliminated, whilst in heterologous competitions cheats had been eliminated from 6/6 replicates in the case of PA01 cheat vs 59.20 cooperator, and 5/6 in the case of 59.20 cheat vs PA01 cooperator. These results indicate that cheats were only cheats with respect to their homologous cooperator.

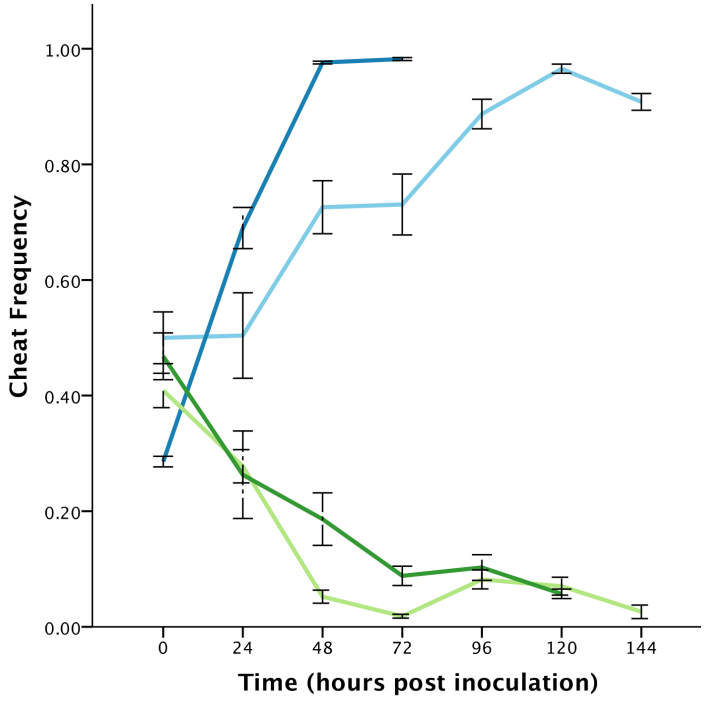

Figure 3. Cheat frequency over time. Cheats dominate the population in the presence of the homologous cooperator from which they were derived (blues), and are eliminated from the population when in competition with a heterologous cooperator (greens). Light blue and light green show 59.20 cheat in the presence of 59.20 cooperator and PA01 cooperator, respectively. Dark blue and dark green show PA01 cheat in the presence of PA01 cooperator and 59.20 cooperator, respectively.

2.4.5 Cooperators with no cheats outcompeted cooperator-cheat combinations

The PAO1 cooperator outcompeted the combination of cooperator and cheat 59.20 (Figure 2; t-test, $t_5 = 3.087$, $p = 0.027$), and went to fixation in 1/6 replicates. The 59.20 cooperator outcompeted the combination of PAO1 cooperator and its cheat (Figure 2; t-test, $t_5 = 7.50$, $p < 0.001$), going to fixation in 6/6 replicates. These results show that the absence of a cheat allows a cooperator strain to outcompete other cooperator-cheat combinations.

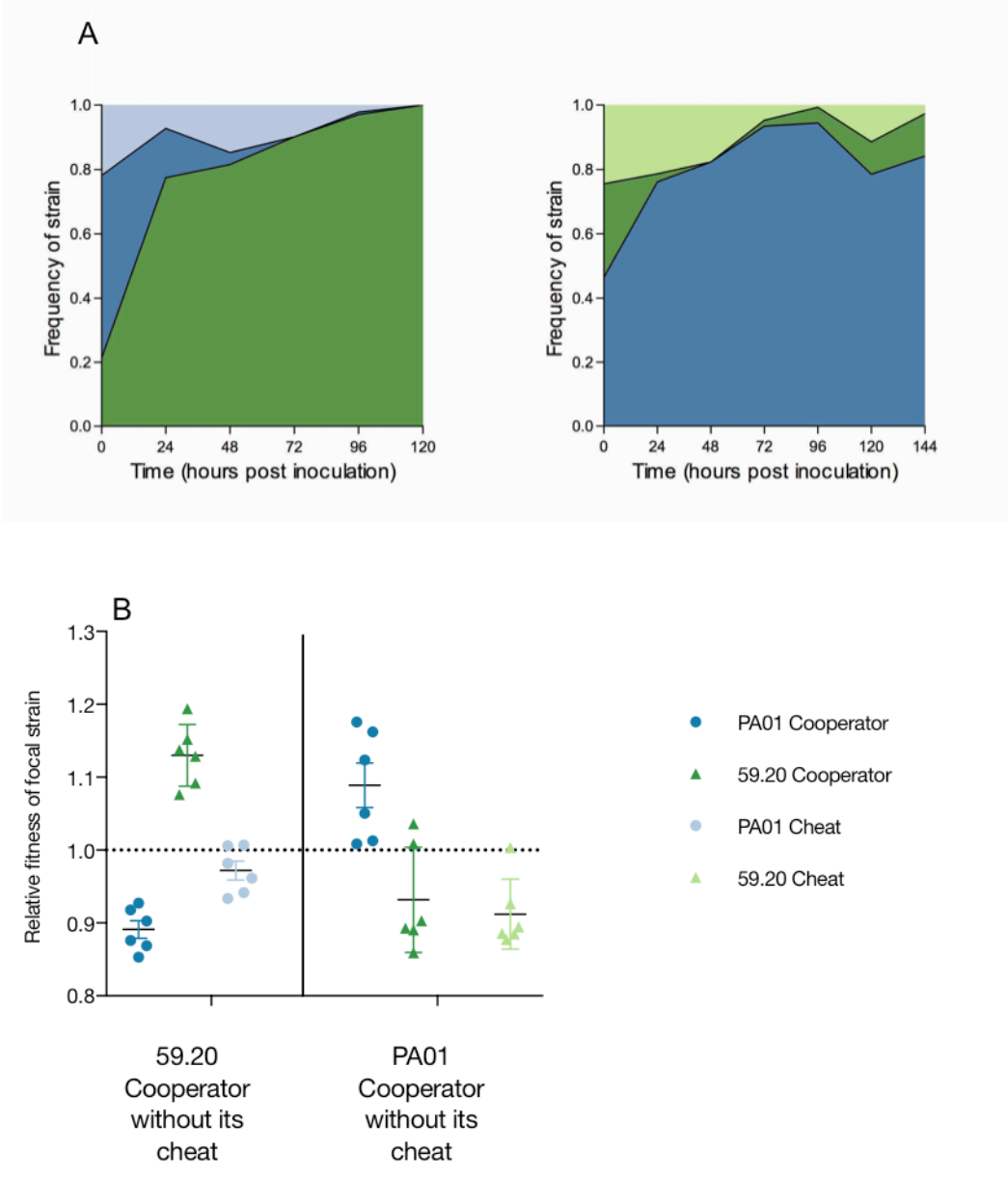

Figure 4. A) Frequency of strains in three-way competitions. The outcome of competition between two cooperating strains is strongly influenced by the type of cheat also present in the population. The frequency of PA01cooperator is shown in dark blue, 59.20 cooperator in dark green, with PA01 and 59.20 cheats in light blue and light green. **B)** relative fitness (Malthusian parameter of focal species relative to the Malthusian parameter of the combination of the other competitors) averaged over time of all strains in the two three-way competitions.

2.4.6 Cheats did not promote within-patch coexistence of siderophore types

The presence of cheats had no effect on the mean proportion of the rarer siderophore type in a population (Figure 3; treatment: $F_{1,21} = 0.4582$, $p = 0.506$). Diversity decreased through time in both treatments (time: $F_{1,142} = 59.576$, $p < 0.001$), with PA01 the dominant strain in all replicates, but we did not observe competitive exclusion in most cases.

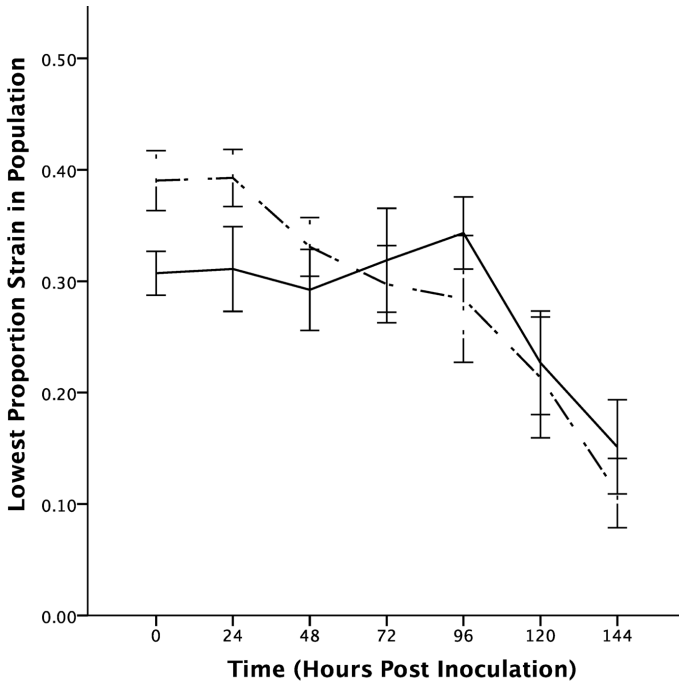

Figure 5. Mean lowest proportion of strain over time. Cooperators and cheats of the same strain type in the "With Cheat" treatments were summed, and the proportions of each strain were calculated for both treatments. The lowest proportion in each replicate was taken as a crude measure of diversity. Dashed line represents populations without cheats, solid line represents populations with cheats. Error bars show mean ±SEM.

2.5 Discussion

Here, we determine whether cheats can potentially maintain the diversity of *P. aeruginosa* pyoverdine "greenbeard" alleles from competition experiments between two types of pyoverdine producer and their respective cheats. Cheats were able to out-compete their homologous cooperators, but cooperators were able to out-compete the heterologous cheats. Moreover, when multiple tag types and cheats are present in a patch, tags without cheats outcompete other strains. Our data suggest that cheating may play an important role in maintaining tag diversity and cooperation (Smith *et al.*, 2005; Rousset & Roze, 2007; Lee *et al.*, 2012) under the assumption of a metapopulation structure, where not all combination of tag types and cooperator/cheat strategies are present in every patch. Such population structure is probable in nature, given bacterial patterns of dispersal (Bell, 2010), and that cheats can readily arise by mutation in public goods traits (Griffin *et al.*, 2004; Harrison *et al.*, 2006; Ross-gillespie *et al.*, 2007; Brockhurst *et al.*, 2008, 2010; Köhler *et al.*, 2009; Biernaskie *et al.*, 2013).

We did not find evidence that presence of cheats acts to maintain tag diversity within single patches. This result is consistent with theory that suggests in the absence of sufficient population structure tag diversity will be eroded (Rousset & Roze, 2007). Our microcosms were spatially structured (they were left static), but any resultant negative frequency dependence was presumably insufficient to overcome the positive

frequency dependent selection associated with tag types (more of one siderophore means greater iron availability for that type), or the intrinsic differences in growth rates between the cooperator strains.

In contrast to our work, recent research has also shown that diversity can be maintained within patches by a single cheat: a so called "loner effect". A dynamic polymorphism of two cooperators with different tags and a single cheat was maintained due to higher costs of cooperation of the non-exploited "loner" strain (Inglis *et al.*, 2016): cheats outcompeted their cooperator strain; the loner strain outcompeted the cheat, and the better cooperator outcompeted the loner strain. The strains used in this experiment are similar to the ones used in the upper panel of figure 2, with a PA01 cooperator/cheat pair, and a type-III siderophore producer; the same type produced by our 59.20 cooperator. The difference between the results probably reflects the relative fitness of cooperators which in our case did not have appropriate values for the loner effect to occur.

Certain strains of *P. aeruginosa*, particularly type-II pyoverdine producers, are able to take-up type-I pyoverdines (De Vos *et al.*, 2001) through a second receptor for type-I pyoverdine, *Fpv-B* (Ghysels *et al.*, 2004), whilst receptors of type-III pyoverdine can recognize pyoverdine type-II (Ghysels *et al.*, 2004). Why the ability to take-up all of a competitor's pyoverdines ("multi-beard") is not a ubiquitous trait is unclear, but one evolutionary constraint

acting on this "multi-bearded" phenotype may be increased susceptibility to bacteriocins. Pyocin S3, a bacteriocin of *P. aeruginosa*, uses pyoverdine type-II as its receptor (Baysse *et al.*, 1999), whilst pyocin S2 kills strains via *FpvA* receptor type-I (Denayer *et al.*, 2007). The functional link between pyocins and pyoverdines in the form of the receptor raises the possibility that the cooperative greenbeard system based around pyoverdines has driven the evolution of pyocin diversity.

Diversity of helping tags are present in other microbial systems, and it is possible that cheating plays a role in their evolutionary maintenance. For example, flocculation – a stress-resistance aggregation phenotype – in *Saccharomyces cerevisiae* is encoded by the highly variable *FLO1* gene with individuals not expressing the gene excluded from the aggregate (Smukalla *et al.*, 2008; Brown & Buckling 2008). Cheating may also play a role in the maintenance of diversity of harming tags. For example, *Escherichia coli* produce a diversity of colicins, a plasmid encoded bacteriocin which kills non-plasmid carrying individuals (James *et al.*, 1996), with positive selection acting at the colicin and immunity loci (Riley, 1993). Immune, but non-colicin producing mutants can invade colicin producers, but are outcompeted by sensitive non-producer (Kerr et al 2002), suggesting that immune, colicin non-producing, greenbeard cheats may also have imposed selection for diversity in colicins (Pagie & Hogeweg, 1999; Biernaskie et al 2013).

3 Greenbeard diversity and spatial structure

3.1 Abstract

Public-goods based cooperation is abound in nature, especially in microorganisms, which secrete into the environment products that many individuals can take up. Explaining the prevalence of these types of behaviours given that cheating confers a growth advantage has been the focus of much theoretical and empirical work. Tag-based cooperation, also known as the greenbeard effect, occurs when the cooperative actor and recipient share a tag, which is used as a cue to direct cooperative behaviour. Cheats can arise; individuals that display the tag but perform no metabolically-costly cooperative behaviour. The diversity of such systems may play a role in maintaining the general behaviour: Whenever cheats dominate, an alternative tag system has an advantage. As with cooperation in general, tag based cooperation requires spatial structure to be maintained. By limiting dispersal and the diffusion of the public-good, spatial structure benefits cooperators, and the benefit to rare cooperators is even greater. Using two strains of the siderophore producing bacterium, *Pseudomonas aeruginosa*, producing two different siderophore types, we test whether spatial structure and the presence of cheats induces the negative frequency dependence required of systems that maintain diversity. Contrary to our

predictions, we found that one strain consistently dominated the other, possibly because it uses its siderophore as a competitive trait.

3.2 Introduction

Cooperation based upon the greenbeard effect, where a gene or linked groups of genes, produces a phenotype, recognises that phenotype in others, and directs cooperative behaviour towards those individuals, is susceptible to cheating by false-beard individuals who display the phenotype but perform none of the cooperative behaviours. Novel phenotypes and associated recognition and directing of behaviour can rescue cooperation, but themselves are susceptible to novel cheats (Hamilton, 1964; Dawkins, 1976). "Beard chromodynamics", a field studying the interactions of different greenbeard phenotypes and their cheats, has proposed that cheats can regulate the frequency of greenbeard cooperators: An environment with a highly frequent greenbeard cooperator will select for its corresponding false-beard cheat, which in turn selects for an alternative greenbeard, and so on (Lee *et al.*, 2012). Using siderophore production in *Pseudomonas aeruginosa* as a model for cooperation, with different greenbeard phenotypes provided by different siderophore types, we have shown that the premise of the model is empirically grounded: Greenbeards are susceptible to their own cheats, and out-grow non-matching cheats. Furthermore, we have shown that in three-way competition, a greenbeard can outcompete another greenbeard, with the

winner entirely dependent on which cheat-type is present. In unstructured environments, we failed to see cheat-regulation of greenbeards, however – diversity was lost when both cooperators were present with both cheats. Here we aim to test whether the addition of spatial structure provides the necessary conditions for cheats to act as regulators of diversity.

By spatially segregating environments, viscosity creates niche heterogeneity. Viscous environments may be expected to support a greater diversity of species, following the competitive exclusion principle (Gause, 1934; Hardin, 1960). Theory suggests this may be the case: By localising the ecological processes of dispersal and between-organism interaction, viscosity can maintain diversity (Durrett & Levin, 1997; Pagie & Hogeweg, 1999b). Empirical demonstrations give the same result (Rainey & Travisano, 1998; Rainey *et al.*, 2000; Habets *et al.*, 2006), though the nature of the between-organism interaction should be taken into consideration, for example, as viscosity limits the ability for cross-feeding, such an interaction results in decreased diversity in viscous environments (Saxer *et al.*, 2009).

For diversity to be maintained in mixed populations each constituent organism must be most fit when rare (Levene, 1953; Rainey & Travisano, 1998; Friesen *et al.*, 2004). In the absence of spatial structure, greenbeard diversity may be lost due to positive frequency dependence: A higher frequency of one greenbeard type in the population increases the frequency

of beneficial encounters for that type, leading to further increase in frequency of that type, as so on to fixation. When public goods are globally distributed, rare cooperators have no advantage, as cheat frequencies vary in direct proportion to cooperator frequencies, and the competition becomes equivalent to that of one cooperator-type vs another, in which positive frequency dependence destroys diversity. As theoretical work has shown, the system fails to meet the requirement of negative frequency dependence, due to the lack of spatial structure (Rousset & Roze, 2007). It is worth noting that even in the absence of positive frequency dependence, with no negative frequency mechanism to maintain diversity, drift would also result in loss of diversity, even if diversity was not already lost due to between-strain intrinsic growth differences.

The addition of structure to the environment will limit the diffusion of the public good, decreasing the opportunity for cheating, increasing the localisation of kin, i.e., increasing the direct and indirect fitness benefits of cooperation (Kümmerli *et al.*, 2009). Spatial structure decreases the chance that a cheat encounters the public good, and when rare this effect is more pronounced; rare regions in which cooperation is high are less likely to be encountered than common regions. With proportionally less cheating upon rare cooperators, negative frequency dependence is possible, and hence the maintenance of diversity.

To test this, we setup competition experiments in spatially structured environments, with two strains of *Pseudomonas aeruginosa,* PA01 and 59.20, producing two different siderophore types (I and III, respectively). Iron is essential for the growth of most organisms (Andrews *et al.*, 2003). In iron limited environments, bacteria secrete siderophores, which have a high affinity for iron. Uptake of the siderophore-iron complex is mediated via receptor binding, with different siderophores having a degree of specificity to their cognate receptors (Meyer *et al.*, 1997). Production of the siderophore incurs a metabolic cost, which becomes a relative fitness cost when in competition against a mutant non-producer. Hence, the production of siderophores can be considered a public-good, and as such has become an extensively used tool for the study of social evolution. We have previously shown that in pair-wise competition, cheats dominate strains producing the siderophore for which they possess the receptor, yet are themselves dominated by strains producing the alternative siderophore. Diversity was not maintained in that system, so we wanted to test whether the addition of spatial structure would alter this outcome. We first compared monoculture growth and investment in cooperation in soil microcosms, and then confirmed that in soil environments, shaking the culture reduces the per-capita iron-chelator activity when cheat are in competition with their corresponding cooperators. We finally test whether the combination of the presence of cheats and population structure (static

soil microcosms) is sufficient to maintain a diversity in the system. We also conducted competitions on agar plates: Here we compared the fitness of each cooperator when rare, in populations with and without cheats. In neither environment did we find a maintenance of diversity, possibly due to siderophores acting in competition with each other.

3.3 Methods

3.3.1 Greenbeards on plates

3.3.1.1 Bacterial strains

Non-siderophore producing mutants were generated from *Pseudomonas aeruginosa* strains PA01, a type one siderophore producer, and 59.20, a type III siderophore producer.

In order to differentiate between the 59.20 cooperator/cheat and PA01 cooperator/cheat, PA01 WT (cooperator) and PA01 Cheat strains were both transformed with the *LacZ* operon according to the protocol described in (Choi *et al.*, 2006). When plated on KB agar supplemented with X-Gal at a final concentration of 50 μg/mL, colonies of PA01 are then discernible from those of 59.20 by their LacZ phenotype. After transformation, six colonies of each strain were picked and grown overnight in KB and stored at -80˚C in 50% vol./vol. glycerol. Experimental populations were derived each from six colonies from streaks

of PA01 and 59.20 cooperators and cheats were picked and grown overnight in Casamino acids media (CAA) CAA:5g casamino acids, 1.18 g $K_2HPO_4 \bullet 3H_2O$, 0.25 g $MgSO_4 \bullet 7H_2O$ in 1 L H_2O).

3.3.1.2 Negative frequency dependence

The underlying mechanism by which a diversity of cooperator types is maintained by the presence of cheats in the population is negative frequency dependence: A cooperator strain must be fittest when rare, and this should occur only in presence of cheats. We tested this by setting up six treatment groups with the following mixes of strains: PA01 cooperator at 1% with 59.20 cooperator at 99%, and vice versa; PA01 cooperator and PA01 cheat each at 1%, with 59.20 cooperator and 59.20 cheat each at 49%, and vice versa. Total cell numbers in each mix were low (~1,000 cells/mL) to allow spatial segregation of strains. To see whether diversity would be maintained to a higher level in the presence of cheats, we also setup two additional treatments with strains at equal frequencies: PA01 cooperator with 59.20 cooperator at 50% each; PA01 cooperator, PA01 cheat, 59.20 cooperator and 59.20 cheat each at 25%. Our previous experiments looking at cooperator diversity with and without cheats were undertaken in static, liquid media, and had shown that diversity in these environments was lost, possibly due to global distribution of the public-good failing to confer an advantage to a rare cooperator. Here, to

understand whether either spatial structure alone, or spatial structure in conjunction with the presence of cheats was sufficient to maintain cooperator diversity, we inoculated these mixes on to iron-limited CAA hard-agar plates (a 10 cm petri-dish containing 25mL iron-limited CAA supplemented with 12 g/L agarose agar) in an iron-limited CAA soft-agar overlay (2.9 mL iron-limited CAA supplemented with 6 g/L agarose agar, and 100 μL of the relevant inoculum). Before addition of the inoculum and apo-transferrin, soft-agar was allowed to cool to 45˚C in a water bath. Six replicates of each treatment were inoculated and plates were then sealed with Parafilm, inverted and incubated at 37˚C. After six days the developed bacterial lawns were rinsed off with a 10 mL volume of M9 salts and collected for plating. Inocula and final time-point samples were plated on KB agar supplemented with X-gal at a final concentration of 50 μg/mL. One-sample t-tests against a hypothesised mean of 1 (equal fitness between competitors) were used to examine rare competitor fitness. Change in the exponent of Shannon entropy was used to compare diversity in the treatments setup at equal frequency. This was calculated as $\exp(H_1) - \exp(H_0)$ where H_0 and H_1 are the Shannon entropies at the time of inoculum and the end of the experiment, respectively. The Shannon entropy was calculated $H = \sum_{i=1}^{s} p_i \cdot \ln p_i$ where p_i is the proportion of individuals belonging to the ith species. The exponent H gives a measure of "effective number of species" (MacArthur .R, 1965).

3.3.2 Greenbeards in soil

3.3.2.1 Bacterial strains

As in the "strains" description in "Greenbeards on plates" the four strains used in this experiment were PA01 WT (cooperator) *LacZ*, PA01 cheat *Lacz*, 59.20 WT (cooperator), and 59.20 cheat. Strains were picked from streaks of -80˚C glycerol

3.3.2.2 Culture media

All the described experiments were undertaken in "soil microcosms": 6 g of compost (John Innes no. 2), was aliquoted into 30mL glass universal tubes and autoclaved. To avoid potential experimental contamination from surviving fungal spores, these microcosms were autoclaved again after 2 days standing at room temperature. 5mL of Milli-Q water was added to each microcosm prior to inoculation.

3.3.2.3 Competition experiments

3.3.2.3.1 Cooperation in structured and unstructured environments

By limiting dispersal and thereby grouping populations of kin, cooperators in static soil microcosm are expected to have higher fitness than shaken, which should result in higher per capita iron chelator activity in static

conditions. To test this, we setup competitions between cheats and their corresponding cooperators (PA01 cooperator and PA01 cheat at 50% each, and 59.20 cooperator and 59.20 cheat at 50% each), as well as competitions between all four previously mentioned genotypes, in six replicate soil microcosms, in shaken (180 r.p.m.) and static conditions. 60 μL of each population was transferred each week for six weeks. Populations were plated after inoculation, at the third transfer, and the final transfer, on KB agar with X-Gal. Per capita iron chelator activity was estimated after the final transfer by use of the CAS assay following the methods described above.

3.3.2.3.2 Diversity in structured environments

Structure in the population limits the dispersal of siderophores, hindering cheat parasitism. In a competition between two cooperators and their cheats, diminishing returns on parasitism may favour rare cooperators, allowing them to recover in frequency. To test whether greater diversity was present in static compared with shaken conditions, six replicate microcosms were inoculated according to four treatment groups; cooperators alone in static and shaken conditions, and cooperators with cheats in either static or shaken (180 r.p.m.) conditions. 60 μL of each population was transferred each week for six weeks. Populations were plated after inoculation, at the

third transfer, and the final transfer, on KB agar with X-Gal. Diversity was estimated by scoring colonies for their *LacZ* phenotype.

3.3.2.4 Monocultures

3.3.2.4.1 Growth

In order to provide a baseline for growth in soil and per capita iron chelator activity for comparison with competition experiments, six replicate soil microcosms inoculated with 60μL overnight culture of each strain were grown in monoculture in shaken (180 r.p.m) and static conditions at 37°C. Each week, for six weeks, 1% of each population was transferred into a fresh soil microcosm. Populations were plated after inoculation, at the third transfer, and at the end of the experiment. Malthusian parameters of each monoculture, estimated at the third and sixth weeks, were compared in a fully-factored REML in JMP v.13 with the factors strain (PA01 and 59.20), population structure (shaken and static), and social strategy (cooperate or cheat), with 'transfer period' and 'replicate' modelled as random factors, with the latter a repeated measure nested within 'strain'. After model simplification, LSMeans test slices were used to investigate differences within interaction effects.

3.3.2.5 Cooperation

The CAS assay was used to determine per capita iron chelator activity at the end of the experiment. Briefly, 20μL of each replicate culture was inoculated into two pseudo-replicate wells of a 96-well plate containing 180μL of iron-limited CAA (CAA supplemented with 100μg/mL human apo-transferrin and 20mM $NaHCO_3$, which is required for iron-chelator activity (both from Sigma)). Iron chelator activities were compared using a GLM in Jmp v.13, with the factors strain (PA01 and 59.20), population structure (shaken and static), and social strategy (cooperate or cheat).

3.4 Results

3.4.1 Greenbeards on plates

3.4.1.1 Negative frequency dependence

For diversity to be maintained, the rare strain must always have a higher relative fitness than the common strain, i.e., have a fitness, relative to that of its competitor, greater than 1. We had hypothesised that the negative fitness impact of cheating would be greater on common cooperator/cheat pairs than rare cooperator/cheat pairs due to their spatial segregation. When rare, strain 59.20 had a relative fitness no different to 1, both in the presence and absence of cheats (one-sample t-test against a hypothesised mean of 1; cheats present, $t_5 = -1.135$, $P = 0.308$; cheats absent, $t_5 = -2.252$,

P = 0.074). Strain PA01 had a higher fitness when rare both in the presence and absence of cheats (one-sample t-test against a hypothesised mean of 1; cheats present, $t_5 = 2.616$, P = 0.047; cheats absent $t_5 = 8.551$, P = 0.0004). Together, these data indicate that diversity will always be lost in these competitions: PA01 will outcompete 59.20 regardless of whether it is rare or common, and regardless of whether cheats are present or absent. See figure 1.

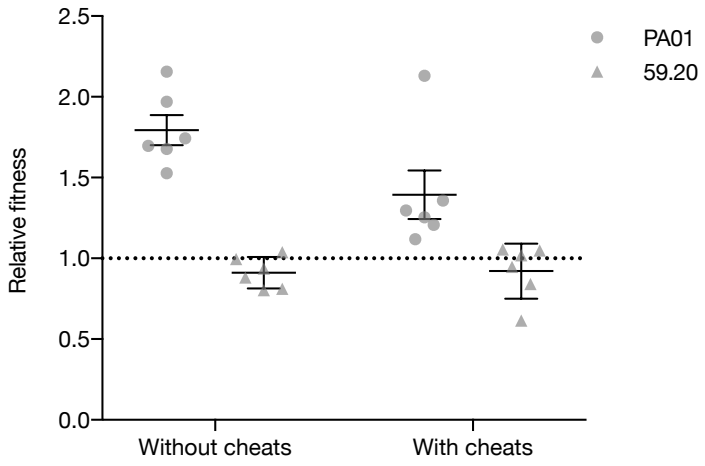

Figure 1. Relative fitness of rare cooperator strains. The horizontal line at 1 indicates equal fitness of the rare and common strain (W = 1). Error bars show mean and ±SEM.

When at equal initial frequency, diversity was lost in all populations, with and without cheats. Since initial diversity was near the maximum possible, this is to be expected. However, no difference in diversity loss was seen between populations with and without cheats (two-sample t-test; $t_{10} = -$

1.625, P = 0.143); the presence of cheats was insufficient to maintain diversity, even in this structured media. See figure 2.

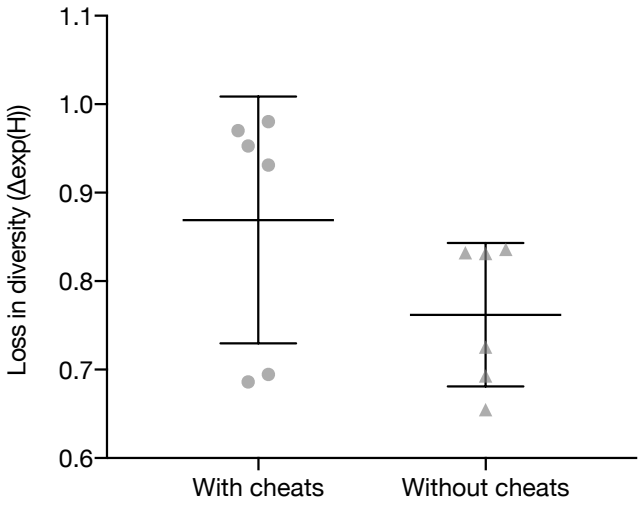

Figure 2. Loss of diversity in treatments with equal initial frequency of strains PA01 and 59.20, with and without cheats. Bars show mean with ±SEM.

3.4.2 Greenbeards in soil

3.4.2.1 Competition experiments

3.4.2.1.1 Cooperation in structured and unstructured environments

In competitions between cooperators and their respective cheats, per capita iron chelator activity was higher in static environments than shaken ($F_{1,20}$ = 75.089, P<0.0001). Within the competitions in shaken environments, there was no difference between the populations with respect to per capita iron chelator activity ($F_{1,20}$ = 0.166, P = 0.689). In static environments, however,

iron chelator activity in the competition between PA01 cooperator and PA01 cheat was higher than in the competition between 59.20 cooperator and 59.20 cheat ($F_{1,20} = 40.413$, $P < 0.0001$). See figure 3.

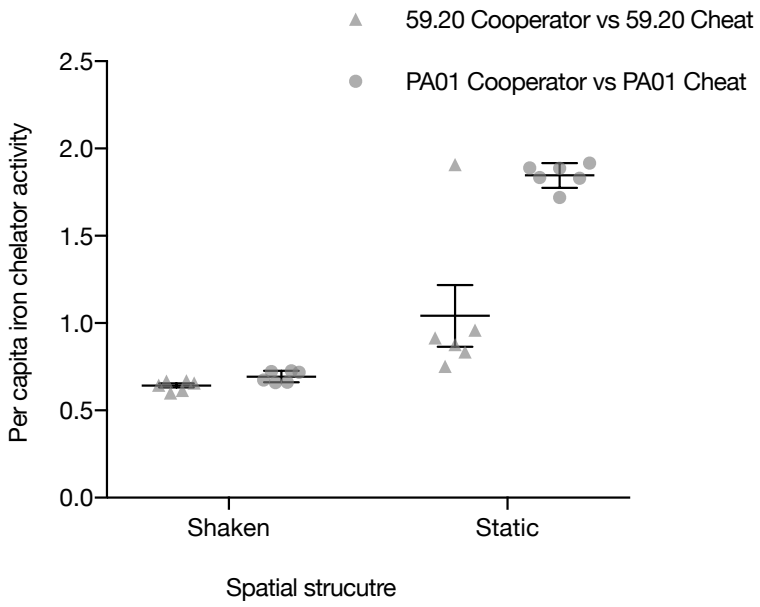

Figure 3. Per capita iron chelator activity of populations from competitions between cooperators and their respective cheats in shaken and static soil microcosms. Data points show the average of two technical replicates. Bars show mean with ±SEM.

In competitions between the two cooperators, with and without their cheats, in shaken environments, the population level iron chelator activity was significantly higher in competitions without cheats ($F_{1,20} = 149.011$, $P < 0.0001$). In static environments cheat presence or absence did not alter per capita chelator activity ($F_{1,20} = 0.166$, $P = 0.688$). However, static environments with cheats had significantly higher per capita iron chelator

activity than shaken environments with cheats ($F_{1,20}$ = 146.382, P < 0.0001). Per capita iron chelator activity in treatments without cheats was no different in shaken and static environments ($F_{1,20}$ = 0.266, P = 0.612). See figure 4

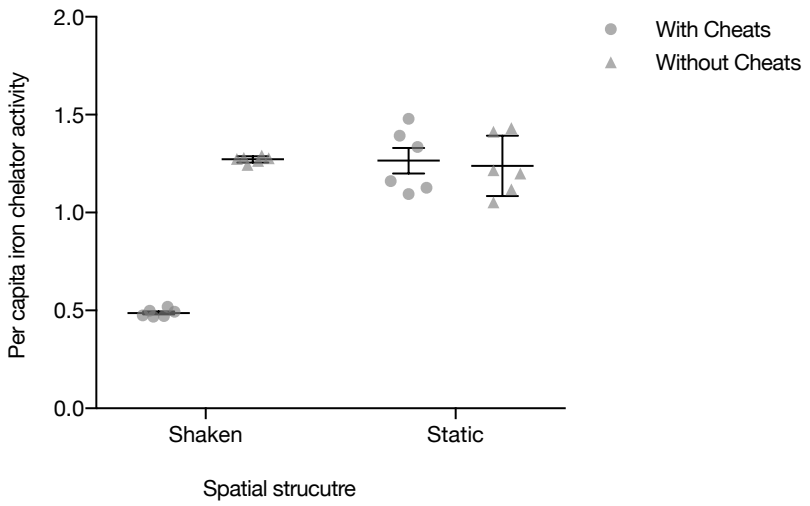

Figure 4. Per capita iron chelator activity of populations from competitions between either just two cooperators, or two cooperators together with two cheats, in shaken and static soil microcosms. Data points show the average of two technical replicates. Bars show mean and ±SEM.

Diversity in structured and unstructured environments

In competitions between the two cooperators, with and without cheats, diversity decreased through time in all populations ($F_{1,47}$ = 146.393, P < 0.0001). The rate of diversity loss was not altered by shaking the environments ($F_{1,46}$ = 0.042, P = 0.839), the presence of cheats ($F_{1,45}$ =

0.000, P = 0.995), nor any effect of the presence of cheats acting differently in shaking or static environments ($F_{1,44}$ = 0.003, P = 0.957). Population structure alone had no effect on diversity ($F_{1,21}$ = 0.109, P = 0.745), regardless of the presence of cheats ($F_{1,20}$ = 0.068, P = 0.979), and neither did the presence of cheats have any effect by itself ($F_{1,22}$ = 0.128, P = 0.724). See figure 5.

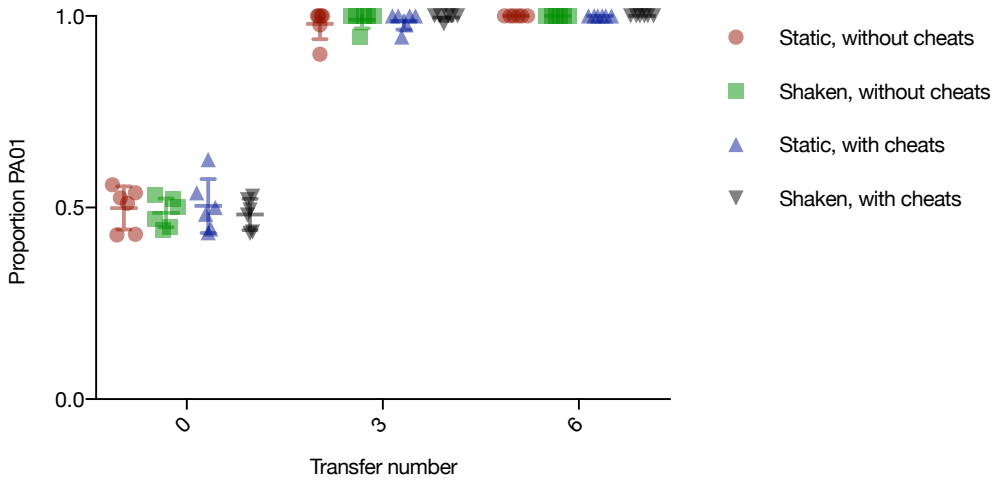

Figure 5. Diversity, shown as PA01 proportion of population, with and without cheats in shaken and static soil microcosms. Bars show mean with ±SEM.

3.4.2.2 Monocultures

3.4.2.2.1 Growth

Removing structure from the environment effected strain growth differently ('strain' by 'population structure' interaction, $F_{1,42}$ = 9.468, P = 0.0037). Shaking the environment did not change the growth of 59.20 ($F_{1,42}$

= 3.007, P = 0.0902); yet increased the growth of PA01 ($F_{1,42}$ = 6.852, P = 0.0123). PA01 also grew better in both shaken and static environments than 59.20 (shaken, $F_{1,42}$ = 105.213, P < 0.0001; static, $F_{1,42}$ = 34.877, P < 0.0001). Such differences were not also influenced by social strategy ('strain' by 'population structure' by 'social strategy' interaction $F_{1,40}$ = 0.308, P = 0.539), and nor did social strategy alone effect growth differently depending on the whether the tubes were shaken or static ('social strategy' by 'population structure' interaction, $F_{1,41}$ = 1.266, P = 0.271). Whilst cheats of both strains had lower growth than their cognate cooperators (59.20, $F_{1,42}$ = 270.534, P < 0.0001; PA01, $F_{1,42}$ = 49.614, P < 0.001), the magnitude of the difference in growth between cooperators and their cheats was greater in 59.20 than in PA01 ('social strategy' by 'strain' interaction, $F_{1,42}$ = 44.219, P < 0.0001). PA01 cooperator grew better than 59.20 cooperator ($F_{1,42}$ = 11.421, P = 0.0016), and PA01 cheat grew better than 59.20 cheat ($F_{1,42}$ = 163.421, P < 0.0001), though 59.20 cooperator also grew better than PA01 cheat ($F_{1,42}$ = 13.427, P <0.0001). See figure 6.

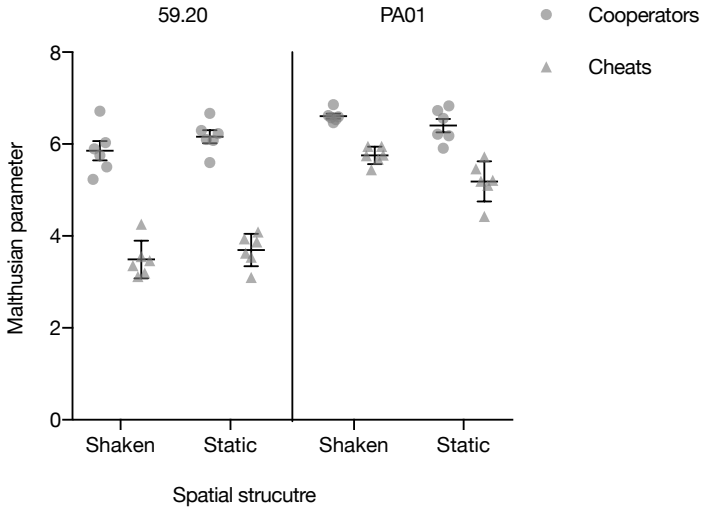

Figure 6. Malthusian parameter of each monoculture in shaken and static soil microcosms. Bars show mean with ±SEM.

3.4.2.2.2 Cooperation

Per capita iron chelator activity was higher in cooperators than cheats ($F_{1,44}$ = 228.228, P < 0.0001). Whilst there was no difference between per capita iron chelator activity in cheats, ($F_{1,44}$ = 1.533, P = 0.222), PA01 cooperator had higher per capita chelator activity than 59.20 cooperator ($F_{1,44}$ = 36.040, P < 0.0001). Per capita chelator activity was not effected by shaking, ($F_{1,43}$ = 0.811, P = 0.373), shaking did not affect the strains in different ways ('strain' by 'population structure' interaction, $F_{1,42}$ = 0.455, P 0.504), and nor did the social strategy affect strains differently in shaking conditions ('strain' by 'populations structure' by 'social strategy' interaction, $F_{1,40}$ = 0.577, P = 0.4521). See figure 7.

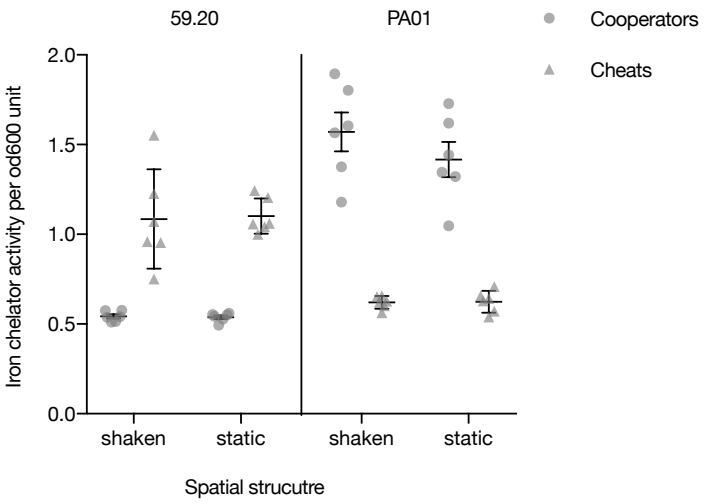

Figure 7. Per capita iron chelator activity of each monoculture in shaken and static environments. Each data point represents the average of two technical replicates. Bars show mean with ±SEM.

3.5 Discussion

Theory suggests that by limiting the dispersal of cooperators and the diffusion of the public good, cheats would reduce the fitness of rare cooperators to a lesser degree than common cooperators, providing the negative frequency dependence required for the maintenance of diversity. In both soil and in semi-solid, iron-limited agar environments, diversity was lost regardless of the presence of cheats. Each strain is required to increase in frequency from rare in order for diversity to be maintained. In solid agar environments, no relative fitness advantage was conferred to one of the rare strains despite the presence of cheats.

Treatments in which PA01 competed against 59.20 consistently demonstrated a vast fitness advantage for PA01, regardless of the presence or absence of cheats. In monoculture, Malthusian parameters showed that in soil conditions, PA01 had a higher growth-rate than 59.20. Per-capita iron chelator activity of this strain was significantly higher than 59.20. Viscous environments have been shown to promote cooperation (Kümmerli *et al.*, 2009), though by reducing diffusion of siderophore, a producer also gains more direct benefit from production. The cooperator producing the greatest amount of siderophore per capita stands to gain the most fitness as viscosity increases, as whilst the direct cost to benefit ratio decreases with viscosity, the indirect benefit to cheat ratio increases with viscosity by grouping kin together with the public-good. This could explain why PA01, which produces a greater amount of siderophore per capita than 59.20, dominated the competitions.

This effect could be further exacerbated by competition between siderophores for iron. Once bound, the siderophore retains iron in the complex, rending it unavailable to another siderophore (Boukhalfa & Crumbliss, 2002; Hider & Kong, 2010). Substantial overproduction by a competitor producing one siderophore type could render an environment uninhabitable to a strain able to take-up only an alternative siderophore type, essentially using siderophore production as a competitive trait. Siderophore production as a competitive trait has recently received some

theoretical attention (Niehus *et al.*, 2017). In structured populations, kin are closer in space than non-kin. Use of public-goods production as a competitive trait therefore requires an excess of production such that it spreads beyond the region in which kin are located, and into the region in which competitors are located. Depending on initial densities, a rare competitor could effectively secure much of the available niche with a substantially greater metabolic investment in siderophore production. In our experiments, the competitor producing the greatest amount of siderophore per capita was PA01. When this strain was common, there was no detectable diversity after six days in viscous environments, yet when rare, diversity increased in treatments both with and without cheats. This substantial overproduction of siderophore may explain why PA01 was able to increase from rare, even in the absence of cheats, and dominated competition experiments when it was common.

Inter- and intra- patch dynamics have been shown in simulation to sustain a diversity of cooperators only when cheats are present (Lee *et al.*, 2012). Our previous work in non-structured environments corroborates this finding. Whilst the same cooperator type in our previous work dominated the population when only cooperators were competing, the lack of viscosity meant that cheats were able to successfully invade a population of their cognate type, yet were unable to invade a population of alternative siderophore-type producers. Environments in which populations are

structured by patches differ from structure through viscosity. Although the distribution of siderophores in both is locally limited, intra-patch viscosity is zero, reducing the direct and indirect benefits of siderophore production and increasing the likelihood of cheat domination, and hence cheat regulation of strain frequencies in the meta-population. Theoretical exploration of beard chromodynamics in patches could easily incorporate the effect of siderophore use as a competitive trait. One could imagine that provided only proximal migration was allowed, with a favourable initial pattern of strains, succession could proceed such that cheats continually "clear a path" for cooperators of an alternative type, and diversity could be maintained in such a system, despite the superior competitive advantage of one cooperator over another. Any degree of random distal migration, however, and the system should collapse in favour of the more competitive cooperator and the cognate cheat.

Overall, we did not see any effect of population structure on the diversity of greenbeard cooperators. Our results suggest that one competitor is far superior to the other, and that structure, rather than increasing diversity, may bias the competition in favour of the competitor investing the greatest amount in public-good per capita. This could be due to a combination of the increase direct and indirect benefit a cooperator gains when in structured populations, together with the ability of siderophores to act as a competitive trait, limiting the ability of the other competitor to secure iron

and hence grow. Deepening our understanding of the biology of siderophore production in *P. aeruginosa*, which is a common pathogen in hospitals, infecting cystic fibrosis patients, burns victims, and other immunocompromised patients, may aid us in developing treatments and improving patient outcomes.

4 Resource heterogeneity and the evolution of public-goods cooperation

Authors

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4.1 Abstract

Heterogeneity in resources is a ubiquitous feature of natural landscapes affecting many aspects of biology. However, the effect of environmental heterogeneity on the evolution of cooperation has been less well studied. Here, using a mixture of theory and experiments measuring siderophore production by the bacterium *Pseudomonas aeruginosa* as a model for public-goods based cooperation, we show that cooperation in metapopulations that were spatially heterogeneous in terms of resources can be maintained at a higher level than in homogeneous metapopulations of the same average resource value. The results can be explained by a positive covariance between fitness of cooperators, population size and resource availability, which allowed cooperators to have a disproportionate advantage within the heterogeneous metapopulations. These results suggest that natural environmental variation may help to maintain cooperation

Key words

Resource heterogeneity, cooperation, siderophores, evolution, microorganisms

4.2 Introduction

The amount of resource available in an environment plays a key role in the evolution of cooperation. Higher resource availability can favour increased cooperation by reducing the marginal costs associated with the behaviour (Brockhurst *et al.*, 2008; Xavier *et al.*, 2011; Connelly *et al.*, 2017), likely due to a trade-off between growth and investment in cooperation (Foster, 2004). Despite the clear importance of resource availability in determining the evolution of cooperation, we currently lack an understanding of the importance of a ubiquitous feature of natural environments: Environments are heterogeneous. It is well established that environmental and genetic heterogeneity in a geographic landscape will alter the (co)evolutionary trajectory of the populations therein (Thompson, 1999, 2005; Vogwill *et al.*, 2009). Here, we investigate the impact of resource heterogeneity on the evolution of social interactions in the opportunistic bacterial pathogen *Pseudomonas aeruginosa*.

One general effect of resource heterogeneity on cooperation likely stems from the positive covariance between cooperation and density: High resource availability patches support more individuals and these individuals are more likely to be cooperators (Brockhurst *et al.*, 2008). Where the

magnitude of dispersal acting to redistribute genotypes across the geographic mosaic is in proportion to the population size of a patch, cooperation may be maintained to a higher degree in heterogeneous environments. This mechanism relies on "hard selection" (Wallace, 1968, 1975; Christiansen, 1975); patches producing a greater number of individuals contribute a greater number of progeny to the meta-population, as opposed to a "soft selection" in which each patch would contribute equally.

Cooperation based around the production of public goods is ubiquitous in microorganisms, usually in the form of a product secreted into the environment, the benefit from which may be received by any local organism. Siderophores are one such example. Iron is necessary for the growth and survival of almost all life on earth (Andrews *et al.*, 2003), and in iron limited conditions many microorganisms will secrete siderophores into the environment. These molecules have a high affinity for iron, and once bound to it can be taken-up by the microorganism usually via receptor binding. Pyoverdine, the siderophore produced by certain *Pseudomonas* species, has been extensively studied in the context of social evolution (Brockhurst *et al.*, 2006; Harrison *et al.*, 2006; Buckling *et al.*, 2007; Harrison & Buckling, 2007; Ross-gillespie *et al.*, 2007; Köhler *et al.*, 2009; Kümmerli *et al.*, 2010; Harrison, 2013; Julou *et al.*, 2013; Zhang & Rainey, 2013). As the individual bacterium producing the pyoverdine compound is not necessarily

the one taking it up, production can be considered a cooperative public good. Non-pyoverdine producing mutants readily evolve in the lab, and in iron limited conditions these increase in frequency beyond their wild-type counterparts and can be considered social cheats (West & Buckling, 2003).

Here we investigate how heterogeneity in resource availability affects levels of cooperation. We first develop an analytical theory, exploring the importance of hard and soft selection under the assumption of a linear relationships between cooperation and resource availability. While recent theory showed that heterogeneity in resource availability increased cooperation through numerical simulation, the mechanisms underlying these results are unclear (Kun & Dieckmann, 2013). After establishing that there was a roughly linear relationship between resource availability and cooperation in terms of pyoverdine production by the bacterium *P. aeruginosa,* experimental evolution of this organism provided empirical support for the key theoretical prediction that heterogeneity in resource availability can promote cooperation under conditions of hard selection. Finally, the results of competition experiments between isogenic pyoverdine cooperators and cheats strongly suggest that siderophore production - and not a linked trait – was a key target of selection during experimental evolution.

4.3 Methods

4.3.1 Theory

Both the methods of and results from theory work were carried out by Andy Gardner.

4.3.1.1 Single population

Assume that bacterial growth comprises a basic rate, proportional to both resource availability and siderophore availability, and an accelerating growth cost of investment into siderophore production. Specifically, $r = Ry\text{-}x^2$, where R is the availability of resources (the same value being experienced by all cells in the population), y is the availability of siderophore (being the average siderophore production of the focal cell's neighbours, which varies from cell to cell) and x is the cell's own investment into siderophore production (which varies from cell to cell). These assumptions mean that the relative cost of producing siderophores decreases with increasing resource availability (Brockhurst *et al.*, 2008)

A focal cell's fitness may then be written as $w_R = \exp(Ry\text{-}x^2)$ and the average fitness of all cells in the population may be written as $w_R = \exp(R\bar{x}\text{-}\bar{x}^2)$, on the simplifying assumption of vanishingly little variation in siderophore production. Accordingly, relative fitness may be defined as $W_R = w_R/\overline{w_R}$ and the criterion for natural selection to favour an increase in siderophore

production in this population is $\frac{dW_R}{dx}|_{x=y=z} > 0$. From the chain rule, $\frac{dW_R}{dx}|_{x=y=z} = \frac{\partial W_R}{\partial x}|_{x=y=z} + \frac{\partial W_R}{dy}|_{x=y=z} \times \frac{dy}{dx}|_{x=y=z} = Rr\text{-}2z$, where $r = \frac{dy}{dx}|_{x=y=z}$ is the average relatedness between a given cell and those cells that make use of its siderophores. The stable level of siderophore production z^* therefore satisfies $Rr\text{-}2z^* = 0$, i.e. $z^* = Rr/2$.

4.3.1.2 Metapopulation

Now consider a metapopulation in which each constituent population may have a different level of resource availability. Assuming that resource availability varies continuously, the probability density of populations having resource availability R may be denoted p_R, satisfying $\int_{Rmin}^{Rmax} p_R dR = 1$. Natural selection favours an increase in siderophore production across the whole metapopulation if $\int_{Rmin}^{Rmax} c_R W_R/dx|_{x=y=z} \mathrm{d}R > 0$, where c_R is the proportion of the ancestry of future generations that is contributed by populations with resource availability R.

4.3.2 Experiment

4.3.2.1 Bacterial strains

P. aeruginosa strain PA01, a wild-type, siderophore producing strain, was used as a social cooperator (referred to as PA01 WT cooperator). A

siderophore knock-out strain, PA01ΔpvdΔpchEF (Ghysels *et al.*, 2004), marked with *LacZ* (using the plasmids and protocol described in (Choi *et al.*, 2006)) was used as a social cheat (referred to as PA01 *LacZ* cheat). Replicates were inoculated with overnight cultures grown each from single colonies, picked from streaks of glycerol freezer stocks.

4.3.2.2 Single population competition experiments

Three medias containing different levels of resource were made: "High", a 1:4 dilution of casamino acids medium (CAA:5g casamino acids, 1.18 g $K_2HPO_4 \bullet 3H_2O$, 0.25 g $MgSO_4 \bullet 7H_2O$ in 1 L H_2O) in M9 salts (M9 salts: 12.8 g $Na_2HPO_4.7H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, in 1 L Millipore H_2O); "Low" a 1:16 dilution of CAA in M9 salts; and "Intermediate" a 1:1 mix of "High" and "Low" medias. To render all environments iron-limited, all medias were supplemented with 100 μg/mL human apo-transferrin (an iron chelator) and sodium bicarbonate at a final concentration of 20 mM (necessary for iron chelator activity (Meyer *et al.*, 1996)). Overnight cultures of PA01 WT cooperator and PA01 *lacZ* cheat grown at 37°C, shaking at 180 r.p.m., in "Intermediate" culture medium, were diluted to OD600 ~0.1 in M9 salts, and 30μL of each was inoculated into 12 replicates of each of the resource medias. Populations were plated both at the beginning of the experiment, and after 24 hours on KB agar with X-Gal (Kings B medium (10 g glycerol, 20 g proteose peptone No.4,

1.5 g $MgSO_4$, 1.5 g K_2HPO_4, in 1L Millipore H_2O) supplemented with 12g bacteriological agar and X-Gal at a final concentration of 50 μg/mL). Colonies were enumerated according to their *LacZ* phenotype, and the relative fitness *(W)* of the cooperative social strategy was estimated by taking the ratio of strain's Malthusian parameters *(m)* (the natural log of a strain's final density over its starting density). Fitness estimates were compared using a one-way ANOVA and post-hoc Tukey-HSD. Cooperator fitness in the intermediate group was tested using a one-sample t-test against the mean of the high and low groups to see whether the relationship between fitness and resource availability was roughly linear. Cooperator fitness in each group was compared to a hypothesised mean of 1 to see whether cooperation had declined in all groups.

4.3.2.3 Experimental evolution in heterogeneous and homogenous metapopulations

Two treatment groups, corresponding to the heterogeneous and homogeneous environments, consisted each of 12 replicate pairs of microcosms, the heterogeneous treatments containing 12 high- and 12 low-resource availability environments, and the homogeneous treatment containing 12 pairs of intermediate resource availability environments. "High", "Low", and "Intermediate" resource availability medias were created as described previously. 6mL of the relevant media was aliquoted into 30mL glass microcosms. Each microcosm was inoculated with 60μL of

PA01 WT cooperator from overnight culture in homogeneous treatment media. Every 48 hours, the matched-pair high- and low-resource cultures in the heterogeneous treatment were mixed, as were the paired cultures in the homogeneous treatment. These mixes were transferred into new media according to treatment. Every 20 transfers a glycerol stock was taken and stored at -80˚C for later assay of population-level iron chelator activity.

4.3.2.3.1 Per capita iron chelator activity assay

60μL of thawed glycerol stock was inoculated into iron-limited CAA medium and grown overnight at 37˚C shaking at 180 r.p.m. From this 1mL of culture was centrifuged at 14,000 rpm in a benchtop microcentrifuge. The iron chelator activity in the supernatant was determined using the chrome azurol S (CAS) assay described in (Schwyn & Neilands, 1987): 100μL of supernatant from centrifuged culture was mixed with 100 μL of CAS solution in a 96 well plate, and incubated for 1 hour in the dark at room temperature. Per capita iron chelator activity is given by: 1-(Ai/Aref)/Density i, where Ai is the absorbance of the ith sample at 630nm, and Aref is the absorbance at 630nm of a reference CAS reaction carried out on the media in which the culture was grown. Density is the sample absorbance at 600nm. To ensure the absorbance at 600nm was measured in the linear range, a 1 in 10 dilution of the sample was used to estimate culture density.

JMP v.9 was used to model log transformed *per capita* iron chelator activity as the response in a fully factorial REML design with the effects "treatment" (homogeneous or heterogeneous) and "time" (transfer number, modelled as a continuous variable), with replicate as a random factor nested within treatment. Post-hoc LS means contrasts were carried out to compare levels of cooperation at the different time points.

4.3.2.4 Competition experiments in heterogeneous and homogenous metapopulations

The initial setup of this experiment regarding homogeneous and heterogeneous treatments and replicates was identical to that of the evolution experiment. Here, however, the 12 microcosms in each treatment were inoculated with 30µl of PA01 WT cooperator and 30µL of PA01 *lacZ* cheat from overnight culture in homogeneous treatment media. All replicates were plated after inoculation on KB agar with X-Gal. Forty-eight hours post inoculation (h.p.i.), the paired cultures were mixed with each other and transferred to fresh media according to the same regime as the evolution experiment. At 96 h.p.i all cultures were plated on KB agar with X-Gal. Cooperator fitness estimates *(W)* were calculated as in the single population competition experiment and were then compared with a t-test in JMP v.9.

4.4 Results

4.4.1 Theory

The analytical model describes a situation in which the cost of cooperation is low in high resource availability environments, and high in low resource availability environments. Under hard selection, the productivity of a population directly determines how many individuals that population will contribute to the next generation. Under soft selection, the productivity of a population is independent of the contribution.

4.4.1.1 Soft selection

In the context of soft selection, all populations contribute the same ancestry to future generations, such that $c_R = p_R$. Accordingly, $\int_{Rmin}^{Rmax} c_R \, dW_R/dx|_{x=y=z} \, dR = \int_{Rmin}^{Rmax} p_R \, (Rr\text{-}2z) \, dR = \bar{R}r\text{-}2z$, and hence $z^* = \bar{R}r/2$, where R is the average availability of resources across the metapopulation. In other words, heterogeneity in resource availability has no impact on the level of competition (Figure 1)

4.4.1.2 Hard selection

In the context of hard selection, each population contributes ancestry to future generations in proportion to its overall growth, such that $c_R = p_R \, \bar{w}_R/\bar{w}$, where $\bar{w} = \int_{Rmin}^{Rmax} p_R \, w_R \, dR$ is the average of fitness across the

metapopulation. Accordingly, $\int_{Rmin}^{Rmax} c_R \, dW_R/dx|_{x=y=z} \, dR = \int_{Rmin}^{Rmax} \exp((R-z)z)(Rr-2z)dR \approx \exp((\bar{R}-z)z)(\bar{R}r-2z) - \frac{1}{2}(\exp((\bar{R}-z)z)z(2z^2-r(2+\bar{R}z)))\sigma^2_R$ and hence $z^* \approx (Rr/2)(1+(r\sigma 2R/2))$. That is, variation in resource availability across the metapopulation ($\sigma 2R > 0$) favours a greater degree of cooperation (higher z*) owing to those populations in which cooperation is most favoured contributing more ancestry to future generations (Figure 1).

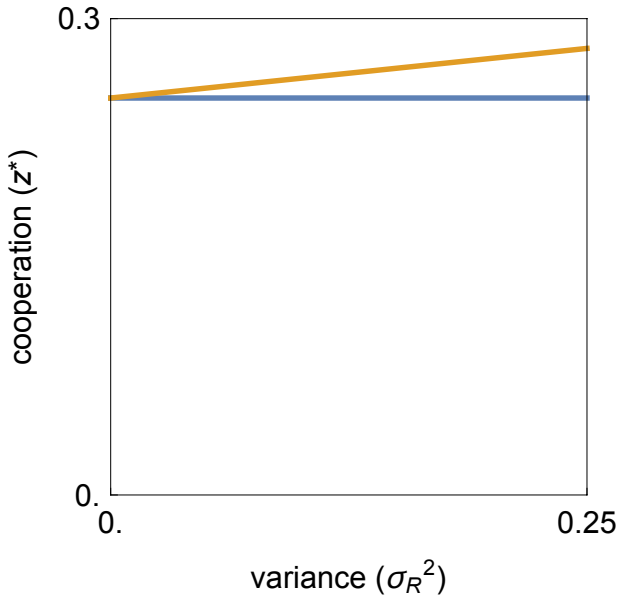

Figure 1. Heterogeneity in resource availability has no effect on levels of cooperation under soft selection (blue). Under hard selection, heterogeneity in resource availability supports higher levels of cooperation (orange). Other quantities: average resource availability $\bar{R} = 0.5$, relatedness $r = 1$.

4.4.1.3 Non-linearity between cooperation and resource availability

For simplicity, the model assumes a linear relationship between resource availability and cooperation. If this assumption is relaxed, heterogeneity in resource availability can both promote or inhibit cooperation under hard selection depending whether cooperation is an accelerating or decelerating function of resource availability, respectively.

4.4.2 Experiment

4.4.2.1 Single population competition experiments

We first established the approximate relationship between resource availability and short-term levels of cooperation. The amount of resource had a significant effect on *P. aeruginosa* siderophore cooperator fitness ($F_{2,33}$ = 20.51, P < 0.0001) when in competition with cheats: Cooperators had higher relative fitness in high resource availability media than in both intermediate (Tukey's HSD, P = 0.0158), and low resource availability medias (Tukey's HSD, P < 0.0001), and cooperators in intermediate resource availability media had higher fitness than in low resources availability media (Tukey's HSD, P = 0.0043).

Cooperator fitness in intermediate resource availability environments was not different to that of the mean of cooperator fitness in high and low resource availability medias, indicating that the relationship between resource availability and cost of cooperation was roughly linear, (one-sample t-test against a hypothesised mean of 0.881, t_{11} = 0.4202, P = 0.6824).

Cooperator fitness in each treatment was significantly lower than that of cheats (one-sample t-tests against a hypothesised mean of 1; high resource availability, t_{11} = -5.033, P = 0.0012; intermediate resource availability, t_{11} =

-8.122, P < 0.0001; low resource availability t_{11} = - 8.411, P < 0.0001). See figure 2.

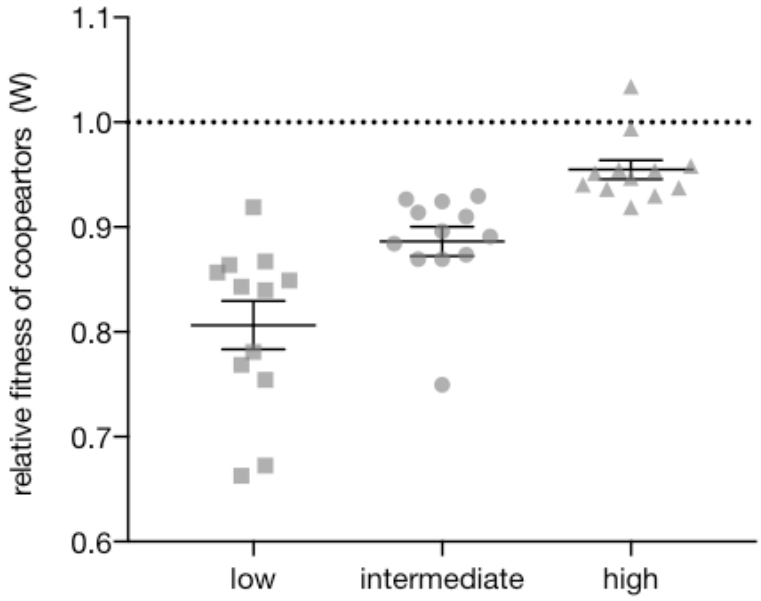

Figure 2. Fitness of cooperators relative to cheats in the three medias over 24 hours of growth. Bars show mean and ±SEM.

4.4.2.2 Experimental evolution in heterogeneous and homogenous metapopulations

Cooperation was maintained to a higher degree in heterogeneous than homogeneous environments ($F_{1,22}$ = 45.565, P<.0001) when initially isogenic wildtype (cooperating) populations were evolved for approximately 40 transfers (~200 generations). In both treatments cooperation decreased over the course of the experiment ($F_{1,46}$ = 121.035, P<.0001), but the rate of decrease in cooperation was greater in homogeneous treatments (F = 4.480, P = 0.040). Post-hoc tests revealed that there was no difference in

per capita iron chelator activity at the start of the experiment ($F_{1,58.32}$ = 0.240, P = 0.626) but that at transfers 20 and 40 there were higher levels of cooperation in the heterogeneous treatments ($F_{1,22}$ = 45.565, P < 0.001, and $F_{1,58.32}$ = 19.641, P < 0.001, respectively). See figure 3.

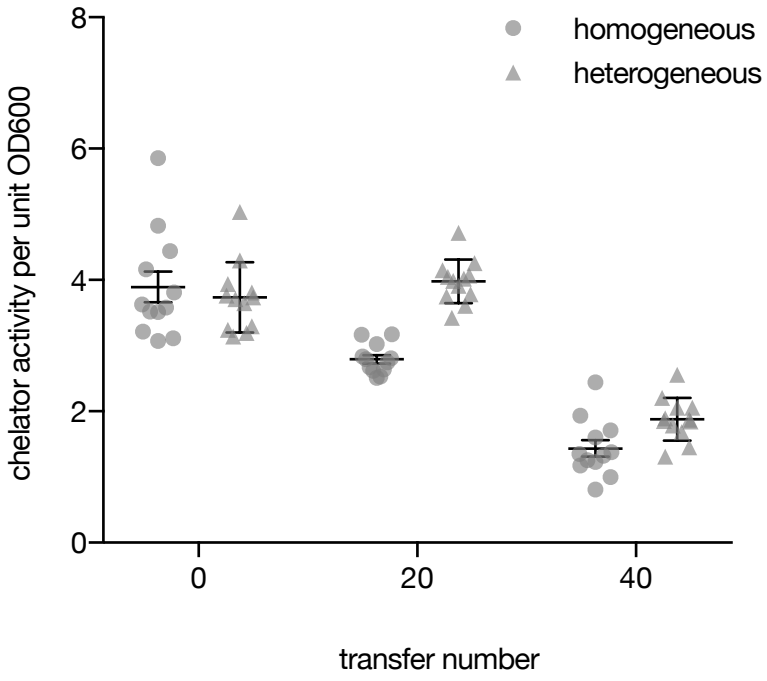

Figure 3. Per capita iron chelator activity across transfers by spatial heterogeneity and homogeneity. Bars show mean and ±SEM.

4.4.2.3 Competition experiments in heterogeneous and homogenous metapopulations

To confirm a causal link between siderophore production and relative fitness under heterogeneous versus homogenous resource availability environments, we conducted competition experiments between isogenic cooperators and cheats under the same conditions as the evolution

experiment. We found that wild-type siderophore-producing cooperators had higher fitness when in heterogeneous environments than in homogeneous (t-test, $t_{22} = -2.426$, $P = 0.0239$). See figure 4.

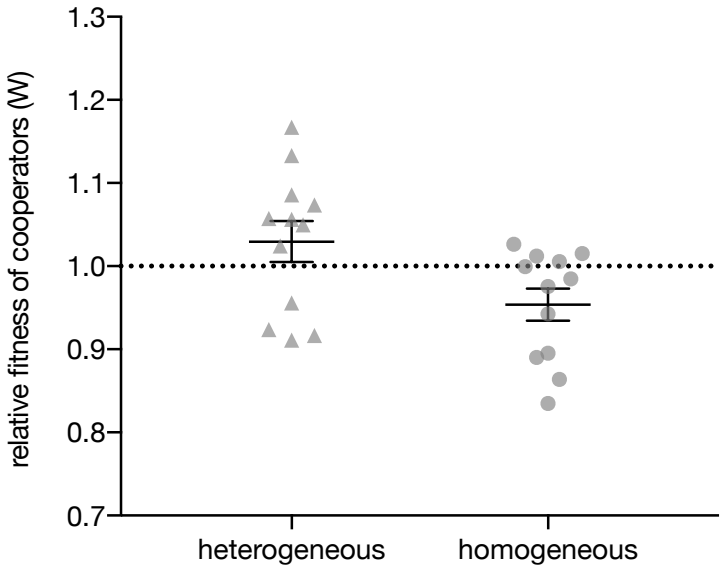

Figure 4. Fitness of cooperators relative to cheats in spatial homogeneity and heterogeneity after four transfers. Bars show mean and ±SEM.

4.5 Discussion

Here we investigated the effect of heterogeneity in resource supply on the evolution of cooperation. We first confirmed the findings of previous work showing that resource availability alters the competitive environment favouring cooperation at higher resource availability in a roughly linear fashion (Brockhurst *et al.*, 2008). We then developed an analytical model in this context showing that hard selection would promote higher levels of

cooperation. Therefore, heterogeneity in resource availability was expected to lead to higher levels of cooperation. We demonstrated this by transferring populations of *P. aeruginosa* over evolutionary time scales and assaying their cooperative investment. Finally, to show that cooperation was the target of selection in our evolution experiment, rather than a linked trait, we ran a short-term competition between a wild-type cooperator and a knock-out cheat. Cooperator fitness was found to be higher in the heterogeneous environments. These findings may reveal an overlooked factor explaining the prevalence of cooperation in biology; where cooperation is less costly, populations are also more productive.

The mechanism underpinning our theoretical and experimental results is the positive covariance between cooperation and productivity resulting from variation in resource availability: In a meta-population consisting of patches that vary in respect to their resource availability, high resource availability patches, containing a higher proportion of cooperators, will contribute a greater number of individuals to the next generation. These findings may be generalizable to any context in which hard selection is in operation and there is a linear relationship between resource availability and cooperation. The most likely scenario to consider in which this relationship ceases to be linear is when resource availability in all patches are so high as to no longer be the limiting factor regarding population size at dispersal, yet the cost of cooperation continues to decrease with a linear function (due to

differing growth-rate disparity between social strategies in different resource availability levels). No difference between heterogeneous and homogeneous environments would be predicted: Patch productivity would be identical, and only within-patch selection would be present. This scenario is akin to that of the prediction for soft selection as in our analytical model. Note that the covariance mechanism operating may still play an important role when there is a non-linear relationship between cooperation and resources levels, but its relative importance in determining net levels of cooperation in heterogeneous environments will be reduced.

Fundamentally linked to heterogeneity, altering resource availability has been shown to affect many aspects of biology, including evolutionary diversification (Hall & Colegrave, 2007), patterns of species' diversity (Eadie & Keast, 1984; Zhou *et al.*, 2002; Horner-Devine *et al.*, 2003) the nature of symbiotic interactions, (Boza & Scheuring, 2004; Bull & Harcombe, 2009; Hom & Murray, 2014; Hoek *et al.*, 2016), and host parasite coevolution (Westra *et al.*, 2015). Investigations of these effects in the context of heterogeneity in resource availability and hard selection may have much to reveal about their ecologies and distributions through the natural world. For example, environmental heterogeneity has been shown to influence population structure and dynamics (Shigesada *et al.*, 1986; Chesson, 2000; Amarasekare, 2003), species' dispersal across habitats (Dewhirst & Lutscher, 2009), population stability (Oliver *et al.*, 2010), and

species diversity and coexistence (Chesson & Warner, 1981; Questad & Foster, 2008; Hortal *et al.*, 2009; Brown *et al.*, 2013). Heterogeneity with specific respect to resource availability has been similarly implicated in playing a crucial role in explaining patterns of diversity (Stevens & Carson, 2002; Maestre & Reynolds, 2006; Eilts *et al.*, 2011; Price *et al.*, 2014; Yang *et al.*, 2015).

P. aeruginosa is an opportunistic human pathogen, particularly in nosocomial contexts, where it causes acute infection in immunocompromised and cystic fibrosis patients (Bodey *et al.*, 1983). Cooperative traits such as siderophore production in *P. aeruginosa* are often linked with virulence (Meyer *et al.*, 1996; Takase *et al.*, 2000; Harrison *et al.*, 2006). Between patient transmission is akin to between-patch migration, and whether populations causing disease are under hard or soft selection will be determined by the mode of transmission which may differ depending on the nature of the infection. Cooperation, and hence virulence, may also be affected by heterogeneity in resource availability between different sites of infection within a host, and the levels of migration between these sites. Increasing our understanding of how hosts function as ecological spaces to affect disease virulence will aid in reducing disease emergence, severity, and potential spread.

5 Temporal heterogeneity in resource availability increases levels of public goods investment in Pseudomonas aeruginosa

Authors

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5.1 Abstract

A universal feature of natural landscapes, affecting many aspects of biology, is that resource availability varies through time and space. The effect of temporal heterogeneity on the evolution of cooperation has not been well studied. Using siderophore production *Pseudomonas aeruginosa* as a model for public-goods based cooperation, we show that temporal heterogeneity in resource availability promotes higher levels of cooperation than in homogeneous environments of the same average resource availability. This finding can be explained by the positive covariance between cooperator fitness, population size, and resource availability; in high resource availability periods, population expansion is greatest, and the relative cost of cooperation is low. As a consequence, periods of high resource availability have a much greater impact than periods of low resource availability in driving co-operator dynamics. These results suggest that natural temporal

variation in resource availability may play a role in the maintenance of cooperation.

Key words

Resource heterogeneity, cooperation, evolution, microorganisms

5.2 Introduction

Temporal variation in environments is thought to affect the prevalence of cooperation (Rubenstein & Lovette, 2007; Jetz & Rubenstein, 2011; Marshall *et al.*, 2016), though the mechanism behind this effect remains unclear. Population dynamics may play an important role (Cockburn & Russell, 2011). For example, temporal variation causes bottlenecks in populations, which can promote cooperation through genetic structuring (Griffin *et al.*, 2004; Brockhurst, 2007; Brockhurst *et al.*, 2007). Despite heterogeneity being a ubiquitous feature of natural landscapes, relatively little consideration has been given to the role of variation in resource availability on the evolution of cooperation. We have previously shown that heterogeneity in resource availability in space can support cooperative behaviour. Here, we investigate the impact of temporal heterogeneity in resource availability on the distribution of social traits in the opportunistic bacterial pathogen, *Pseudomonas aeruginosa*.

The effect of resource availability on investment in social behaviours has received some attention (Foster, 2004; Brockhurst *et al.*, 2008; Xavier *et al.*, 2011; Connelly *et al.*, 2017): As the level of resource increases, so the marginal returns from growth are diminished and investment in cooperative behaviour becomes less costly (Foster, 2004). High resource availability also has the general effect of increasing population size. Considered at the level of the metapopulation, in a landscape with heterogeneous resource availability, the diminished cost of cooperation in environments with higher resource availability means that a greater number of individuals will have originated from patches in which cooperation is less costly. Where the make-up of patch founder populations reflects that of the metapopulation, i.e., patches are colonised under "hard selection" (Wallace, 1968; Christiansen, 1975), heterogeneity in resource availability is likely to maintain cooperative investment to a higher level than where resources are spread homogeneously. Put simply, a greater proportion of patch-founders will have originated in patches in which cooperation was less costly.

We have previous explored the effect of spatial heterogeneity in resource availability on cooperation using a mix of theory and experiment. Our analytical theory showed that increasing resource availability, which has been shown experimentally to decrease the costs of cooperation in our previous work and elsewhere (Brockhurst *et al.*, 2008), leads to higher levels of cooperation when populations are under hard selection. The model

compared the outcome for cooperation in populations under hard and soft selection: Hard selection allows the population dynamic effect to alter the level of cooperation, as a greater number of individuals in the metapopulation originate in high-resource availability regions, in which cooperation has a lower cost. In contrast, soft selection uses an equal number of individuals from each resource level to form the parental generation, allowing only within-patch dynamics to effect of the level of cooperation. We found experimental support for this theory, showing that populations with spatial heterogeneity in resource availability evolved a higher level of cooperation, which in a biological context translates to higher levels of siderophore production in the bacterium, *Pseudomonas aeruginosa.* Almost all forms of life need iron to grow (Andrews *et al.*, 2003), and when iron is limited, microorganisms secrete siderophores into the environment. The siderophore forms a complex with iron, for which it has high affinity, after which it is taken-up by any cell bearing the cognate receptor. Siderophore production incurs a metabolic cost, which is avoided by mutant non-producers, who retain the receptor and hence the benefit of conspecific production; they are social cheats (West & Buckling, 2003). This system has been used extensively to study the evolution of public-goods based cooperation (Harrison & Buckling, 2005; Harrison *et al.*, 2006; Buckling *et al.*, 2007; Brockhurst *et al.*, 2008; Kümmerli *et al.*, 2009; Racey *et al.*, 2010; Harrison, 2013; Zhang & Rainey, 2013). Our analytical

The impact of temporal heterogeneity in resources is however less clear. However, based on our previous work it has the potential to increase cooperation in a similar way to spatial heterogeneity. Specifically, a high productivity period will dominate the dynamics of a low productivity period, due to more population turnover within the high productivity period, i.e., the "founder effect" from a high productivity patch migrating to a low productivity patch will exert a greater influence on the composition of the population than migration events from low productivity patches. However, there are of course caveats to this: 1) nonlinear costs, as mentioned in previous chapter. 2) if temporal variation is sufficiently low, selection will simply be driven by current patch and there will be fluctuations in cooperation through time.

Here we investigate the effect of temporal heterogeneity in resource availability on investment in cooperation. We conducted a competition experiment between isogenic cooperators and cheats, and showed that temporal heterogeneity in resource supply does indeed produce the same general effect as that of spatial heterogeneity. Experiments were conducted in metapopulations, homogenous with respect to resource availability within time points, a condition critical for the maintenance of some cooperation, and crucial to allow us to compare these results with those of spatially varying resource availability in the previous chapter.

5.3 Methods

5.3.1 Bacterial strains

Glycerol stocks of *Pseudomonas aeruginosa* strain PA01 WT (cooperator), or PA01ΔpvdΔpchEF (from now on referred to as PA01 *lacZ* cheat) (Ghysels *et al.*, 2004), marked with LacZ (using the plasmids and protocol described in (Choi *et al.*, 2006)) kept at -80C were streaked onto a KB plate, from which 12 colonies were picked each in to 6mL of "intermediate treatment" media and grown over night at 37˚C in 30mL glass microcosms, shaking at 180 r.p.m. 30μL of each culture was inoculated into each replicate at the start of each experiment.

5.3.2 Competition experiments

Populations of a 50:50 mix of PA01 WT and PA01 *lacZ* cheat were transferred every two days through one of five different resource supply regimes: 1) "High" - four transfers through high-resource media); 2) "Low" - four transfers though through low-resource media; 3) "Intermediate" – four transfers through a 1:1 mix of "High" and "Low" media; 4) "High/Low" – four transfers through alternating "High" and "Low" media; 5) "Low/High" – as the previous treatment but with a reversed order of provisioning. Casamino acids media (CAA: 5g casamino acids, 1.18

g K_2HPO_4•$3H_2O$, 0.25 g $MgSO_4$•$7H_2O$ in 1 L H_2O) was diluted 1:4 in M9 salts (M9 salts: 12.8 g $Na_2HPO_4.7H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, in 1 L Millipore H_2O) to create the high-resource" media, then further diluted 1:4 to create the low-resource media. Media were supplemented with 100μg/mL human apo-transferrin (Sigma) (a strong iron chelator)) and 20mM $NaHCO_3$ (sodium bicarbonate) (required for iron chelator activity). Within each treatment, six matched pairs of populations were pooled at the end of every two-day growth phase, creating a meta-population from which 60μL was transferred into the relevant fresh media. All treatments were grown at 37˚C in 30mL glass microcosms, shaking at 180 r.p.m.

Populations were plated at the beginning of the experiment and at every transfer on KB agar (Kings B medium (10 g glycerol, 20 g proteose peptone No. 4, 1.5 g $MgSO_4$, 1.5 g K_2HPO_4, in 1L Millipore H_2O) supplemented with 12g bacteriological agar and X-Gal at a final concentration of 50μg/mL). Colonies were enumerated by their *LacZ* phenotype and the relative fitness *(W)* of each social strategy was then calculated by taking the ratio of strains' Malthusian parameters *(m)* (the natural log of a strain's final density divided by its starting density).

One-way t-tests with Bonferroni correction against were used to compare cooperator to cheat fitness within each level of resource availability. Fitness

estimates were modelled in a one-way ANOVA and Tukey's HSD post-hoc contrasts were used to compare treatments.

Fitness of cooperators in the temporally heterogeneous resource availability treatments were compared by first constructing a REML model with replicates as repeated measures, nested within the treatment, and then using LSMeans contrasts at each time point.

The Malthusian parameter of populations in the heterogeneous resource availability treatments, estimated after growth in each resource level was modelled in a REML design, with each replicate as random factor, nested within the resource availability of growth phase from which the Malthusian parameter was estimated, with an additional factor describing the pattern of temporal heterogeneity (i.e., whether high-to-low-etc., or low-to-high-etc).

5.4 Results

Cooperator fitness was lower than that of cheats in all levels of resource availability (Bonferroni corrected one-way t-test against hypothesised mean of 1 for homogeneous, high availability, $t_6 = -5.036$, $P = 0.020$; heterogeneous, high/low/high/low availability, $t_6 = -5.435$, $P = 0.0145$; homogeneous, intermediate availability, $t_6 = -11.823$, $P < 0.0005$; heterogeneous low/high/low/high availability, $t_6 = -5.661$, $P = 0.012$; and homogeneous, low availability $t_6 = -15.858$, $P < 0.0005$). See figure 1.

Resource availability had an effect on the fitness of cooperators in competition ($F_{4,25} = 50.238$, $P < 0.0001$). Cooperators in populations with high resource availability, temporally homogeneous environments were fitter than those with intermediate and low resource availability (Tukey's HSD, $P = 0.0003$, and $P < 0.0001$, respectively), and cooperators in intermediate resource environments were fitter than those in low resource environments ($P < 0.0001$). See figure 1.

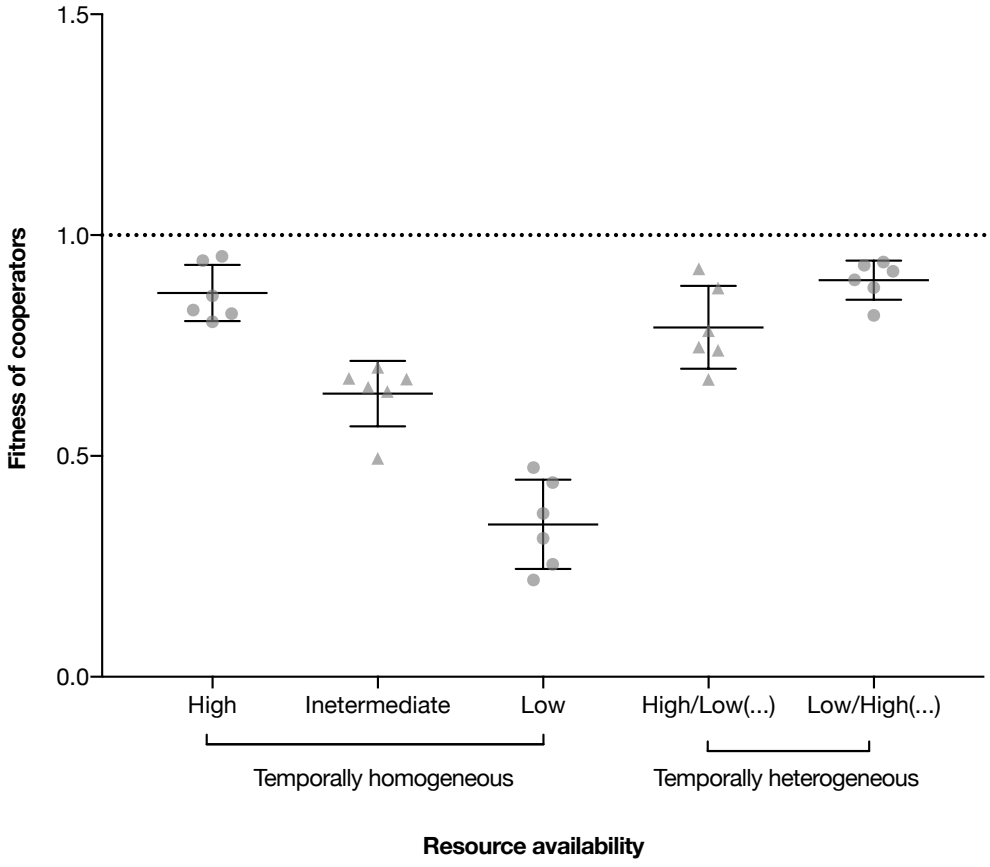

Figure 1. Fitness of cooperators relative to cheats in homogeneous and heterogeneous resource availabilities. Dashed line at relative fitness of 1 shows where each competitor (cheat or cooperator) would have equal fitness. Bars show mean and ±SEM.

Cooperators in heterogeneous environments had a fitness greater than those in intermediate resource environments (Tukey's HSD, $P = 0.0210$, and $P < 0.0001$, for low/high/low/high and high/low/high/low comparisons, respectively) and low resource environments (Tukey's HSD, $P < 0.0001$ for both low/high/low/high and high/low/high/low comparisons), but no different to those in high resource environments Tukey's HSD, $P = 0.440$, and $P = 0.967$, for low/high/low/high and high/low/high/low comparisons, respectively). See figure 1.

To determine the impact of the recent selective environment (i.e., whether the population had experienced high or low resource availability), we followed cooperation frequencies through time in the fluctuating events. Within the temporally heterogeneous treatments, cooperator fitness was higher at the first transfer in the populations that had experienced high resource availability than those that had experienced low, ($F_{43.8} = 4.58$, $P = 0.0379$), but subsequent transfers showed no significant difference. See figure 2.

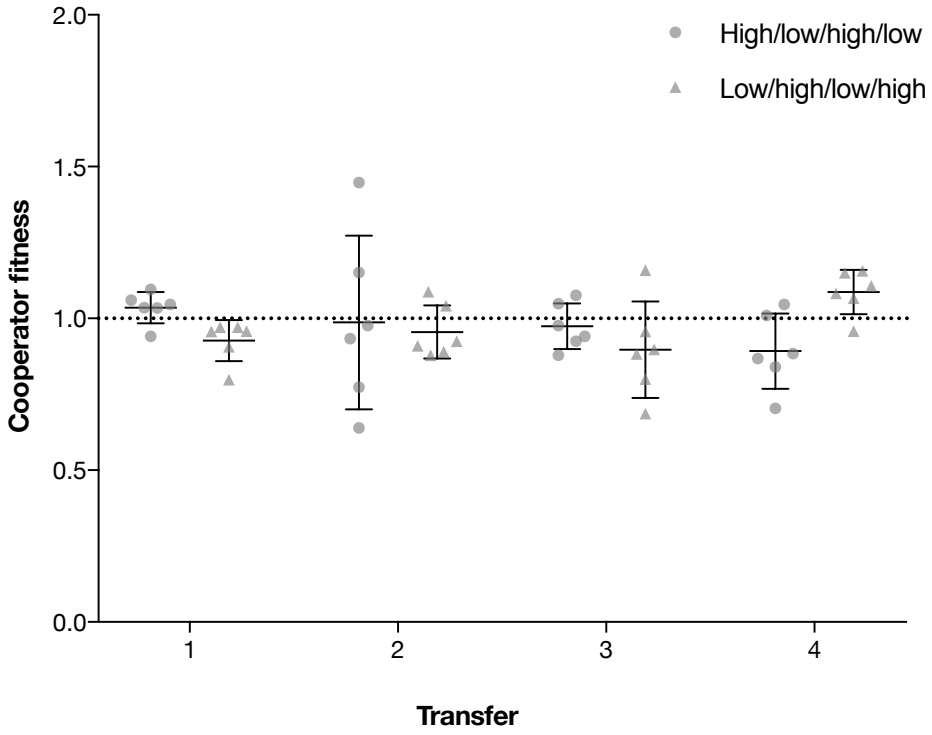

Figure 2. Fitness of cooperators relative to cheats at each time point in temporally heterogeneous resource availabilities. Dashed line at relative fitness of 1 shows where each competitor (cheat or cooperator) would have equal fitness. Bars show mean and ±SEM

To determine whether in heterogeneous resource availability treatments, population expansion was greater after growth in high resource availabilities than in low, we compared the Malthusian parameters of populations after growth in the different resource availabilities. A significant interaction between the pattern of temporal heterogeneity and resource availability was found ($F_{1,20} = 26.009$, $P < 0.0001$). Contrasts showed that growth in low resource medias was lower than high resource medias in both patterns of

temporal heterogeneity (high/low/high/low, $F_{1,20} = 317{,}585$, $P < 0.0001$; low/high/low/high, $F_{1,20} = 112.542$, $P < 0.0001$), but the temporal pattern high/low/high/low had lower growth in low resource availabilities, and higher growth in high resource availabilities than the low/high/low/high pattern (high resource medias, $F_{1,20} = 8.541$, $P = 0.008$; low resource medias, $F_{1,20} = 18.403$, $P = 0.0004$)). See figure 3.

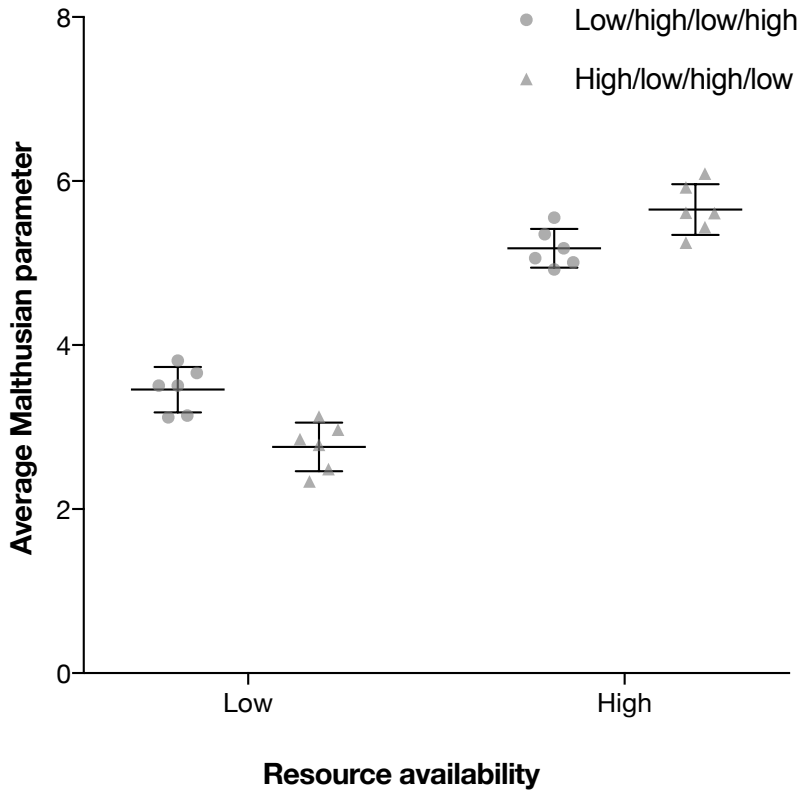

Figure 3. Malthusian parameters from periods of growth in either high or low resource availabilities, showing the two temporally heterogeneous treatments. Data points show the average of each of two growth periods in each resource availability. Bars show mean and ±SEM

5.5 Discussion

Here, I investigated the effect of temporal variation in the availability of resources on the outcome of competition between cooperators and cheats. Despite receiving the same total amount of resource over the course of the experiment, cooperators in populations transferred through heterogeneous resources availabilities had higher fitness than those transferred through homogeneous resources, regardless of the pattern of resource availability. Temporal variation in the environment has been identified as a factor that may be important in explaining the prevalence of cooperation in the natural world (Rubenstein & Lovette, 2007; Jetz & Rubenstein, 2011; Marshall *et al.*, 2016). Our findings show that simple population dynamics may play a large role in explaining the phenomenon: Populations reproduce most in times of high resource availability, which correlate with times in which the cost of cooperative behaviour is comparatively low.

Natural environments will vary both in terms of spatial and temporal resource availability. Taken together, our research shows that the effect of heterogeneity, spatial or temporal, will be to influence the distribution of genotypes at the metapopulation towards that favoured during the most productive times or in the most productive spaces. These results should be generalizable to any system in which hard selection is in operation and a relationship exists between change in a certain factor and population productivity. Resource availability has been shown to affect patterns of

diversity (Eadie & Keast, 1984; Kassen *et al.*, 2000; Zhou *et al.*, 2002; Horner-Devine *et al.*, 2003; Hall & Colegrave, 2007), the relative costs of different immune strategies (Westra *et al.*, 2015), and the nature of symbiotic interactions (Boza & Scheuring, 2004; Bull & Harcombe, 2009; Hom & Murray, 2014; Hoek *et al.*, 2016). Re-examining these findings in the light of hard selection, with temporal and spatial environmental variation may offer great insight to our understanding of species' ecologies and their distributions in the natural world.

Solutions to the problem of cooperation - the persistent advantage of cheating - have been the focus of much empirical and theoretical work (Hamilton, 1964; Smith, 1964; Axelrod & Hamilton, 1981; Queller, 1985; Frank, 1995, 2003). In our experiments, cooperation at all resource levels decreased regardless of temporal variation in resource availability. This is somewhat to be expected, as the heterogeneous treatment did not increase cooperation beyond the level of that in the high resource availability, and the level of cooperation in wild-type PA01 is unlikely to be ideally suited to the novel experimental conditions the organism was exposed to. Nevertheless, the transition from high to low resources and back also bottlenecks the population; this has been shown to favour cooperation by increasing relatedness (Brockhurst, 2007). A long-term evolution experiment mirroring the configuration of the competition executed here may show the extent to which cooperation can be maintained.

This work extends a previous study of spatial heterogeneity in resource availability, which showed a similar effect of maintaining a level of cooperation higher than in homogeneous environments. Both temporal and spatial heterogeneity in resource availability are likely to be a common ecological occurrence in natural settings.

6 Discussion

As individual chapters provide their own self-contained discussions, here I will summarise the main points and findings, before expanding on some of the emerging general issues they raise.

Chapter 2: Cheat mediated evolution of siderophore diversity in *Pseudomonas aeruginosa*

- *P. aeruginosa* strains producing different pyoverdine types can outcompete non-corresponding, non-producing mutants in a competition setting. This confirms that our model system respects one basic, underlying assumption of many theoretical models of tag-based cooperation: That cheats exclusively parasitize their corresponding cooperator, and are outcompeted by non-corresponding cooperator types. Prior cross-feeding experiments revealed a degree of exclusivity, but crucially lack the competition aspect we investigate here.
- The outcome of three-way competitions was strongly predicted by the strategy and tag-types present: Competition between two wild-types is heavily influenced by the additional presence of one cheat type. This indicates that social evolution may play a role in diversity in structured populations.

- In two-way competition between one pyoverdine producing type and another, one type always won, or at least dominated the population. The addition of cheats made no difference to diversity, which was always lost.

Chapter 3: Greenbeards diversity and spatial structure

- As expected, and as has been shown previously (Kümmerli *et al.*, 2009) addition of spatial structure in the form of static soil microcosms supported cooperation in pairwise cooperator/cheat competitions in both *P. aeruginosa* strains.
- On agar plates, or in soil microcosms, static or shaken, and with or without cheats - the outcome of competition experiments was always the same: One strain consistently dominated. Preventing cheats from accessing pyoverdine in spatially structured environment might hinder the mechanism creating negative frequency dependence.
- The dominant strain was also the strain that produced most iron chelator activity per capita – a measure of cooperation. This hints at pyoverdine being used as a competitive trait.

Chapter 4: Resource heterogeneity and the evolution of public goods

- Environmental heterogeneity is known to influence many biological processes. Perhaps the greatest attention in this area has been given

to the generation of diversity. Much less is known about the effect of environmental heterogeneity on the evolution of cooperation.

- Different resource availabilities can affect certain biological processes. For example, cooperation is less costly when resources have high availability (Brockhurst *et al.*, 2008). Crucially, resource levels also impact population growth and density.
- We can link the two above effects: By distributing resources heterogeneously through a landscape and allowing hard selection to operate.
- In this context, we show that spatially heterogeneous landscapes, with respect to resource availability, support higher levels of cooperation than homogeneous landscapes: Cooperation is less costly in the most productive patches, and migration enables these patches to dominate the metapopulation.

Chapter 5: Temporal heterogeneity in resource availability increases levels of public goods investment in *Pseudomonas aeruginosa*

- Environmental heterogeneity can occur through time as well as space. With respect to cooperation, the limited amount of research in this area has found that cooperation correlates with heterogeneous environments (Rubenstein & Lovette, 2007; Jetz & Rubenstein, 2011), though the mechanism behind this is unknown.

- ❖ We experimentally demonstrate that temporal heterogeneity in resource availability supports higher levels of cooperation than temporally homogeneous landscapes.
- ❖ We show that population expansion is greatest in periods of high resource availability, during which the costs of cooperation are lowest.
- ❖ Periodic mortality coinciding with environmental change (i.e., during population transfer) creates population bottlenecks. Over time, bottlenecks are more severe in environments with heterogeneous resource availability, resulting in higher relatedness in heterogeneous environments, further supporting cooperation.

General remarks on greenbeards and diversity

Public-goods, private-goods?

As the iron bound to pyoverdine is unable to be dislocated until uptake (Hider & Kong, 2010), and as uptake is restricted to social partners, production of one pyoverdine type can effectively be used to limit iron available for binding a competitor's own pyoverdine (Niehus *et al.*, 2017). The large differences in per capita iron chelator activity between monocultures of the two wild-type strains in the soil microcosm experiments hint that this concept – that of between-pyoverdine

competition – may explain why the expected diversity was lost in many treatments: In each of those cases, the wild-type investing the greatest amount in pyoverdine production dominated the competition.

The cost of competitive pyoverdine production

Cooperative pyoverdine production incurs a growth cost (West & Buckling, 2003): Competitive over-production should incur a greater cost still, increasing susceptibility to cheats and the rapidity with which they can dominate a competition. Indeed, in chapter 2, figure 3 we see that the cheats of the over-productive strain, PA01, dominate their homologous cooperator more quickly than the cheats of 59.20 do theirs. In patched based population structure this effect may counteract to a degree the competitive advantage that the over-producer has in wild-type/wild-type interactions.

Cheats as drivers of diversity?

No diversity was maintained in the presence of cheats in either non-spatially structured environments, or in static soil microcosms, or on agar plates. Although in chapter 2 we did see that the presence of one or other of the two cheats strongly influenced the outcome of competition between the two wild-types (to the disadvantage of the cheat-cooperator pair), for this to be effective in a natural setting a fairly constricted sequence of

events would be required: As the two competitors cannot initially coexist, for cheat-mediated ecological succession to operate, an initial monoculture would need to be invaded first by its own cheat type, and then by an alternative cooperator. Perhaps this scenario is feasible in certain ecological settings, though this is a question that remains to be answered. Given that the we have seen a consistent competitive advantage of one cooperator over another, the mutation rate and migration rate would need to be balanced in order for diversity to be maintained.

The competitive interactions of many natural isolates of *Pseudomonad* species has recently been investigated (Butaitė *et al.*, 2017). Here, researchers found coexisting producers of different pyoverdine types, with different levels of pyoverdine expression, together with non-producing mutants, sometimes expressing multiple pyoverdine receptors. The outcome of within-environmental sample pairwise competitions and cross-feeding assays suggest that cheats may play a role in maintaining diversity, though the complexity may be render experimental demonstration of any theoretical distillation of these population dynamics impossible: Before even other ecological factors are considered, the effect of competition between pyoverdine types, as well as the effect of pyocins (which target pyoverdine receptors) must also be controlled.

General remarks on spatial and temporal resource heterogeneity

Hard selection

That resource availability can change the selective pressures on organisms is well known (I give many examples in the discussion sections of chapters 4 and 5). Resource availability also affects population productivity. The selection regime, i.e., whether hard or soft, will alter the distribution of genotypes in the metapopulation. Such dynamics have the potential to affect the evolution of many traits.

Bottlenecks in temporally heterogeneous environments

The productivity-resource relationship in temporally varying environments creates more severe population bottlenecks in heterogeneous environments. This has the effect of increasing relatedness. As we have shown, temporally heterogeneous environments also support cooperation via another population dynamic effect: Population expansion is greatest in high resource periods during which the cost of cooperation is lowest. In future experiments, the relative magnitude of these effects could be investigated with an additional factor, with levels prescribing bottleneck size. The effects may cancel out: With a greater fraction of each population transferred, migrant numbers after high-resource availability growth periods will be increasingly closer to the stationary phase density of low resource availability patches. This will increasingly limit the effect of selection in low-

resource availability periods, where very little growth will occur, though it will also decrease relatedness, which disfavours cooperation (Brockhurst, 2007).

General remarks

Whether in natural environments the evolution of diversity in tag-based cooperation is due to the effect of cheats remains an open question. Whilst the success of social evolutionary biology has in large part been due to an underpinning of conceptually simple and clear theory, complexity present in the natural world is often exposed as a hindrance when translating theoretical findings to laboratory models. Studies of pyoverdine production in natural communities would also be problematic due to their complexity, and the relative bluntness of potential experimental manipulation.

Nonetheless, next generation sequencing techniques together with community-level perturbation may provide useful insight into the effects of social evolution in complex scenarios.

Our findings with respect to the effect of temporal and spatial heterogeneity on cooperation give general predictions about the ecological conditions in which we should expect to find a greater prevalence of cooperative behaviour. Correlational studies, for example, Rubenstein & Lovette, (2007) and Jetz & Rubenstein, (2011) are supported by our work,

as we show, using both in theory and experimentation, why we might be expected to see such patterns. A more explicit focus on species' population dynamics in these studies could reveal whether the predicted productivity relationship is in operation.

7 Bibliography

Amarasekare, P. 2003. Competitive coexistence in spatially structured environments: A synthesis. *Ecol. Lett.* **6**: 1109–1122.

Andrews, S.C., Robinson, A.K. & Rodríguez-Quiñones, F. 2003. Bacterial iron homeostasis. *FEMS Microbiol. Rev.* **27**: 215–237. Blackwell Publishing Ltd.

Axelrod, R. & Hamilton, W. 1981. The evolution of cooperation. *Science (80-.).* **211**: 1390–1396.

Baysse, C., Meyer, J.M., Plesiat, P., Geoffroy, V., Michel-Briand, Y. & Cornelis, P. 1999. Uptake of pyocin S3 occurs through the outer membrane ferripyoverdine type II receptor of Pseudomonas aeruginosa. *J. Bacteriol.* **181**: 3849–51.

Bell, T. 2010. Experimental tests of the bacterial distance-decay relationship. *ISME J.* **4**: 1357–65. Nature Publishing Group.

Biernaskie, J.M., Gardner, A. & West, S.A. 2013. Multicoloured greenbeards, bacteriocin diversity and the rock-paper-scissors game. *J. Evol. Biol.* **26**: 2081–2094.

Biernaskie, J.M., West, S. a & Gardner, A. 2011. Are greenbeards intragenomic outlaws? *Evolution* **65**: 2729–42.

Blanquart, F. & Gandon, S. 2011. Evolution of Migration in a Periodically Changing Environment. *Am. Nat.* **177**: 188–201.

Blaustein, A.R. 1983. Kin Recognition Mechanisms: Phenotypic Matching or Recognition Alleles? *Am. Nat.* **121**: 749–754.

Bodey, G.P., Bolivar, R., Fainstein, V. & Jadeja, L. 1983. Infections caused by Pseudomonas aeruginosa. *Rev. Infect. Dis.* **5**: 279–313.

Bodilis, J., Ghysels, B., Osayande, J., Matthijs, S., Pirnay, J.-P., Denayer, S., *et al.* 2009. Distribution and evolution of ferripyoverdine receptors in Pseudomonas aeruginosa. *Environ. Microbiol.* **11**: 2123–35.

Boukhalfa, H. & Crumbliss, A.L. 2002. Chemical aspects of siderophore mediated iron transport. *BioMetals* **15**: 325–339.

Boza, G. & Scheuring, I. 2004. Environmental heterogeneity and the evolution of mutualism. *Ecol. Complex.* **1**: 329–339.

Brockhurst, M.A. 2007. Population bottlenecks promote cooperation in bacterial biofilms. *PLoS One* **2**.

Brockhurst, M.A., Buckling, A. & Gardner, A. 2007. Cooperation Peaks at Intermediate Disturbance. *Curr. Biol.* **17**: 761–765.

Brockhurst, M. a, Buckling, A., Racey, D. & Gardner, A. 2008. Resource supply and the evolution of public-goods cooperation in bacteria. *BMC Biol.* **6**: 20.

Brockhurst, M. a, Habets, M.G.J.L., Libberton, B., Buckling, A. & Gardner, A. 2010. Ecological drivers of the evolution of public-goods cooperation in bacteria. *Ecology* **91**: 334–40.

Brockhurst, M. a, Hochberg, M.E., Bell, T. & Buckling, A. 2006. Character displacement promotes cooperation in bacterial biofilms. *Curr. Biol.* **16**: 2030–4.

Brown, C., Burslem, D.F.R.P., Illian, J.B., Bao, L., Brockelman, W., Cao, M., *et al.* 2013. Multispecies coexistence of trees in tropical forests: spatial signals of topographic niche differentiation increase with environmental heterogeneity. *Proc. R. Soc. London B Biol. Sci.* **280**.

Brown, S.P. & Buckling, A. 2008. A Social Life for Discerning Microbes. *Cell* **135**: 600–603.

Buckling, A., Harrison, F., Vos, M., Brockhurst, M. a, Gardner, A., West, S. a, *et al.* 2007. Siderophore-mediated cooperation and virulence in Pseudomonas aeruginosa. *FEMS Microbiol. Ecol.* **62**: 135–41.

Bull, J.J. & Harcombe, W.R. 2009. Population Dynamics Constrain the Cooperative Evolution of Cross-Feeding. *PLoS One* **4**.

Butaitė, E., Baumgartner, M., Wyder, S. & Kümmerli, R. 2017. Siderophore cheating and cheating resistance shape competition for iron in soil and freshwater Pseudomonas communities. *Nat. Commun.* **8**: 414.

Chesson, P. 2000. Mechanisms of Maintenance of Species Diversity. *Annu. Rev. Ecol. Syst.* **31**: 343–366.

Chesson, P.L. & Warner, R.R. 1981. Environmental Variability Promotes Coexistence in Lottery Competitive Systems. *Am. Nat.* **117**: 923–943.

Chisholm, R.A., Condit, R., Rahman, K.A., Baker, P.J., Bunyavejchewin, S., Chen, Y.Y., *et al.* 2014. Temporal variability of forest communities: Empirical estimates of population change in 4000 tree species. *Ecol. Lett.* **17**: 855–865.

Choi, K.-H., Kumar, A. & Schweizer, H.P. 2006. A 10-min method for preparation of highly electrocompetent Pseudomonas aeruginosa cells: application for DNA fragment transfer between chromosomes and plasmid transformation. *J. Microbiol. Methods* **64**: 391–7.

Christiansen, F.B. 1975. Hard and Soft Selection in a Subdivided Population. *Am. Nat.* **109**: 11–16. [University of Chicago Press, American Society of Naturalists].

Cockburn, A. & Russell, A.F. 2011. Cooperative breeding: A question of climate? *Curr. Biol.* **21**: R195–R197. Elsevier Ltd.

Connelly, B.D., Bruger, E.L., McKinley, P.K. & Waters, C.M. 2017.

Resource abundance and the critical transition to cooperation. *J. Evol. Biol.* **30**: 750–761.

Cornelis, P., Hohnadel, D. & Meyer, J.M. 1989. Evidence for different pyoverdine-mediated iron uptake systems among Pseudomonas aeruginosa strains. *Infect. Immun.* **57**: 3491–7.

Dawkins, R. 1982. *The Extended Phenotype*. Oxford University Press, Oxford.

Dawkins, R. 1976. *The Selfish Gene*. Oxford University Press, Oxford.

De Chial, M., Ghysels, B., Beatson, S. a, Geoffroy, V., Meyer, J.M., Pattery, T., *et al.* 2003. Identification of type II and type III pyoverdine receptors from Pseudomonas aeruginosa. *Microbiology* **149**: 821–831.

De Vos, D., De Chial, M., Cochez, C., Jansen, S., Tümmler, B., Meyer, J.-M., *et al.* 2001. Study of pyoverdine type and production by Pseudomonas aeruginosa isolated from cystic fibrosis patients: prevalence of type II pyoverdine isolates and accumulation of pyoverdine-negative mutations. *Arch. Microbiol.* **175**: 384–388.

Demange, P., Wendenbaum, S., Linget, C., Mertz, C., Cung, M.T., Dell, A., *et al.* 1990. Bacterial siderophores : structure and NMR assignment of pyoverdins Pa, siderophores ofPseudomonas aeruginosa ATCC 15692. *Biol. Met.* **3**: 155–170.

Denayer, S., Matthijs, S. & Cornelis, P. 2007. Pyocin S2 (Sa) kills Pseudomonas aeruginosa strains via the FpvA type I ferripyoverdine receptor. *J. Bacteriol.* **189**: 7663–8.

Dewhirst, S. & Lutscher, F. 2009. Dispersal in heterogeneous habitats: thresholds, spatial scales, and approximate rates of spread. *Ecology* **90**: 1338–1345.

Durrett, R. & Levin, S. 1997. Allelopathy in Spatially Distributed

Populations. *J. Theor. Biol.* **185**: 165–171.

Eadie, J.M. & Keast, A. 1984. Resource heterogeneity and fish species diversity in lakes. *Can. J. Zool.* **62**: 1689–1695. NRC Research Press Ottawa, Canada .

Eilts, J.A., Mittelbach, G.G., Reynolds, H.L. & Gross, K.L. 2011. Resource heterogeneity, soil fertility, and species diversity: effects of clonal species on plant communities. *Am. Nat.* **177**: 574–588.

Forde, S.E., Thompson, J.N. & Bohannan, B.J.M. 2004. Adaptation varies through space and time in a coevolving host–parasitoid interaction. *Nature* **431**: 841–844.

Foster, K.R. 2005. BIOMEDICINE: Hamiltonian Medicine: Why the Social Lives of Pathogens Matter. *Science (80-.).* **308**: 1269–1270.

Foster, K.R. 2004. Diminishing returns in social evolution: The not-so-tragic commons. *J. Evol. Biol.* **17**: 1058–1072.

Frank, S.A. 1995. Mutual policing and repression of competition in the evolution of cooperative groups. *Nature* **377**: 520–522.

Frank, S.A. 2003. Perspective: repression of competition and the evolution of cooperation. *Evolution* **57**: 693–705.

Friesen, M.L., Saxer, G., Travisano, M. & Doebeli, M. 2004. Experimental evidence for sympatric ecological diversification due to frequency-dependent competition in Escherichia coli. *Evolution* **58**: 245–60.

Gardner, A. & West, S. 2010. Greenbeards. *Evolution* **64**: 25–38.

Gause, F. 1934. The Struggle for Existence. *Yale J. Biol. Med.* **7**: 609.

Ghoul, M., Andersen, S.B. & West, S.A. 2017. Sociomics: Using Omic Approaches to Understand Social Evolution. *Trends Genet.* **33**: 408–419.

Elsevier Ltd.

Ghysels, B., Dieu, B.T.M., Beatson, S.A., Pirnay, J.-P., Ochsner, U.A., Vasil, M.L., *et al.* 2004. FpvB, an alternative type I ferripyoverdine receptor of Pseudomonas aeruginosa. *Microbiology* **150**: 1671–80.

Griffin, A., West, S. & Buckling, A. 2004. Cooperation and competition in pathogenic bacteria. *Nature* **430**.

Habets, M.G.J.L., Rozen, D.E., Hoekstra, R.F. & De Visser, J.A.G.M. 2006. The effect of population structure on the adaptive radiation of microbial populations evolving in spatially structured environments. *Ecol. Lett.* **9**: 1041–1048.

Hall, A.R. & Colegrave, N. 2007. How Does Resource Supply Affect Evolutionary Diversification? *Source Proc. Biol. Sci. Proc. R. Soc. B* **274**: 73–78.

Hamilton, W.D. 1964. The genetical evolution of social behaviour. I and II. *J. Theor. Biol.* **7**: 1–16.

Hardin, G. 1960. The competitive exclusion principle. *Science (80-.).* **131**: 1292–1297.

Harrison, F. 2013. Dynamic social behaviour in a bacterium: Pseudomonas aeruginosa partially compensates for siderophore loss to cheats. *J. Evol. Biol.* 1–9.

Harrison, F., Browning, L.E., Vos, M. & Buckling, A. 2006. Cooperation and virulence in acute Pseudomonas aeruginosa infections. *BMC Biol.* **4**: 21.

Harrison, F. & Buckling, A. 2009. Cooperative production of siderophores by Pseudomonas aeruginosa. *Front. Biosci. a J. virtual Libr.* **14**: 4113–4126.

Harrison, F. & Buckling, A. 2007. High relatedness selects against hypermutability in bacterial metapopulations. *Proc. Biol. Sci.* **274**: 1341–7.

Harrison, F. & Buckling, A. 2005. Hypermutability impedes cooperation in pathogenic bacteria. *Curr. Biol.* **15**: 1968–1971.

Hider, R.C. & Kong, X. 2010. Chemistry and biology of siderophores. *Nat. Prod. Rep.* **27**: 637–657. Royal Society of Chemistry.

Hoek, T.A., Axelrod, K., Biancalani, T., Yurtsev, E.A., Liu, J. & Gore, J. 2016. Resource Availability Modulates the Cooperative and Competitive Nature of a Microbial Cross-Feeding Mutualism. *PLOS Biol.* **14**: e1002540. Cambridge University Press.

Hohnadel, D. & Meyer, J. 1988. Specificity of Pyoverdine-Mediated Iron Uptake Pseudomonas Strains. *J. Bacteriol.* **170**: 4865–4873.

Hom, E.F.Y. & Murray, A.W. 2014. Niche engineering demonstrates a latent capacity for fungal-algal mutualism. *Science (80-.).* **345**: 94–98.

Horner-Devine, M.C., Leibold, M.A., Smith, V.H. & Bohannan, B.J.M. 2003. Bacterial diversity patterns along a gradient of primary productivity. *Ecol. Lett.* **6**: 613–622.

Hortal, J., Triantis, K.A., Meiri, S., Thébault, E. & Sfenthourakis, S. 2009. Island Species Richness Increases with Habitat Diversity. *Am. Nat.* **174**: E205–E217.

Inglis, R.F., Biernaskie, J.M., Gardner, A. & Kümmerli, R. 2016. Presence of a loner strain maintains cooperation and diversity in well-mixed bacterial communities. *Proc. R. Soc. B Biol. Sci.* **283**: 20152682.

Inglis, R.F., Gardner, A., Cornelis, P. & Buckling, A. 2009. Spite and virulence in the bacterium Pseudomonas aeruginosa. *Proc. Natl. Acad.*

Sci. U. S. A. **106**: 5703–7.

Inglis, R.F., West, S. & Buckling, A. 2014. An experimental study of strong reciprocity in bacteria.

James, R., Kleanthous, C. & Moore, G.R. 1996. The biology of E colicins: paradigms and paradoxes. *Microbiology* **142**: 1569–1580.

Jansen, V. a a & van Baalen, M. 2006. Altruism through beard chromodynamics. *Nature* **440**: 663–6.

Jetz, W. & Rubenstein, D.R. 2011. Environmental uncertainty and the global biogeography of cooperative breeding in birds. *Curr. Biol.* **21**: 72–78. Elsevier Ltd.

Julou, T., Mora, T., Guillon, L., Croquette, V., Schalk, I.J., Bensimon, D., *et al.* 2013. Cell-cell contacts confine public goods diffusion inside Pseudomonas aeruginosa clonal microcolonies. *Proc. Natl. Acad. Sci. U. S. A.* **110**: 12577–82.

Kassen, R., Buckling, a, Bell, G. & Rainey, P.B. 2000. Diversity peaks at intermediate productivity in a laboratory microcosm. *Nature* **406**: 508–12.

Keller, L. & Ross, K. 1998. Selfish genes: a green beard in the red fire ant. *Nature* **251**: 573–575.

Koedam, N., Wittouck, E., Gaballa, a, Gillis, a, Höfte, M. & Cornelis, P. 1994. Detection and differentiation of microbial siderophores by isoelectric focusing and chrome azurol S overlay. *Biometals* **7**: 287–291.

Köhler, T., Buckling, A. & van Delden, C. 2009. Cooperation and virulence of clinical Pseudomonas aeruginosa populations. *Proc. Natl. Acad. Sci. U. S. A.* **106**: 6339–44.

Kümmerli, R., Griffin, A.S., West, S.A., Buckling, A. & Harrison, F. 2009. Viscous medium promotes cooperation in the pathogenic bacterium *Pseudomonas aeruginosa*. *Proc. R. Soc. B Biol. Sci.* **276**: 3531–3538.

Kümmerli, R., Schiessl, K.T., Waldvogel, T., McNeill, K. & Ackermann, M. 2014. Habitat structure and the evolution of diffusible siderophores in bacteria. *Ecol. Lett.* **17**: 1536–1544.

Kümmerli, R., van den Berg, P., Griffin, A.S., West, S.A. & Gardner, A. 2010. Repression of competition favours cooperation: experimental evidence from bacteria. *J. Evol. Biol.* **23**: 699–706.

Kun, a & Dieckmann, U. 2013. Resource heterogeneity can facilitate cooperation. *Nat Commun* **4**: 2453.

Lee, W., Van Baalen, M. & Jansen, V. a a. 2012. An evolutionary mechanism for diversity in siderophore-producing bacteria. *Ecol. Lett.* **15**: 119–25.

Lehmann, L. & Keller, L. 2006. The evolution of cooperation and altruism--a general framework and a classification of models. *J. Evol. Biol.* **19**: 1365–76.

Leinweber, A., Fredrik Inglis, R. & Kümmerli, R. 2017. Cheating fosters species co-existence in well-mixed bacterial communities. *ISME J.* **11**.

Lenski, R.E., Rose, M.R., Simpson, S.C. & Tadler, S.C. 1991. Long-Term Experimental Evolution in Escherichia coli. I. Adaptation and Divergence During 2,000 Generations. *Am. Nat.* **138**: 1315–1341.

Levene, H. 1953. Genetic Equilibrium When More Than One Ecological Niche is Available. *Am. Nat.* **87**: 331–333.

MacArthur .R. 1965. Patterns of Species Diversity. *Biol. Rev. Camb. Philos. Soc.* **40**: 510–533.

Maestre, F.T. & Reynolds, J.F. 2006. Spatial heterogeneity in soil nutrient supply modulates nutrient and biomass responses to multiple global change drivers in model grassland communities. *Glob. Chang. Biol.* **12**: 2431–2441.

Marshall, H.H., Sanderson, J.L., Mwanghuya, F., Businge, R., Kyabulima, S., Hares, M.C., *et al.* 2016. Variable ecological conditions promote male helping by changing banded mongoose group composition. *Behav. Ecol.* **27**: 978–987.

Mateo, J.M. & Johnston, R.E. 2000. Kin recognition and the "armpit effect": evidence of self-referent phenotype matching. *Proc. R. Soc. B* **267**: 695–700.

Merriman, T., Merriman, M. & Lamont, I. 1995. Nucleotide sequence of pvdD , a pyoverdine biosynthetic gene from Pseudomonas aeruginosa : PvdD Has Similarity to Peptide Synthetases. *J. Bacteriol.* **177**: 252–258.

Meyer, J.M. 2000. Proverdines: Pigments, siderophores and potential taxonomic markers of fluorescent pseudomonas species. *Arch. Microbiol.* **174**: 135–142.

Meyer, J.M., Neely, A., Stintzi, A., Georges, C. & Holder, I. a. 1996. Pyoverdin is essential for virulence of Pseudomonas aeruginosa. *Infect. Immun.* **64**: 518–23. American Society for Microbiology (ASM).

Meyer, J.M., Stintzi, A., De Vos, D., Cornelis, P., Tappe, R., Taraz, K., *et al.* 1997. Use of siderophores to type pseudomonads: The three Pseudomonas aeruginosa pyoverdine systems. *Microbiology* **143**: 35–43.

Niehus, R., Picot, A., Oliveira, N.M., Mitri, S. & Foster, K.R. 2017. The evolution of siderophore production as a competitive trait. *Evolution (N. Y).* **71**: 1443–1455.

Oliver, T., Roy, D.B., Hill, J.K., Brereton, T. & Thomas, C.D. 2010.

Heterogeneous landscapes promote population stability. *Ecol. Lett.* **13**: 473–484.

Pagie, L. & Hogeweg, P. 1999a. Colicin Diversity: a Result of Eco-evolutionary Dynamics. *J. Theor. Biol.* **196**: 251–261.

Pagie, L. & Hogeweg, P. 1999b. Colicin Diversity: a Result of Eco-evolutionary Dynamics. *J. Theor. Biol.* **196**: 251–261.

Price, J.N., Gazol, A., Tamme, R., Hiiesalu, I. & Pärtel, M. 2014. The functional assembly of experimental grasslands in relation to fertility and resource heterogeneity. *Funct. Ecol.* **28**: 509–519.

Queller, D.C. 1985. Kinship, reciprocity and synergism in the evolution of social behaviour. *Nature* **318**: 366–367.

Queller, D.C., Ponte, E., Bozzaro, S. & Strassmann, J.E. 2003. Single-gene greenbeard effects in the social amoeba Dictyostelium discoideum. *Science* **299**: 105–6.

Questad, E.J. & Foster, B.L. 2008. Coexistence through spatio-temporal heterogeneity and species sorting in grassland plant communities. *Ecol. Lett.* **11**: 717–726.

Racey, D., Inglis, R.F., Harrison, F., Oliver, A. & Buckling, A. 2010. The effect of elevated mutation rates on the evolution of cooperation and virulence of pseudomonas aeruginosa. *Evolution (N. Y).* **64**: 515–521.

Rainey, P., Buckling, a, Kassen, R. & Travisano, M. 2000. The emergence and maintenance of diversity: insights from experimental bacterial populations. *Trends Ecol. Evol.* **15**: 243–247.

Rainey, P.B. & Travisano, M. 1998. Adaptive radiation in a heterogeneous environment. *Nature* **394**: 69–72.

Riley, M.A. 1993. Positive selection for colicin diversity in bacteria. *Mol. Biol. Evol.* **10**: 1048–59.

Riley, M. a & Wertz, J.E. 2002. Bacteriocins: evolution, ecology, and application. *Annu. Rev. Microbiol.* **56**: 117–37.

Ross-gillespie, A., Gardner, A., West, S.A. & Griffin, A.S. 2007. Frequency Dependence and Cooperation : Theory and a Test with Bacteria. **170**.

Rousset, F. & Roze, D. 2007. Constraints on the origin and maintenance of genetic kin recognition. *Evolution* **61**: 2320–30.

Rubenstein, D.R. & Lovette, I.J. 2007. Temporal Environmental Variability Drives the Evolution of Cooperative Breeding in Birds. *Curr. Biol.* **17**: 1414–1419.

Saxer, G., Doebeli, M. & Travisano, M. 2009. Spatial structure leads to ecological breakdown and loss of diversity. 2065–2070.

Schwyn, B. & Neilands, J.B. 1987. Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* **160**: 47–56.

Shigesada, N., Kawasaki, K. & Teramoto, E. 1986. Traveling periodic waves in heterogeneous environments. *Theor. Popul. Biol.* **30**: 143–160.

Sinervo, B., Chaine, A., Clobert, J., Calsbeek, R., Hazard, L., Lancaster, L., *et al.* 2006. Self-recognition, color signals, and cycles of greenbeard mutualism and altruism. *Proc. Natl. Acad. Sci. U. S. A.* **103**: 7372–7377.

Smith, E.E., Sims, E.H., Spencer, D.H., Kaul, R. & Olson, M. V. 2005. Evidence for diversifying selection at the pyoverdine locus of Pseudomonas aeruginosa. *J. Bacteriol.* **187**: 2138–47.

Smith, J.M. 1964. Group Selection and Kin Selection. *Nature* **201**: 1145–1147.

Smukalla, S., Caldara, M., Pochet, N., Beauvais, A., Guadagnini, S., Yan, C., *et al.* 2008. FLO1 is a variable green beard gene that drives biofilm-like cooperation in budding yeast. *Cell* **135**: 726–37.

Stevens, M.H.H. & Carson, W.P. 2002. Resource quantity, not resource heterogeneity, maintains plant diversity. *Ecol. Lett.* **5**: 420–426.

Takase, H., Nitanai, H., Hoshino, K. & Otani, T. 2000. Impact of siderophore production on Pseudomonas aeruginosa infections in immunosuppressed mice. *Infect. Immun.* **68**: 1834–9. American Society for Microbiology.

Thompson, J.N. 1999. Specific Hypotheses on the Geographic Mosaic of Coevolution. *Am. Nat.* **153**: S1–S14.

Thompson, J.N. 2005. *The Geographic Mosaic of Coevolution*. University of Chicago Press.

Vogwill, T., Fenton, A., Buckling, A., Hochberg, M.E. & Brockhurst, M.A. 2009. Source Populations Act as Coevolutionary Pacemakers in Experimental Selection Mosaics Containing Hotspots and Coldspots. *Am. Nat.* **173**: E171–E176.

Wallace, B. 1975. Hard and Soft Selection Revisited. *Evolution (N. Y).* **29**: 465–473. [Society for the Study of Evolution, Wiley].

Wallace, B. 1968. Polymorphism, population size, and genetic load. In: *Population Biology and Evolution* (R. C. Lewontin, ed), pp. 87–108. Syracuse University Press, Syracuse, NY.

Waxman, D. & Peck, J.R. 1999. Sex and adaptation in a changing environment. *Genetics* **153**: 1041–1053.

Wendenbaum, S., Demange, P., Dell, A., Meyer, J.M. & Abdallah, M.A. 1983. The structure of pyoverdine Pa, the siderophore of

Pseudomonas aeruginosa. *Tetrahedron Lett.* **24**: 4877–4880.

West, S.A., Griffin, A.S. & Gardner, A. 2007. Evolutionary explanations for cooperation. *Curr. Biol.* **17**: R661-72.

West, S. & Buckling, A. 2003. Cooperation, virulence and siderophore production in bacterial parasites. *Proc. Biol. Sci.* **270**: 37–44.

Westra, E.R., van Houte, S., Oyesiku-Blakemore, S., Makin, B., Broniewski, J.M., Best, A., *et al.* 2015. Parasite Exposure Drives Selective Evolution of Constitutive versus Inducible Defense. *Curr. Biol.* **25**: 1043–1049.

Woolverton, C.J., Sherwood, L. & Willey, J. 2016. *Prescott's Microbiology*. McGraw-Hill Education.

Xavier, J.B., Kim, W. & Foster, K.R. 2011. A molecular mechanism that stabilizes cooperative secretions in Pseudomonas aeruginosa. *Mol. Microbiol.* **79**: 166–179.

Yang, Z., Liu, X., Zhou, M., Ai, D., Wang, G., Wang, Y., *et al.* 2015. The effect of environmental heterogeneity on species richness depends on community position along the environmental gradient. *Sci. Rep.* **5**: 15723. Nature Publishing Group.

Zhang, X. & Rainey, P.B. 2013. Exploring the sociobiology of pyoverdin-producing Pseudomonas. *Evolution* **67**: 3161–74.

Zhou, J., Xia, B., Treves, D.S., Wu, L., Marsh, T.L., Neill, R.V.O., *et al.* 2002. Spatial and Resource Factors Influencing High Microbial Diversity in Soil Spatial and Resource Factors Influencing High Microbial Diversity in Soil. *Appl. Environ. Microbiol.* **Vol. 68**, **N**: 326–334.