QUORUM SENSING AND THE STABILIZATION OF COOPERATIVE BEHAVIOR IN VIBRIO BACTERIA

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ABSTRACT

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This thesis studies the connections between two ubiquitous biological phenomenon: cooperation and communication; and one may debate whether the latter is a specific example of the prior.

More specifically, the questions examined in this document center on bacteria and their common form of chemical communication known as quorum sensing. My broad interests and opinions regarding quorum sensing are that they play an immense role in providing feedbacks that adjust cellular behavior and gene expression to optimize growth in response to a plethora of environmental cues and signals. The more specific topics examined in this document relate to the tendency of quorum sensing to regulate cooperative behaviors such as public goods production. Such cooperative behaviors are susceptible to cheating by non-participants if they can enjoy a benefit that a given behavior produces without contributing to that behavior. We examined the stability of quorum sensing in a variety of conditions, particularly where public goods positively contribute to fitness, and pursue explanations for how cooperative goods production and the overlaying regulation of quorum sensing can be maintained and stabilized in the presence of potential cheats, and how such behavior holds up over evolutionary time.

A few interesting results were revealed from this research. Firstly, it was definitively shown that quorum sensing regulation provides a distinct advantage in the face of cheats compared to unconditional cooperators. Quorum sensers were able to appropriately regulate

cell physiology, including public goods production, in such a way that this strategy had equivalent fitness to an obligate defector in the environment tested, whereas unconditional cooperators paid heavy costs in terms of growth rate, and were thus outcompeted by other strains at low cell densities, and also cheated by defectors at high cell densities. Together, this provides the strongest reported experimental evidence to date that quorum sensing regulation itself can stabilize cooperative behaviors, even in the absence of other layered mechanisms such as policing or positive assortment by clustering.

Additionally, cooperative strategies were found to be able to invade metapopulations of mostly defectors when sufficient assortment of competing types was achieved. This was found to be true for both *Vibrio harveyi* and *Vibrio cholerae*. Maximum evasion of defectors was achieved when cooperator strains were able to both form structures to separate from competitors (by way of dispersal by motility), and the ability to disperse from other cells through motile phenotypes. Again, the most successful strategy in this regard came from the functional wild type quorum senser as compared to an unconditionally cooperating strategy.

Lastly, experimental evolution of *Vibrio harveyi* populations was conducted in minimal media for 2000 generations. These populations underwent a variety of adaptations to the experimental environment, which was examined in detail through high-throughput genomic sequencing. It was discovered that wild type populations sustained higher fractions of bioluminescent cooperators as well as a higher degree of phenotypic diversity in terms of quorum sensing, while nearly all unconditional cooperator populations experienced rapid evolution and sweeps by *de novo* defectors. Additionally, many of the evolved forms in wild type populations appear to be optimizing and exhibit intermediate bioluminescent and protease production phenotypes.

To my family:
To Silas, my chronometer and encyclopedia
To Ella, my goofball and spitfire
To Jess, my rock and compass

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a rich academic environment.

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KEY TO SYMBOLS

QS   Quorum sensing

HCD   High cell density

LCD   Low cell density

WT   Wild type

UC   Unconditional cooperator

AHL   Acyl homoserine lactone signal molecules

CAA   Casamino acids

M9-casein   M9 media containing casein as the sole carbon source

M9-CAA   M9 media containing casamino acids as the sole carbon source

LB   Lysogeny broth (LB), a rich medium for cellular growth

PG   Public good

SN   Signal negative strain, a triple mutant for QS signal synthase genes

AI   Autoinducer, signal molecule that induces quorum sensing

$\Delta luxR$ *Vibrio harveyi* strain with *luxR* gene deleted

$\Delta luxOU$ *Vibrio harveyi* strain with *luxOU* genes deleted

$\Delta luxO$ *Vibrio cholerae* strain with *luxO* gene deleted

$\Delta hapR$ *Vibrio cholerae* strain with *hapR* gene deleted

NAG   N-Acetylglucosamine

NAC   non-ancestral-cooperator,
                evolved variant with reduced bioluminescence phenotype

Chapter 1

Evolutionary mechanisms that

maintain bacterial cooperation

Preface

The contents of this chapter were adapted from an article previously published in the F1000Research journal in 2015 (Citation: Bruger E and Waters C. Sharing the sandbox: Evolutionary mechanisms that maintain bacterial cooperation [version 1; referees: 2 approved]. F1000Research 2015, 4(F1000 Faculty Rev):1504 (doi: 10.12688/f1000research.7363.1)). Some slight modifications have been made from the original published text. The original manuscript was published under a CC BY 4.0 license (details viewable online at https://creativecommons.org/licenses/by/4.0/legalcode). Copyright: ©2015 Bruger E and Waters C. This is an open access article distributed under the terms of the Creative Commons Attribution Licence, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Disclaimer: Creative Commons Corporation ("Creative Commons") is not a law firm and does not provide legal services or legal advice. Distribution of Creative Commons public licenses does not create a lawyer-client or other relationship. Creative Commons makes its licenses and related information available on an âĂIJas-isâĂİ basis. Creative Commons gives no warranties regarding its licenses, any material licensed under their terms and conditions, or any related information. Creative Commons disclaims all liability for damages resulting from their use to the fullest extent possible.

In this chapter, I examine a variety of potential proximate mechanisms that could act to stabilize and preserve cooperation in microbes. Quorum sensing is a central focus in this chapter, as we suspect it has critical roles to play for many cooperative behaviors in bacteria, both on its own and in concert with other mechanisms discussed in the chapter. While we

predict that many instances of such QS-mediated stabilization exist in nature, few examples have been reported at this point in time.

Microbes are now known to participate in an extensive repertoire of cooperative behaviors such as biofilm formation, production of extracellular public-goods, group motility, and higher-ordered multicellular structures. A fundamental question is how these cooperative tasks are maintained in the face of non-cooperating defector cells. Recently, a number of molecular mechanisms including facultative participation, spatial sorting, and policing have been discovered to stabilize cooperation. Often these different mechanisms work in concert to reinforce cooperation. In this review, I describe bacterial cooperation and the current understanding of the molecular mechanisms that maintain it.

1.1 Introduction

Bacteria were once thought to be solitary individuals, but it is now clear that they lead complex social lives (Crespi, 2001, Velicer, 2003). Multicellular bacterial communities termed biofilms are now considered a normal form of bacterial growth. Bacterial chemical communication, including quorum sensing (QS), is ubiquitous (Platt and Fuqua, 2010, Waters and Bassler, 2005), and the molecular underpinnings of multicellular bacterial structures such as Myxobacteria fruiting bodies and *Streptomyces* filaments are also being elucidated (Kroos, 2007, Petrus and Claessen, 2014). With our increased understanding of bacterial sociality comes a further appreciation of the role of cooperation in many bacterial processes. Microbial cooperative behaviors have important impacts on our own lives, including antibiotic resistance (Lee et al., 2010), biofilm formation in chronic infections (Percival et al., 2010), and virulence during acute infections (Diard et al., 2013, 2014). Explaining the evolution of cooperative tasks has long challenged evolutionary biology, as these systems appear ripe for exploitation by non-cooperating defector/cheater cells that receive the benefits of cooperation without paying the cost of production (West et al., 2007). Because of their short generation times, large population sizes, small genomes, and asexual reproduction, bacteria are now recognized as ideal model systems to understand the factors leading to the evolution and persistence of cooperative behaviors (Barrick and Lenski, 2013, Elena and Lenski, 2003, West et al., 2006). In this review, we will summarize from both a conceptual and a mechanistic perspective our understanding of how cooperation is maintained in bacteria.

1.2 Facultative cooperation

Bacteria have evolved complex regulatory circuitry to respond and effectively acclimate to different environments, so it is not surprising that this flexible regulatory circuitry can also be utilized to control cooperative traits. Cooperative behaviors in bacteria, such as the production of extracellular "public good" molecules, defined as resources that can be utilized by both the producers and the non-producers in the community, are exploitable by non-producing cheater/defector cells. One approach to limit cheater invasion is facultative cooperation. Engaging in cooperation at limited times, particularly when the benefit is the greatest, or in environmental conditions where the cost of cooperation is low can limit or prevent cheater invasion (Heilmann et al., 2015, Hense and Schuster, 2015). In this way, bacteria may preserve cooperation in conditions that would otherwise favor its collapse (Cornforth et al., 2012). It is notable, however, that facultative participation only partly mediates the problem of cooperation by limiting the times when a cell must maintain it. Other mechanisms, such as relatedness, are likely required in conjunction with optional participation to preserve cooperation.

For public goods to be effective, they often must exceed a threshold concentration in the extracellular environment (Heilmann et al., 2015). Therefore, there must be a sufficient number of producing cells contributing to the public good. For this reason, production of many public goods such as exoenzymes, proteases, chitinases, and siderophores are regulated by QS (Fig. 1A) (Waters and Bassler, 2005). This process relies on the secretion and detection by bacteria of small chemical signals known as autoinducers into the extracellular environment. As the cell density of a growing culture increases, so does the concentration of autoinducers. This is reinforced by the positive feedback of many QS systems on autoinducer

synthesis (Ng and Bassler, 2009, Williams et al., 2008). At a specific concentration of signal, receptors bind to and sense these autoinducers, allowing the bacteria to switch from a low- to high-cell density state. This is often seen as a transition from non-production to production of cooperative traits such as extracellular public goods. QS itself is an exploitable cooperative behavior, as QS-specific cheaters that do not signal, overproduce signal, or do not respond to signal can evolve (Diggle et al., 2007).

QS regulation of cooperation has been well studied in the bacterium *Pseudomonas aeruginosa*. This bacterium upregulates extracellular proteases in the high-cell density state (Brint and Ohman, 1995). These proteases degrade extracellular proteins, liberating smaller peptides that can be used for growth. Thus, the growth of *P. aeruginosa* in minimal media with the protein casein as a carbon source is dependent upon a functional QS system. Mutants in the QS pathway do not secrete high levels of proteases and cannot grow in this environment, but they receive negative frequency-dependent fitness benefits when mixed with a cooperating strain (Diggle et al., 2007). In other words, QS mutants can invade the wild-type strain when rare but lose their fitness benefits when common, a feature likely to occur in most public goods scenarios (Diggle et al., 2007, Ross-Gillespie et al., 2007, Sandoz et al., 2007). Therefore, although QS limits maximum public good production to high-cell density, it is not sufficient in this case to completely prevent cheater invasion, although it may mediate the extent to which this occurs. Similar results in a casein growth medium were recently described for *Vibrio cholerae* (Katzianer et al., 2015) and *Vibrio harveyi* (unpublished results, Waters laboratory), which also secrete proteases at high-cell density in a QS-dependent manner. The degree of resistance to cheating QS can provide likely depends upon the cost and benefit functions of the behavior, the manner in which regulation is imposed, and the genetic architecture of the QS system.
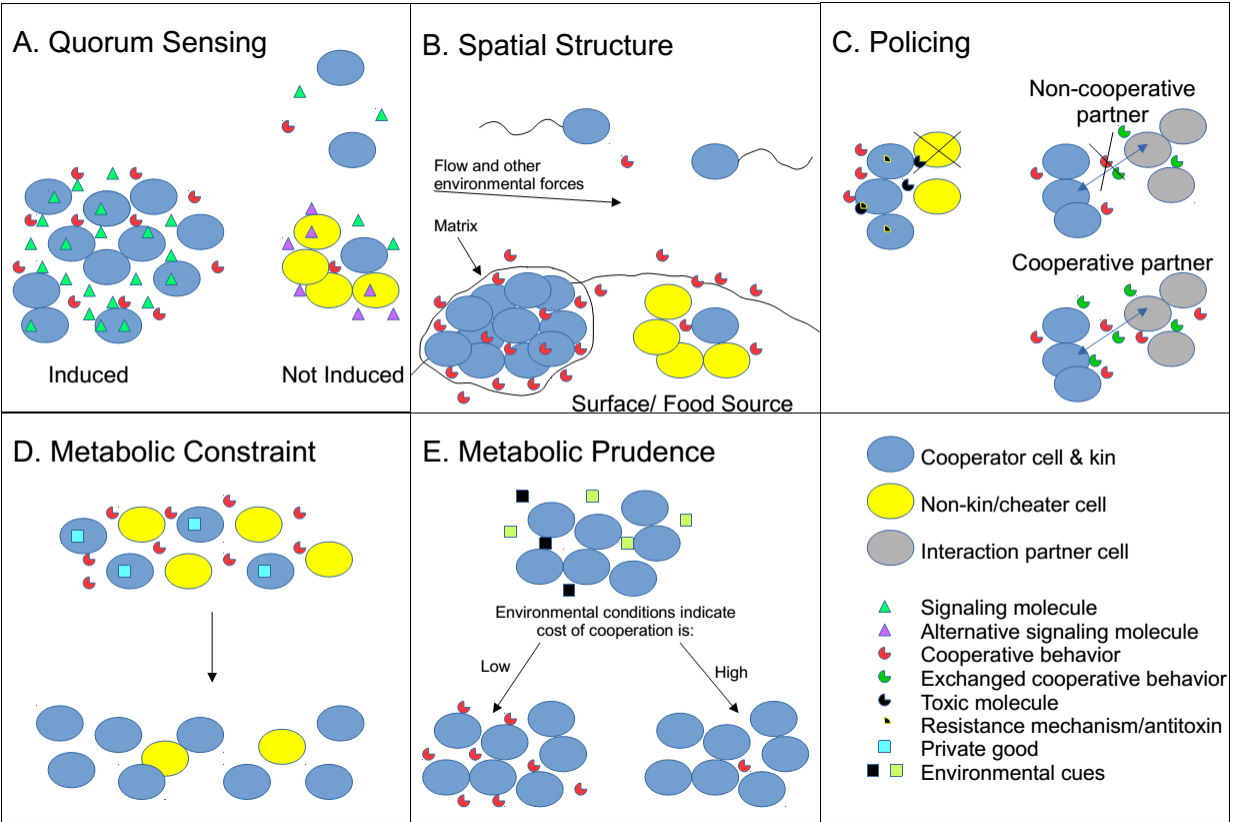
Figure 1.1: **Mechanisms that act to maintain cooperation.** A. Quorum Sensing. The cooperative behavior is induced only when a sufficient amount of signal has accumulated (left). B. Spatial Structure. When cells are able to assort with kin in space, particularly in the case of biofilm formation (bottom left), dispersal of cells and diffusion of public goods are limited and promote the maintenance of cooperative behavior. C. Policing. This mechanism may act through directed harm (left) or restraint of benefits (right). D. Metabolic Constraint. Producers of a cooperative behavior such as a public good also produce an individually retained private good that is beneficial or required for survival and growth in the focal environment. E. Metabolic Prudence. Cells detect nutrients and other cues in their environment to determine whether it is cost effective to cooperate.

As QS itself does not appear sufficient to completely ward off cheaters, at least in these systems, additional mechanisms for cheater prevention are required. One such mechanism, referred to as metabolic constraint, found that the utilization of adenosine was also positively controlled by QS (Dandekar et al., 2012) (Fig. 1D). Unlike protease secretion, which is a public good, adenosine utilization primarily benefits the producing individual, making

this function as a "private" good. The addition of adenosine to the medium inhibited the evolution of QS mutants, as these were not able to effectively use this resource. Therefore, the cooperators were able to access a benefit that was unavailable to the defectors. It was proposed that mutually regulating public and private goods under control of QS could be a mechanism to stabilize QS-controlled cooperative tasks. However, if utilization of adenosine is a private good that benefits the producer in a density-independent manner, it is not clear how this system would have evolved. An alternate hypothesis is that in its natural environment adenosine is primarily present in high-cell density situations. QS could function as an environmental cue to prime *P. aeruginosa* to utilize likely nutrient sources that may be encountered at different cell densities. While QS can explain varying behavioral differences with density, the underlying cooperative behaviors are still promoted when cells have unified interests, such as relatedness. Because of autoinducer specificity, QS provides not just evidence of general density but also the degree of relatedness of the surrounding community. It is therefore our view that metabolic constraint systems will only evolve when utilization of the private good in a density-dependent manner is favored. On a broader note, any condition in which co-regulation of the public and private good is most beneficial could exhibit metabolic constraint.

Another mechanism to limit cheater invasion is to only produce public goods when their cost is minimal, an idea termed metabolic prudence (Fig. 1E). *P. aeruginosa* also produces carbon-rich molecules called rhamnolipids that allow large numbers of these organisms to "swarm" over certain surfaces, such as soft agar plates. Rhamnolipids are a public good that can be exploited by non-producers to swarm. Xavier and colleagues noticed (Xavier et al., 2011), however, that a non-producer did not exhibit higher fitness than a rhamnolipid producer during a swarm assay of chimeric populations, even though a significant portion

of carbon was being directed towards synthesis of this public good. The authors deduced that *P. aeruginosa* only produced rhamnolipids when experiencing an excess of carbon in relation to nitrogen levels. Thus, the cost of production for this public good was minimized and did not lead to reduced fitness versus the non-producer. Interestingly, rhamnolipid production is also regulated by QS, indicating that production only occurs at high density and/or relatedness as well (Latifi et al., 1995).

Metabolic prudence is thus a facultative cooperation mechanism, which illustrates that bacteria integrate the relative cost associated with cooperative traits. Though it has not yet been widely demonstrated, it is likely to occur for additional microbial cooperative behaviors. It is common for complex traits to be controlled by multiple regulatory inputs. For example, the catabolite repressor protein CRP which responds to the presence of phosphotransfer sugars, at least in *Escherichia coli*, regulates 300 genes, which is 7% of its genome (Zheng et al., 2004). It is our opinion that regulatory connections between QS (or other signaling systems), central metabolism, and the control of cooperation will be common, and finding other systems that demonstrate metabolic prudence will be an exciting new avenue of research (Soto and Nishiguchi, 2014).

1.3   Spatial structure and assortment

Spatial structuring of related cooperators is a key mechanism by which cooperation is likely evolved and maintained (Griffin et al., 2004, Lion and Baalen, 2008, West et al., 2007) (Fig. 1B). One critical example of cells actively structuring themselves in an environment that is proposed to encourage cooperation is the production of biofilms (Kreft, 2004). Biofilms are multicellular communities of bacteria encased in an extracellular matrix. A

costly and potentially cheatable behavior itself (Popat et al., 2012), biofilm formation provides a framework for cells to situate themselves in space and direct cooperative benefits preferentially towards clonal offspring and other related kin. Biofilms also restrict diffusion so that public goods, such as extracellular enzymes, remain near the producing cell rather than being dispersed by flow or other forces (Drescher et al., 2014, Persat et al., 2015). This was recently demonstrated as *V. cholerae* biofilms attached to chitin surfaces retain sufficient amounts of the extracellular enzymes chitinases to metabolize this nutrient (Drescher et al., 2014). Cells that do not form biofilms lose higher portions of these public goods due to increased loss via diffusive and advective forces, which likely reflects conditions encountered in natural environments (Drescher et al., 2014, Persat et al., 2015). This means that public goods benefits remain distributed over a narrower, more local range in space that favors their diversion toward neighboring kin cells. Additionally, biofilm formers may even be able to exclude non-producers from colonized nutrient source surfaces (Schluter et al., 2015).

However, this structuring also comes with the potential for more competition between kin, especially if cells don't also possess the capacity to disperse and colonize new patches in their habitat. For example, experimentally evolved lineages that produce more biofilm through an evolved wrinkly spreader lifestyle are able to bind tightly to neighboring cells due to enhanced production of extracellular matrix materials, but this typically comes with a tradeoff for growth potential (Spiers et al., 2002, Spiers, 2007). The tradeoff may be in part restrained by the reduced ability of cells to disperse from a cluster. A similar example of a tradeoff between colonization and dispersal is seen in *V. cholerae*, where biofilm producers compete and grow better on a surface but are less effective at dispersing to new locations in their habitat (Nadell and Bassler, 2011), and natural populations of *Vibrio cyclitrophicus* demonstrate differential specialization for colonization onto and dispersal from particles,

signifying that this phenomenon could be more widespread (Yawata et al., 2014). This suggests that multiple selective pressures are naturally acting upon microbes that can either reinforce or act against other cooperative behaviors.

In the examples described thus far, no assumptions have been made about the ability of cells to recognize the presence or identity of neighboring cells, and this is not always theoretically necessary for spatial structure to enable cooperation (Oliveira et al., 2014, Van Baalen and Jansen, 2006). However, it may be important to be able to recognize neighbors and restrain competition if surrounded primarily by relatives. There are many examples of cells being capable of effectively distinguishing between self and non-self and adjusting behavior accordingly in ways that impact growth outcomes (Mehdiabadi et al., 2006, Rendueles et al., 2015, Strassmann et al., 2011). QS is one such system in bacteria, but other contact-dependent recognition systems such as the contact-dependent growth inhibition (CDI) system of *Burkholderia*, type VI secretion systems, and flocculation in yeast have been described (Anderson et al., 2014, LeRoux et al., 2015, Smukalla et al., 2008). This ability to correctly decipher amongst neighbors and the composition of the surrounding community could greatly encourage the success of cooperation, particularly if production of cooperative behaviors is predicated upon sensing members of a cell's own genotype.

It is worth noting that producer cells at low frequency can in some cases preferentially gain the benefit of public goods production compared with non-producers, even in the absence of higher ordered structure (Gore et al., 2009). This creates a snowdrift scenario whereby the rare type has an advantage. This situation is, however, highly dependent on the parameters of the specific cooperative behavior, but it may contribute to maintenance of cooperative tasks.

1.4   Policing

Policing and related forms of punishment are proposed as another mechanism to stabilize cooperation in the face of potential cheaters (Fig. 1C). In this scenario, an aggressive action that negatively impacts fitness targets cheaters relative to cooperators (Travisano and Velicer, 2004). Policing has been commonly observed among many eukaryotic organisms (Clutton-Brock et al., 1995), including insect workers, birds, and social primates, but the prevalence and diversity of molecular mechanisms underlying bacterial policing are not well characterized. Punishment could be enacted by either restraining benefits directed to a non-contributing partner or by direct harm. In the second case, this behavior may be costly to the enacting individual but still stabilize other cooperative behaviors for cooperating kin in the population as long as the punishment and resulting cooperation are positively correlated (Eldakar and Wilson, 2008, Hamilton, 1970).

One example of policing is the enforcement of sanctions, seen in the interaction of the root-nodule forming bacteria *Rhizobia* with its host plant. Symbionts that do not sufficiently contribute fixed nitrogen to their associated host receive a limited flow of oxygen and nutrients in return (Kiers et al., 2003, West et al., 2002). Limiting benefits or imposing costs to less or non-cooperative partners in this manner should favor more cooperative partners in mutualisms (West et al., 2002, Bull and Rice, 1991). In some systems, such as squid-*Vibrio* symbioses, the host is able to filter and selectively favor suitable partners in such a manner, and host-enforced bottlenecks are also likely to play a strong role in maintaining fidelity in the interaction (Koch et al., 2014, Lee and Ruby, 1994). Bottlenecks may more generally act to stabilize cooperative behavior, regardless of host association (Brockhurst, 2007, Chuang et al., 2009, Cremer et al., 2012). In this way, restraining a benefit in the

face of non-reciprocating partners can have the effect of maintaining the interaction. These sanctions need not be restricted to inter-species interactions and could be imagined to occur, for instance, in populations where cells are exchanging metabolites (Estrela et al., 2012).

Recently, QS in *P. aeruginosa* has also been shown to induce policing that targets QS defectors by regulating cyanide production and resistance (Wang et al., 2015). As described above, QS induction of extracellular proteases is necessary for maximum growth in a minimal media environment with casein as the carbon source. In this case, certain classes of QS defectors are unable to produce co-regulated compounds that counteract the effects of cooperator-produced cyanide and are thus unable to completely invade a QS-proficient population cooperating via extracellular enzyme production that could otherwise be exploited by the potential cheats.

In all likelihood, these types of policing mechanisms may be difficult to maintain and unlikely to be common for maintaining bacterial cooperation (West et al., 2007). Because policing behaviors are costly to perform and may target related kin, they may convey a fitness disadvantage under many conditions. However, this effect may be somewhat alleviated if such traits are only expressed conditionally, as shown in the QS-regulated example. For cooperative partners, from either the same or different species, sanctions may arise more naturally. Due to negative effects on the productivity caused by a poor partner, reciprocal sanctioning effects will more naturally emerge, as fewer partners will be present to repay the favor (Oliveira et al., 2014).

1.5   Division of Labor in Bacteria

A penultimate form of cooperation that is a requirement for the development of higher ordered multicellularity is "division of labor". Division of labor can be defined as cooperating individuals that perform discrete tasks that are themselves costly to the individuals, but the sum total of this task distribution is beneficial to the larger community. Division of labor is clearly evident in complex multicellular eukaryotes. A heart cell has differentiated to perform very different tasks than a liver cell. In these organisms, development and terminal differentiation are keys to driving and maintaining phenotypic heterogeneity. Division of labor has also clearly been observed in bacteria. A classic example is spore formation by Myxobacteria (Shimkets, 1990). Upon starvation, this predatory bacterium aggregates into multicellular mounds, which ultimately form structures called fruiting bodies coated with environmentally resistant Myxobacteria spores that rise above the local surface. These structures are thought to aid in dispersal of the spores to new environments.

Division of labor is also proposed to be a common feature of biofilms, although this is a controversial idea that as of yet has little experimental support. Indeed, five specific *Bacillus subtilis* cell types can be observed in a monospecies biofilm (van Gestel et al., 2015). These subtypes localize to distinct regions of the biofilm, but the adaptive function of these cell types is not clear, as a locked matrix-producing cell type is sufficient to produce a robust biofilm in the lab. However, it is likely that a homogenous biofilm would be maladaptive in the natural life cycle of *B. subtilis*, as the laboratory environment is missing key aspects of the natural world. It also seems unlikely that biofilms will remain as genetically homogenous as liquid cultures due to limited dispersal leading to restriction of competition to local scales. Understanding the evolutionary factors that drive the emergence of phenotypic

heterogeneity must rely on a better understanding of the ecology of these bacteria and ideally guide experiments on these organisms in more naturally relevant systems. Moreover, like all forms of cooperation, identifying the strategies and mechanisms that maintain these interactions in the face of defectors will be an intriguing area of research.

The above examples represent division of labor in monospecies systems, but interspecies division of labor can and does occur as well. In complex microbial communities, we predict that division of labor will be most evident in the cooperation of individuals through metabolic exchanges (Tasoff et al., 2015, Werner et al., 2014). Indeed, this has been observed in clinical cystic fibrosis isolates of *Staphylococcus aureus* (Hammer et al., 2014). This phenomenon has been shown to be possible in synthetic as well as natural communities (Oliveira et al., 2014, Morris et al., 2012, 2014, Pfeiffer and Bonhoeffer, 2004). In mixed communities, members that are not directly involved in the exchange may still impact it providing a degree of separation or assortment of individual partners, a process labeled social insulation (Oliveira et al., 2014, Mitri et al., 2011). With the increasing importance of the human and animal microbiome in health and disease, understanding cooperative division of labor interactions in these communities, and potentially with the host, will be an increasingly important area in microbial evolution.

1.6   Conclusion

As we have seen, microbial cooperation occurs in diverse manners, and the mechanisms guiding its maintenance are likewise diverse. These mechanisms all fundamentally act to mediate the fitness costs imposed by expressing cooperative behavior or by altering the way that benefits are administered. Fitness gains may be directed to the acting party (direct),

to related kin (indirect), or both, and thus act to increase the organism's overall inclusive fitness. Also evident throughout this review, these mechanisms may and often do work in concert with one another. As researchers discover novel examples of microbial cooperation, more mechanisms that direct their maintenance will likely come to light. We are particularly optimistic that many mechanisms of cooperation utilizing optional participation guided by communication will be discovered in experimental and natural communities. The surface has only been scratched in this area of research, and it will be exciting to see what new mechanisms are uncovered, how they may potentially be used for industrial and medical applications, as well as how they may inform what we know about biology at both the micro and macro scale.

Chapter 2

Bacterial quorum sensing stabilizes cooperation by optimizing growth strategies

Abstract and Importance

Communication has been suggested as a mechanism to stabilize cooperation. In bacteria, chemical communication termed quorum sensing (QS) has been hypothesized to fulfill this role, and extracellular public goods are often induced by QS at high cell density. Here we show with the bacterium *Vibrio harveyi* that QS provides strong resistance against invasion of a QS-defector strain by maximizing growth rate at low cell densities while achieving maximum productivity through protease upregulation at high cell density. Alternatively, QS mutants that act as defectors or unconditional cooperators maximize either growth rate or yield, respectively, and are thus less fit than the wild type QS strain. Our findings provide experimental evidence that regulation mediated by microbial communication can optimize growth strategies and stabilize cooperative phenotypes by preventing defector invasion, even in well-mixed conditions. This effect is due to a combination of responsiveness to environmental conditions provided by QS, lowering of competitive costs when QS is not induced, and pleiotropic constraints imposed on defectors that do not perform QS.

Cooperation is a fundamental problem for evolutionary biology to explain. Conditional participation through phenotypic plasticity driven by communication is a potential solution to this dilemma. Thus, among bacteria, QS has been proposed to be a proximate stabilizing mechanism for cooperative behaviors. Here, we empirically demonstrate that QS in *V. harveyi* prevents cheating and subsequent invasion by non-producing defectors by maximizing growth rate at low cell density and growth yield at high cell density, whereas an unconditional cooperator is rapidly driven to extinction by defectors. Our findings provide the first experimental evidence that adding QS regulation prevents the invasion of cooperative populations by QS defectors even in unstructured conditions and strongly support the role

of communication in bacteria as a mechanism that stabilizes cooperative traits.

2.1 Introduction

Cooperative behavior is a widespread phenomenon that pervades all levels of biological organization and has helped to catalyze all major transitions in the history of life (Szathmáry and Smith, 1997). Extensive cooperation is also prevalent in microbes and plays fundamental roles in many bacterial processes including biofilm formation, virulence, bioenergy, host-microbe interactions, as well as forming stable communities that maintain essential ecosystem functions (Crespi, 2001, Velicer, 2003, Diard et al., 2013, Percival et al., 2010, Popat et al., 2012, West et al., 2002). One class of microbial cooperative behaviors is the production of public goods (PG): products that provide benefits to both producers and defectors within a community. In the context of PG, individuals that contribute by producing the good are defined as cooperators, while non-producers are defined as defectors. Importantly, defectors are not always inherently cheaters, but are capable of cheating if they reap fitness advantages by exploiting social behaviors (Zhang and Rainey, 2013, Jones et al., 2015, Ghoul et al., 2014). However, conditional participation through phenotypic plasticity driven by communication could provide a potential solution to this dilemma (Kümmerli et al., 2009).

Quorum sensing (QS) is a ubiquitous form of chemical communication in bacteria that allows cells to sense changes in local cell density to globally alter gene expression (Waters and Bassler, 2005, West et al., 2012). Many QS regulons appear to be enriched for secreted PG products (Popat et al., 2015a), and it has been hypothesized that QS could act as a mechanism to stabilize cooperation (Travisano and Velicer, 2004). Additionally, QS has been shown to interact with other mechanisms that function to stabilize cooperative behaviors, such as metabolic constraint, metabolic prudence, or policing (Bruger and Waters, 2015, Dandekar et al., 2012, Xavier et al., 2011, Wang et al., 2015).

However, the postulated role of QS to innately stabilize cooperative traits has not been widely demonstrated. Most empirical studies of laboratory and natural populations suggest that QS systems can be invaded, and in some cases destabilized, including invasion of both *Pseudomonas* and *Vibrio* species, by QS-defectors (Sandoz et al., 2007, Diggle et al., 2007, Katzianer et al., 2015). In fact, the range of conditions under which cooperation is favored may be quite limited (Oliveira et al., 2014). This raises the question of why QS frequently regulates the expression of PG and whether it is in fact advantageous for maintaining cooperative traits.

Here, we addressed this question by examining a PG commonly regulated by QS: extracellular protease production in the bioluminescent marine bacterium *Vibrio harveyi*. This QS system is ideal to study the role of communication in stabilizing cooperation because both defectors and unconditional cooperators (UC) of the QS pathway can be easily generated. By assessing the competitive outcomes of cooperators and defectors in an environment where fitness is dependent on QS-regulated protease production, we demonstrate that a wild type (WT) strain with a functional QS system prevents defector invasion whereas an UC mutant strain is driven to extinction. QS prevents cheating by regulating a switch in growth strategies from maximizing growth rate at low cell density by restraining from protease production to increasing growth yield at high cell density when available resources become more scarce. Whereas the WT strain can use QS to take advantage of these different strategies at the appropriate densities, the defector and UC maximize only rate or yield, respectively, with a resulting tradeoff in the other capacity. Our results suggest that prudent regulation of metabolism by QS is a mechanism by which this system promotes the maintenance of cooperation.

Strain	Genotype	Strain Description
BB120	ATCC BAA-1116	Wild type (WT) strain, functional QS system
KM669	$\Delta luxR$	Knockout mutant of gene encoding QS master regulator LuxR, acts as a defector
JAF78	$\Delta luxOU$	Knockout mutant of genes encoding LuxO and LuxU, acts as an uncondtional cooperator
KM83	$luxO$ D47E	Point mutant, produces LuxO that constitutively mimics phosphorylated state, defector
JMH634	$\Delta luxM$ $\Delta luxS$ $\Delta cqsA$	Triple signal synthase mutant (SN), unable to induce QS but responds to signal
CW2001	$\Delta luxAB$	Deletion of luciferase enzyme components, functional QS but unable to produce light

Table 2.1: **Strains of *V. harveyi* used in this study.**

2.2   Materials and Methods

2.2.1   Bacterial Strains and Media and Growth Conditions

Bacterial strains used in this study include *Vibrio harveyi* strain ATCC BAA-1116 (Bassler et al., 1997), recently reclassified as V. campbellii (Lin et al., 2010), and derivatives (2.1). Bacteria were grown in LB (Accumedia) broth or in M9 salts (Sigma-Aldrich or Becton, Dickinson and Company) supplemented with sodium chloride (Macron Fine Chemicals) to a final concentration of 2% (w/v) and 0.5% (w/v) of sodium caseinate (Sigma-Aldrich), casamino acids, or tryptone (Becton, Dickinson and Company) as sole carbon sources. Bacteria were routinely grown in a 30 °Celsius shaker at 250 rpm in 16mm borosilicate glass tubes containing 3 mL media. Experimental cultures were initially grown in liquid LB cultures and subsequently acclimated by passaging in M9 liquid media. Cells were collected by centrifugation ($10{,}000xg$) and washed in the equivalent growth medium prior to competition experiments. Supernatants were obtained from cultures grown in M9-casein media for 24 hours. Cells were removed by first pelleting at $10{,}000xg$ and then filtered with 0.22 μm filters (Millipore). Supernatants were added back at the percentage of final culture volume indicated.

2.2.2   Competition Fitness Assays

2.2.2.1   Competition Design

Competitors were assessed for the ability to invade when rare by initiating populations with a range of relative frequencies of the defector and relevant cooperator strains for single growth phase competitions. Populations were grown 24 hours, plated on LB agar, and population compositions were assessed. For serial competition assays, populations were initiated by mixing strains at a ratio of 99:1. Populations were grown for 24 hours, plated on LB agar to assess population composition and productivity, and a subset was diluted 1/1000 to new M9 liquid media followed by 24 hours of growth (10 generations). During competitions, bioluminescence phenotypes were determined in the dark using an AlphaImager HP imaging system (ProteinSimple). Ancestral cooperator strains uniformly produce bright bioluminescent colonies, while all defector strains investigated were non-luminescent in appearance.

2.2.2.2   Fitness Calculations

Where determined, defector relative fitness (w) was calculated as the ratio of Malthusian parameters (m) for strains in pairwise competitions, which also equates to the ratio of competitor doublings over the experimental time interval of 24 hours (Lenski et al., 1991). The Malthusian parameter is calculated as

$$m_i = ln(x_1/x_0)$$

for competitor i, where x_0 and x_1 are the densities of that competitor at the start (0) or end (1) of the experimental period, and

$$w = m_1/m_2,$$

when comparing competitors *1* and *2*.

2.2.3   Protease Assays

Extracellular protease activity was assessed using a protease fluorescence assay (Sigma-Aldrich) with filtered (0.45 μm pore filters, Ambion) supernatants from cultures grown in M9-casein media. Conjugated casein molecules release the fluorophore fluorescein isothiocyanate (FITC) when cleaved, allowing sensitive assessment of protease activity. Fluorescence was measured in an EnVision Multilabel Plate Reader (PerkinElmer) with the excitation wavelength at 485 nm and the emission wavelength at 535 nm. Measurements were normalized to the dilution used for supernatant samples and cell density of the culture, determined by plate counting.

2.2.4   Statistical Analyses

Statistical analyses were conducted using R 3.2.2. Growth of different strains in varying M9 media sources was compared using ANOVA [Population Density Strain Type (cooperator or defector)*Carbon Source] with Tukey's Honestly Significant Difference (HSD) post-hoc test to account for multiple comparisons (Fig. 2.1). Protease production was compared across 3 strains at 24 hours of growth in M9-casein media using ANOVA [Per-capita protease activity Cell Density+Strain Type] (Fig. 2.2, top panel). The effect of supplementing

different supernatants on $\Delta luxR$ growth in M9-casein media was compared using ANOVA [Growth(measured by OD600) Supernatant source*Supernatant concentration] with Tukey's HSD post-hoc test. A linear model was constructed to analyze effects of relative fitness vs. frequency estimates [Competitor relative fitness Competition pairing*Starting competitor frequency] (Fig. 2.4, top panel). In experiments examining the dynamics of growth (Figs. 4-6), data points are bounded with 95% confidence intervals for error bars to allow statistical comparison.

2.3   Results

2.3.1   QS is required for maximum growth of *Vibrio harveyi* utilizing casein

To evaluate the stability of QS and the cooperative behaviors it regulates against defector invasion in *V. harveyi*, we first established conditions in which fitness was dependent upon QS activation of PG production. We found that growth in M9-casein media was highly dependent on the ability to induce QS at high cell density. In this environment, casein breakdown by extracellular protease production is required to reach a maximum growth yield (Natrah et al., 2011, Wilder et al., 2011, Popat et al., 2015a). In *V. harveyi*, the accumulation of autoinducer increases expression of the master transcription factor LuxR, switching gene expression from the low- to high-cell-density state. We observed that only cooperator strains, including the WT conditional cooperator, which induces *luxR* in response to autoinducer as cell density increases, and the UC $\Delta luxOU$ mutant, which constitutively expresses *luxR* and subsequently the high-cell-density QS regulon, reach maximum densities

in this media after 24 hours of growth (Fig. 2.1). Meanwhile, defectors, including the $\Delta luxR$ strain (cannot produce the LuxR master regulator), grew to a significantly lower density of around 4% of the WT in M9-casein media (Fig. 2.1, p<0.001). This outcome was observed for all defector strains tested that cannot induce *luxR* expression including the *luxO* D47E mutant and an autoinducer 'signal negative' production mutant ('SN', Fig. 2.1). The production of bioluminescence did not impact growth in this condition as the $\Delta luxAB$ dark mutant grew identically to the WT strain. In contrast, using predigested carbon sources such as casamino acids or tryptone significantly diminishes the differences in growth between cooperator and defector strains (Fig. 2.1).

2.3.2 Functional and mutant QS strains differ in density-dependent protease activity

To confirm that these growth results were due to extracellular protease activity, we quantified and monitored activity during growth of cultures started at low cell density (Fig. 2.2, top panel). As expected, the $\Delta luxOU$ strain demonstrated high protease activity at all cell densities as this strain is locked in the high-cell-density QS state. The WT strain exhibited QS regulation of protease production, where cells initially exhibited high protease activity immediately upon back-dilution from high cell density. However, protease activity decreased until a quorum was reached, but it ultimately matched the protease activity observed in the $\Delta luxOU$ strain at high cell density (Fig. 2.2, top panel). The $\Delta luxR$ strain exhibited low or undetectable levels of protease activity at all densities examined. Note that the $\Delta luxR$ mutant grows poorly in this medium and does not reach the cell density of the cooperator strains (Fig. 2.1). Our data showing that WT and $\Delta luxOU$ strains of *V. harveyi* have higher
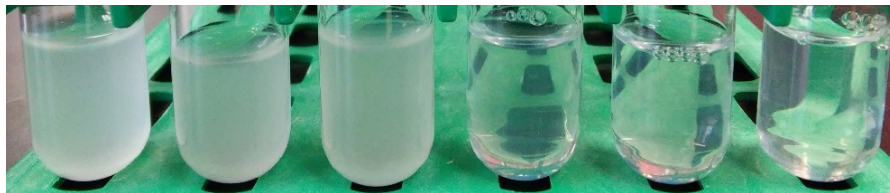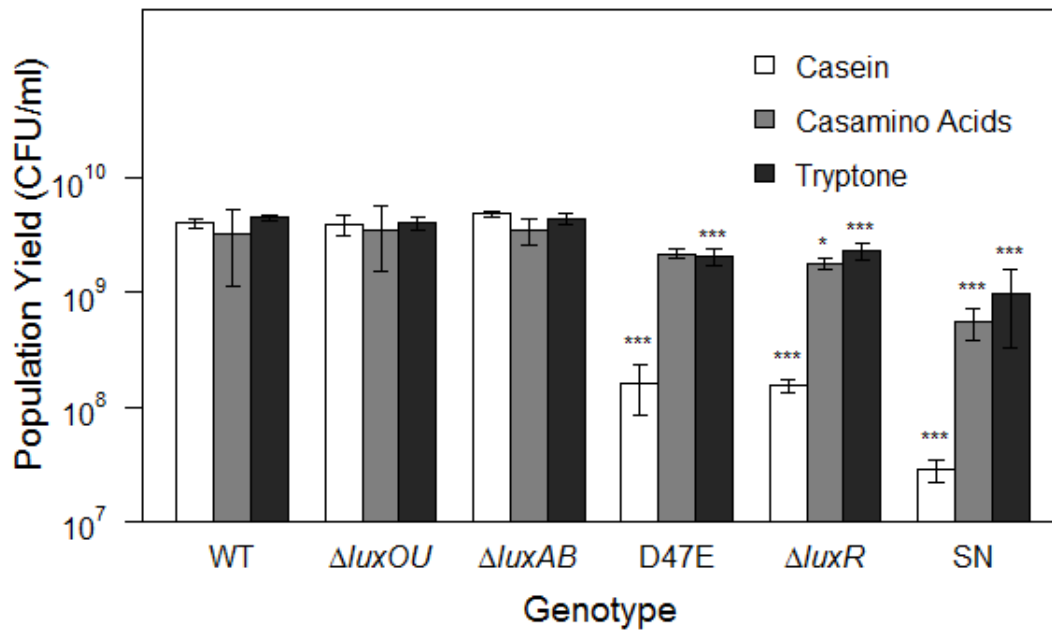
Figure 2.1: ***V. harveyi* requires QS for maximal growth in M9-casein media.** Six strains of *V. harveyi* grown in M9 media with varying sole carbon sources were grown 24 hours and enumerated through viable cell counts (CFU/ml). Top. Bars display the productivity of a given genotype at the culmination of a single growth cycle (24 hours) of four separate biological replicates, and error bars denote the 95% confidence intervals for the mean estimates. Bottom. Images of cooperator and defector genotypes in M9-casein media after 24 hours of growth. Pictures are aligned directly beneath their corresponding label in the top panel. 'WT' = Wild Type strain, 'SN' = Signal Negative strain, 'D47E = *luxO* D47E strain. Differences that were statistically significant from WT in the given media type are reported. * p < 0.05, *** p < 0.001.

levels of protease activity than QS-defector strains agree with earlier work in this and other QS systems (Natrah et al., 2011, Wilder et al., 2011, Popat et al., 2015a).

The poor growth of QS-defector $\Delta luxR$ strain on casein is not due to an inability to use peptides liberated from casein as the growth of $\Delta luxR$ in this environment can be rescued by the addition of cooperator supernatants from the WT or $\Delta luxOU$ strains, but growth is not improved by its own supernatant (Fig. 2.2, bottom panel, p<0.001). It is also worth noting that supernatants from all examined defector strains induced bioluminescence expression of an autoinducer mutant of *V. harveyi* nearly 100-1,000-fold less than cooperator supernatants, a level lower than predicted by growth differences in M9-casein alone, suggesting that these defectors produced low levels of autoinducer and would be unlikely to cheat by inducing cooperators to produce PG (Fig. 2.3).

2.3.3   A functional QS circuit prevents defector invasion

Although higher levels of protease production by the WT and $\Delta luxOU$ strains lead to increased growth in M9-casein media, this behavior is potentially susceptible to cheating by defectors that could benefit from the nutrients liberated by these extracellular proteases. To test whether cooperators that produce higher levels of protease could be cheated, we competed WT and $\Delta luxOU$ strains against the $\Delta luxR$ defector. The competition experiments were performed in M9-casein media, and frequencies of the competing strains were varied. The populations were started at densities significantly lower than the quorum. The competitions consisted of one growth period, and the QS phenotype of isolated colonies was determined by colony bioluminescence phenotypes to quantify defectors and cooperators.

A striking difference in defector invasion was observed depending on which cooperator strain was used. While $\Delta luxR$ increased in frequency against $\Delta luxOU$ at all starting fre-
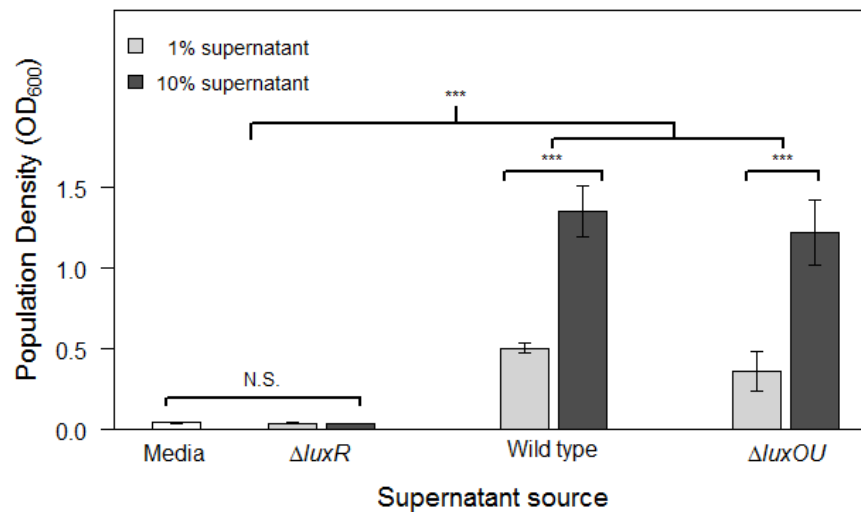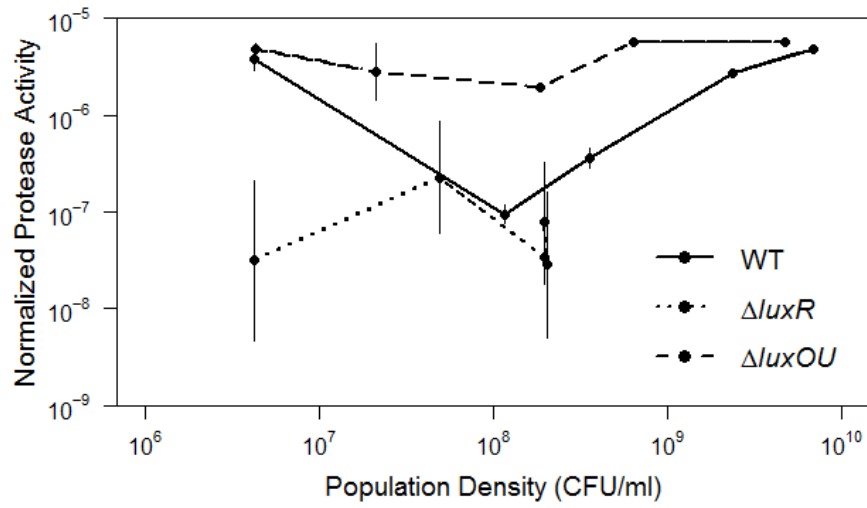
Figure 2.2: **Extracellular Protease activity is regulated by QS in *V. harveyi*.** Top panel. Protease levels between cooperator and defector genotypes grown in M9-casein media are plotted versus the growth as measured by CFU/mL (n = 3). Error bars reflect standard error of the mean. Bottom panel. $\Delta luxR$ cultures were supplemented with supernatants at the indicated concentration from the strains indicated on the x-axis (n = 4). Error bars reflect 95% confidence intervals. *** P < 0.001. N.S.: not significant.
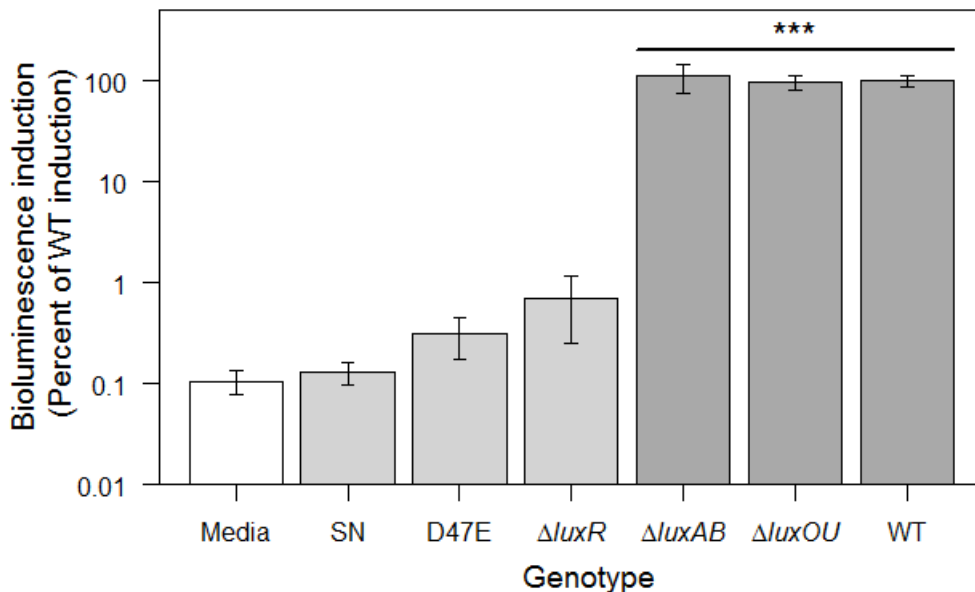
Figure 2.3: ***V. harveyi* requires QS for maximal signal production in M9-casein.** Supernatants from six strains of *V. harveyi* grown in M9-casein media indicated on the x-axis were supplemented to growing cultures of the signal negative (SN) strain (n=9). Bars display the bioluminescence induced by the supernatant of a given strain, normalized to culture growth density measured by OD600, as a percentage of the induction provided by wild type supernatants. Colors differentiate strains considered to be cooperators (dark gray) and defectors (light gray). 'Media' treatment denotes no supernatant added to the culture. Error bars denote the 95% confidence intervals for the mean estimates. 'WT' = Wild Type strain, 'SN' = Signal Negative strain, 'D47E' = *luxO* D47E strain. Differences that were statistically significant from the Media treatment are reported. *** $p < 0.001$.

quencies tested (Fig. 2.4, top panel, red line), it was unable to invade the WT strain at any examined frequency (Fig. 2.4, top panel, black line, Fig. 2.6, top panel). As a result, the WT strain had equivalent fitness to the defector strain across all starting defector frequencies tested, whereas the $\Delta luxOU$ strain had a much lower relative fitness than the defector. We similarly competed the WT strain against $\Delta luxOU$ at multiple frequencies. Like the $\Delta luxR$ defector, WT was able to invade the $\Delta luxOU$ strain leading to higher fitness at all frequencies examined (Fig. 2.4, top panel). Although WT-mixed populations were not invaded by

$\Delta luxR$ at any frequency tested (i.e. the relative fitness of the strains did not vary), these populations exhibited nearly identical frequency-dependence decrease in population yield as the $\Delta luxOU$-mixed populations (Fig. 2.6, bottom panel), indicating that absolute fitness decreased with increasing defector frequency. Furthermore, this phenomenon persists over a wide range of dilutions (and thus a wide range of starting densities) where WT performs well against $\Delta luxR$, while $\Delta luxOU$ performs increasingly worse as the starting dilution (and thus the number of generations that occur before reaching the environment's carrying capacity) increases (Fig. 2.5).

Based on these data, we predicted that $\Delta luxR$ should drive $\Delta luxOU$ to extinction over longer time scales, but it would be unable to invade the WT strain. We tested this prediction in extended pairwise competition experiments in the M9-casein environment between either cooperative genotype (WT or $\Delta luxOU$) against the defector ($\Delta luxR$), performed with one competitor seeded at a low starting frequency of 1% to evaluate its ability to invade. The competitions were extended over several growth cycles with ongoing daily dilution. From either starting condition, defectors rapidly swept and dominated $\Delta luxOU$ in mixed populations (Fig. 2.7, top panel). This resulted in an underutilization of the available nutrient resources and a consequent "tragedy of the commons" where the productivity of the population drops to levels of the defector strain grown in isolation (Fig. 2.7, bottom panel, Hardin, 1968, Rankin et al., 2007, MacLean, 2008). The lack of stable coexistence in either frequency condition tested (defector rare vs. defector common) suggests that these competing strategies ($\Delta luxOU$ or $\Delta luxR$) are unlikely to coexist in this environment.

When the WT cooperator was competed against the $\Delta luxR$ defector in casein-limited M9 media, neither strain significantly invaded the other, as both genotypes maintained their starting frequencies (Fig. 2.7, top panel). Unlike competitions against the $\Delta luxOU$ UC, the
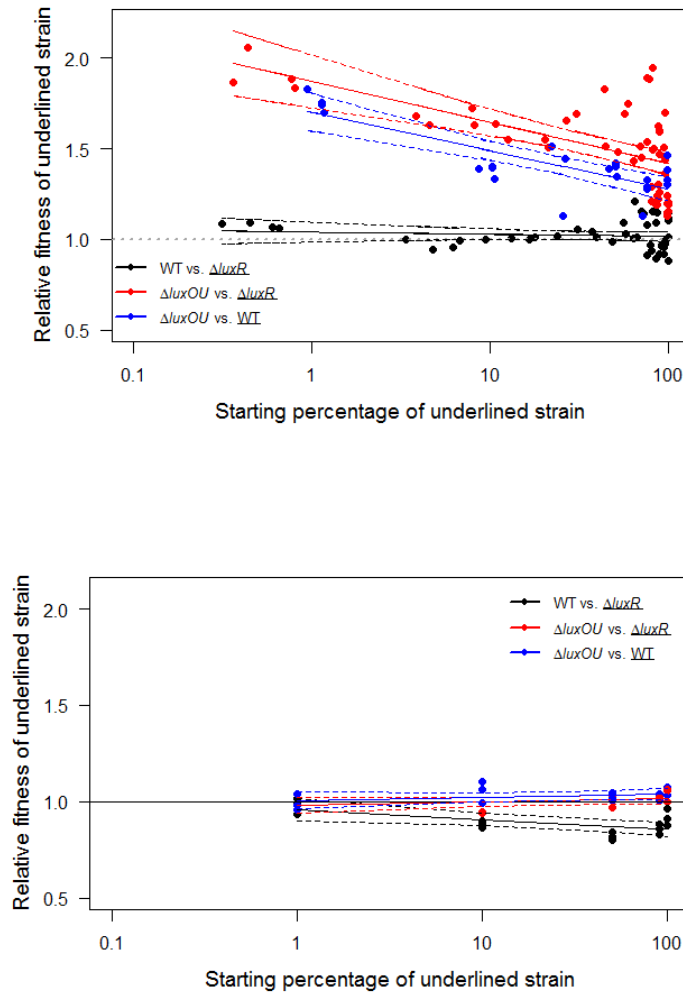
Figure 2.4: **Fitness outcomes from single growth cycle competitions between different *V. harveyi* genotypes vary greatly in M9-casein but not in M9-tryptone** Top. Fitness outcomes in M9-casein. In pairwise competitions between different *V. harveyi* strains, the range of starting competitor frequency was altered and competition experiments were completed in M9-casein media for 24 hours. Fit lines consist of regression fit (solid lines) bounded by 95% confidence intervals (dashed lines) for each competition pairing according to a linear model. For each pairing, the strain whose relative fitness is reported on the y-axis and starting percentage on the x-axis is underlined. Bottom. Fitness outcomes in M9-tryptone. In pairwise competitions between different *V. harveyi* strains, the range of starting competitor frequency was altered and competition experiments were completed in M9-tryptone media for 24 hours. Regression fit lines for the different competition pairs are displayed for each competition pairing according to a linear model. For each pairing, the strain whose relative fitness is reported is underlined.
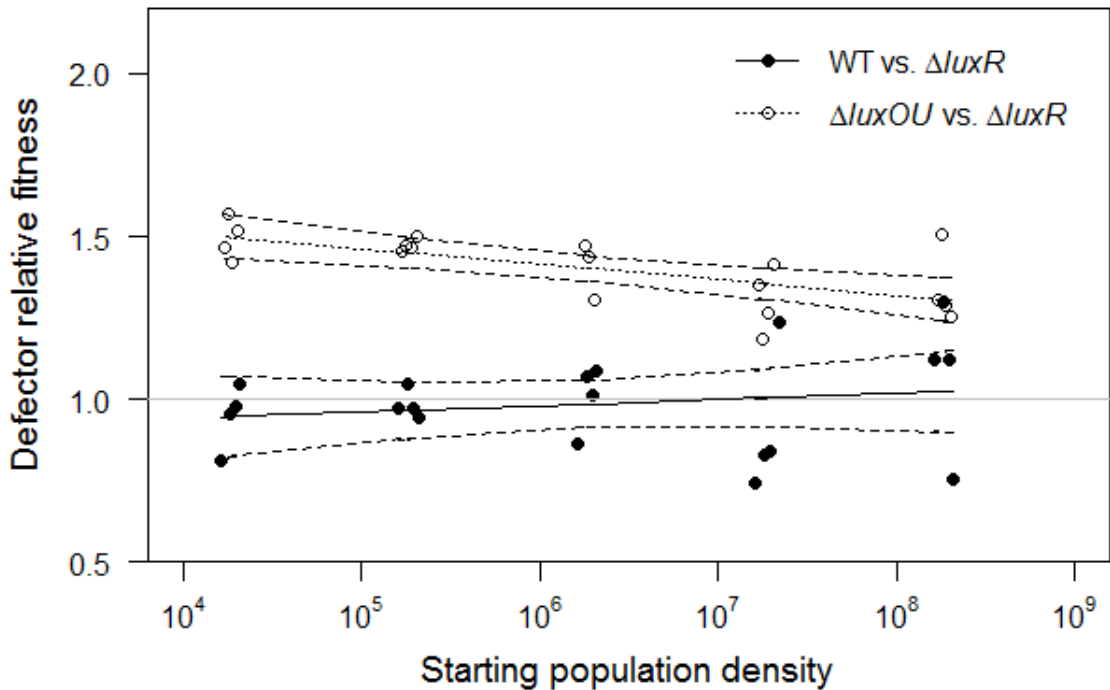
Figure 2.5: **V. harveyi QS provides equivalent fitness against defectors in M9-casein over a range of density conditions.** Growth results from single cycle 24 hour competition experiments in M9-casein of populations initiated at approximately 99% cooperators and 1% defectors at a wide range of densities are reported as the relative fitness of $\Delta luxR$ in relation to the given cooperator strain. For a series of dilutions from initial competition mixes used to achieve a range of densities, for each dilution in the series, N=4 biological replicates per density level. Fit lines consist of regression fit (center lines) bounded by 95% confidence intervals (dashed lines) for each competition pairing according to a linear model.

resulting yield of the WT-mixed populations mirrored the input frequencies and was stable over this time period (Fig. 2.7, bottom panel). In fact, the resulting cell density arrived at in WT versus defector competitions seems to rely strongly upon the initial WT frequency, and a high starting frequency of WT cooperators was able to prevent a tragedy of the commons over this time scale.
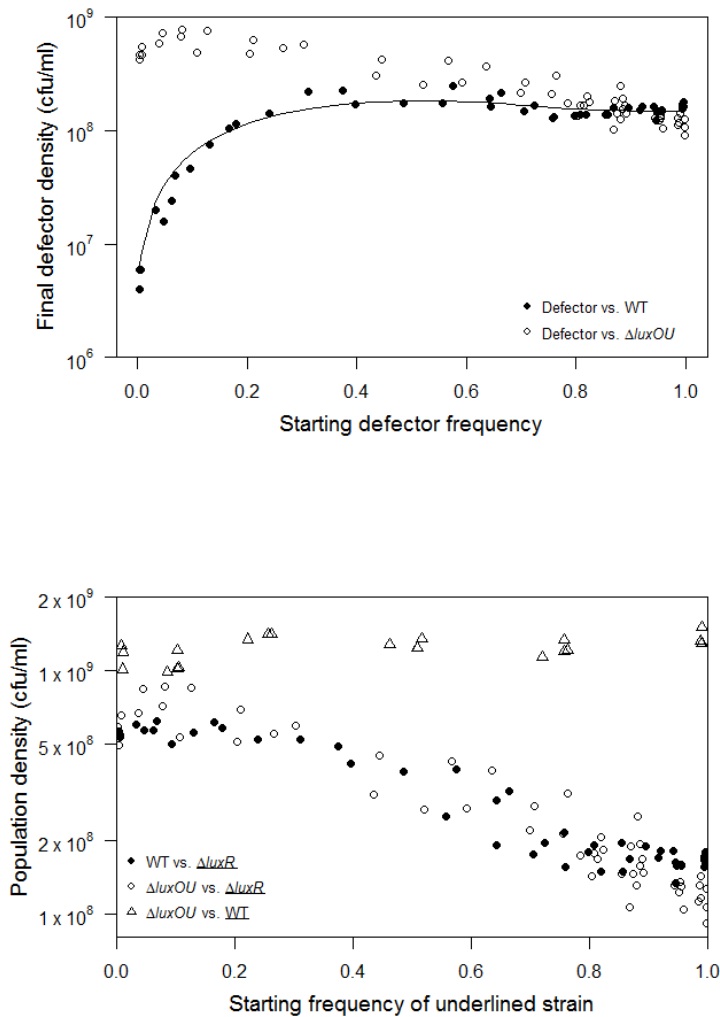
Figure 2.6: **Growth performance of defectors and mixed populations in competition in M9-casein.** Top. Performance of $\Delta luxR$ in mixed populations in M9-casein depends heavily on the competing cooperator strain. Density of the defector strain ($\Delta luxR$) at the completion of a single 24 hour cycle of competition is plotted as a function of the starting defector frequency in the population. Open circles: $\Delta luxOU$ vs. $\Delta luxR$, Filled circles: WT vs. $\Delta luxR$. The black line depicts a smoothed regression fit for the expected defector density if there was no increase in defector frequency, based on the final total population densities. Bottom. Growth performance of mixed populations in competition in M9-casein depends on defector frequencies. Growth results from single cycle 24 hour competition experiments in M9-casein media are reported as total final population densities (both competing strains) in CFU/ml as a function of the starting frequency of the strain underlined in the figure key. Data in both panels are from the same experiment shown in Figure 2.4, top panel.
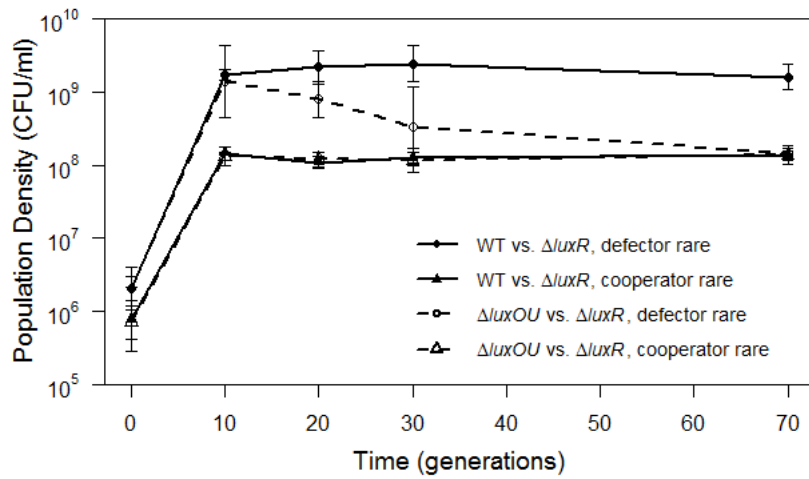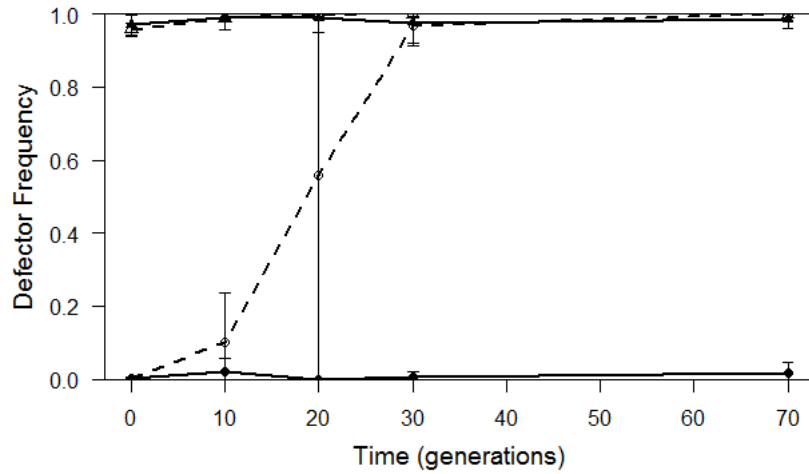
Figure 2.7: **The $\Delta luxR$ defector invades $\Delta luxOU$ but not WT.** Populations contained one of the two cooperator strains, WT or $\Delta luxOU$, and the $\Delta luxR$ defector at a 99:1 mixture or vice versa. Cultures were grown for 24 hours in M9-casein media, diluted 1000-fold and repeated over multiple growth cycles. The legend in B refers to both graphs. Top panel. Defector frequency as determined by non-luminescent colonies is plotted versus generations of growth. Bottom panel. Population productivity measured by plate counts (CFU/ml) is plotted versus generations of growth. Error bars reflect 95% confidence intervals for 3 biological replicates per treatment.
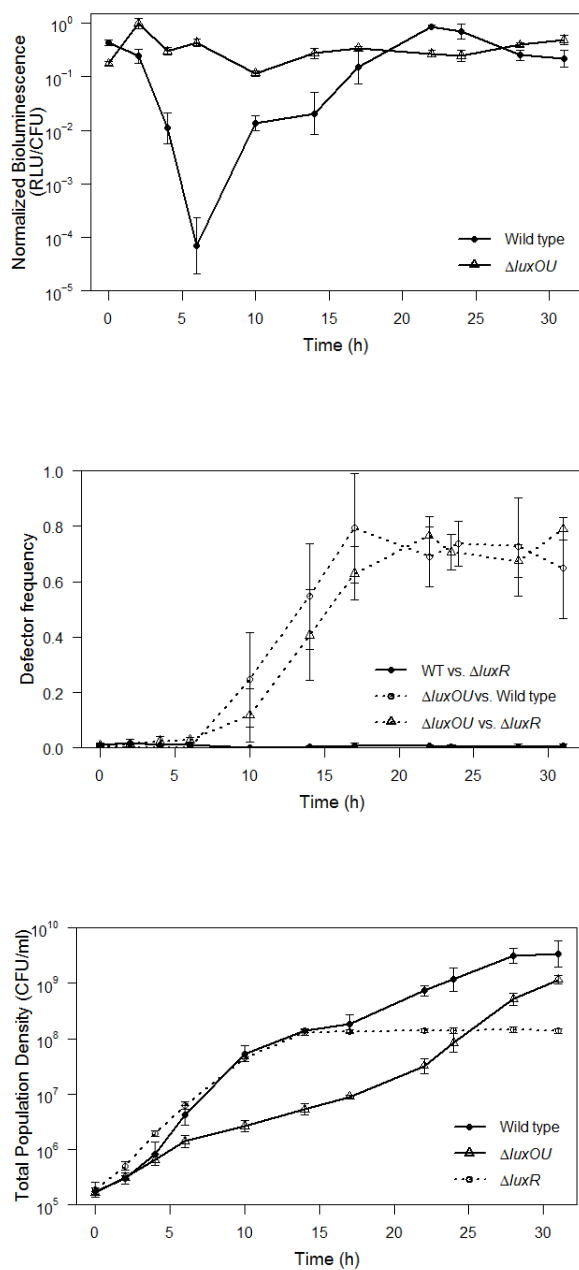
Figure 2.8: **The WT strain optimizes growth strategies at low- and high-cell density.** Top. Bioluminescence induction of the WT and $\Delta luxOU$ in monoculture over the course of a growth cycle in M9-casein media. Middle. Defector frequency over a single growth cycle for $\Delta luxOU$ vs. $\Delta luxR$, $\Delta luxOU$ vs. WT, and WT vs. $\Delta luxR$ with the defector strains underlined started at 1% and competed in M9-casein media. Bottom. Growth of the WT, $\Delta luxR$, and $\Delta luxOU$ in monoculture over the course of a single growth cycle. Error bars represent 95% confidence intervals on four biological replicates per treatment.

2.3.4   A functional QS system prevents defector invasion by modulating growth strategies at different cell densities

As QS is a mechanism that can alter gene expression in response to changes in cell density, we sought to determine the dynamics of QS-induction and the kinetics of defector invasion in the M9-casein environment. To determine the QS induction state at different densities in the M9-casein environment, we monitored per-capita bioluminescence as a proxy for activation of QS. As expected, the WT strain exhibits a strong drop in bioluminescence at low cell density, but beyond 6 hours of growth a quorum is reached and bioluminescence is ultimately induced to maximal levels matching or even exceeding $\Delta luxOU$ (Fig. 2.8, top panel). Alternatively, $\Delta luxOU$ maintains a near constant level of bioluminescence, indicating it is activating QS in an unconditional fashion (Fig. 2.8, top panel). The regulation of bioluminescence in these strains mirrors that seen for protease activity, as would be expected for two phenotypes similarly induced in the high-cell-density state (Fig. 2.2, top panel).

To determine at what point during growth defectors invade cooperators, we measured the frequency of defectors started at 1% of three pairwise competitions ($\Delta luxOU$/WT, WT/$\Delta luxR$, and $\Delta luxOU$/$\Delta luxR$, the defector strains are underlined) over the course of one growth cycle. We observed that significant invasion of $\Delta luxOU$ by both WT and $\Delta luxR$ began to occur as the population reached quorum, at which point these strains rapidly increased to high frequencies (Fig. 2.8, middle panel). At higher cell densities, the invasion of both of these strains plateaued and $\Delta luxOU$ maintained a minor frequency in the population. This indicates that invasion primarily occurred at the transition from low to high cell densities. As seen in earlier results, the $\Delta luxR$ defector could not invade WT at any densities examined (Fig. 2.8, middle panel, Fig. 2.9).
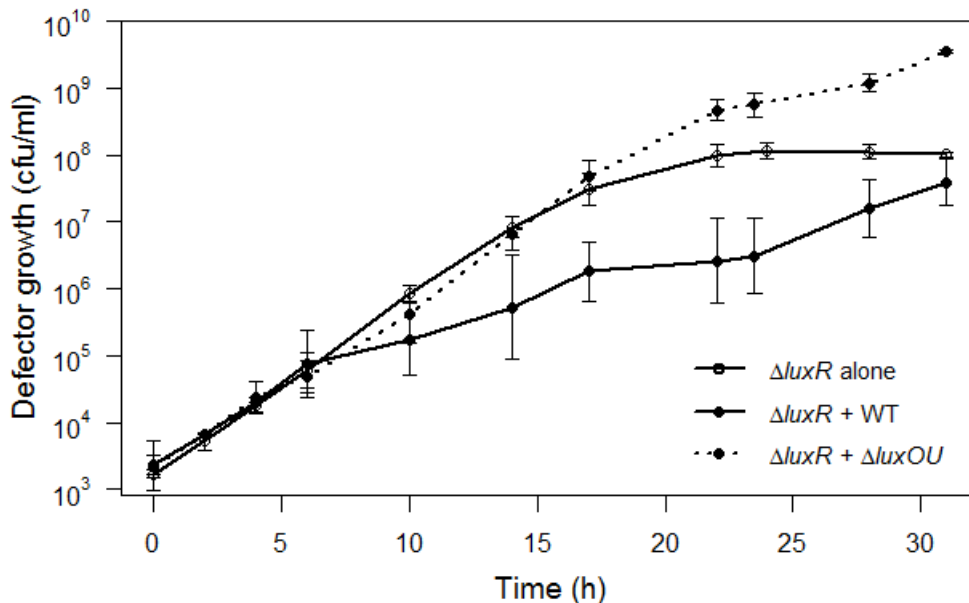
Figure 2.9: **Growth performance of $\Delta luxR$ defector in the presence and absence of competing strains.** Growth results from monoculture and competition experiments in M9-casein media over the course of a single growth cycle. For competition mixed populations, data are from the same experiment as Figure 2.8, and the numbers reported are the $\Delta luxR$ portion of those populations. For monoculture results, matching densities of $\Delta luxR$ alone were initiated and tracked in M9-casein media.

To further understand these dynamics, we measured the growth of WT, $\Delta luxR$, and $\Delta luxOU$ over the course of one growth cycle in monoculture. Both the WT and $\Delta luxR$ strains exhibited equivalent rapid growth at low cell density compared with the unconditional $\Delta luxOU$ mutant (Fig. 2.8, bottom panel). This difference was especially evident at the 5-hour time point, matching the time period during which $\Delta luxOU$ is invaded and an increase in defector frequency becomes readily apparent in competitions (Fig. 2.8, middle panel). As the WT strain grows to higher cell densities, it transitions to a new growth strategy, allowing growth to resume and cell numbers to increase until the carrying capacity is reached. This portion of growth more closely resembles the $\Delta luxOU$ mutant, which experiences an increase

in growth rate at high cell density and ultimately reaches the same yield as the WT strain (Fig. 2.1). This, in combination with the observation that $\Delta luxR$ plateaus in growth before reaching high cell density, suggests that this phase of growth is largely dependent upon QS-regulated PG. Because the $\Delta luxR$ mutant is unable to alter its growth strategy like the WT to produce PG necessary for additional growth, it obtains a significantly lower population yield than either cooperator strain.

The slow growth rate of the $\Delta luxOU$ at low cell density is specific to this environment as $\Delta luxOU$ exhibits equivalent fitness to the WT and $\Delta luxR$ mutant when grown in M9 minimal medium supplemented with tryptone (Fig. 2.4, bottom panel). Furthermore, the higher fitness of $\Delta luxR$ compared to $\Delta luxOU$ is not simply due to its higher individual growth rate, but rather is social in nature as $\Delta luxR$ grows to higher densities when competed with $\Delta luxOU$ than it can by itself in monoculture, demonstrating cheating is occurring in these conditions (Ross-Gillespie et al., 2009; Fig. 2.6, top panel, Fig. 2.9). Conversely, $\Delta luxR$ does slightly worse in mixed populations with WT than in monoculture in both M9-casein and M9-tryptone environments (Fig. 2.9, Fig. 2.4, bottom panel).

2.4   Discussion

QS has been proposed as a mechanism to stabilize cooperation, but only modest experimental evidence exists to support this proposition (Popat et al., 2012, Bruger and Waters, 2015, Allen et al., 2016, Pai and You, 2009, Heilmann et al., 2015, Hense and Schuster, 2015). Because non-producing defectors do not pay the cost to produce PG but can still reap the resulting benefits, a fundamental question is how QS systems, and the cooperative behaviors they regulate, are stably maintained in bacterial populations. Most evidence addressing this

question is based on theory and simulations with little experimental population dynamic data supporting it.

Although it has recently been shown that QS can serve to modulate investment in cooperative goods, this study demonstrates that QS allows cooperators to match the fitness of defectors even in well-mixed environments in the absence of any apparent spatial structure or policing by cooperators (Allen et al., 2016). More specifically, while previous studies have suggested the value of functional QS in comparison to a modeled or simulated constitutive producer (Allen et al., 2016, Schluter et al., 2016), this is the first study to explicitly and definitively show how vast the difference is between QS-producers and UC producers by experimental analysis. Moreover, comparing QS *P. aeruginosa* versus a simulated constitutive cooperator, Allen et. al. concluded that there are regimes where all forms of cooperation are disfavored (Allen et al., 2016). However, our experimental results demonstrate this is not the case in *V. harveyi*. The generality of this effect is unknown, and any differences are likely to depend on specifics of the system, such as signal redundancy and stability, within-population heterogeneity, or QS architecture (Cornforth et al., 2014, Anetzberger et al., 2009, Ng and Bassler, 2009, Even-Tov et al., 2016). In this case, conditional PG production provided by QS would act as a proximate mechanism to stabilize cooperation, allowing more ultimate stabilizing mechanisms of cooperation such as kin selection to act (Scott-Phillips et al., 2011). Indeed, QS has been suggested to identify the proportion of kin in mixed-species communities, and our finding that defector strains produce less signal corroborate the ability to identify the number of cooperators in mixed single-species populations (Schluter et al., 2016, Fig. 2.3).

We first established M9-casein media as an environment where fitness outcomes were dependent upon the ability of *V. harveyi* to activate the cell's QS system and produce

regulated PG (Fig. 2.1). Interestingly, though contrary to our expectations, while $\Delta luxR$ consistently and extensively exploited populations of the UC $\Delta luxOU$ mutant, it was unable to do the same against WT (Fig. 2.4 and Fig. 2.7). These results bear ecological significance as non-luminescent, $luxR$ defectors, and unconditional $luxO$ mutants have all been identified from natural $Vibrio$ populations (O'Grady and Wimpee, 2008, Wang et al., 2011, Nealson and Hastings, 1979). Importantly, this occurred in well-mixed conditions in which no biofilms or cell aggregates were observed and neither assortment of competitors nor privatization of goods would be likely. The equivalent fitness of the WT strain compared to the defector at all frequencies examined demonstrates that functional QS can promote the maintenance of cooperative behaviors, providing a solution to the PG dilemma when in mixed communities (Brown, 1999).

An important consideration of our results is the role of pleiotropy as the QS regulon of $V.\ harveyi$ is extensive (Nealson et al., 1970, Mok et al., 2003, van Kessel et al., 2013). Pleiotropy has been shown as a potential mechanism to inhibit cheating due to costs imposed against defectors (Dandekar et al., 2012, Foster et al., 2004, Asfahl et al., 2015, Mitri and Foster, 2016, Jiricny et al., 2010). $V.\ harveyi$ defectors do pay such costs, as all defector strains tested exhibited growth yield defects that spanned multiple environmental conditions, including those where no proteolytic breakdown of nutrients was required (Fig. 2.1, p<0.001, Fig. 2.4, bottom panel, black line). This is a likely result of the QS system's regulation of a large portion of the cell's genome and interconnectedness with the metabolism of the cell to appropriately tune growth across a wide range of environments (Dandekar et al., 2012, Pai and You, 2009, Mellbye and Schuster, 2014). Competition results between WT and $\Delta luxR$ in M9-tryptone and at lower densities in M9-casein suggest that WT may indeed be a better nutrient competitor (Fig. 2.9, Fig. 2.4, bottom panel). The effect may be explained by the

regulation of *V. harveyi* QS of multiple genes related to transport (van Kessel et al., 2013). And while WT appeared to be neutrally competitive against Δ*luxR* in the shaken conditions tested and was unable to invade when rare (Fig. 2.7, top panel), it might be predicted that adding a variable such as structure that allows assortment between competing genotypes could foster an advantage for WT (Killingback et al., 1999).

Pleiotropy is likely to play a role in the diminished growth rate of the Δ*luxOU* strain as well. Protease production is not the only trait upregulated by QS, and expression of other public and private goods are also likely to impact growth performance. The WT strain may not only be appropriately expressing proteases, but other goods, both public and private. Indeed, regulation of cooperation by QS may even be an exaptation with the original selection on regulated private goods, but regardless it is significant for the persistence of these now-regulated cooperative behaviors. However, the Δ*luxOU* mutant is not maladaptive in all selective conditions, as this mutant excels in monoculture or when high nutrient levels are freely available.

The WT strain was also able to exploit and invade the unconditional Δ*luxOU* cooperator (Fig. 2.4, bottom panel, and Fig. 2.8, middle panel). This demonstrates that such unconditional cooperation can also be exploited by more restrained forms of cooperation. Indeed, the WT strain resembles the defector strategy at low densities. Competitive exclusion of Δ*luxOU* by both Δ*luxR* and WT exhibits negative frequency-dependence, demonstrating some diminishing returns as less Δ*luxOU* is present. This is likely due to reduced opportunities for cheating, although this dependence does not elicit coexistence (Fig. 2.4, top panel; Foster, 2004, Ross-Gillespie et al., 2007). Instead, at no point does this cheating cease to be favored, characteristic of a prisoner's dilemma (Turner and Chao, 1999, Greig and Travisano, 2004). Similar experiments with *Pseudomonas aeruginosa* observed an intermediate

frequency at which the cooperators and defectors had equivalent fitness (Diggle et al., 2007), illustrating these QS systems are not functioning equivalently.

Alternatively, the WT strain appears to perform in a frequency-independent manner when competed with the $\Delta luxR$ defector, suggesting the defector's performance here is not a result of cheating on PG (Fig. 2.4, top panel, Fig. 2.9). This is also supported by the inability of $\Delta luxR$ to grow beyond monoculture yields in the presence of WT (Fig. 2.6, top panel, Fig. 2.9). All signs of frequency-dependence were removed when these same strains were competed in M9-tryptone media where casein has been enzymatically digested (Fig. 2.4, bottom panel). In these conditions, $\Delta luxOU$ exhibited equivalent fitness to the strains that invaded it in M9-casein media. Therefore, our results are not simply due to $\Delta luxOU$ exhibiting poor fitness in all environments examined.

The competition results obtained from the *V. harveyi* QS system are different from those previously described in other systems, especially with regard to its apparent ability to withstand invasion by a defector strain that is unable to induce the high-density QS state. For example, a *lasR* mutant (another QS-defector) of *P. aeruginosa* was able to successfully invade a WT QS strain under conditions that required proteolysis (Dandekar et al., 2012, Sandoz et al., 2007). Invasion of WT by defectors has even been reported for *Vibrio cholerae*, as a deletion strain of *hapR*, a homolog to the *V. harveyi luxR*, is able to invade and supplant the WT strain in an M9-casein environment (Katzianer et al., 2015). Whereas in these other systems the WT strains are readily invaded by the corresponding defector, and in some cases swept to extinction, the *V. harveyi* WT strain is able to withstand analogous invasion (Fig. 2.4, top panel, and Fig. 2.7). The results of this study demonstrate that QS systems and environmental conditions exist where QS bacteria can resist the invasion of QS-defector mutants. Thus QS-regulated cooperation is here shown to be a more efficient,

robust alternative to unconditional cooperation, allowing populations to resist invasion by within-species defectors (Heilmann et al., 2015, Hense and Schuster, 2015).

Examining growth dynamics revealed that a functional QS system allowed the WT strain to conditionally switch between low investment in QS-regulated goods when nutrients are readily available, and allow cooperators to only invest in the production of PG such as proteases when it is prudent due to larger numbers of cooperator cells. This has the effect of maximizing growth rate at low-cell-density while also achieving maximum growth yields by producing protease PG at high-cell-density, presumably when surrounded by a large number of kin. This relatively high fitness afforded in both states preserves cooperative behaviors associated with QS. Such phenotypic plasticity has been similarly shown to provide fitness benefits for other cooperative goods (Kümmerli et al., 2009). Although the $\Delta luxR$ defector is able to rapidly invade the UC in pairwise competitions because it is able to achieve the higher growth rate strategy, it is unable to maximize yield in monocultures like a cooperator. Alternatively, the $\Delta luxOU$ mutant maximizes growth yield at the expense of a lower overall growth rate, particularly at low cell density, allowing it to perform well in monoculture but rendering it susceptible to defector invasion. This contrasting growth strategy has relevance to previous investigations into tradeoffs between growth rate and yield, and the plasticity conferred by QS may allow cells to evade this tradeoff by matching growth strategies appropriately to the prevailing environment conditions (MacLean and Gudelj, 2006, Pfeiffer et al., 2001, Novak et al., 2006).

The delay observed in activating cooperation by QS-regulation in the WT strain is consistent with proposed forms of restraint such as metabolic prudence and generalized reciprocity, in which investment in cooperative traits expression is tuned to be more economical for the producing organism (Xavier et al., 2011, Allen et al., 2016, Darch et al., 2012). In our case,

cost is lowered for WT during early growth when signal density is too low to induce QS. In this state, production of the PG is not efficient as the concentration of protease will be too low to yield a net benefit (Heilmann et al., 2015). This situation could naturally occur when a pure culture of *V. harveyi* is at low cell density or if *V. harveyi* is in a mixed community surrounded by non-kin or defectors, when investment in competitive strategies is more beneficial than high levels of cooperation. However, we also note that invasion of $\Delta luxR$ into the unconditional $\Delta luxOU$ ceases at higher densities (Fig. 2.8, top panel). Following the reasoning of metabolic prudence (Xavier et al., 2011), it is possible that under these density conditions it is less costly to express and produce proteases. It is also possible that protease enzymes may persist in the environment and thus measuring their levels reflects past as well as current production, causing an overestimation of real costs to cooperator strains at high cell density.

In summary, we have experimentally shown in *V. harveyi* that QS limits cooperative goods production to when the benefit of making these products outweighs production costs, and could allow cooperation to resist defector invasion in environments where initial densities are low, which are likely common in its natural aquatic habitat (Pai et al., 2012). QS-regulation may be particularly advantageous for goods that exhibit accelerating benefits and would best be expressed at higher cell densities (Heilmann et al., 2015, Cornforth et al., 2012). Therefore, it is advantageous to delay cooperation until density reaches sufficiently high levels, where it is known that production is more beneficial (Darch et al., 2012), or particularly at the transition from low to high density, precisely where QS is predicted to play an important role (Hense and Schuster, 2015). However, as functional quorum sensers appear to perform well against defectors across a wide range of density conditions (Fig. 2.5), QS may provide the environmental sensitivity to allow cells to act as density generalists,

responding particularly to kin numbers in a given environment. The shift in responses to density we observed is resemblant of shifts between individual and group fitness maxima, and the expectation that organisms will behave as if to maximize their inclusive fitness (West et al., 2012, Brown and Johnstone, 2001). QS could play an important role in effectively maximizing inclusive fitness. Together, these results suggest that cells with a fully functional QS system possess a significant advantage over systems that do not appropriately regulate cooperative behaviors and are more resistant to cheating by optimizing growth strategies in a density-dependent manner, providing support for the idea that communication can act as a form of cheating control (Velicer, 2003, Travisano and Velicer, 2004).

Chapter 3

Dispersal Maintains Quorum Sensing

in Bacteria

via a Simpson's Paradox

Abstract

Quorum sensing (QS) is a mechanism of bacterial chemical communication that regulates many genes including those that encode cooperative behaviors. Although mechanisms exist that can help to stabilize QS, it is not always clear if or how cooperators may increase in a population of primarily defectors. We hypothesized that discontinuities between local and global fitness trends could allow QS cooperators to invade a QS defector population, even though the cooperators do not ever individually exhibit higher relative fitnesses in local subpopulations. This type of discontinuity is classified as a Simpson's paradox. To test this hypothesis, mixed metapopulations of conditional, unconditional, and defector QS strains of *Vibrio harveyi* and *Vibrio cholerae* were diluted, fragmented into subpopulations, and grown in an environment that requires QS induction of public goods production for maximum growth. We found that separation of these strains into small populations via strong bottlenecks led to a global invasion of both facultative and unconditional cooperators into a majority defector population. Examination of the motility of mixed populations revealed that cooperators are able to disperse and outcompete defectors at the edge of motile colonies. Our results suggest that not only does spatial assortment of cooperator and defector genotypes stabilize QS cooperators, the dispersal of cooperative cells to colonize new environments can also increase overall cooperative fitness. Therefore, the ability to modulate colonization and dispersal may provide a mechanism for the maintenance of QS systems.

3.1 Introduction

Explaining the ongoing persistence of cooperative behavior has been a long-standing challenge for evolutionary biology (Hamilton, 1963, 1964, Axelrod, 2006). Cooperative behaviors are not expected to be evolutionarily stable, and thus require additional mechanisms to explain their existence and persistence in nature (Nowak, 2006, Bruger and Waters, 2015). Nonetheless, cooperation is widely found at all levels of biological organization. One particular class of cooperative behaviors is the production of public goods; these are fitness-enhancing products that require individual investment but whose resulting benefits can be shared across members of a population. Public good production provides an opportunity for non-producing defectors to cheat contributing cooperators within a mixed population of bacterial cells, increasing their frequency while simultaneously destabilizing productivity of the whole population leading to a tragedy of the commons (Hardin, 1968, Rankin et al., 2007).

One mechanism contributing to the limitation of defectors is facultative production of public goods when the benefit is increased or the cost is reduced (Foster, 2004). Bacteria often regulate public good production in a density-dependent manner by using extracellular chemical communication known as quorum sensing (QS). In *Vibrio harveyi* and *Vibrio cholerae*, the QS circuit consists of a phosphorelay pathway that is controlled by exogenous chemical signals known as autoinducers (Waters & Bassler, Ng & Bassler). At low cell density, when autoinducers are low, membrane bound kinases funnel phosphate into this pathway, leading to phosphorylation of the response regulator LuxO, which ultimately represses expression of the high-cell-density master regulators LuxR for *V. harveyi* and HapR for *V. cholerae*. At high cell density, the autoinducers bind to their cognate receptors and

activate their phosphatase activity. This leads to dephosphorylation of LuxO and expression of LuxR/HapR, which induces genes expressed at high cell density. The *Vibrio* QS circuit is a powerful system to study the evolution of QS as a constitutive cooperating strain can be generated by mutation to *luxO*, which locks the cells into the high-cell-density state, while a defector genotype locking the cells into the low-cell-density state can be generated by deleting *luxR/hapR*.

We previously showed that the cooperative production of extracellular proteases, public goods susceptible to cheating by non-producing defectors, was stabilized in the marine bacterium *Vibrio harveyi* by facultative expression via QS regulation (Chapter 2). This regulation afforded cooperator cells the ability to eliminate production costs at low densities and not express costly cooperative behaviors until a sufficient number of cooperative bacteria was present. Alternatively, a $\Delta luxOU$ mutant strain which is an unconditional producer of protease locked in the high-density QS state was rapidly invaded by non-cooperating cells ($\Delta luxR$) and driven to extinction (Chapter 2).

However, even though QS regulation stabilized this cooperative behavior, cooperator cells were unable to invade a population of mostly defectors in an environment that required proteolytic activity for maximum productivity (M9 media with casein as the limiting carbon source). In addition, *V. cholerae* locked high-cell-density strains that are presumably unconditional cooperators (UC) have been isolated from natural environments (Wang et al., 2011). This raises the question of how QS is preserved in natural environments, and whether conditions exist that enable cooperators to succeed and invade into a population of mostly defectors.

We hypothesized that QS-proficient strains could invade a population of defectors via a Simpson's paradox (Simpson, 1951, Blyth, 1972). Generally speaking, this term describes

a statistical phenomenon in which a given trend is evident in multiple groups (e.g. A>B), but the reverse trend is observed when all of those groups are combined (i.e. B>A). With regard to cooperative traits, this paradox describes a situation where defectors outperform cooperators on local scales due to higher relative fitness, but cooperators ultimately outperform defectors on global scales due to a higher absolute fitness, either due to advantages in growth rate or growth yield.

To test the role of dispersal and population bottlenecks on the performance of QS and QS-regulated cooperative behaviors, we adopted an approach that included cycles of global cell mixing as a metapopulation (Levins 1969), high levels of dilution, fragmentation into subpopulations and subpopulation regrowth, followed by combination of the subpopulations into a new metapopulation. We hypothesized that defectors should be favored over cooperators within mixed subpopulations, but subpopulations that by chance had more cooperators present would be favorably weighted in the metapopulation due to higher growth yields (Chuang et al., 2009, Cremer et al., 2012). If the difference is great enough, this would act to overcome the relative disadvantage of cooperators to preserve or even increase their total proportion in the metapopulation. Under this regime, we observed that both the WT strain and the UC strain invade defectors in metapopulations. Additionally, although experimental results from the related bacterial pathogen *V. cholerae* have shown that $\Delta hapR$ defectors can effectively invade and dominate the WT strain in a mixed population (Katzianer et al., 2015), we observed invasion of different *V. cholerae* cooperator genotypes into the $\Delta hapR$ defector with our experimental approach. Moreover, competing cooperator and defector strains in a low-agar motility assay (Kearns, 2010) generated an assortment of cooperator genotypes and improves their competitive outcomes. Our results demonstrate the frequency of QS in mixed *Vibrio* populations can be increased by a Simpson's paradox through popu-

lation bottlenecks, dispersal events, and self-structuring that leads to genotypic assortment, illustrating that high growth yield cooperative strategies can outcompete high growth rate defector strategies on global scales given the appropriate ecological conditions (Pfeiffer and Bonhoeffer, 2004, Kreft and Bonhoeffer, 2005).

3.2   Materials and Methods

3.2.1   Strains and Media

V. harveyi strains BB120 (WT, ATCC BAA-1116), and derivative strains JAF78 ($\Delta luxOU$), and KM669 ($\Delta luxR$) were grown at 30 °C as indicated. *V. cholerae* strains used included C6706 (WT), and derivative strains SLS349 ($\Delta luxO$), and BH1543 ($\Delta hapR$) and were grown at 35 °C. Strains were grown in 'M9 casein', a liquid media based upon M9 salts (Sigma-Aldrich or Beckton, Dickinson, and Company) supplemented with sodium chloride (Macron) to 2% (w/v) final concentration and sodium caseinate (Sigma-Aldrich) to 0.5% (w/v) to form M9 casein media. Strains were streaked from frozen stocks, and individual colonies were passaged for 24 hours in LB before passage in M9-casein media. For growth in static M9-casein cultures (Fig. 3.2), cultures were allowed to grow 48 hours unshaken at 30 degrees Celsius between transfers.

3.2.2   Metapopulation Growth

Strains were passaged in monoculture in M9 casein media for 24 hours before mixing. During initial mixing, competing strains were washed, diluted to equivalent densities, and then combined. Upon mixing, cells were diluted and divided into subpopulations in a 96-

well plate and passaged for growth. Dilution estimates were made on the basis of OD600, such that the target density was 1-2 cells per subpopulation. Estimates of average starting subpopulation were additionally corroborated by counting the number of empty wells (determined as wells with no significant increase in OD600 over background) present and fitting that number to a Poisson distribution (Chuang et al., 2009). Plates were grown for 3 days in 30 degrees Celsius for *V. harveyi* and for 2 days in 35 degrees Celsius for *V. cholerae* between transfers. Plates were incubated on orbital shakers to facilitate even mixing within subpopulations. After the period of growth, subpopulations were measured for individual growth in a SpectraMax M5 plate reader, mixed, and the resulting metapopulation was measured for overall growth using both OD600 and viable cell counts on LB agar plates. The frequency of cooperators was determined by quantifying bioluminescent colonies for *V. harveyi* while the frequency of defectors for *V. cholerae* was determined by quantifying rugose/smooth colonies indicative of the $\Delta hapR$ strain and other competitor strains.

3.2.3   Motility plates

Strains were grown 24 hours in LB, then transferred and grown in M9-casein for 24 hours to acclimate. Competing strains were mixed in a 1:1 ratio and approximately 10^6 cells were plated on the surface of M9-casein petri plates containing 0.3% agar. Plates containing strains of *V. harveyi* were incubated at 30 degrees Celsius and plates containing *V. cholerae* strains were incubated at 35 degrees Celsius. Plates were imaged and sampled from daily. Edge samples were obtained from the visible edge boundary of the colonies. When whole colonies were sampled (Fig. 3.6), the entire dispersed colony was harvested, suspended in M9-casein liquid media, vigorously vortexed until an even consistency was obtained, then diluted and plated to get count estimates. Cells were phenotyped according to colony morphology

(smooth/rugose) for *V. cholerae* and colony bioluminescence phenotype (luminescent/non-luminescent) for *V. harveyi*.

3.3   Results

3.3.1   Comparing growth of *Vibrio* cooperators and defectors in M9-casein media

We have previously shown that when cooperators and defectors of *V. harveyi* QS are mixed and competed in well-mixed conditions, $\Delta luxOU$ suffers from a prisoner's dilemma and is invaded by both the WT strain and $\Delta luxR$ defector at all starting frequencies, while the WT exhibits equivalent fitness to $\Delta luxR$ at all starting defector frequencies (Chapter 2). Importantly, however, the WT strain was unable to invade the $\Delta luxR$ defector even when present in low abundance. Therefore, we wondered how the WT QS system maintains its abundance in the natural world.

The first step to address this question was examining cooperator and defector growth in monoculture in a minimal medium in which casein was the sole carbon source (M9-casein). As we have previously reported, *V. harveyi* cooperator strains that can express the high-cell-density QS state (WT and $\Delta luxOU$) are able to grow to high yields in this environment. This is due to the induction of extracellular protease production by QS in *V. harveyi*, making enzymes that are required to digest the extracellular casein liberating nutrients for growth. Alternatively, the $\Delta luxR$ mutant is unable to grow to a high yield in M9-casein due to an inability to produce extracellular proteases. Here, we extended the time of this experiment and still observed poor yield of the $\Delta luxR$ at 48 and 72 hours compared to the WT and UC

(Fig. 3.1). Analogous QS strains of *V. cholerae* were similarly examined: WT (facultative
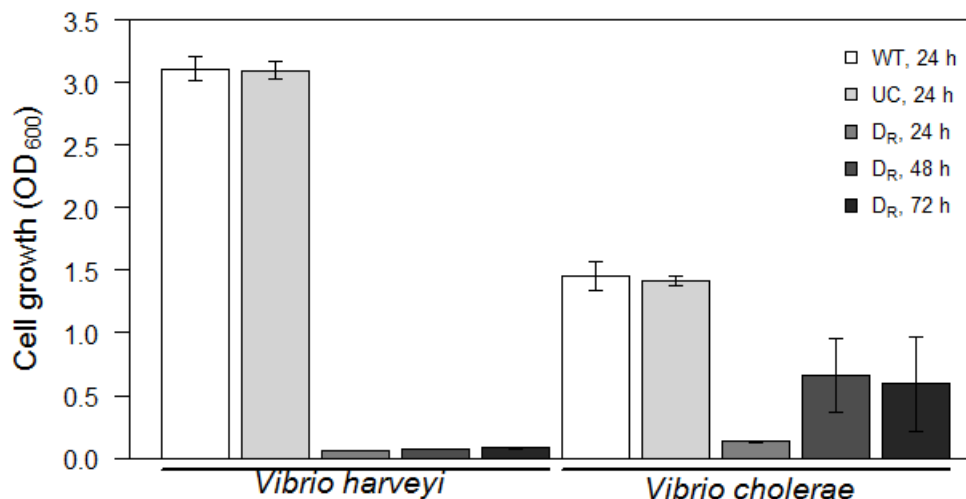
Figure 3.1: **Growth of *V. harveyi* and *V. cholerae* strains in M9-casein media.** Strains were grown in M9-casein media and measured at daily intervals, using absorbance at 600 nm in a spectrophotometer. Cooperator strains showed no further changes in absorbance after 24 hours. 'WT': wild type strain; 'UC': unconditional cooperator strain; 'D_R': QS response defector strain. Error bars represent 95% confidence intervals on N=3 biological replicates.

cooperator), $\Delta luxO$ (UC), and $\Delta hapR$ (defector). Like *V. harveyi*, strains of *V. cholerae* that could activate the high-density QS state exhibited higher absolute fitness, demonstrated by increased growth yield compared to the defector strains (Fig. 3.1). Although the $\Delta hapR$ defector exhibited poor growth at 24 hours, one difference we noted between the two *Vibrio* species was that the $\Delta hapR$ defector of *V. cholerae* was able to grow to significant cell densities at 48 and 72 hours.

3.3.2 A metapopulation approach selects for increases of *Vibrio* cooperator strains

We hypothesized that environments that allowed sufficient assortment to prevent exploitation of cooperators could allow the frequency of cooperator strains to increase. One such ecological condition that could allow this would be local assortment of cooperating genotype afforded by spatial structure, either imposed by the physical environment or produced by cells themselves. To test this hypothesis, we performed competition experiments between either the facultative cooperator or the UC versus the defector in M9-casein minimal media in static liquid cultures or in cultures with low concentrations of agar added to provide greater opportunities for spatial structure to emerge. This experiment was performed with the cooperator genotypes at both rare (1%) and common (99%) starting frequencies. Under conditions of unshaken media to allow more possible assortment (Kassen et al., 2000, Srivastava et al., 2004, Rainey and Travisano, 1998), we still observed that the UC was competitively excluded at both frequency treatment conditions tested, and that neither $\Delta luxR$ nor WT could invade the other when rare in a mixed population (Fig. 3.2). Identical results were obtained with cultures in which a low concentration of agar was added to the liquid medium to prevent free diffusion of the bacteria. Therefore, in the conditions that we tested, parameters that enabled increased assortment were not sufficient for QS cooperating populations to increase in frequency. We next considered the possibility of a Simpson's paradox in which cooperator frequency could increase without ever having a relative fitness higher than the defectors if most interactions between cells are with their own genotype. In this case, we hypothesized that dispersion of mixed metapopulations into multiple small subpopulations through bottlenecks could increase the frequency of cooperators. This is because populations
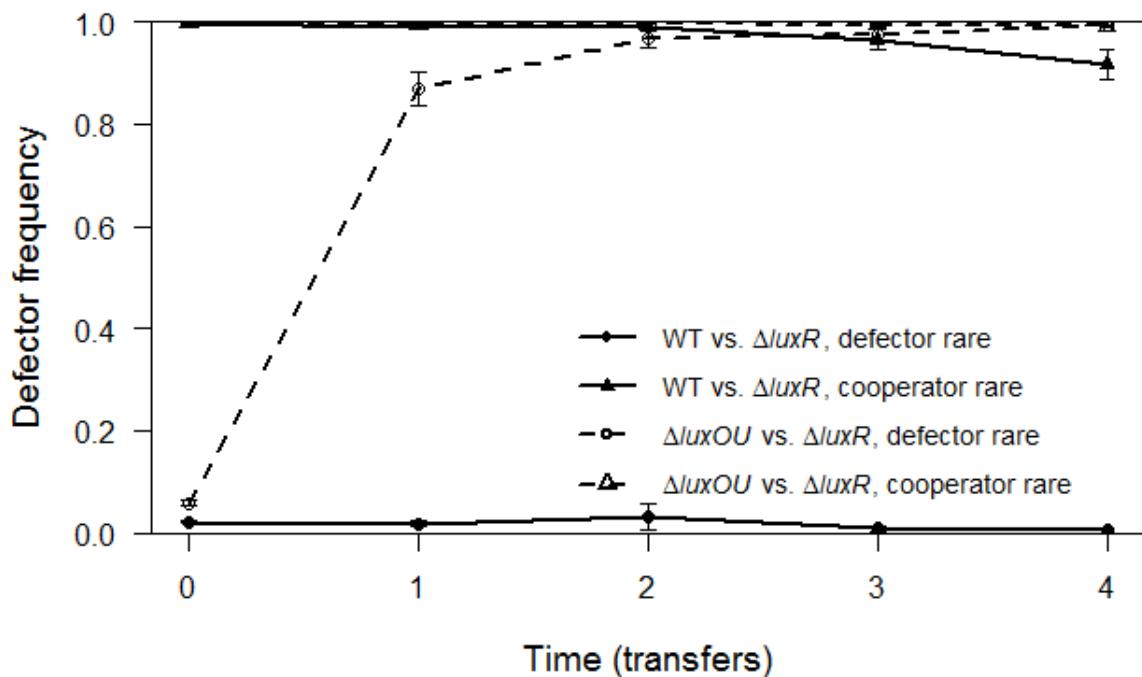
Figure 3.2: **Competitive outcomes of mixed _V. harveyi_ cooperators and defectors in unmixed M9-casein media.** Cooperator strains of _Vibrio harveyi_ were mixed with Δ_luxR_ at 99:1% or 1:99% ratios and allowed to grow for a series of steps. Each transfer grows for 2-3 days before diluting into new media. Error bars represent 95% confidence intervals on 3 biological replicates.

randomly enriched for cooperator genotypes would have higher yields relative to populations randomly enriched for defector genotypes. Thus we adjusted our experimental approach to test this hypothesis (Cremer et al., 2012). This consisted of mixing either the conditional or UC cooperator strains into a predominantly (∼90%) defector population in M9-casein media, followed by diluting and dividing the mixture into subpopulations in 96-well plates to promote assortment. The dilutions were carried out so that each population was seeded with an average of 1-2 founding cells (see Materials and Methods). After a period of regrowth, the subpopulations were recombined, and the ratio of cooperator genotypes versus defectors was

determined by plating for viable cell counts and quantifying the number of bioluminescent colonies. This cycle was repeated 7 times.

Indeed, in these experimental conditions we observed a distinct and uniform increase in the frequency of both facultative and UC strains of *V. harveyi* compared to the defector genotype, effectively fixing for both cooperator genotypes (Fig. 3.3, top panel). These results demonstrated that even the UC that was distinctly disadvantaged in growth rate and driven to extinction by defectors in well-mixed conditions was favored in these conditions.

We then examined the generality of this result by performing analogous experiments with *V. cholerae*. We considered *V. cholerae* a more stringent test of the generality of our experimental approach for two reasons. First, previous results demonstrated the WT strain of *V. cholerae* was invaded by a $\Delta hapR$ defector mutant. In contrast, we have shown that WT *V. harveyi* exhibits equivalent fitness to the $\Delta luxR$ mutant (Katzianer et al., 2015). Second, even though *V. cholerae* defectors suffer from a growth deficiency in M9-casein media, they recover a substantial amount of growth by 48 hours, unlike the *V. harveyi* defectors that never significantly recovered from their growth defect at 24 hours in the M9-casein environment (Fig. 3.1). Nonetheless, for both *V. cholerae* WT and $\Delta luxO$ UC, we saw a strong invasion of cooperative strains into a $\Delta hapR$ defector-majority metapopulations with increases from 10% to 80% or 95% for the WT and $\Delta luxO$ mutants, respectively (Fig. 3.3, bottom panel). To more closely examine the dynamics of WT and $\Delta luxOU$ invasion, we determined QS activity (measured in proxy by bioluminescence normalized to cell growth) and growth (measured by OD600) of all subpopulations of *V. harveyi*. The results for the first and final transfer are shown (Fig. 3.4). Interestingly, even though both cooperator strains are able to effectively fix in these metapopulations, the WT strain exhibits more homogenous outcomes with many more subpopulations reaching higher densities and high (but not maximum at the time of
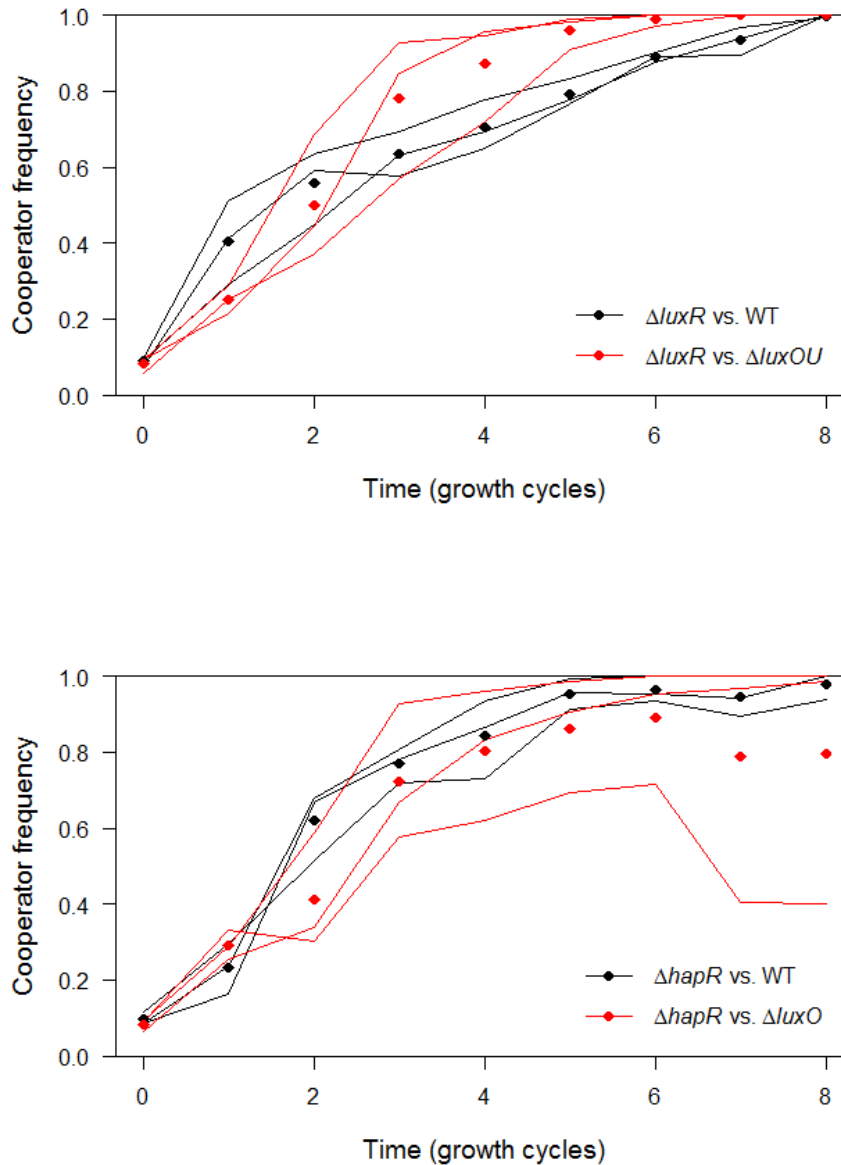
Figure 3.3: **Demonstration of Simpson's paradox in metapopulations of *V. harveyi* and *V. cholerae* grown in M9-casein.** Simpson's Paradox results in increasing fractions of cooperators in metapopulations of *Vibrio* bacteria. Top panel. For *V. harveyi*, growth time between transfers is 3 days, and Bottom panel. for *V. cholerae* it is 2 days. $\Delta luxR$ is the *V. harveyi* defector strain, and $\Delta hapR$ is the *V. cholerae* defector strain. Lines follow the trajectories of individual metapopulations and data points indicate the mean cooperator frequency of the 3 replicate metapopulations per treatment.

measurement) bioluminescence with relatively few populations present in intermediate states. This is consistent with previous findings that the WT strain is more effective at maximizing growth by appropriately regulating production of a fitness-impacting public good (Chapter 2). Alternatively, the $\Delta luxOU$ subpopulations display a continuum of growth phenotypic profiles, with many populations at low to intermediate population densities yet with very high (near maximum) bioluminescence. Even after the last growth cycle, when cooperators in both treatments are near fixation in their metapopulations, subpopulations from the $\Delta luxOU$ treatment measured along this continuum while the majority of WT subpopulations reached a saturating density (Fig. 3.4). This difference between strains is a likely result of WT utilizing superior growth strategies in response to QS signaling to more rapidly reach high densities, while $\Delta luxOU$ inefficiently induces QS at low densities, which in turn delays its growth rate and causes a slower rebound in cell numbers from the very small starting sizes experienced in these subpopulations (Chapter 2).

3.3.3 Combined dispersal by motility and strong growth increases cooperator frequency in M9-casein

The observed increases of cooperators in our metapopulation experiments demonstrated that the cooperator strains in the study could invade defector-majority populations if they encountered sufficient assortment from defectors to realize the growth yield advantages they enjoyed in M9-casein (Fig. 3.1, Fig. 3.3). We additionally measured the dispersal capabilities of *Vibrio* strains in isolation by plating monocultures on M9-casein motility plates and measuring the total visible dispersal area over time. We found that for both species tested, the defector strain had the largest dispersal area, although it was not nearly as dense as
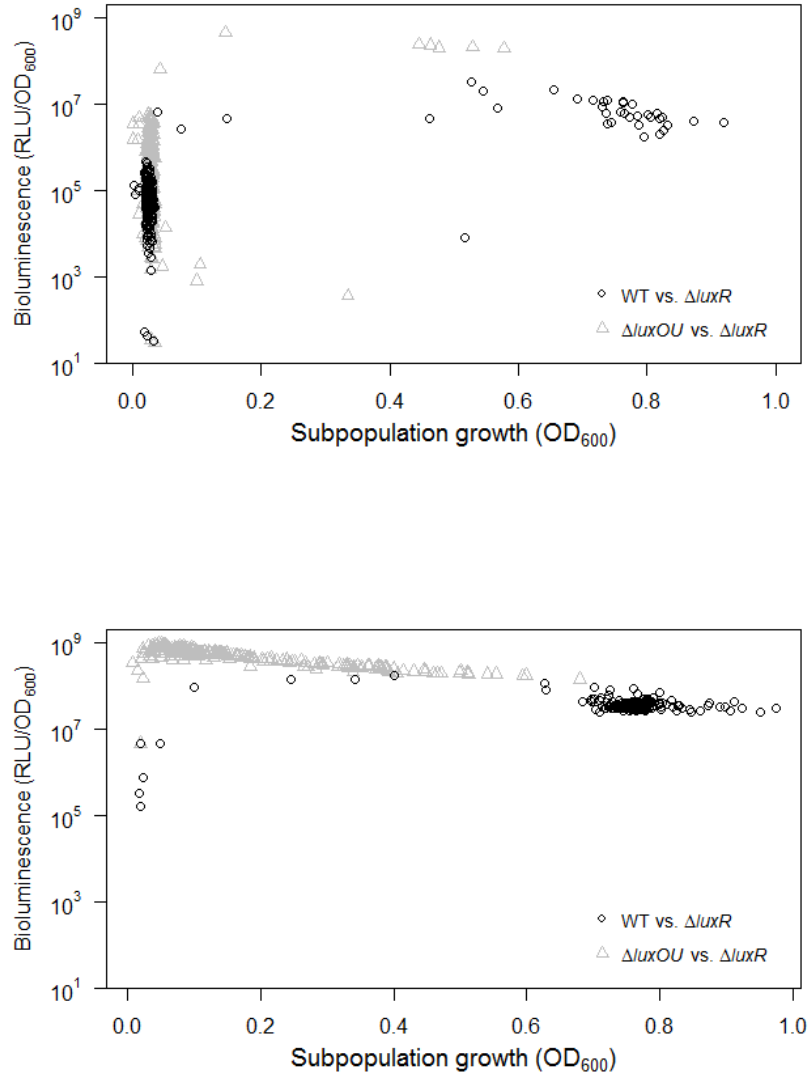
Figure 3.4: **Phenotypic profiles of subpopulations of *V. harveyi*.** Phenotypic profiles of subpopulations of *V. harveyi* over the course of the experiment, shown on the first transfer day (top panel) and the final day of growth (bottom panel), demonstrate different patterns in the relationship between QS activity (measured in proxy by bioluminescence normalized to cell growth) and growth (measured by culture absorbance (OD_{600})). Interestingly, even though both cooperator strains are able to effectively fix in these metapopulations, these results display more of continuum with a negative correlation for $\Delta luxOU$ and more of a saturating relationship for WT with many more subpopulations reaching higher densities. This is likely a result of the ability of WT to resist cheating even when in mixed populations with defectors (Chapter 2).

the colonies produced by either cooperator strain (Fig. 3.5). This was closely followed by the WT strain, and in both cases the UC strain performed worst, despite claims that QS positively impacts motility (Yang and Defoirdt, 2015, Waters and Bassler, 2005, Srivastava and Waters, 2012). Because the *V. harveyi* UC strain has been previously shown to be relatively slow-growing in M9-casein (Chapter 2), this growth defect would mask any potential advantage it has in motility. The combined growth and motility results led us to predict
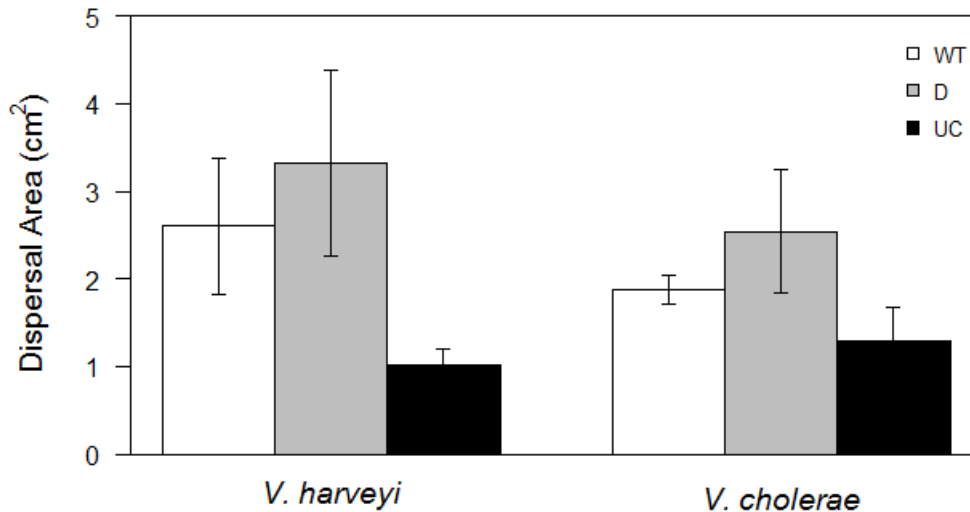
Figure 3.5: ***Vibrio* strains exhibit a colonization-dispersal tradeoff in M9-casein media.** Strains were seeded on motility (0.3%) agar plates with starting population sizes equal to $\sim 1/1000$ of the defector strain carrying capacity in M9-casein media ($\sim 10^5$-10^6 cells). Motility was calculated by measuring the total area of the region over which cells had traversed from their initial inoculation spot, determined by the fringe of visible cell growth, at 24 hours post-inoculation. Error bars represent 95% confidence intervals for 3 biological replicates.

that dispersal and colonizing of new environmental patches could favor cooperating genotypes that were proficient at both motility and growth in a given environment. To do this, we mixed populations of cooperator and defector strains (1:1 mixtures) and allowed them to

compete on M9-casein motility plates, where both growth of cells and dispersal would be allowed. Indeed, we observed that QS positively impacts growth of *V. harveyi* and *V. cholerae* cooperators in dispersing colonies compared to the corresponding defector genotypes, particularly at the colony edges (Fig. 3.6). Because much larger seeding populations are used in this experiment (around 10^6 cells), this is a much more stringent test of the potential for the Simpson's paradox to aid cooperative behaviors like protease production in nature. In the colony centers, both WT strains perform better in the presence of defectors than the corresponding UC strain (Fig. 3.6). This difference was most pronounced between *V. harveyi* strains, consistent with our earlier results in well-mixed conditions (Chapter 2). The WT strains also constitute majorities of the cell populations on the colony edges, while the UC strains struggle to make it to the colony exterior (Fig. 3.6). Thus, while *Vibrio* cooperators performed similarly well in experimental metapopulations, WT and UC strains perform very differently from one another when competed in motility plates. Harvesting entire dispersed colonies from motility plates revealed that it is possible for *V. cholerae* strains competed against $\Delta hapR$ to increase in frequency, but only in certain cases (Fig. 3.6). Only the WT strain of *V. cholerae* showed clear and consistent increases in their frequencies within these colonies, while all strains that lacked either functional *luxO* or *flrA* were very deficient in competition and experienced uniformly low fitnesses against $\Delta hapR$ (Fig. 3.6, Fig. 3.7). Differences were also detected between *V. harveyi* cooperator strains competed against the $\Delta luxR$ mutant, with WT showing no significant drop from its starting frequency of 50%, while the $\Delta luxOU$ competitor was largely outcompeted within dispersed colonies by the end of the experiment (Fig. 3.6). Together, these experiments provide evidence that the effects of an unimpaired QS system provides upon growth and dispersal make WT strains of either species more competitive in the environments tested, even allowing the *V. cholerae* WT

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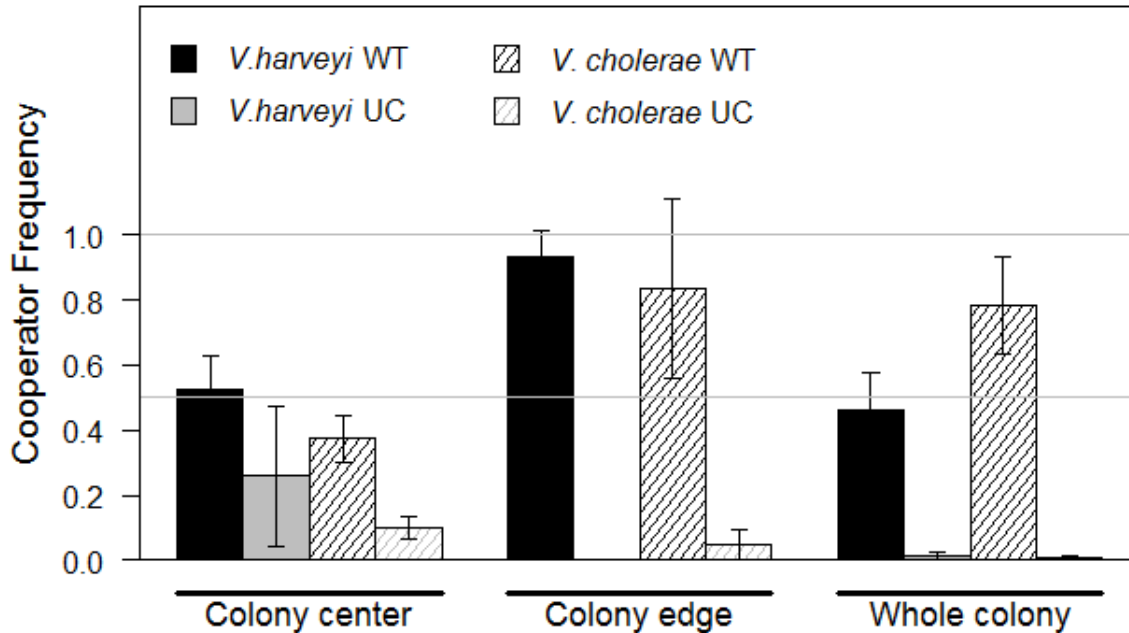
Figure 3.6: **Competitive outcomes in motility plate competitions.** During growth in M9+casein motility (0.3% agar) plates, samples were taken from the edge of colony growth, diluted and plated to determine the composition of these populations. At the completion of the experiment, the entire colony was harvested, homogenized, diluted, and plated to determine the composition of these populations. Cooperator strains were competed against $\Delta luxR$ for *V. harveyi*, and $\Delta hapR$ for *V. cholerae*, respectively. The UC strains refer to $\Delta luxOU$ for *V. harveyi*, and $\Delta luxO$ for *V. cholerae*, respectively. Error bars represent 95% confidence intervals for N=4 biological replicates per strain mix.

strain to increase in mixed populations with defectors.

3.4   Discussion

Here we have demonstrated that a Simpson's paradox occurs to increase the frequency

of functional QS systems in metapopulations of mixed cooperating and defecting genotypes

when the ability to cooperate provides an advantage in cell yield. Previous results have shown
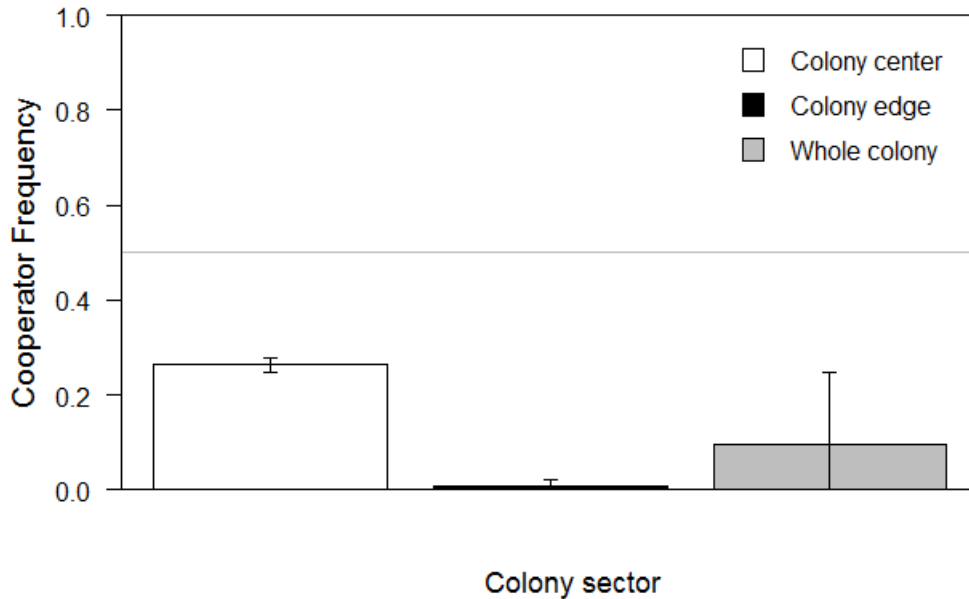
Figure 3.7: **Competitive outcomes of Δ*flrA* in motility plate competitions.** The *V. cholerae* Δ*flrA* strain was competed against Δ*hapR* in M9+casein motility (0.3% agar) plates. During growth, samples were taken from the edge of colony growth, diluted and plated to determine the composition of these populations. At the completion of the experiment, the entire colony was harvested, homogenized, diluted, and plated to determine the composition of these populations. Error bars represent 95% confidence intervals for N=4 biological replicates per strain mix.

that Simpson's paradox can be observed in a synthetic system due to differences between the growth rates of producers and non-producers of a synthetic genetic circuit that regulates a public good (Chuang et al., 2009). In that system, a signal molecule itself is considered the public good and its reception regulates a gene that allows growth in the presence of an antibiotic.

Here, we tested if a Simpson's paradox stabilizes cooperation using a non-engineered cooperative system, focusing on protease production under the regulation of QS as the public good of interest. These results are ecologically relevant as this interaction between bacteria is found in natural environments and is based upon differences in carrying capac-

ity/productivity, which are likely to commonly occur among strains that elicit cooperative behaviors to differing degrees (Foster et al., 2007).

In addition, we extended our investigations to a more stringent experiment that included large starting populations of mixed cooperator and defector strains in which dispersal was driven by the bacteria themselves. In this M9-casein-agar environment, functional quorum sensers of both *Vibrio* species used motility to perform substantially better than the corresponding UC strain, leading to increases of cooperators at the edge of an expanding motile population (Fig. 3.6). In the case of *V. cholerae*, the combined effects of QS on growth and motility even increased WT abundance through the entire colony (Fig. 3.6). The fact that this increase is less extensive than those seen in the metapopulation experiments is consistent with other metapopulation experiments that manipulated starting population sizes (Chuang et al., 2009) and previous findings that cell density affects the distribution of cells and extent of cooperation found within biofilms (van Gestel et al., 2014).

Examining outcomes in motility plates confirmed our suspicions that while UC strains performed quite well in earlier metapopulation experiments, they would not do as well in a larger, more mixed population. Indeed, for both *V. harveyi* and *V. cholerae*, the WT did significantly better in the colony center and this improvement in competitive growth impacted its ability to reach and succeed at the colony edges (Fig. 3.6). This result is consistent with observations in other systems that cooperators can outpace defectors at the leading edge of range expansions (Morgan et al., 2012). Visual examination of bioluminescence in *V. harveyi* colonies also demonstrates this difference (Fig. 3.8). While both WT and $\Delta luxOU$ *V. harveyi* strains exhibit strong bioluminescence in monoculture dispersing colonies, they react very differently when competed in motility colonies against $\Delta luxR$. While neither appear to fully overtakes $\Delta luxR$ at the colony edges (Fig. 3.6), the outcomes are very stark,

with $\Delta luxOU$ being swept within the first day of the competition, while WT appears to gain

an advantage at the colony edge against $\Delta luxR$ defectors (Fig. 3.8, Fig. 3.6). This is also

corroborated by data from the entire mixed colonies, where WT retains similar abundances

throughout the entire dispersing population over the course of the experiment, while $\Delta luxOU$

was outcompeted and drastically underrepresented in these populations by the experiment's

end (Fig. 3.6). Further examination of the importance of motility in *V. cholerae* cooperators
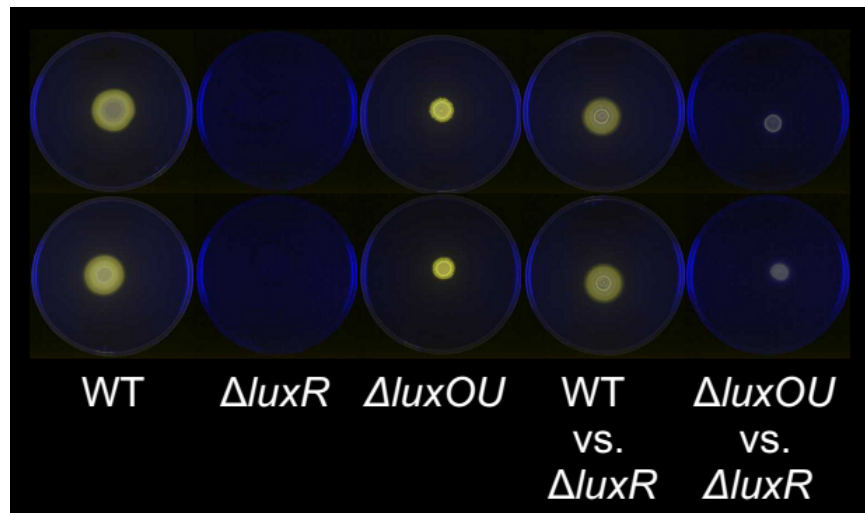
Figure 3.8: **Dispersal of *V. harveyi* populations in monoculture and in competition in M9-casein motility plates.** Pictures were taken of motility plates after 24 hours of growth. Images are overlays of pictures taken with and without visible light applied to detect bioluminescence of colony. In these images, a stronger yellow indicates more bioluminescence and stronger blue indicates less bioluminescence.

during these competitions with defectors revealed that functional motility was necessary for

competitive success in the motility agar plates, as $\Delta flrA$ mutants also did poorly in motility

plate competition assays (Fig. 3.7). While $\Delta flrA$ performed better against $\Delta hapR$ than the

$\Delta luxO$ strain in mixed colonies, it stills experienced significant drops in population frequen-

cies over the course of the competitions, an expected response due to its inability to disperse

further out in the growing colonies.

The observed patterns of growth and motility of strains in isolation (Figs. 3.1 and 3.5) and

in competition (Fig. 3.6), suggest that the *Vibrio* strains examined provide a demonstration of a tradeoff in their potentials for colonization and dispersal, and may predict coexistence between the competing types in the experimental environment. Previous population genetic results for production of siderophores, another public good, in *Vibrios* demonstrated co-existence between producers and non-producers, and our results from motility plates also demonstrate mixed populations at the colony frontiers (Cordero et al., 2012, Cordero and Polz, 2014, Fig. 3.6). We have shown that strong growth and the ability to disperse are both requisites for success in competitions with defectors in motility plates (Fig. 3.6, Fig. 3.8). While previous reports of tradeoffs between colonization and dispersal, even in *Vibrios*, has been reported, the flexibility in gene regulation and resulting growth responses afforded to the WT strains by QS may help somewhat mediate this tradeoff and provide strong competitiveness under a variety of selective pressures (Nadell and Bassler, 2011, Yawata et al., 2014, Chapter 2).

The experiments in motility agar still constitute a continuous resource-rich environment. A patchier, more varied environment (such as particles in natural aquatic environments) should have the effect of placing more weight on the ability to both disperse rapidly to reach new patches and also strongly grow on patches of resource once colonized (Cadotte et al., 2006, Yu et al., 2004, Muller-Landau, 2010). This suggests that these defector strains could be acting as a "fugitive species" which possessed stronger dispersal capabilities in M9-casein plates (Fig. 3.5), while the cooperator strains would represent a competitively superior species due to their increased capacity for growth (Hutchinson, 1951). This would also suggest that fugitives and stronger competitors could coexist in more complex environments that can support multiple niches, the fugitive by dispersing more effectively to open patches and the other cooperator strains by being more fecund in this environment.

Together, we have demonstrated that a Simpson's paradox can be observed in experimental metapopulations that favors cooperators utilizing QS when competing strains undergo strong population bottlenecks. While bottlenecks have been appreciated as potentially important to maintaining cooperative behaviors in populations (Brockhurst, 2007, Waite et al., 2015), this shows that even in large populations, cooperation can be promoted if there is sufficient assortment between cooperators and non-cooperators. One mechanism by which this assortment can be achieved is by active dispersal through cellular motility. Indeed, strains utilizing QS instead of unregulated strategies achieve better combined growth and motility outcomes that allows them to succeed in the presence of high levels of defectors in expanding colonies. This dispersal combined with facultative cooperation controlled by QS enables increased fitness of cooperators even in the presence of non-cooperating defectors. The potential impacts of these findings are not limited to protease production in *Vibrios* examined in this study, but could play a role in preserving many cooperative behaviors that positively impact growth. These phenomena could be especially important within biofilms where the assortment needed to allow enhanced cooperator growth is realized, or in heterogeneous environments where dispersal from and colonization of different patches is highly beneficial (Drescher et al., 2014, Mágori et al., 2003). Previous investigations have suggested that the Simpson's paradox doesn't function to stabilize cooperation within biofilms (Penn et al., 2012), but it is possible that different outcomes may occur in biofilms composed of alternative species such as *Vibrios*. Biofilms have been shown to promote cooperative behaviors and function to localize public goods for *V. cholerae* (Kreft, 2004, Kreft and Bonhoeffer, 2005, Nadell and Bassler, 2011, Drescher et al., 2014). Additionally, *Vibrios* also begin to repress biofilm formation and activate motility at high cell densities, which should promote dispersal. However, it is also important that a competing cooperator strain experiences suf-

ficient growth to seed that dispersal and succeed in its environment. In this manner, it is possible that the *Vibrio* strains with QS in this study undergo cycles of growth and dispersal, thus making them strong competitors in a variety of environments and even increasing cooperative behaviors under their regulation.

Chapter 4

Experimental evolution of quorum sensing in *Vibrio harveyi*

Preface

Previous chapters have focused on relatively short-term outcomes of selection and have examined pre-defined competing strains of *V. harveyi* and *V. cholerae*. The defector strains used in those studies were constructed by engineered deletions of the QS master regulator genes *luxR/hapR* from the genome, conveying a complete loss of the expression of high-cell-density genes such as protease production. Likewise, the unconditional cooperator (UC) strains were engineered by mutation to the gene encoding the response regulator *luxO*. While natural mutants of QS have been detected, they are likely to be different in source than the genetically engineered strains that were utilized in Chapters 2 and 3. This chapter seeks to evaluate whether defectors can evolve from parental cooperating genotypes *de novo* in experimental populations and how the evolution of defectors depends on both the genotype from which it evolved and the cooperator genotypes it encounters in its environment.

4.1 Introduction

The degree to which quorum sensing (QS) is under selection in nature is an open question. It has been suggested that investment in signaling should be highest in intermediate frequency states of cooperators and defectors (Brown and Johnstone, 2001), and that selection should favor decreased investment in QS over time (Popat et al., 2015b). It has also been observed that natural populations of bacteria often have mixed populations of producers and non-producers of public goods and QS cooperators and defectors (Wang et al., 2011, Cordero et al., 2012), suggesting that selection favors the evolution of defecting types and may also foster the coexistence of these different strategists.

I have so far shown that functional QS in *Vibrios* plays important roles in stabilizing cooperative behaviors in the presence of engineered defectors in mixed populations (Chapter 2) and can also promote the increase of cooperative behaviors when competing strains undergo dispersal events or population bottlenecks (Chapter 3). However, it is possible that different, fitter defectors than the $\Delta luxR$ mutant we used as the prototypical defector in those studies could evolve if enough time were provided.

Our previous investigations into cooperation and defection primarily utilized staged competitions of mixed populations with the $\Delta luxR$ strain. Individual cells of this mutant strain should behave as defectors for cooperative behaviors positively regulated by QS, such as extracellular protease production, and could possibly exploit cooperators expressing this behavior in their presence. This type of cheating behavior by QS-defectors has been reported in other experimental systems such as *Pseudomonas aeruginosa* and *Vibrio cholerae* (Sandoz et al., 2007, Katzianer et al., 2015, Diggle et al., 2007). However, master regulator knockout mutants are also likely to bear significant pleiotropic fitness costs as they often regulate large

numbers of genes, and the estimated regulon of *luxR* in *V. harveyi* is around 10% of the genome (van Kessel et al., 2013, Schuster and Greenberg, 2006).

Therefore, we hypothesized that fitter defectors could evolve and invade *V. harveyi* cooperator strains during the course of extended growth in M9-casein, an environment in which absolute fitness is dependent on the extent of QS-regulated protease production. Our prediction was that defectors would evolve and become detectable in M9 media with limited carbon sources if given sufficient time. Due to the dramatic success of $\Delta luxR$ when competed against $\Delta luxOU$ in M9-casein, we also predicted that it would also be more common to see defectors evolve and increase in the presence of UC than those restrained by QS. To examine this, we initiated replicate populations of *V. harveyi* cells from two cooperator genotypes, a WT strain with functional QS and an UC, $\Delta luxOU$, that expresses QS as though induced at high density regardless of the actual signal or density conditions in which it is present. These strains were evolved in M9 media environments for 2000 generations. As expected, we found that a functional QS system delayed the invasion of defector genotypes. Whereas the $\Delta luxOU$ UC genotype was rapidly invaded by non-luminescent defectors that also do not produce protease, significantly higher numbers of cooperators persisted in the majority of WT populations providing further evidence that QS control of cooperation stabilizes these phenotypes. Many of these persistent cooperators exhibited a dim phenotype in which QS was maintained but dampened, similar to our earlier competition experiments with added signals. Overall, these results provide further support that maintaining functional QS has the effect of preserving cooperative behaviors under its regulation, even over longer evolutionary time scales.

4.2 Materials and Methods

4.2.1 Competitions with supplemented autoinducers

Competitors were assessed for the ability to invade when rare by initiating mixed populations of cooperator and defector strains, and population composition was assessed at daily intervals. For these serial competition assays, populations were initiated by mixing strains at 99% of a cooperator strain and 1% of a potential defector strain (defector rare), or vice versa (cooperator rare). Populations were grown in M9 minimal media containing 0.5% (w/v) sodium caseinate (Sigma) as the sole carbon source for 24 hours, plated on LB agar to assess population composition and productivity, and a subset was transferred (a 1000-fold dilution, allowing approximately 10 generations daily) to new M9 liquid media. Competitions treatments including exogenous autoinducer were conducted in the same manner as other serial competition experiments, except that media was supplemented with the acyl homoserine lactone (AHL) signal molecule 3-hydroxybutanoyl-L-homoserine lactone (HAI-1) and the furanosyl borate diester signal molecule 3A-methyl-5,6-dihydro-furo(2,3-D)(1,3,2)diox-aborole-2,2,6,6A-tetraol (AI-2, provided in the form of 4,5-dihydroxy 2,3 pentanedione (DPD), generously contributed by Bonnie Bassler) to a final concentration of 10 μM.

4.2.2 Evolution Experiments

Twelve replicate populations were initiated from stocks of wild type (BB120) and $\Delta luxOU$ (JAF78) and corresponding *lacZ*-tagged strains (ELB714 and ELB738) *V. harveyi* strains and passaged in M9 salts based media (Sigma or Difco) with two percent sodium chloride

(Sigma) and casamino acids (Difco) or sodium caseinate (Sigma) used as the sole carbon source (M9-casein and M9-CAA). Cultures were passaged in 1 mL cultures in 96 deep-well plates. Cultures were daily diluted 1000-fold into homologous media, allowing ∼9.97 (i.e. approximately 10) generations per day. Stocks were made periodically through the course of the experiment and stored at -80 degrees Celsius. Populations were periodically sampled to determine absorbance, cell counts and QS phenotypes. If any contamination of cultures was experienced, samples were reinitiated from the most recent frozen stock available.

4.2.3   Productivity estimates

Measures of cell growth were made by periodically taking samples of the replicate populations, diluting, and plating on LB agar plates, and enumerating through viable cell counts. More frequent measures of growth were also made by measuring absorbances of population subsamples in a Beckman Coulter spectrophotometer at a wavelength of 600 nm.

4.2.4   Phenotype measurements

Protease production was determined by FITC-caseinase assays (Sigma) as previously described (Chapter 2). In Figure 4.4 lower panel, protease concentrations were estimated by normalizing measurements to a trypsin standard curve. The clones that were analyzed were taken from plated samples of the evolved populations at generation 870 of the experiment. Monocultures of these clones were first passaged in LB for 24 hours, diluted 1000-fold into M9-casein media, grown for 24 hours, and then extracellular protease levels were measured from culture supernatants. Bioluminescence was measured at the population level by taking culture subsamples (100 μL undiluted, or diluted if luminescence levels approached

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saturation) and measuring in an Envision Multilabel Plate Reader (PerkinElmer). Biolumi-nescence at the clonal level was qualitatively assessed by examining colonies on LB agar in an AlphaImager HP light box (ProteinSimple) through the chemiluminescence filter and no visible light applied to observe only light produced by cells growing on the plates. Clonal bioluminescence was also measured quantitatively by propagating isolated colonies from pop-ulations in M9-casein and measuring light output in an EnVision Multilabel Plate Reader (PerkinElmer).

To determine the strain background of screened defectors from the short-term selection experiment (Fig. 4.1), $\Delta luxOU$ and $\Delta luxR$ cells or their evolved descendants were deter-mined by screening for resistant colonies on LB with chloramphenicol (10 μM). The WT strain is sensitive to chloramphenicol. The $\Delta luxOU$ strain was distinguished from $\Delta luxR$ by colony PCR of select clones.

4.3   Results

4.3.1   Exogenous autoinducer increases selection for defectors, especially cooperator-derived defectors

Our previous results (Chapter 2) indicated that a functional QS system could prevent invasion by defector cells whereas a UC strain was rapidly invaded. One primary explana-tion of this result is that premature production of protease by the $\Delta luxOU$ unconditional cooperator at low densities enhanced defector invasion. Likewise, timely restraint of public goods production by the WT strain was predicted to prevent defector invasion. To test this possibility, we reasoned that premature induction of the high-density QS state in the WT

strain by addition of exogenous autoinducer (AI) signal molecules that activate the QS response should decrease its ability to prevent defector invasion by prematurely activating QS in a manner decoupled from the actual cell density conditions, which is resemblant of the QS phenotype of the $\Delta luxOU$ strain. Therefore, the WT strain was supplemented with 10 μM concentrations of HAI-1 and AI-2, the two predominant AIs in *V. harveyi* at the initiation of the culture (Waters and Bassler, 2006). Addition of these AI molecules resulted in WT bioluminescence being virtually indistinguishable from the $\Delta luxOU$ strain at all densities during growth, demonstrating that QS was induced by these signals. We repeated the competitions of WT with the $\Delta luxR$ defector in the presence and absence of exogenous HAI-1 and AI-2 and observed different outcomes upon AI supplementation (Fig. 4.1, top left). As observed previously, the defector strain rapidly invaded the $\Delta luxOU$ strain, and this invasion was accompanied by a drop in the productivity of the population as a whole (Fig. 4.1, top panels, solid red lines). In the case of the WT treatment with added signal, no significant invasion occurred for the first 20 generations, at which point non-luminescent defectors and "dims" (variants that maintain reduced but still detectable bioluminescence) that we label as NACs (for "non-ancestral-cooperators"), began to increase and ultimately became nearly 90% of the population (Fig. 4.1, top left panel, blue lines). By the end of the experiment, the WT treatment with added AIs also exhibited a significant drop in population productivity, but still remained more productive than the $\Delta luxOU$ treatment populations (Fig. 4.1, top right panel, blue versus red line). In this particular experiment, the WT populations without added signal also experienced a modest invasion by NACs as well by the end of the experiment, although to a much lesser extent than the other conditions examined (Fig. 4.1, top left panel, solid black line). Additionally, this invasion was not enough to upset the productivity of the populations (Fig. 4.1, top right panel, solid black line). These results
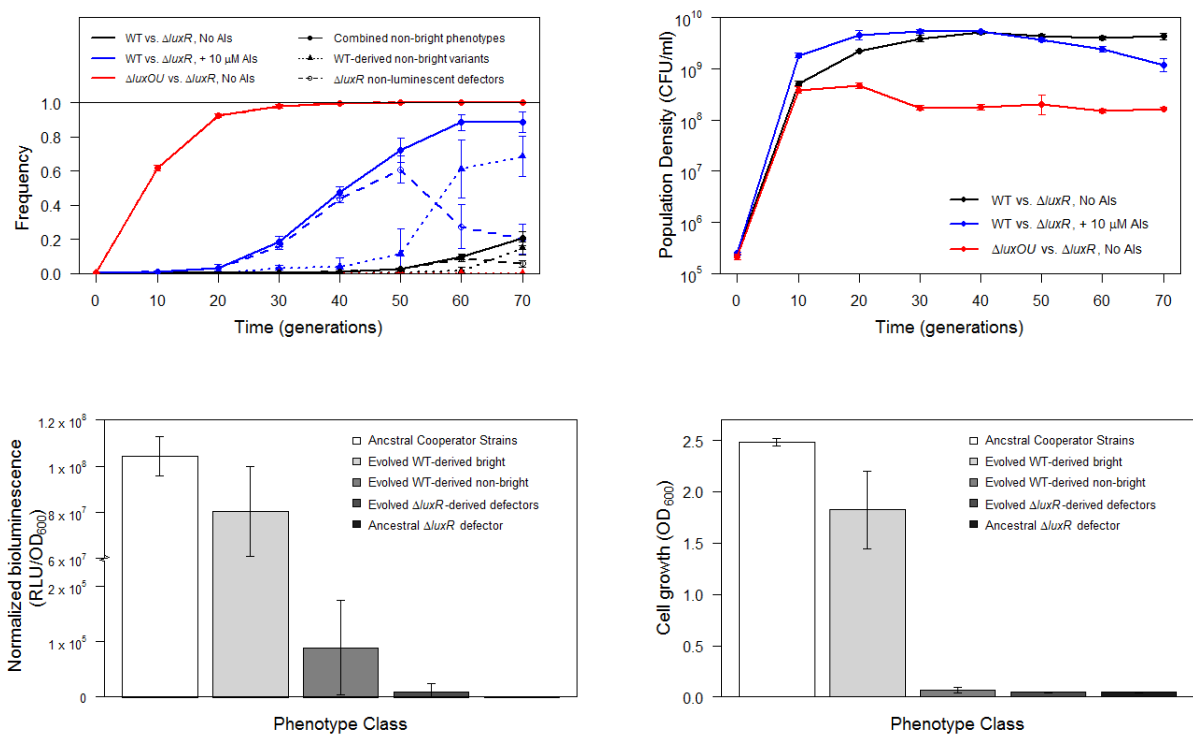
Figure 4.1: **Competitions of *V. harveyi* strains in M9-casein media in the presence and absence of supplemented autoinducers.** *V. harveyi* cooperator strains WT and $\Delta luxOU$ were competed against $\Delta luxR$ in M9-casein media, with or without the addition of 10 μM autoinducer HAI-1 and AI-2. Top panels. Defector frequency (top left panel) and population productivity (top right panel) were measured on a daily basis. Defector background was determined by chloramphenicol phenotype. For top panels, error bar represent 95% confidence intervals. Bottom panels. At the conclusion of the selection experiment, clones from different phenotypic classes (classified by colony bioluminescence) were isolated from the experimental populations and analyzed for light production (bottom left panel) and growth in M9-casein media (bottom right panel). Growth was measured by absorbance at 600 nm in a spectrophotometer. Bioluminescence was estimated using a EnVision Plate Reader. Error bars for the lower panels represent 95% error bars for each phenotypic class. The ancestral non-luminescent control strain used was $\Delta luxR$ and the ancestral luminescent control strains included both WT and $\Delta luxOU$.

support our hypothesis that premature induction of protease can promote defector invasion, although it appears that even in the presence of supplemented AIs at concentrations beyond that required for typical QS induction, the WT strain suffers less invasion than the $\Delta luxOU$ UC.

Defectors took much longer to invade in competitions with WT than in competitions with $\Delta luxOU$, suggesting defectors evolved during the experiment rather than reflecting an invasion of the seeded $\Delta luxR$ defector. Therefore, we hypothesized that the competitions with WT selected for a different subset of defectors than the competitions between $\Delta luxOU$ and $\Delta luxR$. Because the $\Delta luxR$ strain is chloramphenicol-resistant, we distinguished if the defectors that arose in competitions with WT with or without AIs were derived from the ancestral WT strain or the $\Delta luxR$ mutant strain. Indeed, in both cases, the majority of NACs were chloramphenicol-sensitive, suggesting these evolved from the WT ancestor rather than the $\Delta luxR$ defector (Fig. 4.1, top left panel, black and blue dotted lines). Alternatively, the rapid sweep of $\Delta luxOU$ in competition suggested that these defectors are descended from the seeded $\Delta luxR$ mutant, and indeed, PCR confirmed this to be the case for every defector tested in this condition (data not shown). These results suggest that the WT strain maintaining the ability to communicate has more adaptive potential to evolve fitter defector variants that outcompete both the WT ancestor and the seeded $\Delta luxR$ defectors in mixed populations, whereas the unconditional $\Delta luxOU$ strain is more evolutionarily constrained.

When we did a closer analysis of the invading strains, we observed a qualitative difference between the different treatment groups. Whereas all defectors in the $\Delta luxOU$ treatment displayed a non-luminescent phenotype, as would be expected as these are derived from the $\Delta luxR$ mutant (Fig. 4.1, top left panel), the evolved types in the WT with added AI condition and those that evolved without AI addition were markedly different. The dynamics proceeded as such: $\Delta luxR$ levels in the WT with added AI populations began to rise after 20 generations of growth in M9-casein media and reached maximum frequencies around generation 50 (Fig. 4.1, top left panel, dashed blue line). At this point, we observed the emergence of a WT-evolved subpopulation, that displayed a dim phenotype, which began to

appear at higher levels and were selected to become the major member of the populations by generation 70 (blue dotted line, Fig. 4.1, top left panel). Because WT populations with added signal do not reach the maximum population density observed in the experiment until days two through four of the experiment (Fig. 4.1, top right panel, blue line), we hypothesized that this reduced density delayed invasion of $\Delta luxR$ defectors during the initial generations of the experiment, as it is at high densities where defectors are most likely to be able to exploit WT cells. When $\Delta luxR$ defectors reached their maximum frequencies corresponds directly with when WT-derived defectors and dims began to rapidly increase in frequency in the populations, displaying dynamics resembling clonal interference (Hill and Robertson, 1966, Kao and Sherlock, 2008, Fogle et al., 2008, Lang et al., 2013). This increase of WT-derived dim cells indicates that these mutants were more fit than $\Delta luxR$ lineages they encountered in this environment. While these waves of invasion served to reduce the ancestral strain, it was not completely swept from the population and was present at 10% at generation 70. During this period of time, the density of these populations dropped roughly three- to four-fold, but still maintained around a seven- to eight-fold increase over the densities of the $\Delta luxOU$ populations that were swept by $\Delta luxR$ (Fig. 4.1, top right panel). Importantly, the WT-derived dims maintained the ability to QS as they expressed dim luminescence, and even in the presence of exogenous AI, 80% of the cells in the WT with added AI condition expressed QS to some degree. In total, this suggests that the dim variants that evolved from the WT genotype are fitter than $\Delta luxR$ defectors in M9-casein and that their evolution prevents the more extensive tragedy of the commons that was observed in $\Delta luxOU$ lineages. A subset of clones that maintained a bright phenotype, dim phenotype, or dark phenotype were isolated from the WT with AI selection experiment and analyzed for bioluminescence and growth in monoculture (Fig. 4.1, bottom panels). The

results indicate differing levels of bioluminescence with the dims exhibiting nearly 3-orders of magnitude less bioluminescence than the evolved brights. This decreased investment in QS was further exhibited by severely reduced growth in monoculture, indicating that these dim mutants are likely defecting relative to the cooperator strains. We also observed reduced bioluminescence for the evolved bright clones compared to the parental strain, suggesting that even population members lacking a strong dim phenotype might be under selection for decreased QS investment under the high signal level condition (Fig. 4.1, bottom panels).
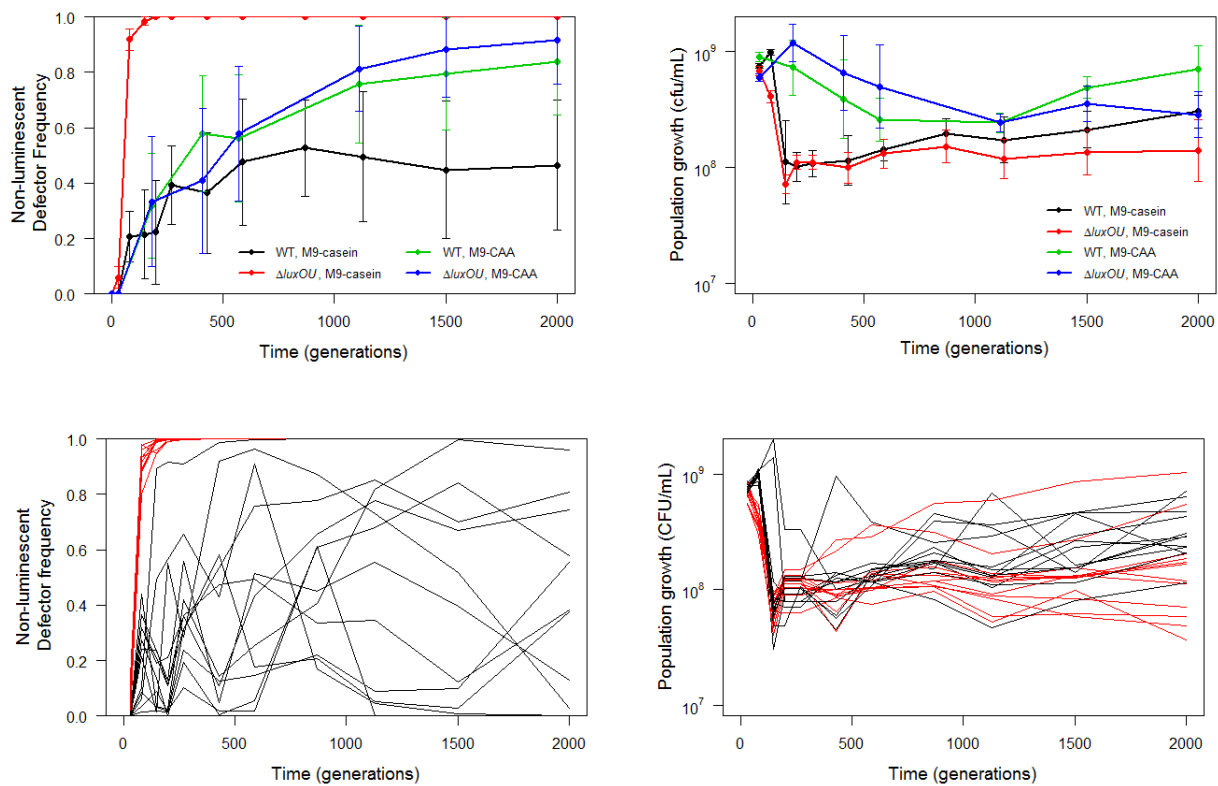
Figure 4.2: **Frequency and growth results from experimentally evolved populations of *V. harveyi*.** The figure depicts observed defector frequency (left panels) and population density achieved at 24 hours of growth (right panels) as mean estimates across treatment groups (top panels) or for individual populations over time (bottom panels). All four treatment groups are depicted in mean estimate plots, but only M9-casein populations are displayed in the tracking of individual populations. Error bars in top panels represent 95% confidence intervals per treatment.

4.3.2   Long-term experimental evolution of the WT and UC QS strains

Based on the results of these experiments indicating that alternate defector strategies could evolve in our experimental conditions, we initiated a longer-term evolution experiment to study the evolution of QS systems in different backgrounds and genetic conditions. Twelve replicate populations each were started from two strains: WT *V. harveyi* which has a functional QS system, and the $\Delta luxOU$ UC that constitutively expresses the QS high-density regulon. This experiment was performed in two different environments: M9-casein in which QS is required for a high growth yield, and M9 media with casamino acids (M9-CAA) in which QS is not required for a high growth yield. These populations were passaged for 2,000 total generations.

We expected to observe the evolution of defectors, defined as colonies that do not produce any visible bioluminescence, in M9-casein but not in M9-CAA. However, during the course of the experiment, defectors and NACs evolved in all four treatment groups, although the patterns of evolution of these defectors was quite different between groups (Fig. 4.2, top left panel). We additionally saw the evolution of dim variants occur in many of the experimental populations. Because the dynamic patterns were clearer and of more interest regarding QS in M9-casein, we focused the majority of our analyses on these lineages. These defectors began to appear at detectable frequencies early in the experiment, within 100 generations for M9-casein populations (Fig. 4.2, left panels). As expected, completely non-luminescent defectors evolved rapidly in $\Delta luxOU$ M9-casein populations, increased, and fixed in all $\Delta luxOU$ populations, with two exceptions discussed below (Fig. 4.2, left panels, red lines, Fig. 4.3). Alternatively, shortly after the appearance of defectors in the UC

lineages, non-luminescent defectors became detectable in WT M9-casein populations as well (Fig. 4.2, left panels, black lines). However, defectors evolved to on average 46% membership of the WT M9-casein populations, and although there was high variability between experimental populations, most populations were not swept by these defectors. The difference between the two genotype treatments in M9-casein is also quite visually striking when these populations are diluted and plated on petri plates to allow growth of individual population members in isolation. While $\Delta luxOU$ populations rapidly and irreversibly lose all bioluminescent cooperators, most WT populations contain significant fractions of cooperators even at generation 2000 (Fig. 4.3).
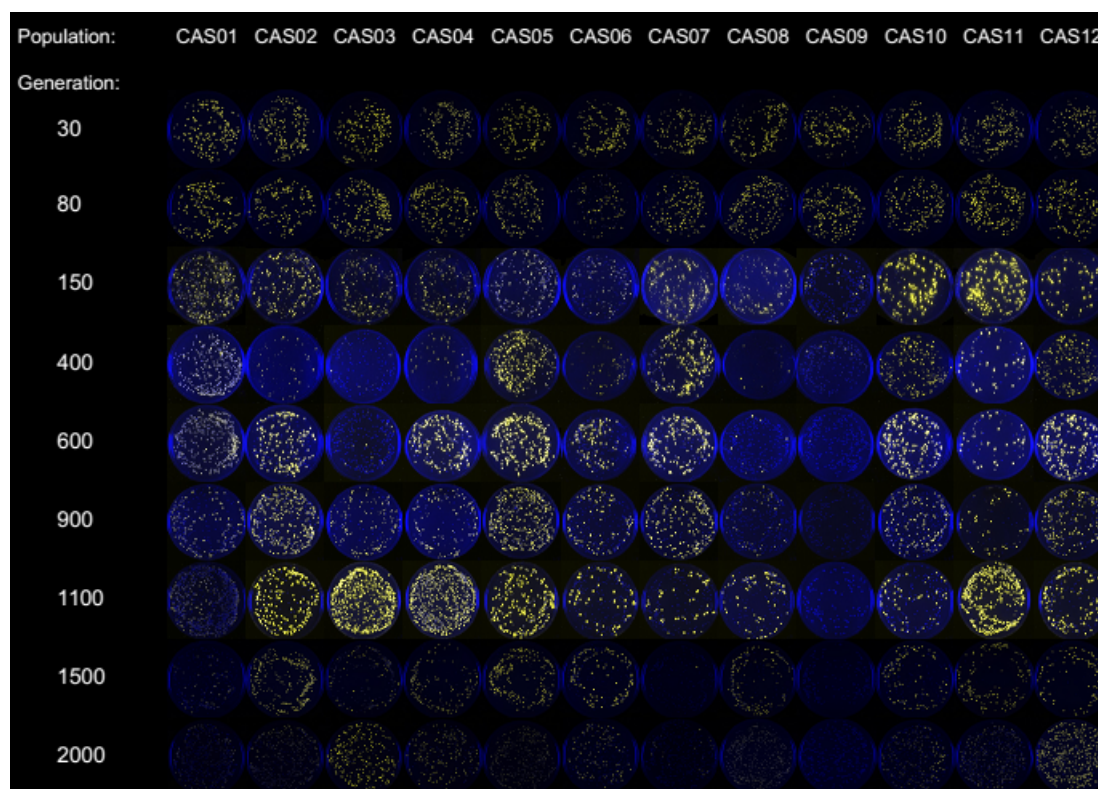
Figure 4.3: **Experimental lineages of *V. harveyi* strains evolved in M9-casein media over 2000 generations examined on petri plates.** Pictures were taken of plated subsamples of the experimental populations after sufficient growth time to allow visible colonies to form, usually 24-48 hours of growth. Images are overlays of pictures taken with and without visible light applied to detect bioluminescence of colony. In these images, a stronger yellow indicates more bioluminescence and stronger blue indicates less bioluminescence. Each column corresponds to a single population, name at top, sampled at multiple time points during the experiment and observed for bioluminescent colonies on petri plates. The numbers on the left side correspond to the generational time point at which the sampling took place. Populations CAS01-12 (top panel) were initiated from WT clones and passaged in M9-casein media for the duration of the experiment; populations CAS13-24 (bottom panel) were initiated from $\Delta luxOU$ clones and passaged in M9-casein media for the duration of the experiment.

Figure 4.3 (cont'd)

Because defection impacts nutrient breakdown in the M9-casein environment, the rise of defectors in M9-casein populations has the effect of dropping the potential productivity of that population. This is indeed what was seen in the M9-casein treatment, with populations from both genotype backgrounds dropping in population densities achieved once defectors became detectable (Fig. 4.2, right panels). This drop in density in the WT populations was different than what was observed when short-term experiments were performed with WT with added AI, in which population density remained high (Fig. 4.1, top right panel). However, the different cooperating genotypes in the M9-casein treatments responded very differently to this drop in population density. We predict that the $\Delta luxOU$ UC populations should continue to express protease production and other behaviors positively regulated by LuxR regardless of actual cell densities because they are locked in the high-cell-density QS state, thus leading to their being rapidly outcompeted by defectors, which was observed (Fig. 4.1, top left panel, Fig. 4.2, left panels, Fig. 4.3). While defectors did invade to detectable levels in the WT M9-casein populations, they only appeared to sweep one of them, with eleven of the twelve replicate lineages maintaining detectable levels of cooperators at the 2000 generation time point of the experiment (Fig. 4.2, left panels, Fig. 4.3). Rather, in most cases cooperators and defectors phenotypic variants coexisted together, which has been observed in other experimental settings (Gore et al., 2009, MacLean and Gudelj, 2006, Pollak et al., 2016, Turner and Chao, 2003). Indeed, in many WT lineages, there was hardly any defector invasion experienced over the course of the experiment, and the extent to which the evolved defectors did invade varied greatly between lineages (WT M9-casein populations generation 2000 defector frequency COV = 0.793, Fig. 4.2, lower left panel).

4.3.3 Defector levels have strong effects on population level phenotypes in M9-casein

Because these populations were started from clonal cooperators, they started out with similarly high levels of bioluminescence and reached high densities in both experimental media conditions. In Figure 4.3, we show that all the experimental populations near the start of the experiment (10 generations into experiment) had similar high levels of both growth yield (in CFU/mL) and in bioluminescence (in RLU/mL), which we interpret as a proxy for high levels of QS activity in the populations (Fig. 4.3, triangles). But over the course of the experiment, the populations diverged in both properties. All populations experienced drops in density from starting points, particularly in M9-casein where defectors had stronger effects on productivity (Fig. 4.2, right panels). After these early drops in density, $\Delta luxOU$ M9-casein populations remain fairly level in population densities for the remainder of the experiment, with the exception of two populations which experienced large increases in population yields, with ending population yields reached at the 2000 generation point among the highest of the experimental populations (Fig. 4.2, right panels). These two populations (CAS16 and CAS19) also have higher levels of bioluminescence than any of the other $\Delta luxOU$ M9-casein populations; this bioluminescence is difficult and in some cases not possible to detect on agar plates, but it can be detected above background levels in a sensitive plate reader (Fig. 4.3). At 2000 generations, the other ten $\Delta luxOU$ M9-casein populations did not have detectable bioluminescence by either plate phenotypes or by plate reader measurements. These populations were phenotypically indistinguishable in both growth and bioluminescence from our control defector strains $\Delta luxR$, luxO D47E, and SN (APPENDIX Table A.1).

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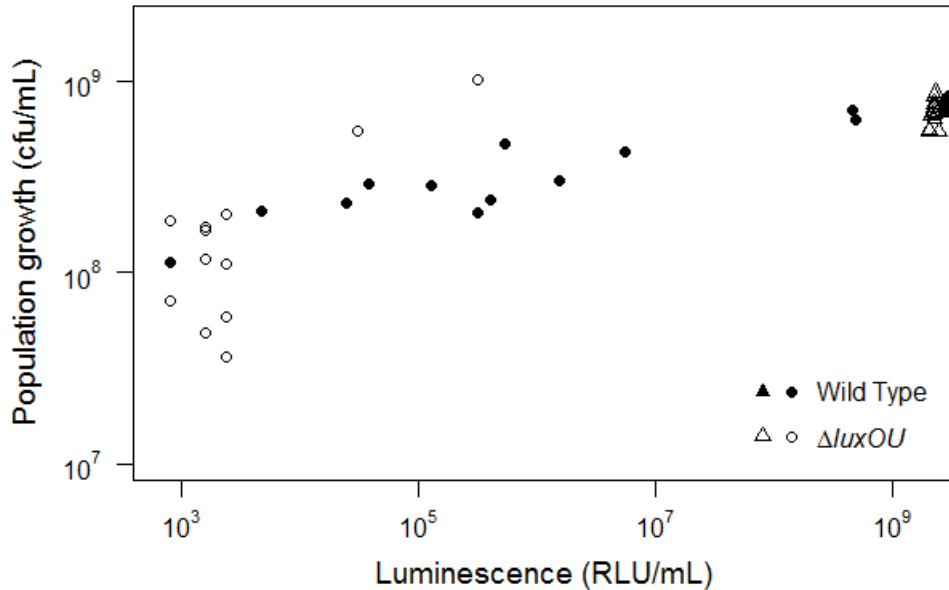
Figure 4.3: **Growth and bioluminescence of _V. harveyi_ populations evolved in M9-casein media.** Populations were sampled at generations 10 and 2000 and measured for growth by viable cell counting and for bioluminescence in a plate reader. Each point corresponds to a single population. M9-casein populations (CAS01-CAS24) are displayed. Triangles represent samples from generation 10 and circles represent samples from generation 2000.

Meanwhile, as previously mentioned, eleven of the twelve WT M9-casein populations still contained detectable levels of luminescent cooperators at the end of the experiments (Fig. 4.2, lower right panel, Fig. 4.3). A single population completely lost detectable bioluminescent cooperators fairly early in the experiment (CAS09), although one other appeared to nearly have lost cooperators at 2000 generations as well (CAS07) (Fig. 4.2, lower right panel). However, though the other WT populations maintain cooperators, they display a diverse range of population-level bioluminescence at 2000 generations (Fig. 4.3, closed circles). There was also a strong positive relationship between population bioluminescence, which we take as a measure of QS activity, and growth outcomes for WT M9-casein populations (correlation between growth and luminescence in WT M9-casein populations at generation 2000

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= 0.9, Fig. 4.3, closed circles). This was not the case for $\Delta luxOU$ M9-casein populations, where most populations exhibited low bioluminescence and growth yield reflective of the evolved defectors that overtook those populations.

The effects seen in M9-CAA populations were different than those seen in M9-casein, especially in terms how defector impact depended on genotypic background. For either genotype, QS did not contribute as much to growth in the M9-CAA environment, and defectors increased in frequency in most populations of both treatment groups (Fig. 4.2, upper left panel). The positive relationship between QS activity and growth yield in M9-CAA is also absent (data not shown), further suggesting that QS activity does not bolster growth in this environment to the extent it does in M9-casein. While QS did not impact growth yields as strongly in M9-CAA, it should be noted that M9-CAA populations did experience drops in growth yields during the experiment, again not as severely as in the M9-casein populations (Fig. 4.2, upper right panel). This is predicted to be due to slight (2-3x) drops in productivity that QS-defectors experience in M9-CAA and because selection is stronger upon growth rates than growth yields.

4.3.4 Evolved non-luminescent defectors possess multiple phenotypic differences from ancestral cooperator strains

As noted, the rise of non-luminescent defectors in M9-casein populations led to large drops in productivity (Fig. 4.2, upper right panel). Because bioluminescence and protease production are similarly induced at high cell density, and based upon previous measurements of protease production and bioluminescence in defectors (Fig. 2.2 in Chapter 2), we hypothesized that the decreased population productivity resulted because these defectors also exhib-

ited low to non-measurable levels of protease production, similar to our engineered $\Delta luxR$ strain. However, it is possible that bioluminescence evolved independently from protease production. To test this possibility, single clones from each of the different bioluminescence phenotypic classes (bright, dim, dark) present in a given population at generation 870 of the experiment were chosen from all 48 experimental populations based on their colony bioluminescent phenotype. The clones were subsequently grown in M9-casein media, and measured for protease activity. These measurements of protease production taken after cultures were grown 24 hours to allow them to reach their carrying capacity in M9-casein did indeed confirm that all non-luminescent clones tested produced similar low levels of protease to the $\Delta luxR$ defector control (Fig. 4.4). The low levels of protease production also confirmed that they are in fact general QS-defectors, and not only harboring mutations specific to bioluminescence. We interpret these results to mean that defectors displaying corresponding drops in both properties are due to genetic changes affecting the QS regulatory machinery rather than multiple separate mutations specific to both individual processes. Though not specifically measured here, signal measurements of other control strains showed that QS defectors tend to also produce less signal molecules, and this trend could also be true for these evolved defectors (Chapter 2 Fig. 2.3).

To determine if varying levels of protease activity impacted growth, we characterized growth phenotypes of theses clones in M9-casein when grown in monocultures and compared it to their protease production phenotypes (Fig. 4.4, lower panel). We also measured several replicates of the control genotypes WT, $\Delta luxOU$ and $\Delta luxR$ for comparison. We found that there was a saturating relationship between the two properties, where clones that produced more protease also grew better in M9-casein media. A certain amount of extracellular protease production, measuring at around 50 nM present in culture supernatants, corresponded
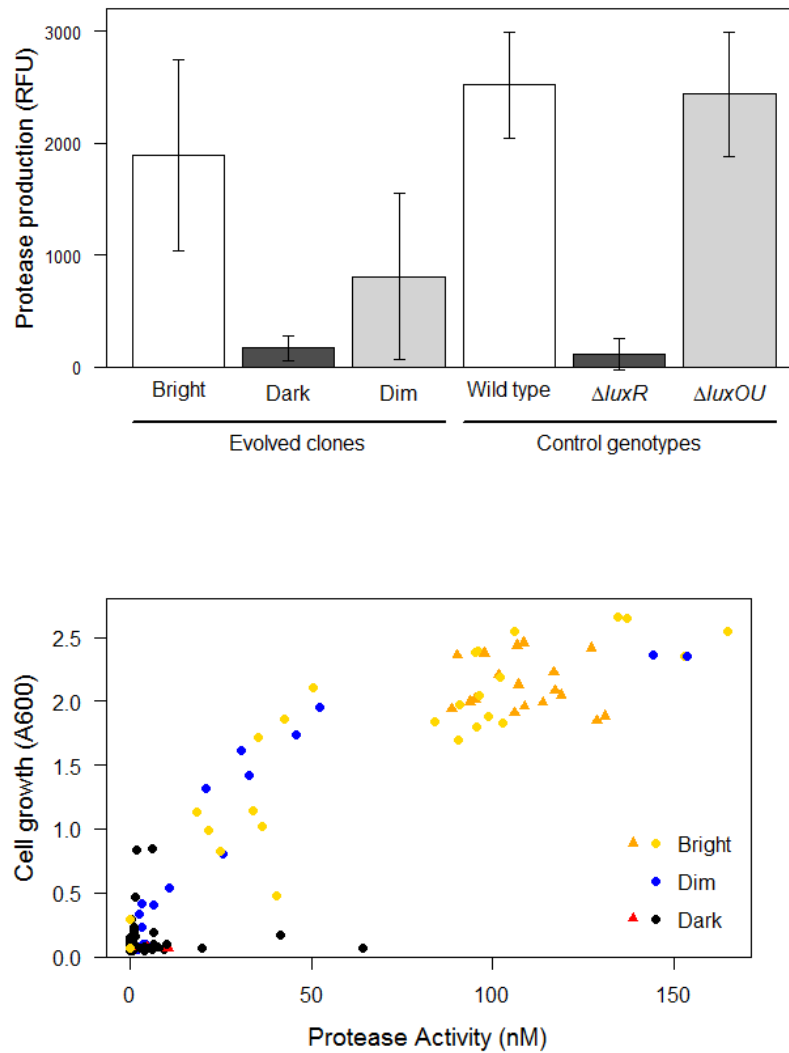
Figure 4.4: **Examination of growth and protease production of evolved clones in M9-casein.** Clones from different phenotypic classes (gauged by colony bioluminescence) were taken from experimental populations at generation 870. The clones were grown for 24 hours in M9-casein. Growth was measured by absorbance at 600 nm in a spectrophotometer. Protease production was estimated using a FITC-casein cleavage assay with culture supernatants. Fluorescence measurements were binned by phenotypic class (top panel), or normalized to a trypsin standard curve and plotted against growth measurements (bottom panel.) Error bars in top panel represent 95% error bars. In bottom panel, circles represent evolved clones and triangles represent control strains. The non-luminescent control strain used was $\Delta luxR$ and luminescent control strains included both WT and $\Delta luxOU$.

to nearly maximum growth yield at 24 hours, and beyond that level of production additional benefits in growth yield were minimal. As expected, the $\Delta luxR$ strain (Fig. 4.4, red triangles) displayed relatively low levels of both quantities and the ancestral cooperator strains (Fig. 4.4, orange triangles) both possessed high levels of protease production and growth in M9-casein media. Likewise, all evolved defector clones assayed had lower levels of growth in the media and produced lower levels of protease. Interestingly, evolved clones characterized as brights or dims were found to have a wide range of growth and protease phenotypes. Some had similar levels of protease production and growth as the ancestral cooperator strains, some displayed even higher levels than the ancestral cooperator strains, and others had decreased capacities for both (Fig. 4.4, lower panel). Those that had decreased levels of each seemed to produce a smaller fraction of protease production but experience the majority of overall growth in comparison with the yields of the ancestor strains. The clones that exhibited this combination of phenotypes were mainly clones evolved from the WT M9-casein treatment, and possessed a large proportion of dim clones.

4.4   Discussion

In Chapter 2, we showed that QS prevents defector invasion on short time scales. In this chapter, I extended these studies to address whether QS stabilizes cooperation in the presence of exogenous AI and on longer evolutionary time scales. These experiments further addressed questions about the links between QS and the cooperative behaviors that it often regulates. While the WT strain of *V. harveyi* has previously been seen to resist invasion by the $\Delta luxR$ defector, that particular strain was also observed to have pleiotropic costs and may have been constrained in its ability to further adapt to the M9-casein environment (Chapter 2). Even

when we attempted to coerce the WT strain into behaving like a constitutive cooperator by adding exogenous signal to the environment, we saw that WT was not outcompeted in the same fashion as $\Delta luxOU$, and the environment even elicited selection for novel intermediate phenotypes (Fig. 4.1).

We wondered, if defectors were given the opportunity to evolve *de novo*, rather than be genetically engineered and forcibly introduced into experimental populations, would they evolve and would this occur along the same paths and impact the same mutational targets? Although cooperation is a theoretical challenge for evolutionary biology to explain (Hamilton, 1963, 1964, West et al., 2007), we considered the possibility that defectors would be unlikely to evolve in the presence of WT due to the results found in Chapter 2. This prediction was only partly correct, as defectors did evolve and increase in the presence of WT, but in reduced proportion compared to the $\Delta luxOU$ ancestor (Fig. 4.2, upper left panel). All $\Delta luxOU$ populations were rapidly invaded and ultimately swept by evolved variants with reduced QS activity (Fig. 4.2, lower left panel). In ten of the twelve replicate lines, these defectors were indistinguishable in phenotype from the engineered defector strains used as controls. However, in two other lineages (CAS16 and CAS19), after appearing to be completely swept by dark defectors, extremely dim variants emerged and proceeded to dominate the population. (Fig. 4.3). It will be interesting to determine the genetic nature of these defectors. This broad trend of sweeps of the UC by evolved defectors corroborates past competition results indicating that $\Delta luxOU$ is much less adept at preventing cheating by defection in the M9-casein environment than the WT strain. Our results also predict that the dim variants that evolved were fitter against the WT ancestor than the $\Delta luxR$ strain, as they were able to invade WT populations in many cases while the $\Delta luxR$ strain could not (Chapter 2 results, Fig. 4.1, upper left panel).

The level of QS participation of the experimental populations measured by population bioluminescence impacted growth the most in the WT M9-casein treatment evolved for 2000 generations (Fig. 4.3). It was also the case that the two $\Delta luxOU$ M9-casein populations that exhibited stronger QS activity grew much better than the other ten replicates, which measured low in both bioluminescence and growth in M9-casein. The trend between QS participation and growth yields was not observed in the asocial M9-CAA condition. Because of this relationship between our phenotypes of interest and the stronger dynamic trends of defector evolution, we focused more of our analyses on lineages passaged in the M9-casein environment. Specifically, the strong differences observed between defector dynamics in the two genotypic backgrounds in M9-casein support our prior claim that functioning QS can have strong impacts on stability of cooperative behaviors over timescales ranging to 2000 generations, and likely much further. These results were observed in well-mixed populations in which spatial structural was unlikely (biofilms were not observed) and migration was not possible, variables that would be more commonplace in natural environments and could further extend this stabilizing effect QS has on cooperative behaviors.

When defectors invaded in M9-casein treatments, population productivity was sacrificed (Fig. 4.2, right panels), but for two different reasons. In $\Delta luxOU$ populations, this decreased productivity is because cooperator genotypes were replaced by defector types with lower carrying capacities in this environment. In WT populations, the density decreased to below levels needed to fully induce QS (Fig. 4.2, right panels). A drop of density in the WT parent grown in M9-casein should have a two-fold effect; it should a) lead to less activation of QS due to lower densities, and b) we have previously seen that in many cases QS-defectors also produce lower per capita signal levels due to regulatory feedbacks (Ng and Bassler, 2009, Chapter 2 Fig. 2.3). The capacity to turn off QS in the appropriate conditions, particularly

in response to drops in density and/or rises in defector frequencies provides the ability for WT to withstand even evolved defectors from overtaking a population. However, many WT M9-casein lineages did experience some gains in population productivity over the remainder of the experiment (Fig. 4.2, lower right panel). This could be due to further beneficial mutations by both cooperators and defectors within these populations, and the extent to which either occurs will be a question of interest moving forward.

While we don't expect complete QS defectors such as $\Delta luxR$ to coerce QS induction of WT cooperators due to their lower overall levels of signal production (Haas, 2006, Czárán and Hoekstra, 2009), we were able to artificially simulate this scenario by adding exogenous signal molecules at high concentrations to M9-casein environments. In this way, we attempted to recapitulate the constitutive QS phenotype observed from the unconditional $\Delta luxOU$ strain, which expresses QS-associated genes regardless of actual signal concentrations, in the WT background (Waters and Bassler, 2006). Interestingly, even when we do this, the invasion dynamics of $\Delta luxR$ into WT-majority populations are quite different from those seen against the UC strain (Fig. 4.1). Though we supplemented the two autoinducers responsible for the majority of QS activation (autoinducers 1 and 2, HAI-1 and AI-2), it appears that $\Delta luxR$ only begins to invade after 2 days when populations reach their carrying capacity before the 24-hour transfer point. Even more interesting, once $\Delta luxR$ reaches a sizable portion of the population, defectors evolved from the WT background were rapidly selected for and led to a decrease in the frequency of $\Delta luxR$ in the populations (Fig. 4.1, upper left panel). Many of these evolved variants tested harbored intermediate phenotypes with regard to both bioluminescence (indicative of decreased QS activity) and growth in M9-casein, mostly likely due to decreases in protease production (Fig. 4.1, lower panels). The fact that these variants did not cause the same population productivity drop as $\Delta luxR$, even when these types did

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become the major population member also suggests that they harbor an intermediate QS phenotype, rather than being QS-defectors to the same extent as $\Delta luxR$ (Fig. 4.1, top panels). This suggests that even if there is a decrease in the levels of investment into QS expended by these strains, those levels are still markedly higher than full QS-defector strains.

There were also a large number of dim variants that evolved during the long-term evolution experiment, and these were especially enriched in the WT M9-casein treatment, with 8 of those 12 lineages possessing some notable dimming or harboring a dim type within these populations by generation 2000 of the experiment. This effect was substantially diminished among $\Delta luxOU$ lineages, with only 2 lineages displaying any bioluminescence, and this bioluminescence was extremely difficult to visually detect. Growth in M9-CAA also appears to have diminished this effect, with only 1 $\Delta luxOU$ and 5 WT populations displaying any dim variants, two of the WT populations in which again the bioluminescence was inconsistently observable.

Comparing the growth and protease production of evolved clones from these populations was informative about the relationship between QS investment and growth in M9-casein. When plotted against each other, it forms a non-linear saturating curve (Fig. 4.4, lower panel). This curve is reminiscent of descriptions of Michaelis-Menton or Monod dynamics for resources well described elsewhere (Tilman, 1981). The edge where many dim clones are located is in the area of the curve where increases in growth obtained as a function of protease production begins to decrease. This curve is also resemblant of a Pareto front (Satterwhite and Cooper, 2015, Shoval et al., 2012) and suggests that the evolved WT lineages, especially the dims, were selected for types that were more optimized to the experimental environment through decreased investment in protease production, likely through diminished QS investment, but without completely losing all of the benefits of QS, as full QS-defectors do. In this

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way, evolved dims with reduced but not fully-eliminated QS activity should be able to better conserve resources, withstand defector exploitation, and maximize growth rates in M9-casein media compared to their more cooperative ancestors. While this resembles defection, and the dim variants could potentially exploit more aggressively cooperating genotypes in similar fashion to the outcome between WT and UC strains (Chapter 2), this principally should act as a process of optimization to the selection regime, where populations that best balance pressures to maximize growth rates, minimize exploitation (namely, maximizing inclusive fitness, Hamilton, 1964, West et al., 2012), and maximize the likelihood to gain additional beneficial mutations are likely to perform well over time. Reducing investment in costly cellular processes such as protease production would aid in both increasing cellular growth rates and in minimizing exploitation by cheaters, but by not completely eliminating this production, it also minimizes losses in population yields, and thus increases the likelihood of the population acquiring more beneficial mutations (Sniegowski and Gerrish, 2010). In this way, dims may be expected to evolve and take over all populations grown in M9-casein if given sufficient time.

In sum, I have presented further evidence that QS can sustain cooperative behaviors it regulates in mixed populations with defectors over longer evolutionary time scales. We saw that defectors evolved in all genotypic backgrounds and experimental environments tested, although they were most strongly selected for in M9-casein media and in the $\Delta luxOU$ genetic background. While defectors do evolve in the WT lineages, their paths are more variable and they are much less likely to sweep their resident populations, and the general relationship between QS activity and growth productivity of the populations is maintained. Some of this resistance to exploitation must be related to our previous results showing that QS allows cells to maximize growth rates at low densities and postpone production of costly secreted

products until signal and density levels are high enough to help mediate that cost. However, we also saw that in many WT M9-casein lineages, while QS was maintained (measured by luminescence proxy), the apparent investment levels in QS decreased through evolution of variants that exhibit reductions in bioluminescence, protease production, and growth yield phenotypes (Fig. 4.2, Fig. 4.3). These dim variants may be optimized to the M9-casein experimental environment. Importantly, though, the dim variants still maintain the cooperative behavior and could serve as a reservoir for this phenotype over time (Pollak et al., 2016). Overall, while defection is the stable strategy in M9-casein in the presence of $\Delta luxOU$, in the WT M9-casein treatment mixed populations of cooperators and defectors were the dominant trend, and QS and the cooperative traits it regulates decreased in both function-level and frequency in the populations, but were not wholly eliminated, again demonstrating how functional QS can stabilize cooperative behaviors.

Chapter 5

Quorum sensing in *Vibrio harveyi* and growth promoted by low cell density regulation

Preface

Previous chapters in this dissertation have primarily focused on the impacts of *Vibrio* QS on the stability of cooperative behaviors. This was mainly limited to studies of extracellular protease production, a behavior that is positively regulated by QS and is maximally produced at higher cell densities. However, *V. harveyi* QS regulates hundreds of genes in the genome, many of which are not positively regulated by QS. This in in contrast to other QS systems, like *Pseudomonas aeruginosa*, in which the vast majority of genes regulated by QS are induced at high cell density. Many of these genes represent intracellular private goods, but interestingly there are also genes that contribute to the production of public goods that are negatively regulated by QS (i.e. expressed at low cell density). Given some of these trends, we wondered if there are environmental conditions that would allow QS-defective strains to outperform QS-functional strains.

5.1 Introduction

A number of studies have show that QS effectively acts to maximize fitness benefits at high density, efficiently transition between different density states, and in many cases preferentially regulates secreted products (Darch et al., 2012, Hense and Schuster, 2015, Heilmann et al., 2015, Popat et al., 2015a, Schluter et al., 2015). Secreted products are often cheatable public goods. However, it is also true that the *V. harveyi* QS system has significant effects on the regulation of hundreds of genes, estimates ranging as high as 10% of the genome (Schuster and Greenberg, 2006, van Kessel et al., 2013r). This set of genes is wide-spanning in predicted functions, and only a small portion of these genes affect public goods production.

On the whole, it appears that QS in *Vibrios* regulate more genes at high densities than at low densities, as the low density master regulator *aphA* regulates far fewer genes than the high cell density master regulator *luxR* (Rutherford et al., 2011, van Kessel et al., 2013). But both contribute to large-scale differences in regulation at low and high cell densities. The previous three chapters examined interactions with respect to a single limiting carbon source, casein, where growth is positively impacted by the ability to turn on the QS circuit. But because many genes are downregulated by QS, we hypothesized that we could identify limiting carbon sources where growth is negatively correlated with a strain's ability to turn on the QS circuit. This net effect could occur by repressing a behavior at high densities or by activating it at low densities. In environments with carbon sources that required these downregulated behaviors to be utilized, we hypothesize that locked low-cell-density mutants would actually outperform locked high-cell-density mutants in monoculture, and if growth depends on public goods downregulated by QS, then there may be cheating upon QS-

defective strains by QS-functional strains, the reverse of what we have reported in Chapters 2-4. To probe this hypothesis, we examined a number of different carbon sources to see whether growth was affected by the ability to activate the QS circuit.

5.2   Materials and Methods

Cultures of *Vibrio harveyi* were grown M9 liquid media with a range of single carbon sources. These were grown either in Biolog 96-well format plates or in borosilicate glass test tubes at 30 degrees Celsius with 250 rpm on an orbital shaker. DNA provided for growth was herring sperm DNA (Invitrogen). At daily intervals, growth in 96-well plates were measured in a SpectraMax M5 plate reader at and growth in test tubes was enumerated by measuring optical densities in a Beckman Coulter spectrometer or by viable cell counts on agar plates.

5.3   Results

Many carbon sources were examined, guided by those available (via Biolog plates) and with reasoning led by known downregulated targets from results of microarray and RNAseq studies (Rutherford et al., 2011, van Kessel et al., 2013). We were able to successfully identify several carbon sources that showed enhanced growth for QS-defective strains over QS-functional strains. This pattern for such carbon sources was that QS-defective strains grew to a maximum level among tested strains by 24 hours of growth, WT grew slightly worse to equally well as the QS-defective strain, and the locked high-cell-density $\Delta luxOU$ mutant grew significantly worse than all other strains tested. The specific carbon sources that met this outcome included glycerol (Fig. 5.1, top panel), mannitol (Fig. 5.1, bottom panel), and glutamate (Fig. 5.2). Several genes related to glycerol metabolism are downregulated by

Figure 5.1: **Growth of *V. harveyi* strains in M9 media with different sugar alcohols as the limiting carbon source.** Strains were grown in M9 media with 0.5% glycerol (top panel) or mannitol (bottom panel) supplemented as the sole carbon source for 24 hours. Cell growth was measured in a spectrophotometer by absorbance of the cultures at 600 nm. Error bars represent 95% confidence intervals on N=3 biological replicates.

V. harveyi QS, consistent with our observed result (van Kessel et al., 2013). Interestingly though, genes related to mannitol were predicted to be upregulated by QS, but growth on mannitol still appeared to be most favored by QS-defective strains. Mannitol repressor protein is positively regulated by QS, which could explain this outcome (van Kessel et al., 2013).

Glutamate is also an interesting case, because though it was provided as the sole carbon source, it can also be used by cells as a nitrogen source. Glutamate is also used to generate other amino acids in the cell. In fact, it has been estimated that as much as 85% of cellular nitrogen is derived from glutamate, making it an incredibly important nutrient source (Magasanik, 1993). This is because the most important pathway of assimilation is the ubiquitous glutamine synthetase/glutamate synthetase (GS/GOGAT) pathway (Merrick and Edwards, 1995). Together, glutamate and ammonia, the other nitrogen source present in the M9 media used for growth, are combined to form glutamine by the enzyme glutamine synthetase (GS). This compound is then combined with alpha-keto-glutarate and NADPH to form two molecules of glutamate by glutamate synthetase (GOGAT). Alternatively, because of the large ammonia concentrations present that may provide a feedback on this circuit preventing effective processing of glutamate, this could have negative impacts on using glutamate as a carbon source. It has been suggested elsewhere that QS could act as a control point for cells effectively processing nitrogen from the environment (DeAngelis et al., 2008). Consistent with expectations based on our analyses of *Vibrio harveyi* protease production, in BIOLOG plates we found that QS-defectors were universally impaired for growth on di-peptides as the limiting nitrogen source in comparison with either cooperator strain. There were two exceptions where $\Delta luxOU$ was impaired more than the other two strains, glycine-aspartate and glycine-glutamate. The constitutive $\Delta luxOU$ appeared
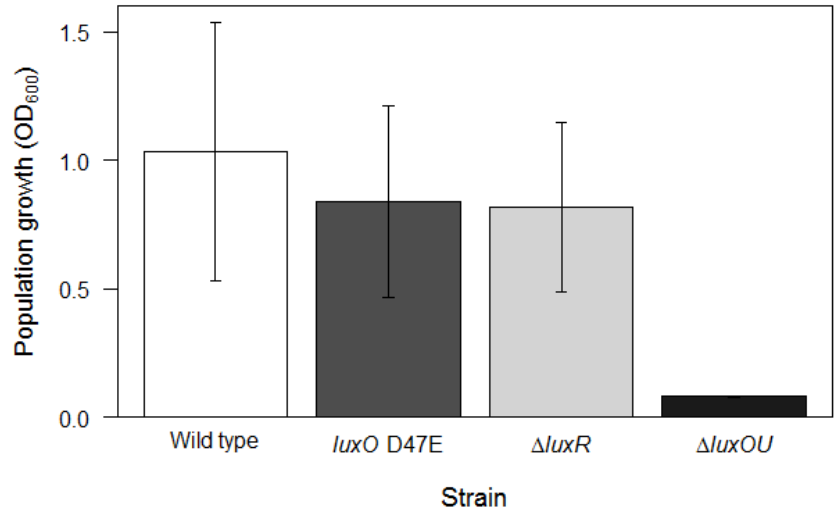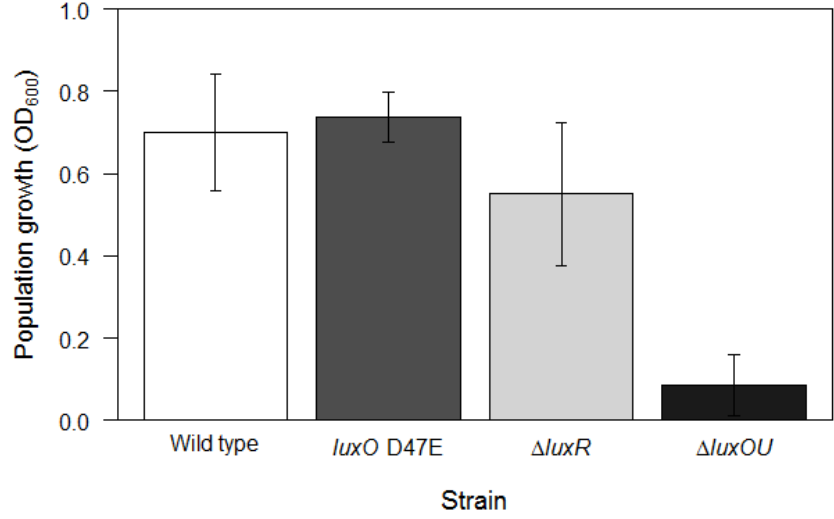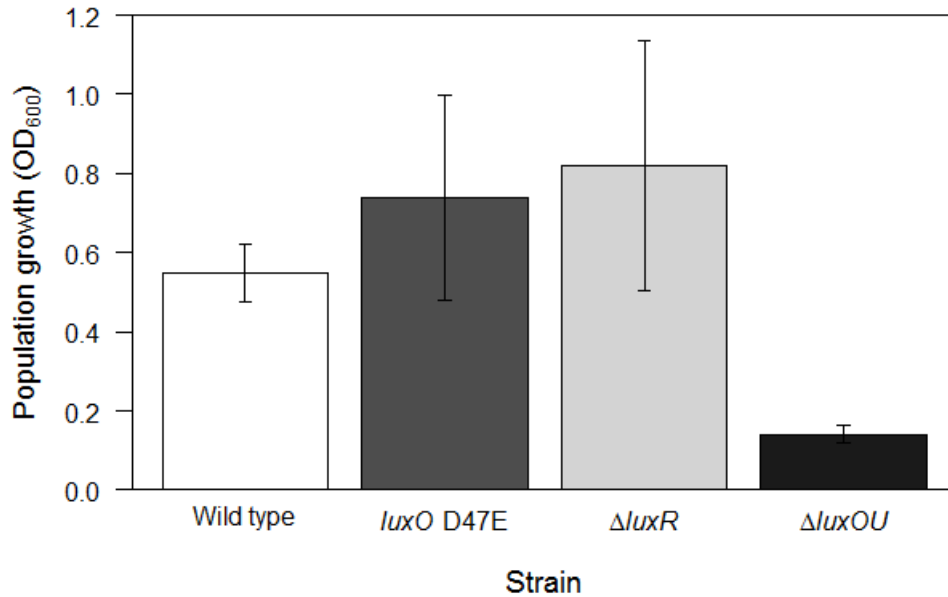
Figure 5.2: **Growth of *V. harveyi* strains in M9 media with glutamate as the limiting carbon source.** Strains were grown in M9 media with 0.5% glutamate supplemented as the sole carbon source for 24 hours. Cell growth was measured in a spectrophotometer by absorbance of the cultures at 600 nm. Error bars represent 95% confidence intervals on N=3 biological replicates.

inhibited on a number of nitrogen sources relevant to the GS/GOGAT cycle, including L-glutamine and L-glutamate. Beyond these compounds, $\Delta luxOU$ was also less competent than the *luxO* D47E defector strain when inorganic salts like nitrate or nitrite were included as the limiting nitrogen source, suggesting that $\Delta luxOU$ may experience some defects in processing nitrogen. This also included to a lesser extent ammonia, the limiting nitrogen source in M9 media. Lastly, $\Delta luxOU$ was deficient for growth on the DNA-related compounds adenine and guanosine. Because it was suggested that DNase production was also downregulated by QS (Blokesch and Schoolnik, 2008), we also examined growth on DNA as a sole phosphorus source. With glucose held constant as the carbon source, phosphate was provided to cultures of cells from inorganic phosphate salts or organic (DNA) sources. It

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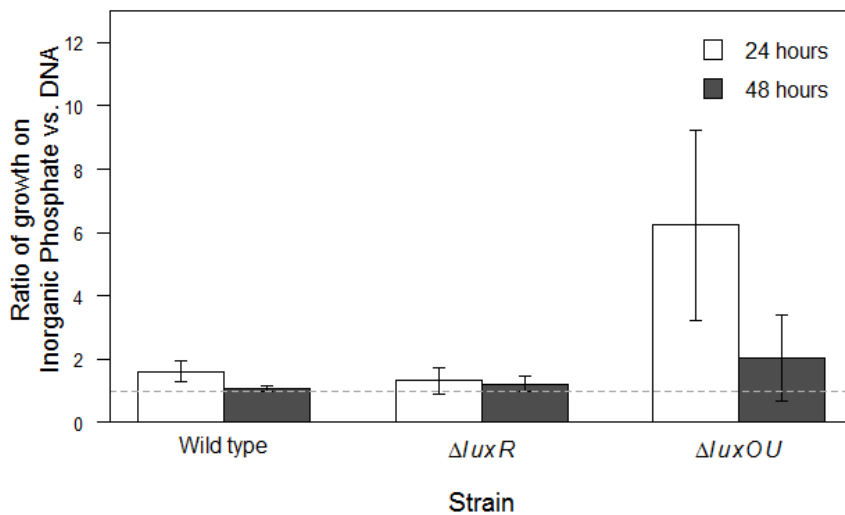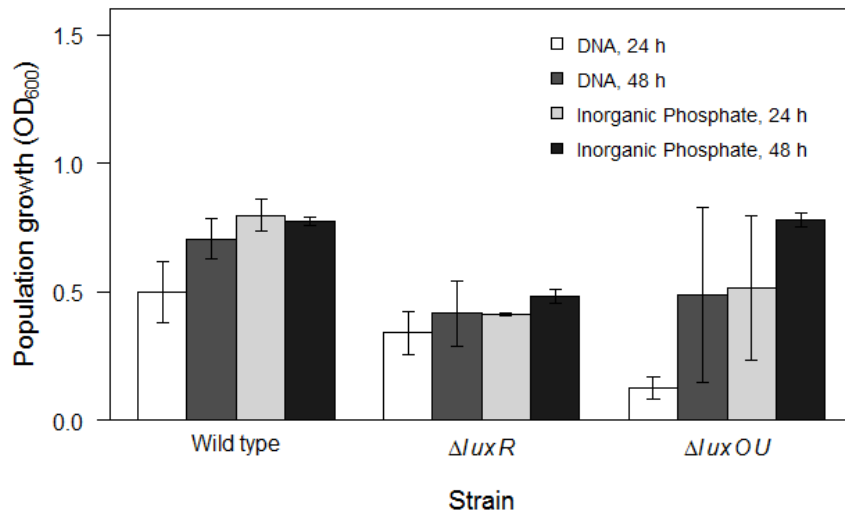
Figure 5.3: **Growth of *V. harveyi* strains in M9-glucose media supplemented with different phosphorus sources.** Strains were grown in M9 media with 0.5% glucose media supplemented with DNA or inorganic phosphate salts provided as the phosphorus source for 24 hours. Cell growth was measured in a spectrophotometer by absorbance of the cultures, and displayed as the optical density at 600 nm (top panel) or as the ratio of absorbances between the two conditions (growth with organic phosphate/growth with DNA, bottom panel). Error bars represent 95% confidence intervals on N=3 biological replicates.
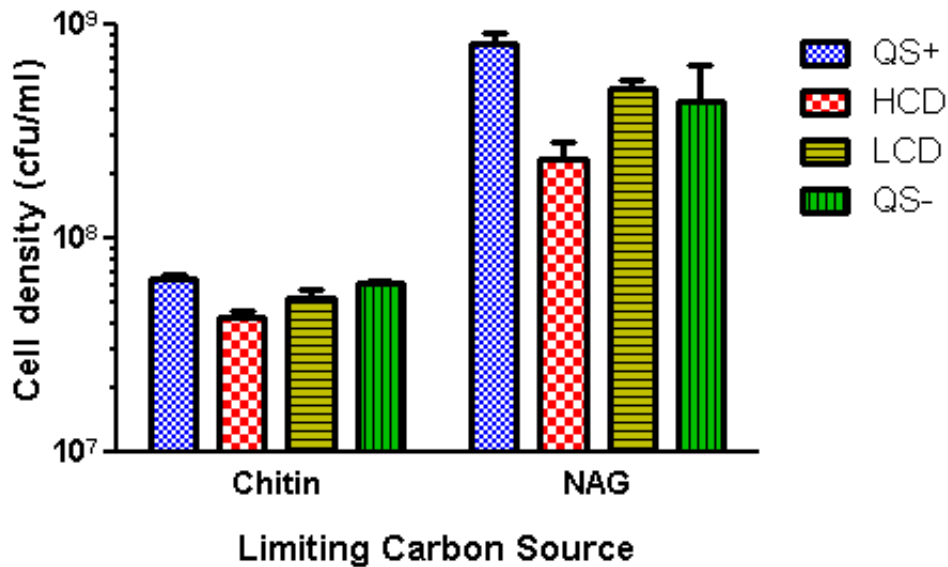
Figure 5.4: **Growth of *V. harveyi* strains in M9 media with chitin or NAG supplemented as the sole carbon source.** Strains were grown in M9 media with 0.5% (w/v) chitin or NAG supplemented as the sole carbon source for 24 hours. Cell growth was measured by viable cell counts on petri plates. *V. harveyi* strains used were WT ('QS+'), $\Delta luxOU$ ('HCD'), *luxO* D47E ('LCD'), and $\Delta luxR$ ('QS-'). Error bars represent 95% confidence intervals on N=3 biological replicates.

was observed that $\Delta luxOU$ experienced a definite delay in growth when provided DNA as

the sole phosphorus source (Fig. 5.3). This is presumably due to the negative regulation of

two extracellular DNases, *dns* and *xds*, which could be thought of as public goods. This is

interesting because if $\Delta luxOU$ is deficient in the production of these public goods, it lays

out the potential scenario where in pairwise competitions, $\Delta luxOU$ could cheat other strains

when grown on DNA as a phosphorus source, a stark change from the outcome observed in

M9-casein. In similar fashion chitinases, which can also qualify as public goods (Drescher

et al., 2014), are negatively regulated by QS as well (Defoirdt et al., 2010). Chitin is an ex-

tremely common biotic polymer found in aquatic environments, consisting of many units of

N-acetyl-D-glucosamine (or 'NAG'). Growth on chitin granules and on NAG were enhanced

in QS-defective strains, and most notably $\Delta luxOU$ grew worse than any of the strains tested

in these conditions (Fig. 5.4).

5.4 Discussion

The results of this chapter reaffirm that QS is a global regulatory system that controls many behaviors beyond just the regulation of public goods. We identified environments and nutrient sources in which QS activation is inhibitory to reaching maximum growth in that environment. This negative regulation must be especially strong in situations where WT, which we think behaves as a density generalist that can acclimate and perform well in most environments (Fig. 2.5; unpublished data, Will Soto, Waters laboratory), is inhibited for growth as was seen in M9-glutamate. It is likely, and perhaps unsurprising, that a gene circuit that impacts the expression of so many genes from the genome would have strong impacts on metabolism (Davenport et al., 2015), and as we saw the nutrient source revealing the hypothesized growth patterns were diverse in form. My results suggest that the low-cell-density response is beneficial for nitrogen metabolism and catabolism of several nitrogen-rich polymers. This study also reveals how the particular context in which a limiting nutrient is used can be important. For instance, in earlier chapters, we focused on the dynamics occurring when casein is used as the limiting carbon source in an environment. It is possible that the use of casein as the single nitrogen source or both a limiting carbon and nitrogen source would shift the types of growth outcomes that occur.

Of greatest interest to emerge from this work is the idea that QS might be impacting nitrogen metabolism, something that has not been directly examined in other chapters of this thesis. It appears that the locked high cell density strain grows worse than WT or QS-defector strains when grown with major components of the GS/GOGAT cycle as the

limiting nitrogen source: glutamine, glutamate, ammonia. This strain also appears to grow worse on nitrate and nitrite, suggesting overall impairment for nitrogen processing in the cell. Independent experiments showed that growth on glucose with casein as the limiting nitrogen source led to yield decreases for all strains tested except $\Delta luxOU$, suggesting that some decoupling of feedbacks between carbon and nitrogen processing occurs in this strain.

Growth and competition experiments between different QS strains growing on chitin were performed but not extensively characterized. Growth on granular chitin presents complicating factors, with more special restrictions imposed in the growth media and slower growth rates overall for all strains due to the need to first adhere to a chitin particle. The general growth pattern appears similar on its subunit NAG, with the UC strain performing the poorest in monoculture. The fact that $\Delta luxOU$ grows poorly on this subunit suggests that it would not be able to cheat on other strains in the presence of this limiting nutrient source. However, this assay evaluates growth productivity more than growth rate, and only direct competitions will reveal whether cheating dynamics occur.

Extracellular DNA is unique among these compounds because it can be utilized for carbon, nitrogen, or phosphorus. We have so far only conducted preliminary analyses with DNA as the sole phosphorus source. The results were striking supporting the idea that the low-cell-density state aids in the breakdown of polymeric DNA for uptake through DNase production, and that DNase could be a cheatable public good produced by low cell density cells that could be cheated by cells in a high cell density state. In this respect, it will be interesting to see what competitive outcomes result when $\Delta luxOU$ is competed against a QS-defector on limiting DNA. Another important control is to test growth on nucleotides rather than a DNA polymer to evaluate whether strains like $\Delta luxOU$ are intrinsically restricted for growth on these types of nutrients. Because $\Delta luxOU$ did recover somewhat in growth on

DNA by 48 hours, it is predicted that this is an issue of DNase production rate rather than a defect for nutrient uptake.

Impacts on growth by the negative regulation of QS in *Vibrios* could have important implications for competition in natural environments, and in cases like the DNA growth results, shift our ideas about what genotypes will behave as cooperators and defectors, and this may shift given the resource environment. Many different polymers should be encountered in heterogeneous environments, and among the most prevalent that *Vibrio* encounter in marine environments is chitin, and we have shown that growth on chitin is reduced for strains with more active QS activity (Fig. 5.4). In total, these results may ultimately help explain the tremendous diversity observed among environmental bacteria like the *Vibrios* and help us work toward a framework for predicting which QS strategies will win out in various conditions.

Chapter 6

Concluding Remarks

6.1   Introduction

The overall aim of this dissertation has been to use *Vibrio* species as a model system to examine if and how chemical communication stabilizes cooperative social behaviors. My central hypothesis was that QS can act as a stabilizing mechanism for cooperative behavior. The findings published here extend our understanding of the roles that QS plays in cells, to not only mediate density-dependent changes in gene expression, but to also appropriately align growth strategies to the environment of a population and thereby provide fitness gains and stabilization of QS-controlled behaviors. My results do not imply that the role of QS is restricted to regulating cooperative behaviors, or even that it evolved for this purpose (though it does seem to be one of the strong advantages of having a QS system), but rather QS is likely affecting a wide range of behaviors, both public and private, in *Vibrios*.

6.2   Stabilization of *Vibrio* cooperative protease production by QS

6.2.1   Conclusions

As seen throughout Chapters 2 of this thesis, in the *Vibrios* examined, having functional QS positively impacts the stability of the cooperative behavior of protease production, even in conditions that would generally be predicted to disfavor the maintenance of cooperation. More generally, it also influences cellular fitness on a wide range of growth substrates. We demonstrated that the protease production of *V. harveyi* is potentially exploitable, but that the functional QS system of the wild type strain acts to minimize cheating in the social M9-

casein environment, in many instances completely abolishing it, by restraining expression of protease production until high density after other available nutrients have been depleted. Because QS is quite ubiquitous among bacteria (LaSarre and Federle, 2013, van Kessel et al., 2013) and large QS regulons are not uncommon (Chugani et al., 2012, Majerczyk et al., 2014, Schuster and Greenberg, 2006), we anticipate this result is generalizable. Broadly speaking, further examples of communication stabilizing cooperative behaviors in many other organisms will emerge, in many cases being a requirement for the stable existence of cooperation. While historically modelers in evolutionary game theory and other biological fields tended to think of cooperation and defection in terms of fixed strategies, this is likely not the common norm for the majority of natural systems (Axelrod, 2006, Doebeli et al., 2004, Killingback et al., 1999, Killingback and Doebeli, 2002). Especially when a given behavior is costly, using more information to guide expression of that behavior, such as surrounding cell density, would be beneficial to respond conditionally in a direct or probabilistic way (Anetzberger et al., 2009). In addition, we demonstrated for the first time that QS itself is an adaptable trait and can be modified, and not lost from populations, to decrease investment in cooperation to more appropriately grow and prevent defection over long term evolution. Therefore, I conclude that QS helps to stabilize cooperative behaviors over longer time scales (Bruger and Waters, 2015).

Most historical work done on QS and cooperation has shown that even WT strains were invaded by master regulator mutant strains analogous to the $\Delta luxR$ defector strain that we employed (Diggle et al., 2007, Katzianer et al., 2015, Sandoz et al., 2007). This makes our system unique with respect to how resistant it was to defector invasion. The particular reasons for this difference likely reflect the architecture of the QS system, and other details of the genome, and will be worthwhile for future examination. However, we predict that

more cases in which the benefits of possessing regulation by QS over an unregulated strategy will emerge.

While some modeling and simulation work have predicted that QS could diminish exploitation by cheaters (Allen et al., 2016, Schluter et al., 2016), this is the strongest empirical evidence to support this claim to date. Our experimental system is highly advantageous because: a) the genetics underlying have been well-described, b) the tightly QS-regulated bioluminescence gene expression serves as a built-in reporter of QS activity, and c) we have an engineered strain ($\Delta luxOU$) that activates QS constitutively, but not at maximum levels different from the QS-induced WT strain, which allows us to ask specific questions about the value of QS-regulation over the expression of its regulon. As a result, we were able to test our hypothesis in a manner that has not been possible in other experimental systems where appropriate constitutive strains have not been identified. As QS has also been reported to act as a reporter of kinship (Schluter et al., 2016), our results also agree with long-standing knowledge that kin selection can stabilize cooperation in other organisms such as social animals (Nowak, 2006, West et al., 2007, Hamilton, 1964). Because QS is very ubiquitous in bacteria, our results are very likely generalizable to other organisms and cooperative behaviors beyond protease production to explain their stability in natural communities. Furthermore, our results lend support to the idea that the information provided by communication is critical to stabilizing cooperative behaviors. In the future, it may come to be seen as the norm that not only are cooperative behaviors commonly regulated by communication systems such as QS, but that communication is required to make cooperation evolutionarily stable.

6.2.2   Future directions

A number of questions remain about the impacts of social and non-social behaviors in preventing cheating on the WT strain. This could be approached by performing fitness assays in a wide range of environments, from those where absolute fitness is heavily dependent on the QS system (e.g. M9-casein or M9-BSA) to those where fitness depends less on QS (e.g. M9-tryptone, M9-CAA, or LB), and along a gradient where these medias are mixed at different ratios. Particularly in M9-BSA, growth does not rapidly reach high densities and there appears to be very low levels of available nutrients without proteolysis so this may constitute an environment of extreme fitness dependence on a cooperative trait.

It would also be informative to do expression analysis such as RNAseq on the different strains used in these studies grown in M9-casein medium to see the differences in expression and whether they are limited to mainly cooperative good genes or extend broadly. Because we have observed pleiotropic effects when the QS system is mutated, the expectation is that there will be broad-reaching effects in gene expression changes that will also include many intracellular private goods and affect other central cellular processes such as metabolism, and it will be particularly interesting which impart the strongest effects in our experimental social conditions (M9-casein). Genes that specifically impact interactions between cells in a population are another potential target. For example, Type VI secretion has been studied in other bacteria, including other *Vibrio* species, but it has not been extensively studied in *V. harveyi* and could play a role in the interactions that occur between cooperators and defectors if QS at all affects the expression of the T6SS genes (Shao and Bassler, 2014, Majerczyk et al., 2016).

While QS provided stability to the maintenance of protease production over a range of

densities in closed-system batch cultures, the reasons for this ability to prevent defector invasion remain slightly unclear. Our understanding suggests that at low densities, the WT strain is able to achieve equivalent fitness against defectors because it saves on fitness costs by turning QS off. However, the null expectation was that defectors would still receive added benefits in the presence of cooperators, including WT, at high cell densities. Indeed, even in competitions with WT in M9-casein, there are hints that in some competition replicates the defector strain begins to do slightly better at higher densities (Fig. 2.5 from Chapter 2), though not enough so to make great gains before populations reach carrying capacity. Testing fitnesses in batch cultures at high cell density would be difficult because very few divisions could occur before population carrying capacities are reached. However, it would be informative to look at analogous competitions in the same media environments under fixed density conditions by use of either chemostats or turbidostats. My prediction for such experiments is that QS provides protection against cheating, especially at low densities where QS is turned off, and that this advantage would diminish as the density increases. Particularly if conditions could be established where the long term steady state density is above that required for QS-induction (e.g. $> 10^8$ CFU/mL), defectors may be able to invade even the WT strain. But again, this may be difficult to carry out, as invading defectors would be predicted to cause population-wide drops in cell densities in environments where growth yields depend on QS, such as in M9-casein.

We have examined fitness outcomes over a fairly wide range of frequencies, but over a narrower range of starting densities. The dynamics that occurred between cooperators and defectors at different starting population sizes were only just explored (Fig. 2.5). Expanding similar experiments to a wider swath of densities would allow more complete construction of a fitness landscape as a function of density and frequency between cooperator and defector

strains, and a deeper understanding of all the conditions in which one strategy or the other is favored. Again, these outcomes were mainly restricted to M9-casein media where fitness outcomes were closely linked to a social behavior; this could also be expanded to examine environments and growth sources which are predicted to depend less (or not at all) on social traits.

These experiments were conducted using a single substrate as a limiting carbon source. It would be interesting to see if similar dynamics persist when a cooperative trait affecting fitness is related to a limiting nitrogen or other nutrient source. Casein is a unique compound in this respect as it could be employed in a given media as a carbon or nitrogen source. These experimental regimes could be conducted in isolation, or even be oscillated over time to shift selection pressures, such as switching between casein as the limiting carbon source and the limiting nitrogen source by the addition of additional nutrients. Of interest to this point, while we observed similar growth outcomes between WT and the UC strains of *V. harveyi* in M9-casein media, preliminary results suggest differences in M9 media with glucose as a carbon source and casein provided as the limiting nitrogen source (Fig. 6.1), with UC achieving densities as much as an order of magnitude more than the WT (and more than when casein is the sole carbon source). Alternatively, QS-defectors do worse in this condition than in M9-casein. This suggests some interference between uptake of these nutrient sources under the low cell density regulation program that is alleviated with QS activation at higher densities. Our initial examination into the competitive outcomes between cooperators and defectors focused primarily on different QS genotypes in M9-casein media. An alternate approach is to place the expression of the public good, the extracellular protease, outside of the QS regulon. I attempted this experiment by overexpressing the metalloprotease gene VIBHAR_RS11785 in the WT strain to mimic the constitutive $\Delta luxOU$ mutant, but this
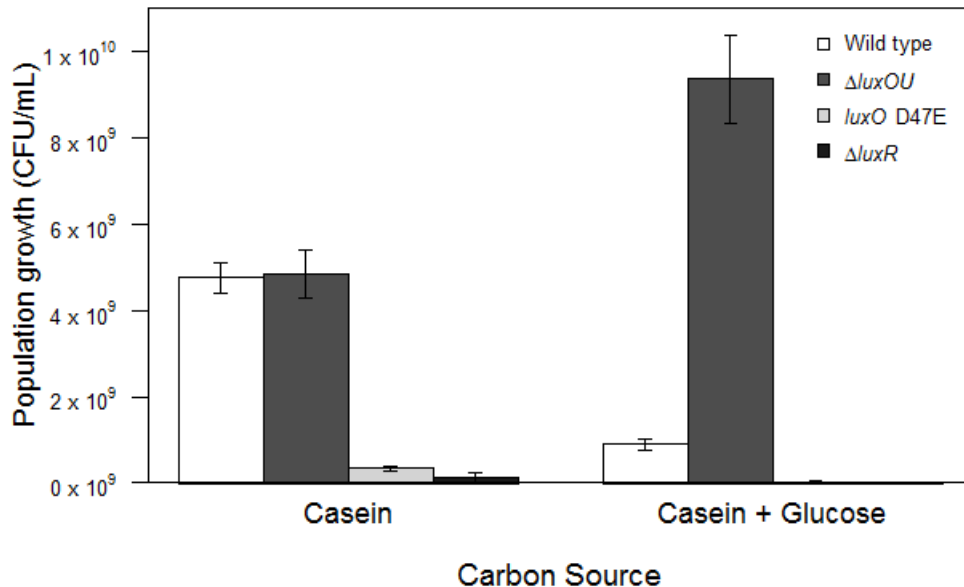
Figure 6.1: **Growth of *V. harveyi* strains in M9-casein media in the presence and absence of glucose.** Strains were grown in M9-casein media with or without 0.5% glucose supplemented for 24 hours and enumerated with viable cell counts on LB petri plates. Error bars represent 95% confidence intervals on N=3 biological replicates.

experiment led to severe growth deficiencies. An alternate strategy could move the protease gene to the genome under control of a constitutive promoter in order to avoid the selection and copy number issues associated with a plasmid. Additionally, it would be useful to create a deletion strain of VIBHAR_RS11785 to help isolate the fitness effects that are specifically due to protease production from those that are due to other genes under QS control. However, there is another protease (peptidase M4) encoded in the *V. harveyi* genome with high identity to the protease encoded by VIBHAR_RS11785 (hemagglutinin), which may contribute to the overall protease phenotype of a strain and also explain why we did not observe specific protease-negative variants in our evolution experiment.

While in most cases I had a readily distinguishable phenotype to differentiate between cooperators and defectors, such as bioluminescence or colony morphology, in other condi-

tions, there may be subtler or unobservable phenotypic differences in these strains, or stains may have similar starting phenotypes. One common example from this work is in differentiating WT and $\Delta luxOU$ *V. harveyi* strains. Because both capably activate QS at high densities, they cannot be differentiated by colony bioluminescence phenotype. Historically, these have been differentiable on plates due to the $\Delta luxOU$ strain harboring a chloramphenicol resistance cassette in its genome. During the course of my work, I have also introduced a *lacZ* cassette to a neutral genomic site via a Tn7 vector system which could also be used to distinguish these strains. This is useful because it simplifies identification of competing strains, but this system could be extended to more uses. Specifically, it would be most useful to tag different fluorescent protein markers using this same system, which would then allow us to differentiate large numbers of competing strains in high-throughput fashion via flow cytometry.

6.3   Simpson's Paradox in *Vibrio* bacteria

6.3.1   Conclusions

In Chapter 3 of this dissertation, we extended previous studies conducted in well-mixed liquid cultures to examine whether we could identify conditions where cooperation would be favored and could increase in the presence of defectors. It was previously demonstrated using modeling and synthetically engineered systems that a Simpson's paradox could lead to dynamics that favored cooperation at the global population level despite cooperators having lower relative fitnesses locally in the presence of defectors (Chuang et al., 2009, Cremer et al., 2012). This occurred because differences between cooperator and defector growth outcomes,

where cooperators experienced either increased growth rate or growth yield that resulted in enhanced growth for groups that contained higher proportions of cooperators.

These studies utilized a metapopulation approach that we adopted for use in our system. We found that when we applied stringent bottlenecks to our metapopulations, we observed increases of cooperator strains of both *V. harveyi* and *V. cholerae*, even for the unconditional cooperating strains used. This served as confirmation of the previous results, and extended them further by applying the approach to a naturally evolved QS system. These findings are in support of the well-established idea that assortment of cooperators from defectors can aid in the stabilization of cooperation (Nowak and May, 1992, Killingback and Doebeli, 1998).

It was striking that even the UC strains dramatically increased, even fixing in some experimental metapopulations, and led us to explore the range of conditions where both the WT and UC could invade. We next examined an opposite extreme that involved mixing and seeding large numbers of cooperators and defectors in a viscous media that would allow dispersal and possible separation between strains. Under this regime, we did indeed see strong differences between cooperator strains for both *Vibrio* species examined. In both cases, the UC performed worse against defectors than the corresponding WT strain, particularly when frequencies were measured from the bulk population. Both WT strains performed especially well on the colony edge, where they could be found to be the dominant fraction of cells present. When entire colonies were mixed and enumerated, the WT *V. cholerae* strain was found to have significantly increased from starting frequencies, demonstrating that we could recreate the outcome seen in metapopulations on these plates. The WT *V. harveyi* strain did not change from its starting frequency in the population, possibly as a result of the inability of *V. harveyi* strains to form robust biofilm structures that could lead to separation of competing strains.

6.3.2   Future Directions

While the investigation into the Simpson's paradox suggested that providing assortment between competing strains could enable an advantage for cooperators in metapopulations, this study also introduced other complications that could use further clarification. Specifically, while the experiments conducted in Chapter 2 used isolated populations with no migration between them, one of the key steps in the metapopulation approach incorporated in Chapter 3 was mixing of subpopulations, which introduced migration between lineages in addition to imposing bottlenecks. To separate these two phenomenon, these experiments could be completed with altered migration regimes, by either allowing decreased, non-global migration or imposing no migration by only bottlenecking within individual subpopulations and no mixing between. This last approach would have the drawback of likely limiting cooperator increases because the metapopulations were started with a majority of defectors and subpopulations that start with defectors are likely to remain dominated by defectors. Because of the genetic nature of the strains used in these studies (mutants have gene deletions), it is possible for cooperator strains to spontaneously mutate to a defector state, but in the absence of horizontal gene transfer it is impossible to get revertants of *V. harveyi* Δ*luxR* or *V. cholerae* Δ*hapR* mutant strains that switch to a cooperative state. Thus, the best approach to get at this question would be to use a range of sub-global migration rates.

We predict that the differences between *V. cholerae* and *V. harveyi* WT strains in motility plates was due in part to differences in the ability to form biofilm structures, and more rigorous testing of this prediction could be an informative experimental follow-up. The *V. harveyi* strain and derivatives used do not form detectable biofilms due to insertion sequences present in genes required for biofilm formation (unpublished data, Waters laboratory). To

examine this prediction, a follow-up set of competitions could be performed with *V. harveyi* strains that are expressing biofilm genes from *V. cholerae*, either on a plasmid or from a neutral site in the genome, which can be tagged using a Tn7-derived system (Choi et al., 2005). An alternative approach could be to use a *V. cholerae* defector strain that does not produce biofilms. The $\Delta hapR$ strain used is known to be a strong biofilm former (Hammer and Bassler, 2003). While biofilm formation did not seem to make a strong difference in this environment, as vpsL strains that do not form biofilms still performed well, all of these strains were competed against the $\Delta hapR$ defector. To get more deeply at this question, these experiments would have to be repeated with a $\Delta hapR$ $\Delta vpsL$ defector strain. This mutant strain does not have the distinctive rugose colony morphology of the $\Delta hapR$ strain, and currently we do not have a marker in place to differentiate it from the cooperator strains. In order to carry out these experiments, strains must first be engineered to harbor a gene that would allow us to distinguish competitors on petri plates, such as *lacZ* or the *V. harveyi lux* genes, or by using fluorescent protein encoding genes that would allow differentiation on an individual cell basis in a flow cytometer or at the bulk population level in a plate reader.

While we were able to observe a Simpson's paradox with the metapopulation approach used, and also recapitulated that result for *V. cholerae* on motility plates, the range of parameters for which this works is still not well defined. We correctly predicted and observed that while UC strains performed well in metapopulations, they did much worse than WT strains in the large mixed populations initiated on motility plates. However, at which population size the outcomes begin to differ between WT and UC strains is still unknown. To make predictions about where this might occur, it would be advantageous to initially examine this in a system that is easier to manipulate and a wider parameter range can more easily be investigated. We propose carrying out analogous experiments using the *in silico* Avida

evolutionary platform to better understand where the differences between quorum sensing and unconditionally cooperating strategies may occur and be most important for competitive outcomes.

6.4   Evolutionary outcomes of *Vibrio harveyi*

6.4.1   Conclusions

When we did not see the invasion of WT quorum sensers by our engineered defector strain that was predicted from the broader literature on microbial social evolution, we next examined the evolutionary trajectories of *Vibrio harveyi* in two laboratory environments, one meant to be social (M9-casein) and another meant to be non-social (M9-CAA) in an attempt to place different selective pressures on the function of QS, and in the WT strain and the constitutive $\Delta luxOU$ strain. To our knowledge, this is the first long term evolution experiment that aimed to probe the effects of QS on the regulation of cooperative behaviors.

Over time, we saw that QS-defectors did evolve in all treatment groups, and that this effect was more striking, consistent, and revealed the strongest differences between ancestral genotypes in the M9-casein environment. While defectors also evolved in the WT M9-casein populations, the dynamics were more varied between replicate populations and all were very different from the $\Delta luxOU$ M9-casein populations. The defectors were tolerated better by WT than $\Delta luxOU$ and did not rapidly sweep their resident populations. Thus the degree of diversity in QS function within the experimental populations was maintained at higher levels in WT populations, with multiple lineages with different levels of QS activity able to persist together over time. As a result, the majority of WT populations were not swept by

defectors, and in fact maintained high frequencies of cooperators within their lineages.

There were also a few interesting exceptions: one WT lineage that lost cooperators (CAS09), another that seemed to be in the process of losing cooperators (CAS07), and two $\Delta luxOU$ populations experienced high levels of growth and extremely low, but still detectable, levels of bioluminescence (CAS16 and CAS19). These results demonstrate that, while there were definite overarching trends observed in the experiment, there was also diversity in the range of population outcomes observed.

6.4.2   Future Directions

The scope of potential directions to extend and follow-up on this initial experiment is extensive. Whole research programs have been established upon evolution experiments (Lenski et al., 1991), and there are many unique specific questions that could be asked about QS and *Vibrios*, and more general questions about microbial evolution as a whole. Our remaining questions relate to both the genotypes and environments used (Cooper and Lenski, 2010, Blount et al., 2008), and the dynamics and interactions occurring within the experimental populations (Rendueles et al., 2015, Fiegna et al., 2015, Martin et al., 2016).

We have conducted genomic sequences of the evolved M9-casein populations and are in the process of determining what evolutionary paths different lineages have undertaken (Barrick and Lenski, 2013). Many questions remain about the results of this experiment, including:

1.Do different types of mutations underlie the defectors that evolve in the different genotypic backgrounds? Determining this would tell us more about whether the genotypes used are differently constrained in terms of the beneficial mutations that can occur.

2. Is there parallelism that occurred in the evolution of defectors that occurred within and

swept $\Delta luxOU$ M9-casein populations, occurred within but did not sweep WT M9-casein populations, or among the dim variants that evolved especially often within the WT M9-casein populations? Knowing this would tell us more about the range and types of genetic changes that can give rise to these phenotypes, and potentially expand our knowledge of what genes are impacting the QS system of *V. harveyi*.

3.From what we learn of the sequencing process, we can examine more closely what is occurring within particular experimental populations. The dimming effect that has occurred in many populations did not observably happen in all populations. Is this a matter of these populations not sampling a dim mutation yet, or because some fitter variant evolved instead? It might be predicted that QS allows cells to act as generalists in terms of density-dependent strategies. Does this turn out to be the case? Do populations that lack dim variants instead have two coexisting specialists, a defector and a cooperator? How does the occurrence of dims impact the prevalence of more non-functional defectors within these populations?

For the dim source mutations that are discovered, these can then be remade in our control strains to see how much of the change observed in fitness is due to these specific mutations. Dims can also be competed against engineered defectors and ancestral cooperator strains to determine how they perform in the presence of either. My prediction is that dims will outcompete all ancestral cooperator strains in M9-casein, and possibly the $\Delta luxR$ defector as well. In any case, I would not expect the evolved dim variants to perform worse in the presence of defectors than the WT strain. We have thus far primarily limited our genomic analyses to the M9-casein treatments. It would shed further light on the selective pressures on the *V. harveyi* QS system in different environmental conditions by conducting similar genomic analyses of the populations that were evolved in the M9-CAA environment. My prediction there is that QS should be under less stringent selection in M9-CAA and thus

that changes that occur in the genome will be more often unrelated to the QS system, and when they are, are likely to be less parallel and consistent in nature.

We have made the claim that dims are optimizing to the M9-casein environment, and this is supported by the diminished but still detectable levels of protease production and bioluminescence in these strains, and in the increased growth potential in M9-casein they possess over complete QS-defectors. If the experiment were continued, it is possible that dims could evolve in all populations that did not completely lose cooperation. An interesting extension of this question is: will dims continue to be selected for decreased investment in QS, or will that level stabilize? This could possibly be modeled from an adaptive dynamics or game theoretical standpoint to make advanced prediction of what we could expect to see by extending the evolution experiment.

We also made the claim that the evolved dims and defectors exhibited general drops in QS-regulated behaviors and should display a corresponding drop in signal production. This still needs to be experimentally measured, either directly with mass spectrometry, or indirectly by seeing how well these strains induce bioluminescence from the SN reporter strain. It is possible, but not likely, that some strains could still produce higher signal levels, and thereby elicit expression of cooperative behaviors from others in their populations in a manner reflective of deceitful cheaters (Ghoul et al., 2014, Backwell et al., 2000).

Different selection regimes should also lead to different evolutionary trajectories, especially related to QS and in different QS ancestral genotype backgrounds. This could be selection in M9 media with casein as a sole nitrogen source or as a sole carbon and nitrogen source, selection in a more stringent condition dependent on protease activity such as M9-BSA, or in alternating nutrient conditions. Particularly in variable and shifting environmental conditions, we predict that the WT strain with functional QS will be more

evolutionarily stable than any engineered mutant strains with altered QS function.

The observed evolved variants were selected for in the presence of a broader population of different types. It would be interesting to see how evolution would proceed if they were isolated from their resident populations and used to form new experimental populations. The selection for decreased QS investment might be lightened in this case, and further defector variants may not rapidly evolve, again suggesting that these strains are optimizing to the experimental environment and might be approaching an evolutionarily stable strategy ("ESS", Smith, 1982).

By altering experimental environments from the one in which populations were evolved, for example by evolving M9-casein populations in M9-CAA and vice versa, we could examine whether evolution in a social condition (M9-casein) leads to fitness costs in an asocial condition (M9-CAA) and whether this relationship is reciprocal. This could reveal whether trade-offs occur when cells invest more in social behaviors versus non-social behaviors over the course of evolution.

Most evolved populations have a mixed composition. When competed against an ancestor strain, I predict that the defectors present would benefit the most, but this needs to be validated through in-depth examination of competitions of all the evolved lineages. Additionally, it is unclear whether these lineages display a constantly increasing fitness against ancestors (Wiser et al., 2013), or whether the cooperator-defector dynamics present within the populations mean that fitness is only increased relative to the immediately preceding population. This can be examined by competing population samples from different time points against the ancestor as well as against one another.

We could examine potentially evolved interactions, by examining the growth performance of isolated phenotypes compared to mixed populations. Evolving interactions could be ei-

ther synergistic, signaling the infancy stages of mutualisms, or antagonistic, such as often observed red-queen dynamics (Pfeiffer and Bonhoeffer, 2004, Ewald, 1987, Dercole et al., 2006). When multiple types coexist for extended periods of time, it is possible for novel forms of cooperation such as cross-feeding to develop and enhance the overall growth of all populations

The exceptional population cases that do not reflect the more general trends are also quite interesting. It is possible that in the CAS09 lineage where WT cooperators went extinct there just happened to be more beneficial mutations that defectors acquired, or possibly a defector variant evolved that was better at exploiting WT but rarer to evolve due to a restricted mutational target. It is also intriguing that the $\Delta luxOU$-evolved CAS16 and CAS19 populations grew very well in M9-casein despite exhibiting extremely low (but still detectable) levels of QS investment. Identifying and recreating these variants will shed light upon the causes of their success in this experiment, and perhaps tell us more about how QS does (and sometimes does not) provide adaptive advantages in this M9-casein environment.

6.5   Low-cell-density behaviors

6.5.1   Conclusions

QS has historically been thought of as a density-dependent form of control over gene expression, with particular emphasis on turning genes on appropriately once populations reach high cell densities (Swift et al., 2001, Fuqua et al., 1994, Darch et al., 2012). However, the QS regulons are often extensive, and also cause changes in gene expression at low cell densities (van Kessel et al., 2013, Rutherford et al., 2011). This regulation at low cell densities

should also contribute to cellular fitness in natural environments. We identified several conditions where, rather than observing that QS-defectors exhibit negative pleiotropic effects as in environments such as M9-casein and M9-CAA media, it is the QS-cooperators that appear to experience growth defects. More specifically, the constitutive $\Delta luxOU$ strain of $V.$ $harveyi$ that is locked in a high cell density state of QS regulation performed poorly on some limiting carbon sources, while the WT strain does similarly well to QS-defector strains. These nutrient sources were diverse, ranging from sugar alcohols including glycerol and mannitol, the amino acid glutamate, to varying polymers that require public goods for breakdown into digestible subunits. Especially for polymer breakdown, it would be very interesting to confirm that the public good enzymes responsible for this degradation are downregulated by QS. If confirmed, this would predict that in some environments "cooperators" actually behave as defectors and could cheat "defectors" that are actually producing more of a given public good molecule.

6.5.2 Future directions

While it is clear that such environments exist whereby the $\Delta luxOU$ locked high-cell-density mutant displays growth deficiencies, it is unclear why this would be, particularly on resources that required breakdown by extracellular public goods. It has been suggested elsewhere that cooperation should actually not be favored to occur at high density number due to the risk of cheating (Zhang and Hui, 2011), which could explain this pattern. But it is not clear why some public goods (e.g. extracellular proteases and amylases) seem to be favorably produced at higher cell densities for $V.\ harveyi$, while others do not (e.g. chitinases and DNases). To get a better idea of why this might be, more knowledge about the fitness of cooperators and defectors in the presence of different polymers is needed by performing

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competitions on single polymers, as well as mixtures of starch, DNA, chitin, and protein sources.

Similar to the evolution experiments conducted in M9-casein media, selection experiments of *V. harveyi* strains in conditions where growth is favored by the repression of QS would tell us more about the favored evolutionary paths that occur in these conditions, and the types of strategies that emerge. It is possible that *luxO* mutants could evolve, and function as defectors, under an extended selective regime in M9 media with chitin as a limiting carbon source or in M9 media with DNA as a limiting nutrient source.

In addition, oscillating growth in M9-casein and in the presence of one of these QS-repressed polymer substrates (chitin or DNA) may reveal additional population dynamics. It is possible that shifting between these environments could give rise to long term coexistence between cooperators and defectors, even between UC and QS-defector strains. However, we would also predict that the WT strain, which can access both low and high-cell-density behaviors, could out-compete both the defector and UC in changing or variable environments. Additionally, because DNA can serve as a limiting carbon, nitrogen, or phosphorus source, it could be differently used in media and possibly lead to different competitive dynamics dependent on which nutrient source(s) it is providing.

6.6   Summary

In conclusion, this dissertation has added to our knowledge of the ecological and evolutionary dynamics facing populations of QS bacteria. Though QS often regulates cooperative goods production, direct evidence that QS is directly needed to stabilize these behaviors within populations was lacking. We have clearly shown that *Vibrio* protease production

works as an exploitable public good. However, we demonstrated that QS provides added stability against not only defined obligate defectors, but also against subtler defectors that evolve directly from QS-proficient strains. In many cases, the strains that ultimately emerge in the conditions examined have been selected for lower investment in QS, but not a complete cessation. We showed that QS allows cooperative behaviors to be maintained in well-mixed populations, even in the presence of defectors. Beyond the stalemate that occurs against defectors in well-mixed conditions, QS also allows the frequency of cooperation to increase when environmental conditions provide assortment of cooperators from defectors. This phenomenon could also be extended over thousands of generations, where we saw that WT lineages, unlike UC lineages, could preserve cooperation within experimental populations, often at high frequencies. Together, we see that in multiple ways regulation through QS provides fitness advantages beyond those provided by unconditional strategies.

APPENDIX

V. harveyi strains			
Strain	Description	QS phenotype	Reference
BB120 (ATCC BAA-1116)	Wild type (WT) strain	Functional QS system	Bassler et al., 1997
KM669	$\Delta luxR$	Non-functional	Pompeani et al., 2008
JAF78	$\Delta luxOU$	Locked in high cell density state	Miller et al., 2002
KM83	$luxO$ D47E	Locked in low cell density state	Tu and Bassler, 2007
JMH634	$\Delta luxM$ $\Delta luxS$ $\Delta cqsA$	Non-signaling, but signal-responsive	Waters and Bassler, 2006
CW2001	$\Delta luxAB$	Functional, non-luminescent	Waters and Bassler, 2006
ELB707	$luxO$ D47E $glmS::Tn7-lacZ$	Locked in low cell density state	This study
ELB714	$glmS::Tn7-lacZ$	Functional	This study
ELB717	$\Delta luxR$ $glmS::Tn7-lacZ$	Non-functional	This study
ELB738	$\Delta luxOU$ $glmS::Tn7-lacZ$	Locked in high cell density state	This study
V. cholerae strains			
Strain	Description	QS phenotype	Reference
C6706str2 (V. cholerae El Tor strain)	Wild type	Functional	Waters et al., 2008
BH1543	$\Delta hapR$	Non-functional	Hammer and Bassler, 2003
SLS349	$\Delta luxO$	Locked in high cell density state	Hammer and Bassler, 2003
DS13	$\Delta flrA$	Functional, lacks master regulator of motility	Srivastava et al., 2013

Table A.1: **Strains of *V. harveyi* and *V. cholerae* used in this work.**

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