Using experimental evolution to evaluate diversification of *Pseudomonas fluorescens* SBW25 in complex environments

Dissertation

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SUMMARY

Understanding the ecological conditions promoting diversification of lineages has been one of the major challenges in the field of evolutionary ecology. The ecological theory of adaptive radiation identified two potential conditions to divergence; selection resulting from differences between environments and from the interaction between species. So far, the best explained factor is differences between environmental features giving rise to divergence of lineages. Most of the other cases on adaptive radiation indicated that resource competition is an important process underlying diversification, although the roles of the other species interactions are not explicitly tested. Predation, for instance, is still underexplored, despite having both ecological and evolutionary impacts on prey populations. Role of predation on diversification remained unsettled, as field studies are challenging and experimental studies showed its modifying effect when selection is driven by resource competition. Importantly, the interacting effects of different ecological processes on diversification should be evaluated in detail. The aim of this thesis is to evaluate the importance of predation, and its effect on diversification when predation and resource competition are both present.

The first chapter investigated the relative contributions of predation and resource competition to diversification when they act simultaneously. In our experimental evolution study, initially isogenic populations of bacterial prey grew either in the presence or absence of predation in two different resource levels. We determined the major classes of colony morphologies (i.e. morphotypes), which we observed over time in replicate populations. Then, we used the changes in the frequency of these morphotypes to estimate phenotypic diversity over time. We found that predation was the main ecological process driving diversification of bacterial populations. In the absence of predation, resource competition did not lead to diversification. Importantly, the resource level of the environment generated an eco-evolutionary feedback. The differences in the resource level led to differences in the number of predators per prey, which changed the strength of selection, which in the end determined the level of diversity. As a result, the resource level of the environment had a significant contribution to diversification in the presence of predation leading to a higher level of prey diversity in the low resource environment. It has been challenging to disentangle

the inputs of different species interactions, however, our experiment presented how the resource level of the environment can contribute both to resource competition and predation leading to differences in selection through eco-evolutionary feedbacks.

The second chapter aimed to understand how predation selected for different bacterial morphotypes in environments with different resource levels. Here, we investigated ecologically relevant phenotypic traits and measured the fitness of morphotypes in the presence and absence of predation. We discovered that morphotypes, which were evolved and coexisted in the low resource environment in the presence of predation, showed clumping and cell-chaining traits. Although having these different defense strategies, the coexisting morphotypes had similar defense levels with different competitive abilities. These data suggested that difference in defense trait was not solely selected by predation, but by predation in interaction with resource competition. In the high resource environment, on the other hand, only clumping morphotypes were found. Our data demonstrated that predation generated strong directional selection for defense evolution favoring one defensive phenotype in the high resource environment, where resources were abundant and resource competition was not effecting the selection directly. This chapter provided insight into how different ecological conditions favored different traits.

The third chapter examined the roles of ecological processes acting in our evolution experiment on stable coexistence. We investigated the ecological mechanisms which could potentially lead to stable coexistence of two morphotypes in the low resource environment and in the presence of predation. By measuring the fitness of morphotypes in different ecological conditions, we showed that predation operated as an equalizing mechanism and decreased the strength of resource competition and thus allowed morphotypes to coexist stably. Additionally, although identifying the degree of niche overlap experimentally is difficult in such complex ecological conditions, multiple lines of evidence in our study supported possible niche differentiation. Specifically, we presented potential niche differentiation by demonstrating how morphotypes differed in ecologically relevant traits and by following their frequency over time from the early stages of diversification on. We concluded that interacting ecological processes play an important role in stable coexistence. Importantly, our findings highlighted the

importance of investigating both types of coexistence mechanisms, before inferring that high degree of niche overlap limits stable coexistence.

ZUSAMMENFASSUNG

Eine der fundamentalen Fragen der Evolutionsökologie untersucht die Rolle ökologischer Bedingungen bei der Aufspaltung von Arten. Die Theorie der adaptiven Radiation benennt zwei mögliche Szenarien für diese Divergenz: Selektionsprozesse basierend auf der Interaktion zwischen den Arten sowie durch unterschiedliche Umweltbedingungen. Unterschiedliche Umweltbedingungen, die zur Artbildung führen sind bisher am besten erforscht. Die meisten anderen Szenarien adaptiver Radiation postulieren Ressourcenknappheit als weitere Hauptursache, die zur Aufsplittung von Arten führt, auch wenn die Rolle der Interaktionen zwischen den Arten bisher nicht explizit untersucht wurde. So ist beispielsweise ist die Rolle des Räuberverhalten trotz ihres großen ökologischen Einflusses auf Beutepopulationen in diesem Kontext weitestgehend unerforscht. Feldstudien zur Bedeutung von Räuber-Beute-Beziehung im Artbilungsprozess sind sehr schwierig durchzuführen, experimentelle Untersuchungen weisen auf darauf hin, dass der divergierende Einfluss primär dann zum tragen kommt, wenn Selektion durch Ressourcenknappheit getrieben wird. Besonders wichtig ist hierbei die Bedeutung möglicher Interaktionen verschiedener Selektionsprozesse im Detail zu analysieren. Das Ziel der hier vorliegenden Doktorarbeit ist es die Bedeutung von Räuber-Beute-Verhalten und seiner Bedeutung auf Artbildungsprozesse im Kontext von Ressourcenknappheit zu erforschen.

Das erste Kapitel beschäftigt sich mit dem relativen Anteil gleichzeitig stattfindenden Räuber-Beute-Verhaltens und Ressourcenknappheit auf die Aufsplittungsprozesse. In unserem Experiment zu experimenteller Evolution evolvieren ursprünglich genetisch identische Populationen bakterieller Beute unter Vorhandensein oder Fehlen von Räubern in zwei Szenarien mit unterschiedlichem Ressourcenanteil unterschiedlich. Hierbei konnten wir Klassen unterschiedlicher Kolonie Morphologien (i.e. Morphotypen) bestimmen, deren Formierung sich im Zeitverlauf verlässlich replizieren ließen. Wir nutzten die Frequenzänderungen der unterschiedlichen Morphotypen um Diversität im Zeitverlauf messen und charakterisieren zu können. Hier konnten wir Räuberverhalten als ökologischen Hauptfaktor identifizieren, welcher die Diversifizierung bakterieller Populationen vorantreibt. In Abwesenheit von Räuberverhalten führte Ressourcenknappheit nicht zur Aufsplittung.

Ressourcenverfügbarkeit der Umwelt bedingten folgendes evolutionsökologische feedback: Unterschiede in der Ressourcenverfügbarkeit führten zu Unterschieden in Verhältnis Räuber zu Beute, was die Selektionsstärke auf Aufsplittungsprozesse verändert, und letztendlich den Grad der Diversität bedingt. Das Vorhandensein von Ressourcen in der Umwelt hat einen signifikanten Einfluss auf die Aufsplittung in Anwesenheit von Räubern und führt in Umweltszenarien mit Ressourcenknappheit zu einem hohen Grad an Beutediversität. Den Einfluss verschiedener zwischenartlicher Interaktionen aufzufächern stellt eine große Herausforderung dar. Mit unserem experimentellen Ansatz konnten wir jedoch zeigen, dass das Ressourcenniveau der Umwelt sowohl zum Streit über Ressourcen, als auch zu verstärktem Räuberverhalten führen kann, was letztendlich zu Unterschieden in Selektionsprozessen durch evolutionsökologische Feedbacks führt.

Das zweite Kapitel entstand aus der Motivation verstehen zu wollen, wie Räuberverhalten in Umweltszenarien mit unterschiedlichem Ressourcenvorkommen zur Selektion verschiedener Bakterien Morphotypen führen kann. Hierfür untersuchten wir ökologisch relevante phänotypische Merkmale und bestimmten Fitnessparameter der Morphotypen bezüglich Wachstum und Abwehr. In Umweltszenarien mit Ressourcenknappheit unter denen klumpende und kettenbildende Zelltypen co-existieren, konnten wir die Entstehung verschiedener Abwehrmechanismen beobachteten. Trotz unterschiedlicher Abwehrmechanismen wiesen co-existierende Morphotypen ähnliche Abwehrstufen mit unterschiedlichen Wettbewerbsfähigkeiten auf. Dies lässt vermuten, dass Unterschiede in Abwehrmechanismen nicht ausschließlich durch Räuberdruck, sondern im Zusammenspiel mit Ressourcenkonkurrenz selektiert werden. Divergente Selektion könnte sich daher auf die Konkurrenzfähigkeit co-existierender Verteidigungs-Morphotypen auswirken. Unter Umweltbedingungen mit Ressourcenüberschuss wurden ausschließlich klumpenbildende Morphotypen gefunden. Unsere Ergebnisse weisen darauf hin, dass Räuberverhalten zu starker gerichteter Selektion verteidigender Phänotypen in Umweltbedingen mit Ressourcenüberschuss führt, unter denen Konkurrenz über Ressourcen keinen direkten Einfluss auf Selektion zeigt. Dieses Kapitel liefert einen umfassenden Überblick über den Einfluss unterschiedlicher ökologische Szenarien auf die Favorisierung verschiedener Merkmale.

Im dritten Kapitel wird die Rolle ökologischer Prozesse im Experiment bei stabiler co-Existenz näher erforscht. Wir untersuchten ob klumpenbildende und zellketten bildende Morphotypen in Umweltbedingungen mit Ressourcenknappheit und in Abwesenheit von Räuberdruck stabil co-existieren konnten. Unter Räuberdruck waren die Fitnessparameter der wettbewerbsstärkerem klumpenden Morphotypen und der unterlegenen zellformenden Morphotypen vergleichbar. Wir konnten zeigen, dass Räuberdruck als ausgleichender Mechanismus agierte und die mittleren Fitnessunterschiede zwischen beiden Morphotypen verringert. Demzufolge können beide Morphotypen stabil co-existieren, selbst bei geringste Nischendifferenzierung unter Feinddruck. Wir konnten keinen stabilisierenden Mechanismus, der die Nischenüberlappung verkleinert, erkennen. Dennoch konnten wir bei Fokussierung auf Fitnesslevel potenzielle Nischendifferenzierung aufzeigen, und so ökologisch relevante Merkmale identifizieren. Auch wenn die Bestimmung des Überlappungsgrades der Nischen unter derart komplexen ökologischen Bedingungen extreme schwierig ist, unterstützen unserer Studie mögliche Nischendifferenzierung. Wir schlussfolgern hieraus, dass interagierende ökologische Prozesse eine wichtige Rolle bei co-Existenz spielen. Hervorzuheben ist speziell die Wichtigkeit beide Mechanismen für co-Existenz zu betrachten, insbesondere können ausgleichende Mechanismen die Fitnessunterschiede interagierender Arten minimieren, statt anzunehmen, dass ein hoher Grad an Nischenüberlappung stabile co-Existenz limitiert.

INTRODUCTION

Adaptation underlies diversity

The theory of evolution by natural selection was developed to explain the existence of vast number of life forms, which are different from each other, and yet depend on each other within complex interactions. Darwin and Wallace independently suggested everlasting adaptive evolution as a process responsible for the extinction, emergence and persistence of species (Darwin & Wallace 1858). Since then adaptive radiation, which is the divergence of a single lineage into multiple adaptive lineages, has accounted for life's diversity (Schluter 2000).

The neutral theory of diversity was proposed as an alternative hypothesis that could also give rise to the life's diversity (Hubbell 2001). This theory assumes that differences among individuals are irrelevant to their adaptive success, and diversity results from a stochastic balance between speciation and extinction or immigration and extinction. The neutral theory of diversity has been refuted mainly due to two observations. First, there have been significant correlations between variation in heritable traits and variation in fitness in various studies (reviewed in Schluter 2000). And second, the distribution and abundance of species in different habitats was not in line with the theoretical expectations of stochastic balance (e.g. Dornelas et al. 2006). Consequently, diversification is predominantly recognized as a result of adaptive evolution.

Diversity depends on ecology

According to the ecological theory of adaptive radiation, the main mechanism for phenotypic differentiation (i.e. diversification) is divergent selection. Between allopatric populations, diversification takes place via divergent selection acting on different traits in different environments (Schluter 2009). This extensively studied process is called ecological speciation (Schluter 2001). Some of the well-known examples in nature are three-spine stickleback speciation arising from adaptation to either freshwater or marine habitats (McKinnon & Rundle 2002), butterfly speciation caused by color pattern mimicry (Jiggins et al. 2001) and pea aphid speciation through host-pathogen specialization (Peccoud et al. 2009).

The mutation-order model proposes another way for diversification in allopatry. In this model reproductive isolation occurs not by selection but by chance, because different mutations can be fixed in isolated populations adapting to similar selection pressures (Schluter 2009). There are only few experimental and natural examples supporting this view like the speciation of Monkey flower (Fishman & Willis 2006) and the evolution of cell size in replicate *E. coli* populations (Travisano et al. 1995), therefore the mutation-order model is accepted as a minor mechanism for diversification (Schluter 2009).

The second major mechanism of adaptive radiation is disruptive selection due to interspecific competition. Disruptive selection reduces resource competition via selecting for extreme phenotypes over intermediate ones. Such diverse trait evolution (i.e. character displacement) occurs where depletion of resources leads populations to specialize in new resources (Schluter 2000). Adaptive radiation of Darwin's finches is one of the well-studied examples of character displacement in nature. When small and medium finches occur in sympatry, different beak sizes, which are specialized to different seed sizes, are selected for to reduce interspecific resource competition (Schluter et al. 1985). Other cases of character displacement have also been experimentally shown such as the *Cnemidophorus* lizards (Radtkey et al. 1997) and the Scarlet Gilia wildflowers (Caruso 2000). Although character displacement has been mainly related to competition for resources (reviewed in Pfennig & Pfennig 2009), other ecological interactions might theoretically enable phenotypic differentiation through divergent selection as well (Holt 1977; Day et al. 2002; Abrams 2000a). How these different ecological interactions drive diversification is yet to be explored.

Role of predation in diversification

Predation is an important ecological interaction, having an impact on the abundance, distribution and evolution of prey populations (Abrams 2000b; Day et al. 2002; Langerhans 2006; Casini et al. 2012; also reviewed in Koch et al. 2014). The effects of predation on prey diversification are variable; predation might promote or constrain phenotypic differentiation of prey. Differences in predation regimes among environments have been suggested to be responsible from the majority of diversity in prey's traits as a result of divergent selection (e.g. Nosil & Crespi 2006; Diabaté et al.

2008; Ingram et al. 2012; Swaegers et al. 2017). Predation might theoretically enable trait divergence also by generating disruptive selection, in which intermediate types are selected against (e.g. Doebeli & Dieckmann 2000). Predation might hinder diversification as well, if it decreases the density of prey populations to a level that the strength of resource competition becomes insignificant for diversification (e.g. Buckling & Rainey 2002).

Moreover, anti-predator defense strategies can involve various traits related to morphology, behavior, and life history of an organism. This might lead to reduction in other traits due to limited resources, genetic and/or physiological constraints (i.e. trade-offs). It has been shown that defense strategies can be selected at the cost of competitiveness (Wellborn 2002; Yoshida et al. 2004), and since frequency-dependent selection based on a trade-off might lead to diversification (Friesen et al. 2004), predation by leading growth-defense trade-offs can mediate diversification (Olendorf et al. 2006).

Previous studies suggest that predators might drive diversification in a similar way as resource competition does (Abrams 2000a; Buckling & Rainey 2002b; also reviewed in Vamosi 2005). However, even in well-established model systems, such as Trinidadian guppies (Reznick et al. 1996) and stick insects (Nosil & Crespi 2006), there is not enough quantitative data showing diversification through selection posed solely by predators. As a result, there has been no complete case showing predation-mediated diversification despite the accumulating arguments.

Maintenance of diversity in sympatry

Once sympatric speciation occurred, there will be direct competition between phenotypes and the expected outcome would be that the best-adapted phenotype eventually outcompetes others (Gause 1934; MacArthur & Levins 1967; Tilman et al. 1982). However, phenotypes using similar resources in an environment can coexist according to the modern coexistence theory (Chesson 2000).

Every phenotype with traits unique to them occupies a particular niche in the environment. If there are several niches in a given environment, selection favors diversification to fill those available niches (e.g. Phillimore & Price 2008, also reviewed in Stroud & Losos 2016). Coexistence of phenotypes in sympatry depends mostly on the niche overlap of competing species (Chesson 2000). Stabilizing mechanisms such as resource partitioning and frequency-dependent predation are crucial for coexistence because they reduce niche overlap and decrease interspecific competition relative to intraspecific competition. Stable coexistence might also be achieved even if the degree of niche overlap is high, when equalizing mechanisms minimizes fitness differences between coexisting phenotypes.

Ecological interactions such as resource competition, predation, and mutualism are considered to play a major role for creating and altering the equalizing and stabilizing processes in an environment (Chesson 2000; Chesson & Kuang 2008). Disentangling the interaction between different ecological processes requires investigating ecologically relevant traits by taking both equalizing and stabilizing mechanisms into consideration.

Significance of simultaneously acting ecological interactions

The role of ongoing evolution on shaping life's diversity is difficult to assess without ecological understanding (Gavrilets & Losos 2009; Simoes et al. 2016). Both for diversification and maintenance of the diversity, demonstrating relative contributions of different ecological interactions is essential, because in complex environment selection results from multiple stressors simultaneously and acts on several traits at once (e.g. Rohr et al. 2004; Coors & De Meester 2008; Gunderson et al. 2016). However, limitation of detecting the early stages of diversification in natural populations makes it difficult to determine the importance of different ecological interactions on the process of diversification. Consequently, our understanding of diversification is limited to selection arising from resource competition between species. Nevertheless, studying concurrent ecological interactions might provide new insights into evolutionary mechanisms driving diversification.

Advantage of experimental evolution

Experimental evolution is the study of evolution under controlled environments with organisms that have short generation times and large population sizes. Experimental evolution makes it possible to investigate how adaptation changes in response to changes in the experimental treatments. This approach has been proven to be insightful, providing a substantial amount of empirical evidence for the drivers and the underlying mechanisms of evolutionary processes (reviewed in Kawecki et al. 2012; Kassen 2014). The difficulty of investigating the contribution of different ecological interactions to diversification, and demonstrating the maintenance of diversity could be overcome via experimental evolution approach.

Model system

Unicellular eukaryote *Tetrahymena thermophila* is one of the fastest growing protists with a generation time between 2-3 hours when it reproduces asexually under laboratory conditions (Cassidy-Hanley 2012). *T. thermophila* can be cultured in synthetic media without bacteria, and kept naïve regarding prey handling. Therefore, its rapid generation time and convenient maintenance makes *T. thermophila* one of the frequently used model for experimental evolution studies (Ketola et al. 2003; Meyer & Kassen 2007; Hall et al. 2008; Friman et al. 2008; Ketola et al. 2013; Friman & Buckling 2013; Friman et al. 2013; Hiltunen & Becks 2014; Hiltunen et al. 2015). Gram-negative bacterium *Pseudomonas fluorescens* SBW25 is a well-studied bacterial model for adaptive radiation in microcosm experiments (Rainey & Travisano 1998; Kassen et al. 2000; Buckling & Rainey 2002b; Fukami et al. 2007; Hall & Colegrave 2007; Bailey et al. 2013; Brockhurst et al. 2004; Flohr et al. 2013). Specifically, in the absence of predation, the bacteria have been shown to diversify rapidly into niche specialists producing distinct colony morphologies when plated onto agar plates (i.e. morphotypes) and colonizing different niches of a microcosm (Fig. 1B; Rainey & Travisano 1998). In this experiment, competition for limiting resources (i.e. oxygen) led to diversification of the initially isogenic bacteria in the spatially structured environment (i.e. static incubation of microcosms containing liquid medium). In our study, we used the well-established ciliate *T. thermophila* and bacterium *P. fluorescens* SBW25 as a microbial predator-prey

system to study diversification of the bacterial prey in the presence of the ciliated predators.

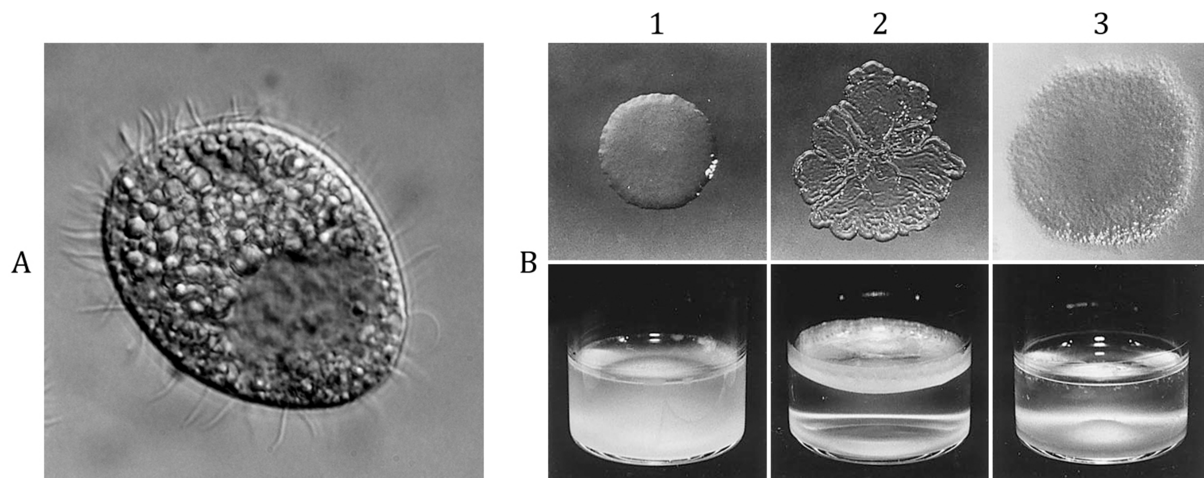

Figure 1 | A microbial predator-prey system

A. The ciliate *Tetrahymena thermophila* (photograph adapted from Lutz Becks), and **B.** colony morphologies and niche preference of the different morphotypes of the bacterium *Pseudomonas fluorescens* SBW25 (photographs adapted from Rainey & Travisano 1998). **B1.** Ancestral smooth morph colonizing the broth of the microcosm diversifies into different morphotype classes of **B2.** wrinkly spreaders colonizing the air-liquid interface, and **B3.** fuzzy spreaders colonizing the bottom of the microcosm after 72 hours in spatially structured environment, in full strength medium and in the absence of predation.

Thesis outline

The ecological theory of diversity ensures a well-structured explanation for the emergence and the maintenance of diversity, nevertheless some parts of it still requires support through empirical data. Over the last few decades, there has been an increasing interest to use microbial evolution experiments to test the hypotheses and underlying mechanisms of diversification and its maintenance. These experiments supported that resource competition is the main driver of diversification, yet they mostly excluded the role of other ecological interactions. Although it has been argued that ecological interactions determine the extent of diversity, there has been little attempt to investigate the effects of interacting ecological factors. The objective of this thesis is to evaluate the interacting role of resource competition and predation on the diversification of prey in microbial experiments.

The first chapter focuses on the contributions of predation and resource competition to prey's diversification. We performed an experimental evolution study started with initially isogenic populations of bacterial prey propagated both in the presence and absence of predation in two different resource levels. In our experiment, we showed that predation alone leads to diversification. More importantly, we found that resource level of the environment and predation interacts and determines the extent of diversity.

The objective of the second chapter is to characterize the morphotypes evolved in the experiment described in Chapter 1. We investigated ecologically relevant phenotypic traits and measured the fitness of morphotypes regarding growth and defense. We found that similar defense levels were achieved through different defense traits as a result of selection generated by interacting effects of predation and resource competition. This chapter provides a comprehensive understanding about the different traits favored by different ecological conditions, and reveals the link between ecology and evolution.

The coexistence of evolved morphotypes was the focus of the third chapter. Particularly, we investigated the role of ecological interactions acting in our evolution experiment as stabilizing and equalizing mechanisms. We measured the fitness of coexisting morphotypes in a series of experimental environments. We found that the stable coexistence of evolved morphotypes might be possible through an equalizing effect of predation, which decreased the fitness difference between coexisting morphotypes substantially and allow them to coexist even though they might have a large niche overlap.

CHAPTER 1

Predation promotes diversification of prey

INTRODUCTION

Species interactions have been recognized as one of the major ecological factors driving adaptive differences both within and between species (Schluter 2000). Such differences have been suggested to give rise to diversification among populations leading ultimately to their speciation in nature (Vermeij 1994; McPeek 1996; Thompson 1999; Doebeli & Dieckmann 2000; Schluter 2001; Hoskin & Higgie 2010). Most of the studies on natural populations limits our understanding how simultaneously acting species interactions lead to diversification as these studies cannot investigate the underlying factors during early stages of diversification. Controlled experiments with microbes, however, have been proven to be a powerful tool to broaden our knowledge on diversification (reviewed in Kassen 2014).

The majority of experimental studies identified resource competition as the main driver of diversification (reviewed in Kassen 2014). Direct evidence from different model organisms showed that the use of same resources (i.e. complete niche overlap) give rise to strong resource competition and result in diversification to utilize available ecological opportunity (i.e. underused resources). For instance, Rainey and Travisano (1998) showed that the isogenic ancestral bacterium diversifies rapidly (within 100 generations) into different niches through competition for limited resources (i.e. oxygen). The evolution of niche specialists in their experiment depended on ecological opportunity generated by environmental heterogeneity (i.e. unshaken microcosm).

Predation is also recognized as an important species interaction affecting diversification. For instance, an experimental evolution study on diversification of *Pseudomonas fluorescens* SBW25 in the presence of predator *Tetrahymena thermophila* showed that predation slowed down diversification by decreasing the strength of resource competition, and selected for higher frequencies of one of the niche specialists (i.e. wrinkly spreader) (Meyer & Kassen 2007). Another study with this predator-prey system demonstrated that predation has a modifying effect on the extent of diversity by changing the frequency of wrinkly spreaders (Introduction for details) depending on the resource level of the environment (Hall et al. 2008). Overall, these experiments as

well as other studies suggest that predation and resource competition can interact with each other, and result in differences in diversification (Chesson & Kuang 2008; Chase et al. 2002).

Additionally, such an interaction between predation and resource competition might depend on and change with ecological components of the environment. For instance, the level of resources available to the prey could influence both prey and predator densities. As a result, resource level of the environment might simultaneously alter different ecological processes; (1) the resource competition within the prey and (2) the strength of predator-prey interaction. When the resource level of the environment only affects resource competition, similar diversification patterns could be observed both with and without predation. If the resource level of the environment only alters predation pressure, diversification could be observed only in the presence of predation and with similar patterns regardless of the resource level of the environment. Furthermore, both predation and resource competition can have modifying effects on diversification. If resource competition is the main driver and predation has a modifying role, diversification occurs regardless of predation, but the extent of diversity (e.g. number of types coexisting) is expected to be different with predation. If predation is the main driver and resource competition is a modifier, diversity is expected only in the presence of predation, but the extent of diversity differs between resource levels. These predictions require empirical evidence showing how differences in the interaction between resource competition and predation affect diversification. We propose that using the *Tetrahymena-Pseudomonas* system we can explore the varying effects of simultaneously acting species interactions (i.e. resource competition and predation) on diversification and whether resource level of the environment as an ecological factor changes these effects.

We performed an experimental evolution study in which replicate populations of initially isogenic bacterium grew either in the presence or in the absence of predation in two different resource levels. We showed that predation was the major ecological interaction leading to diversification independent of the resource level of the environment. Importantly, different resource levels gave rise to different predator-prey ratios, which altered the selection by predation and led to differences in the level of

diversity between both environments. We found that higher level of diversity was reached when both predation and resource competition contribute to selection. Unlike the common view that resources available to the prey affects diversity solely by changing resource competition, our data reveal the importance of indirect effects of the resource level on predation-mediated selection.

MATERIAL AND METHODS

A ciliate predator and a bacterial prey

We used a well-established microbial system consisting of a protist predator, *Tetrahymena thermophila* 1630/1U (CCAP), and a bacterial prey, *Pseudomonas fluorescens* SBW25 (Meyer & Kassen 2007; Hall et al. 2008; Friman et al. 2008; Friman & Buckling 2013; Friman et al. 2013; Hiltunen & Becks 2014; Hiltunen et al. 2015) Unlike many other predator models, *T. thermophila* can be cultured axenically (i.e. bacteria free) prior to experiments, and thus stock cultures can be kept naïve regarding prey handling (Cassidy-Hanley 2012). Prior to the experiment, ciliate stocks were maintained axenically in PPY medium containing 20 g proteose peptone (Sigma-Aldrich) and 2.5 g yeast extract (Sigma-Aldrich) in 1 l deionized water (dH_2O) at 20°C. *P. fluorescens* SBW25 was cryopreserved in 45 % ($^v/_v$) glycerol saline at -80°C.

Evolution experiment

In this experiment bacteria were propagated either in the presence of predation (PP) or in the absence of predation (AP) in two different resource levels. We used two concentrations of the bacterial growth media consisting of M9 salts and King's B nutrients (thereafter M9KB Medium). Concentrations were prepared as 5% (LRE: Low Resource Environment) and 20% (HRE: High Resource Environment) of the full-strength medium (20 g proteose peptone (Biomol), 10 g glycerol (Sigma-Aldrich), 11.28 g M9 minimal salts 5x (Sigma-Aldrich) in 1 l dH_2O). We tested growth of ciliates in the LRE and HRE in the absence of bacteria, and we did not find a significant increase in their growth in neither of the environments (data not shown). Thus, we confirmed that in our experimental set-up, ciliates could grow only by preying upon bacteria in the environment.

We used 20 ml glass vials containing 6 ml liquid medium as microcosms. To start the experiment, we inoculated ~10^5 bacteria from overnight incubation of a single colony of *P. fluorescens* SBW25 (200 rpm, 28°C for 16 hours in full-strength M9KB medium) to each microcosm. We added ~6300 ciliates to each microcosm in the PP treatments. Before adding ciliates, PPY medium was removed by centrifugation (3000 rpm, 8

minutes, 0°C) and the remaining pellet containing the ciliates was re-suspended in the test medium (either in 5% or 20% M9KB media). Each treatment was established as five independent replicate populations (i.e. microcosms). Every second day, we transferred 10% of each microcosm to a new vial after vortexing for 45 seconds at maximum speed. We propagated these lines for thirty days under static conditions at 28 °C. Before transferring to the new microcosm, we obtained the density of ciliates and bacteria and cryopreserved subsamples from each microcosm at -80°C in 45 % ($^{v}/_{v}$) glycerol saline for fitness and diversity assays. Since ciliates do not survive this procedure we only obtained bacterial populations, when we retrieved populations from the cryopreserved stocks.

Isolating and preserving ciliates from PP treatment

We isolated *Tetrahymena thermophila* at 10-day intervals from both resource levels of the PP treatment. We preserved these ciliates in a slow-growth and bacteria-free condition for fitness assay. In order to remove bacteria from samples taken from the microcosms, one liter of PPY medium was supplemented with 10 ml of 100x Antibiotic Antimycotic Solution (Sigma-Aldrich) containing 10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml (thereafter selective medium). We inoculated 1 ml of each replicate (on day 10, 20 and 30) into 24 ml of selective medium and incubated these for one week at 20°C to eliminate bacteria. Importantly, we confirmed that these ciliate cultures were free from bacteria before transferring them to slow-growth condition. To test for removal of bacteria, we inoculated 1 ml from selective cultures into 24 ml full-strength M9KB, and incubated for 16 hours at 200 rpm, at 28°C, and then plated 100 µl onto M9KB agar plates. After confirming that cultures were bacteria-free, we took samples from selective cultures and removed the selective medium by centrifugation (3000 rpm, 8 minutes, 0°C) and re-suspended ciliates into 1 ml PPY medium. These bacteria and drug-free cultures were then transferred to the slow-growth condition consisting of 40 ml PPY-agar and 160 ml PPY medium in a 250 ml culture flask. This growth condition and the low incubation temperature (20°C) ensured slower growth of ciliates (Dr. T. Hiltunen, personal communication). We maintained the slow-growing ciliate cultures for thirty days at 20°C before testing their fitness (see "Measuring the grazing evolution of ciliate populations" for details). Slow-

growth of ciliates minimized the accumulation of mutations during preservation of ciliates over thirty days until they were tested for fitness. Also, gradual degradation of additional resources from PPY-agar into the liquid medium over time allowed us to keep these cultures undisturbed in the long-term (i.e. no bottleneck effect of refreshing the cultures).

Development of the flow cytometer for the enumeration of ciliates

We developed a high-throughput and accurate enumeration method for *Tetrahymena thermophila*. Measurements by flow cytometer (FCM) have been shown to be efficient and accurate for quantification of unicellular organisms in the previous studies. We developed a protocol based on the study of Lindström et al. (2002), in which the authors optimized FCM to count small ciliates. In order to optimize FACSCalibur FCM (Becton Dickinson) settings, we used several samples from axenically growing cell cultures of ciliates with different densities, ciliates together with different known densities of bacteria, and ciliates with different phenotypes of bacteria. The latter to test whether clumping of bacteria will generate false signals due to their size. All samples were preserved with formaldehyde (Sigma-Aldrich) at 4°C, and counted under microscope for comparison. Knowing the density of ciliates in each sample, we optimized our settings for the CellQuest software (Becton Dickinson) to accurately quantify the number of ciliates in cultures regardless of the content of a sample (i.e. with/without bacteria, different concentrations and phenotypes of bacteria). Also, we optimized the settings for the well-plate set-up of the FACSCalibur (Becton Dickinson) to count 96 samples per plate using the auto-sampler of the machine. Prior to counting, 200 µl samples within each well were stained with 1 µl of 100x concentration of SYBER Green I (Sigma-Aldrich), and incubated in dark for one hour at 24°C. Each well mixed three times, and 10 µl of each sample was counted per well. Importantly, we found that for accurate counting number of ciliates should not exceed ~1500 in 10 µl. Thus, if the first count of a sample from the experiment exceeds this threshold, we repeated the measurement after dilution. For the dilution of samples, we used FACSFlow (Becton Dickinson).

Finally, we determined to use the following settings for the CellQuest software (Becton Dickinson); forward scatter (FSC) was set to E-1 at log scale to measure cell size, side scatter (SSC) was set to 240 nm at log scale to measure cell complexity, photomultiplier tubes (PMT) was set to 270 nm at log scale to measure fluorescent intensity. We set PMT to 85 nm to adjust the threshold.

Diversity assay for bacterial populations

We determined the bacterial diversity over time by following the frequency of morphotypes, i.e. different colony morphologies on agar plates (Chapter 2 for details). For each replicate population growing either in the presence or absence of predation, and in the low and high resource environment (LRE and HRE, respectively), we plated cryopreserved samples at 6-day intervals. We excluded overnight incubation prior to plating bacterial populations to prevent the loss of rare morphotypes. Therefore, cryopreserved samples of bacteria were dissolved directly in Ringer's Solution (Sigma-Aldrich), and further dilutions were made to obtain 100-300 colony-forming units (CFUs) per plate. We counted CFUs, and calculated the frequency of each major morphotype (i.e. more than 1% of the total CFUs per plate at a given time point) for each replicate population. Plating was repeated at least three times on independent days for each replicate population. We calculated the Simpson's Diversity Index over time from these counts. The Simpson's Diversity Index takes into account the number of different types (i.e. richness) and the relative abundance of each type (i.e. evenness) to calculate the diversity index (Simpson 1949). According to the Simpson's Diversity Index, $S=(\Sigma\ n\ (n-1))/N\ (N-1)$, where n is the number of individuals for a given type in a population and N is the number of total individuals of all types in the population, diversity (S) ranges between 0 and 1. When the S value equals to 0 diversity is infinite, whereas 1 means no diversity. In order to make it more intuitive, we used 1-S in our diversity index.

Measuring the defense evolution in bacterial populations

We determined the defense of bacterial populations over time at 6-day intervals by using ciliate growth as a proxy for resistance against grazing (Hiltunen & Becks 2014).

We used 5% M9KB as the test environment for replicate populations growing over thirty days in the LRE either in the presence or absence of predation, while we used 20% M9KB as the test environment for replicate populations growing in the HRE either in the presence or absence of predation. For each test environment, we also assayed the defense level of ancestral bacteria. This allowed us to calculate the defense level of bacterial populations relative to the ancestral bacteria. We excluded overnight incubation to prevent the loss of rare morphotypes. Specifically, a subsample of cryopreserved samples were retrieved in Ringer's solution, and from that suspension we inoculated 2 µl into 2 ml of 5% or 20% M9KB media in 24-well plates as three technical replicates per population and time point. We then added ~2100 ciliates from stock cultures into each well. Before adding ciliates, PPY medium was removed from the ciliate stock used for the experiment by centrifugation (3,000 rpm, 8 minutes, 0°C) and the remaining pellet containing the ciliates was re-suspended in the test medium (either 5% or 20% M9KB media). After 48 hours, ciliates were counted using Flow Cytometer, and differences in final ciliate densities were taken as an estimate for the bacterial defense levels. This approach gives direct information about the strength of the trophic link between prey and predator. Defense level of bacteria were calculated relative to ancestor by using the formula of $D=1-(CD_{evolved}/CD_{ancestor})$, where $CD_{evolved}$ is the density of naive ciliates fed on evolved bacteria, and $CD_{ancestor}$ the density of naive ciliates fed on the ancestral bacteria. When D equals to 0, the bacteria populations' defense level is equal to the defense level of the ancestral bacteria. D values greater than 1 indicate higher levels of defense relative to the ancestor, and vice versa.

Measuring the growth parameters of bacterial populations

We obtained growth curves of each bacterial population by kinetic measurements of OD_{600} for 50 hours. In order to compare competitive abilities of populations evolved in the PP and AP treatments, we incubated the populations grew in the presence of predation in both resource levels without predators. After retrieving a subsample of cryopreserved samples in Ringer's solution, we inoculated 2 µl into 198 µl of 5% or 20% M9KB medium into each well of a 96-well plate with three technical replicates. This assay was replicated three times with samples in randomized positions within 96-well plates. Kinetic measurements of OD_{600} were obtained every five minutes at 28°C with

Epoch-2 plate reader (Biotek). Prior to every measurement, the plate was shaken for 5 seconds in orbital mode with a frequency of 282 cpm. Overall, we had growth curves for 120 populations, and we analyzed several growth parameters after log transformation of the OD_{600} values.

First, we calculated Area Under Curve (AUC) using the R package MESS (Ekstrom 2017). AUC summarizes the growth rate and the density of a population as one value. We also calculated AUCs for the ancestral bacteria both in 5% and 20% M9KB media. Then, we converted these values to relative AUC by using the formula $AUC_{relative}=AUC_{evolved}/AUC_{ancestor}$. When the $AUC_{relative}$ value equals to 1, it means that the AUC equals to the ancestral AUC. $AUC_{relative}$ values greater than 1 indicate higher level of area under curve relative to the ancestor, and vice versa.

Second, we acquired the duration of lag phase (LP) for each replicate population using the R package grofit (Kahm et al. 2010) from log transformed OD_{600} values. We calculated relative LP by using the formula of $LP_{relative}=LP_{evolved}/LP_{ancestor}$. When the $LP_{relative}$ value equals to 1, it means that LP equals to ancestral LP. $LP_{relative}$ values greater than 1 indicate higher level of lag phase relative to the ancestor, and vice versa. We used the same package and calculations to obtain relative growth rates ($GR_{relative}$) of replicate populations. Therefore, when the $GR_{relative}$ value equals to 1, it means that GR equals to the GR of ancestor. $GR_{relative}$ values greater than 1 indicate higher level of growth rate relative to the ancestor, and vice versa.

Measuring the grazing evolution of ciliate populations

We isolated ciliates at 10-day intervals from both resource levels to test for their grazing efficiency. We used 5% M9KB as the test environment for ciliates isolated from the LRE, while we used 20% M9KB as the test environment for ciliates isolated from the HRE. For each test environment, we also assayed the growth rate of stock ciliates, i.e. naïve ciliates regarding prey handling. This allowed us to calculate grazing efficiency of ciliates isolated from the experiment relative to the naive ciliate stock. A subsample of cryopreserved ancestral bacteria was retrieved in Ringer's solution, and from that suspension we inoculated 2 μl into 2 ml of 5% or 20% M9KB medium into each well of a

24-well plate. Then, we added ~2100 ciliates from each ciliate culture; isolates maintained in slow-growth condition and naïve ciliate stock with three technical replicates. Before adding ciliates, PPY medium was removed by centrifugation (3,000 rpm, 8 minutes, 0°C) and the remaining pellet containing the ciliates was re-suspended in the test medium (either 5% or 20% M9KB medium). After 48 hours, ciliates were counted using FCM (see "Development of the flow cytometer for the enumeration of ciliates" for details). We then calculated growth rate (GR) of a ciliate culture by using the formula of $GR=(\ln(Ciliate_{t48})-\ln(Ciliate_{t0}))/48$, where $Ciliate_{t0}$ is the initial density of ciliates in 2 ml (i.e. 2100 individuals), and $Ciliate_{t48}$ is the density of ciliates after 48 hours feeding on the ancestral bacteria in 2ml. Then, we convert these values to relative growth rate by using the formula of $GR_{relative}=GR_{isolate}/GR_{naive}$. When the $GR_{relative}$ value equals to 1, it means that the GR equals to the GR of naïve ciliates. $GR_{relative}$ values greater than 1 indicate higher level of growth rate relative to the naïve ciliates, and vice versa.

Following population dynamics of ciliate and bacteria during evolution experiment

We obtained population density of bacteria over time for both resource levels of the PP and AP treatments by plating dilutions onto M9KB agar plates for each replicate population at 6-day intervals. Also, we quantified the number of ciliates with FCM at 6-day intervals to obtain density of ciliates for both resource levels of the PP treatment. Using ciliate and bacteria densities of the PP treatments, we calculated predator-prey ratio over time for each resource level to obtain information about the strength of predation.

Measuring short-term predator-prey ratio

We performed a short-term experiment and followed the same procedure as in the evolution experiment to measure predation pressure over 72 hours in both resource levels with three replicate microcosms each. During 72 hours, we did not perform serial transfers to new microcosms. We obtained densities of ciliate and bacteria every 6 or 12 hours, and calculated predator-prey ratio over time.

Statistical analyses

Statistical analyses were performed with R (R Core Team, 2015) in Rstudio (Version 1.0.143), using the geepack package (Halekoh et al. 2006). To test for differences in the diversity, defense level and growth parameters across treatments we used generalized estimating equations generalized linear model (GEEGLM) with predation, resource level and time as explanatory variables. To test for differences in densities of bacteria and ciliate as well as predator-prey ratio across treatments we use GEEGLM with resource level and time as well as their interaction as explanatory variable. For all tests, the time points coming from one replicate population were grouped to correct for the temporal autocorrelation between data points. We used family Gamma for the analyses. We used model simplification to determine the significance of predation, resource level, time and their interactions.

RESULTS

Predation promotes diversification of prey

We followed the frequency of major morphotypes over time (Fig. 1; Chapter 2 for the detailed characterization of evolved morphotypes). Different classes of morphotypes determined based on the colony morphology of bacteria on agar plates (Methods for details). We found that Morphotype 1, which was evolved in the absence of predation and in the low resource environment, and Morphotype 2, which was evolved in the absence of predation and in the high resource environment, were similar to the ancestor (Fig. 1A and B). We found that predation promoted diversification of ancestral bacteria into a range of different morphotypes (Fig. 1C). Specifically, Morphotype 4 and 6 coexisted in the populations of the low resource environment, and Morphotype 3 was found together with Morphotypes 4 and 6 in one replicate population (Fig. 1C). Additionally, most of the replicate populations of the high resource environment were dominated either by Morphotype 4 or 5, and both coexisted in one replicate population (Fig. 1C).

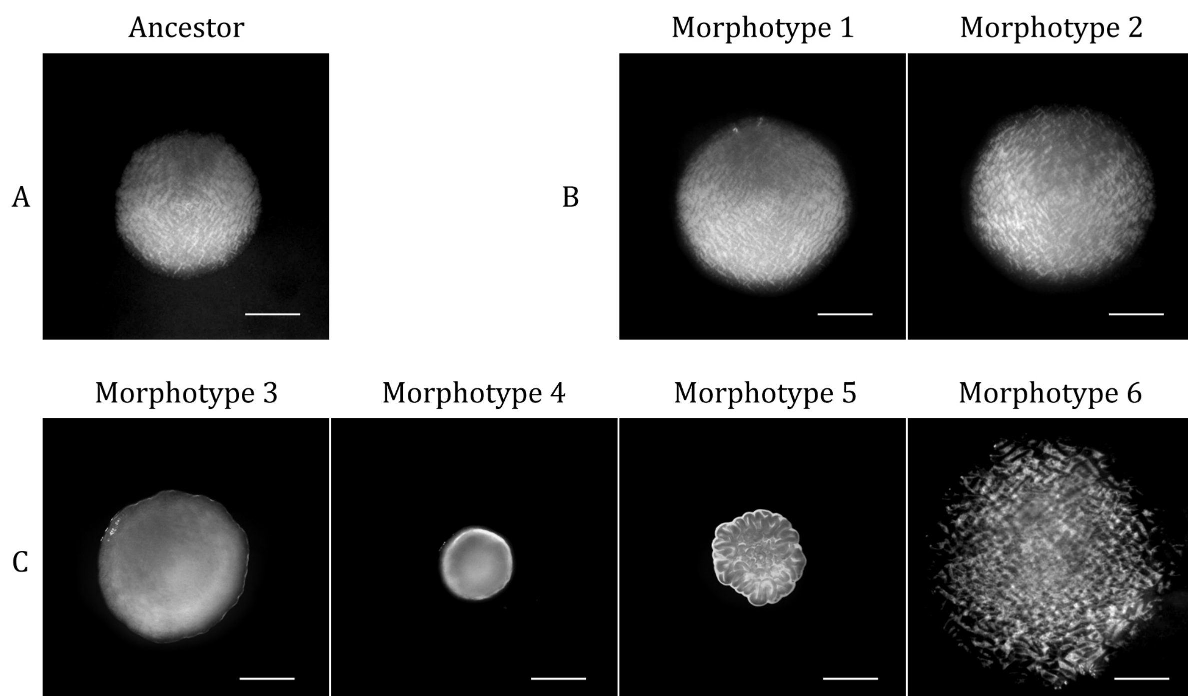

Figure 1 | Colony morphology of ancestor and evolved morphotypes

A. Ancestor, **B.** morphotypes isolated from replicate populations evolved in the absence of predation, and **C.** morphotypes isolated from replicate populations evolved in the presence of predation. Scale bars represent 1 mm.

Predation leads to higher level of diversity in the low resource environment

We calculated the Simpson's Diversity Index for bacterial populations evolved either in the presence or absence of predation and in two different resource levels. Simpson's Diversity Index was calculated based on the frequency of major morphotypes over time for each replicate population (Methods for details). We found that predation, resource level, time and each interaction had a significant effect on the diversity of bacterial populations (Fig. 2; Table S1 for statistics). Predation promoted diversification and there was a significant difference in diversity between the low and high resource environments (Fig. 2; Table S1 for statistics). The extent of diversity, which changed over time, depended on the resource level of the environment (Fig. 2; Table S1 for statistics).

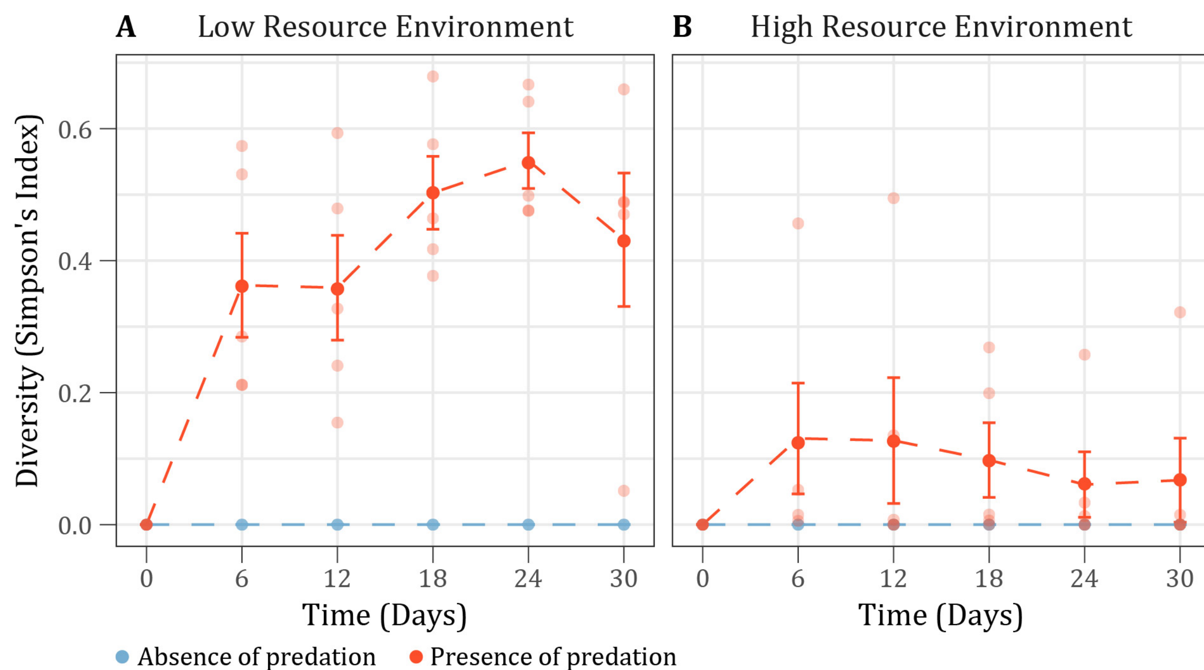

Figure 2 | Diversification of the initially isogenic populations of bacteria

Diversity shown in blue for populations evolved in the absence of predation, and in red for populations evolved in the presence of predation **A.** in the low resource environment, **B.** in the high resource environment. Translucent points: replicate populations, solid points: mean of replicate populations, error bars: standard error of mean.

Predation selects for defense evolution

To test whether adaptation to predation differs between two resource levels, we measured the defense relative to the ancestor for bacterial populations evolved either in the presence or absence of predation and in two different resource levels. Relative defense was calculated by measuring the final densities of predators feeding both on the ancestral and evolved bacterial populations (Methods for details). Populations evolved in the presence of predation showed significantly higher defense levels compared to the populations evolved in the absence of predation (Fig. 3; Table S2 for statistics). Moreover, this increase was significantly altered by the difference in resource level of the environment (Fig. 3; Table S2 for statistics).

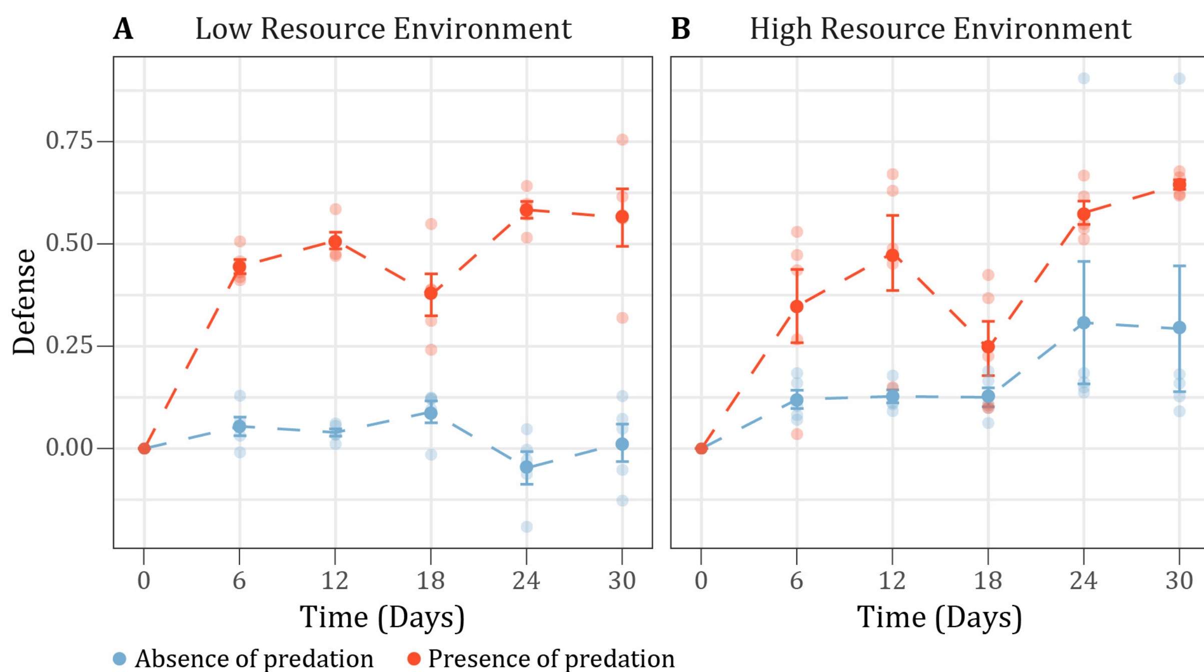

Figure 3 | Evolution of defense against predation in the bacterial populations

Defense shown in blue for populations evolved in the absence of predation, and in red for populations evolved in the presence of predation **A.** in the low resource environment, **B.** in the high resource environment. Translucent points: replicate populations, solid points: mean of replicate populations, error bars: standard error of mean. Defense = 0 indicates ancestral defense.

Adaptation to predation leads to reduction in growth

Growth curves of populations evolved either in the presence or absence of predation and coming from different time points were obtained by kinetic measurement of optical density at 600 nm without predators (Fig. 4; Methods for details).

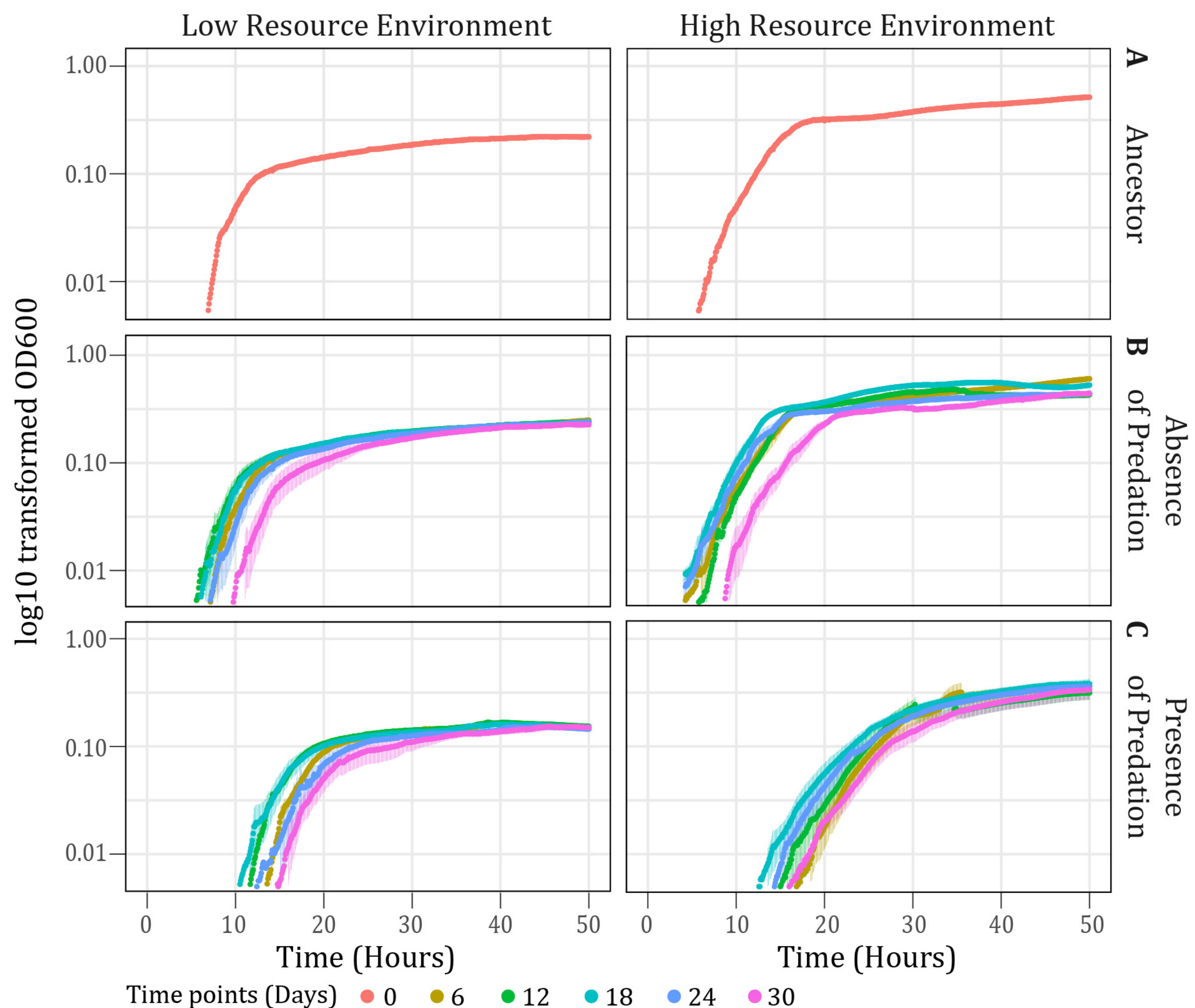

Figure 4 | Growth curves of the bacterial populations

Different colors represent bacterial populations coming from different time points of the evolution experiment; **A.** growth curve of the ancestor (i.e. Day 0) in low and high resource levels, **B.** growth curves of populations evolved in the absence of predation (from Day 6 till Day 30) in two resource levels, **C.** growth curves of populations evolved in the presence of predation (from Day 6 till Day 30) in two resource levels. Lag phase, which starts at 0 hour and ends with the start of the exponential phase in each growth curve, is not depicted.

We calculated the Area Under Curve (AUC) relative to the ancestor for each population to summarize the growth rate and population density from the growth curves (Methods for details). We found that the AUC of populations evolved in the presence of predation decreased significantly compared to the AUC of populations evolved in the absence of predation (Fig. 5; Table S3 for statistics). The difference in AUC between the populations evolved in the presence and absence of predation was altered significantly by the difference in resource level of the environment (Fig. 5; Table S3 for statistics).

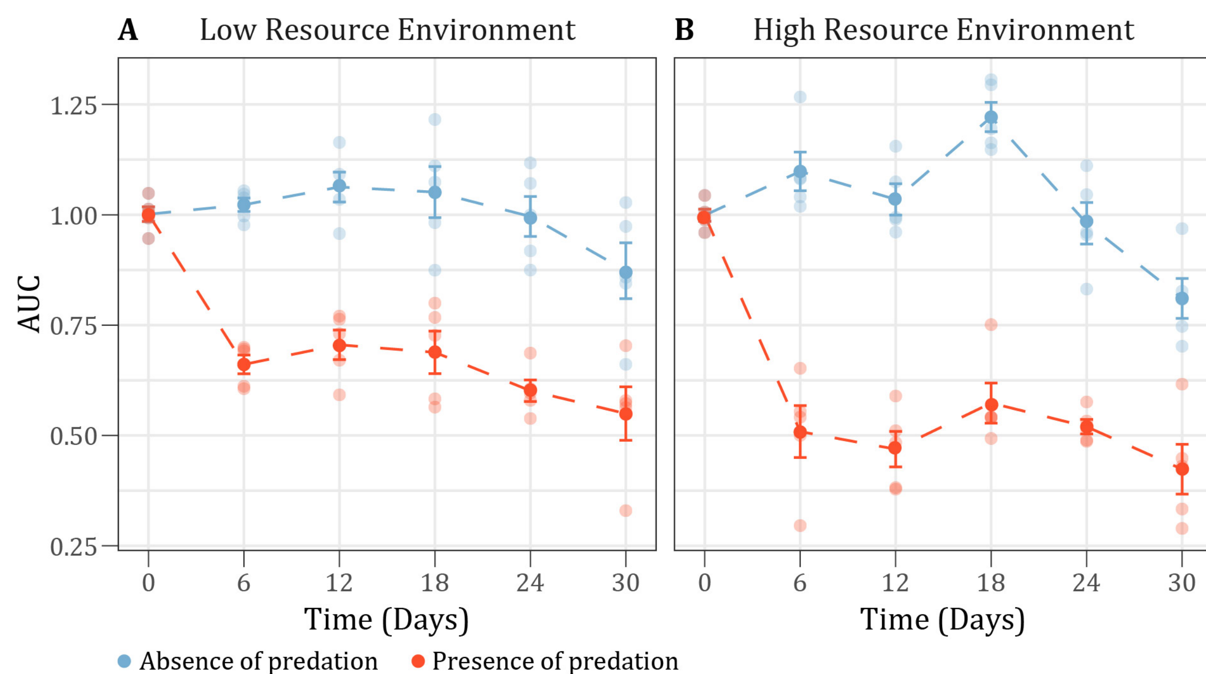

Figure 5 | Growth of the bacterial populations

AUC shown in blue for populations evolved in the absence of predation, and in red for populations evolved in the presence of predation **A.** in the low resource environment, **B.** in the high resource environment. Translucent points: replicate populations, solid points: mean of replicate populations, error bars: standard error of mean. Relative AUC = 1 indicates ancestral AUC.

From the growth curves, we obtained the lag phase of each population evolved either in the presence or absence of predation, and in both resource levels, which demonstrates the duration before the population starts the exponential phase. We found a significant increase in the lag phase of populations evolved in the presence of predation. Lag phase of populations evolved in the high resource environment was significantly lower than the lag phase of populations evolved in the low resource environment (Fig. 6, Table S4 for statistics).

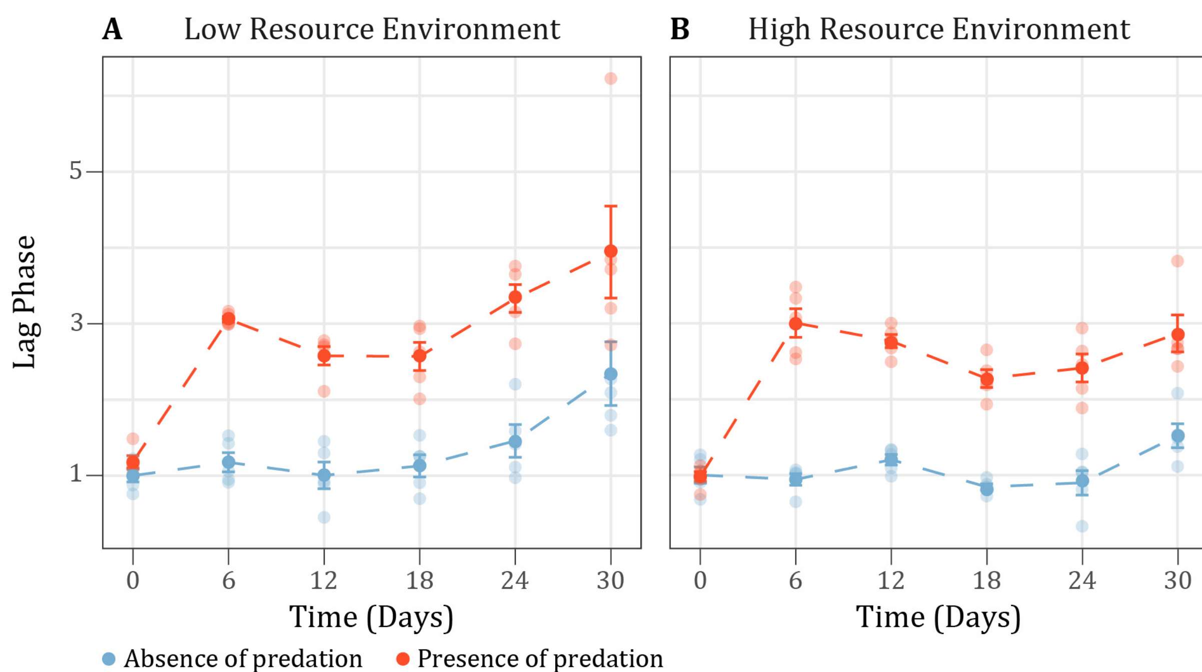

Figure 6 | Lag phase of the bacterial populations

Lag phase shown in blue for populations evolved in the absence of predation, and in red for populations evolved in the presence of predation **A.** in the low resource environment, **B.** in the high resource environment. Translucent points: replicate populations, solid points: mean of replicate populations, error bars: standard error of mean. Lag phase = 1 indicates ancestral lag phase.

Also, from the growth curves, we calculated growth rates of each population evolved either in the presence or absence of predation, and in both resource levels. We found that there was a significant effect of resources between the growth rate of bacteria evolved in the presence of predation and the growth rate of bacteria evolved in the absence of predation (Fig. 7, Table S5 for statistics).

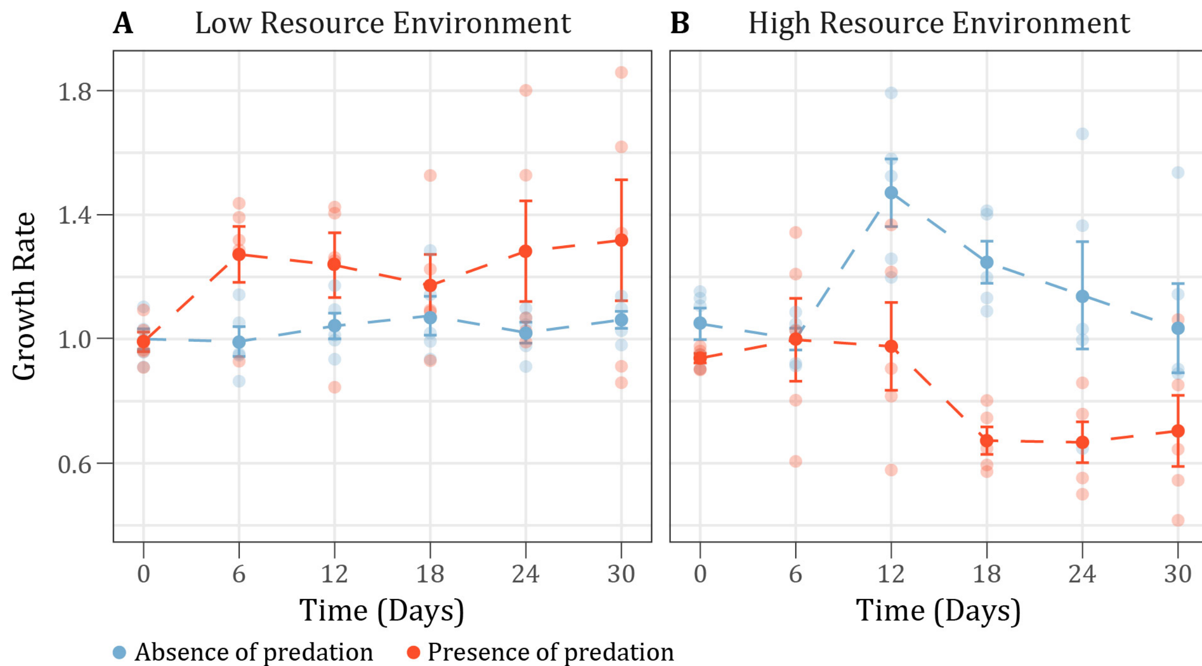

Figure 7 | Growth rate of the bacterial populations

Growth rate shown in blue for populations evolved in the absence of predation, and in red for populations evolved in the presence of predation **A.** in the low resource environment, **B.** in the high resource environment. Translucent points: replicate populations, solid points: mean of replicate populations, error bars: standard error of mean. Growth rate = 1 indicates ancestral growth rate.

Ciliates did not coevolve with bacteria

To test whether ciliates coevolved with bacteria over time, we measured the growth rate of ciliates isolated from both resource levels and from different time points. We did not find any significant difference in growth rates of isolated ciliates relative to ancestral ciliates in neither of the resource levels (growth rate of ancestral ciliates when fed by ancestral bacteria in the LRE=0,069420±0,004293; in the HRE=0,101964±0,001043; growth rate of ciliates from day 30 when fed by ancestral bacteria in the LRE=0,056351±0,002087; in the HRE=0,097154±0,004954)

Resource levels determine the strength of predation

We followed the changes in densities of predator (ciliates) and prey (bacteria) both in the low and high resource environment (LRE and HRE, respectively) at 6-day intervals over thirty days. We found a significant effect of predation on the density of prey populations, which resulted in lower prey density in the presence of predation in both resource levels (average density of prey in the absence of predation in the LRE=1,1x10^8 and in the HRE=3,5x10^8; Table S6 for statistics). Predation decreased the densities of prey populations in the HRE significantly more than it decreased the densities of prey populations in the LRE (Fig. 8; Table S6 for statistics). Furthermore, predator densities were significantly higher in the HRE than in the LRE (Fig. 8; Table S7 for statistics).

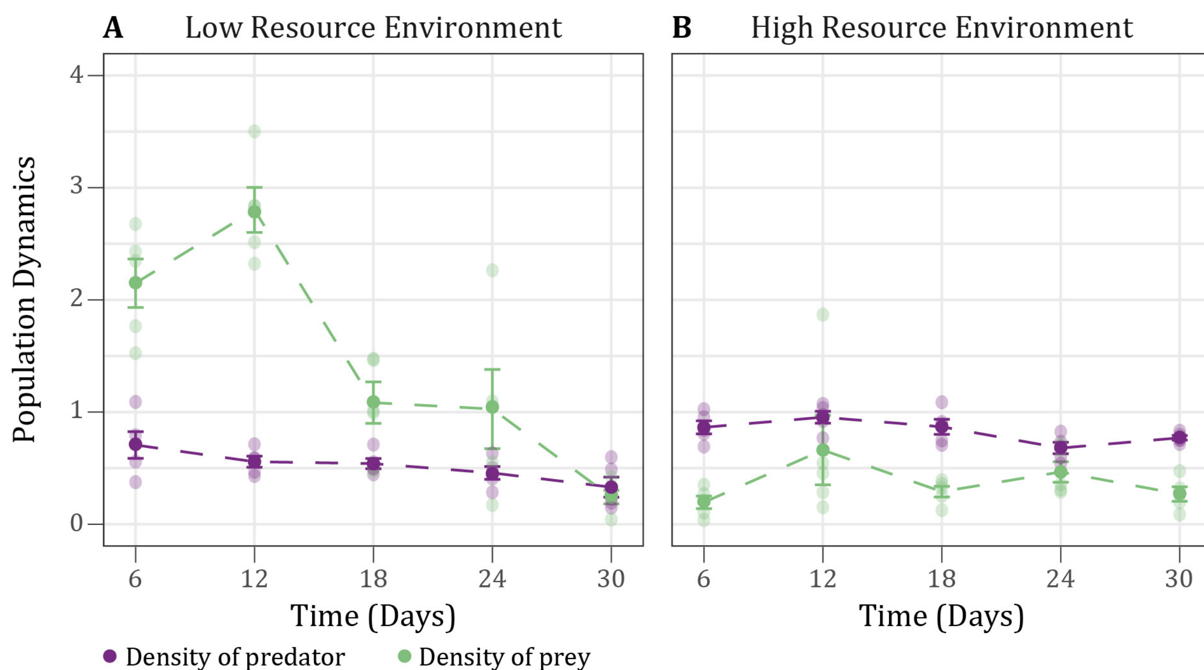

Figure 8 | Predator-prey dynamics during the evolution experiment

Density of predator shown in purple and density of prey shown in green **A.** in the low resource environment, and **B.** in the high resource environment. Densities as 8x10^4 ciliates and 4x10^7 bacterial cells per ml.

Consequently, the predator-prey ratio (i.e. strength of predation) was significantly higher in the HRE than in the LRE during the evolution experiment (Fig. 9B; Table S8 for statistics). We performed a short-term experiment, in which we followed the changes in densities of predator and prey in both resource levels over 72 hours (Fig. S1), and calculated the predator-prey ratios. We found that over time the predator-prey ratios were significantly different between both resource levels (Fig. 9A; Table S9 for statistics).

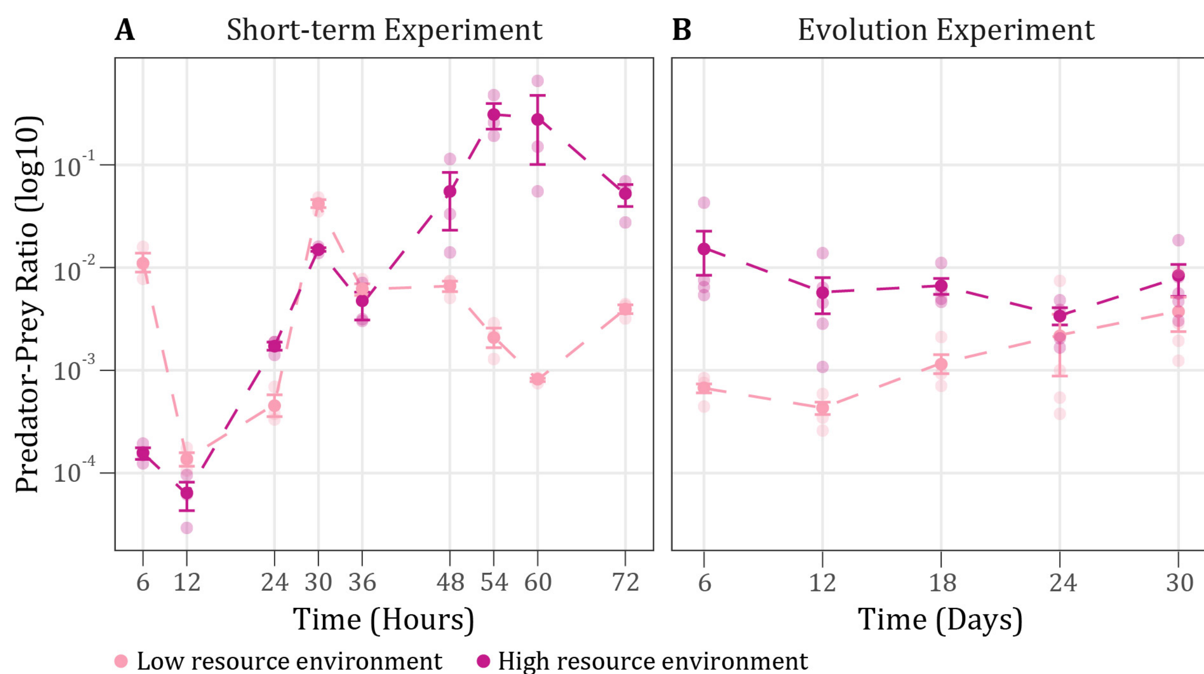

Figure 9 | Predator-prey ratio

Predator-prey ratio during **A.** the short-term experiment, and **B.** the evolution experiment in both resource levels (Methods for details). Predator-prey ratio shown in pink for the low resource environment, and in magenta for the high resource environment. Translucent points: replicate populations, solid points: mean of replicate populations, error bars: standard error of mean.

DISCUSSION

Previous studies on diversification emphasized the importance of resource competition without explicitly testing the effect of interplay between different types of species interactions. In particular, despite having various effects on prey communities (Abrams 2000b; Day et al. 2002; Langerhans 2006; Casini et al. 2012; also reviewed in Koch et al. 2014), predation has been accepted as a minor player for diversification (reviewed in Vamosi 2005). Simultaneously acting ecological processes might have, however, diverse effects on diversification depending on the relative strengths of the different species interactions. Resource level of the environment can generate differences in the relative contributions of predation and resource competition, as it might have ecological effects on both predator and prey populations. Here, we studied the role of predation on diversification, and in combination with resource competition when environments differ in the resource levels.

We showed that predation selected for high level of defense and led to diversification of ancestral bacteria into different colony morphologies (i.e. morphotypes) both in the low and high resource environment. Bacterial populations evolved in the presence of predation showed rapid diversification, as most of the evolved morphotypes were observed already on day 6. Interestingly, different morphotypes emerged in different resource levels and we found higher diversity in the low resource environment compared to the high resource environment. In the absence of predation, however, diversification occurred neither in the low nor in the high resource environment. Specifically, morphotypes observed at the end of the evolution experiment were similar to the ancestral colony morphology. Hence, the strength of resource competition was not strong enough to generate diversification in the absence of predation. Collectively, these data suggest that in this experiment the presence of predation gave rise to diversification of bacteria, and the differences in the extent of diversity between the two resource levels could be due to the effect of resource level of the environment on selection by predation.

We investigated the contribution of the resource levels to predation by following predator-prey ratio in replicate populations over time, as a direct measure for the

strength of predation. We found that in the high resource environment the strength of predation was significantly greater over time compared to the low resource environment during the evolution experiment. Besides, we performed a short-term experiment, and followed the predator-prey ratio in both resource levels. We discovered that the strength of predation was also greater in the high resource environment compared to the low resource environment after 48 hours during the short-term experiment. Thus, we confirmed that higher predator-prey ratio resulted from increased resources, and it was not due to the evolution in prey and/or predator. Thereby, we discovered that the resource level of the environment led to an eco-evolutionary feedback; differences in the resource level led to differences in the number of predators per prey, which changed the strength of selection for diversification, which determined the level of diversity.

To the best of our knowledge, there has been no direct evidence showing predation as the main ecological process driving diversification (but see Meyer and Kassen 2007). Also, resource level of the environment has been suggested to alter largely resource competition. For instance, it has been shown that diversity was highest at intermediate resource availabilities (Kassen et al. 2000; Hall & Colegrave 2007; Friman et al. 2008; Hall et al. 2008). We showed that resource level of the environment affects also the strength of predator-prey interaction, and thus the extent of diversity. Specifically, increasing the resources available to the prey increased the predator-prey ratio and resulted in higher selection by predation, which in turn selected for lower level of diversity. Thus, our experiment demonstrated an indirect effect of resource level of the environment on diversity.

CHAPTER 2

Predation in interaction with resource competition leads to divergence in defense traits

INTRODUCTION

Bacterial strains differ in many traits like any other organism. Biofilm formation, which is an ecologically important characteristic observed at various strains, is a way of surface colonization. In addition to biofilm formation, there are also other ways to colonize surfaces such as surface motility and fruiting body development (Harshey 2003). However, one trait might have several advantages depending on the environmental conditions. For instance, it has been shown that biofilm formation increases the resource capture, resistance against natural enemies and efficiency of horizontal gene transfer (reviewed in Stoodley et al. 2002 and Flemming et al. 2016). Since environments are often complex with different selection pressures changing over time, when one trait is favored over another is not well understood.

Both predation and resource competition have important ecological and evolutionary consequences on bacterial communities by changing their density, diversity and stability (Jürgens & Matz 2002; Corno & Jürgens 2006; Saleem et al. 2013; Litchman et al. 2015; Ferenci 2016; Sexton & Schuster 2017). Specifically, predation might favor diverse defense traits such as aggregation of cells, increased motility and toxin production (reviwed in Matz & Kjelleberg 2005, Pernthaler 2005 and Jousset 2012). These different defense strategies might result from the differences in predator-prey interaction such as feeding mode of predator and strength of predation pressure. However, it is also likely that evolution of such diverse defense traits depends on other species interactions in the environment. For instance, these different defense strategies might associate with different growth costs; consequently their evolution might also depend on the strength of resource competition in the environment. Since it has been challenging to study the effect of interplay between species interactions on selection, assembled bacterial communities were often used to infer the underlying ecological pressures responsible from observed trait differences. How trait divergence is initiated in the presence of predation and resource competition has remained unexplored.

In Chapter 1, we showed that predation promoted diversification of bacteria (i.e. evolution of different colony morphologies), and that the resource level of the environment altered the extent of diversity in the presence of predation. Thus, our

evolution experiment offered an invaluable opportunity to provide insight into trait evolution under multiple selection pressures. Several studies showed that colony morphology is a complex trait, which is determined by various cellular phenotypes such as cell shape, motility and differential exopolymer expression (Shapiro 1998; Watnick et al. 2001; Rakhimova et al. 2008; Workentine et al. 2010).

Here, we investigated ecologically relevant phenotypic traits to characterize the evolved morphotypes and determined their fitness (growth and defense) in different resource levels to test whether and how their evolutionary advantage changed depending on the ecological conditions. We showed that morphotypes evolved in the absence of predation did not show a divergence in any trait compared to the ancestor. Morphotypes evolved in the presence of predation, however, had high level of defense and displayed size-related defense traits. Specifically, different defense traits such as exoploymer overproduction and cell-chaining evolved and coexisted in the low resource environment as a result of selection generated by interacting effects of predation and resource competition. Our study demonstrated that combination of predation and resource competition changed the fitness landscape over time, and led to the evolution of different defense traits.

MATERIAL AND METHODS

Isolating evolved morphotypes

In order to study the phenotypic and genotypic characteristics of evolved bacteria, we isolated 10 clones for each morphotype across different treatments and replicate populations at the end of the evolution experiment (on day 30). Dilution of cryopreserved samples of replicate populations from day 30 were plated onto M9KB agar plates directly from glycerol stocks. We incubated these plates for 48 hours at 28°C. Then we picked single clones from each type and streaked them onto M9KB agar plates (i.e. purification plates). We took photographs (VisiCam, VWR) under a dissection microscope after 48 hours of incubation at 28°C to confirm and record the colony morphology. Then, we isolated single clones from these plates and prepared overnight cultures. After 16 hours of incubation at 220 rpm and 28°C in the M9KB medium, isolated morphotypes were cryopreserved in 45 % ($^{v}/_{v}$) glycerol saline and stored at -80°C (Table 1). We used other strains of *Pseudomonas fluorescens* SBW25 with known genotypes (i.e. wrinkly spreader and fuzzy spreader, obtained from Dr. Jenna Gallie) and characteristics as control for the assays described below.

Table 1 | Evolutionary history of isolates used for identifying morphotypes

Isolate	Morphotype	Resource level	Predation	Population
*1	M1	LRE	Absent	R1
2	M1	LRE	Absent	R1
*3	M1	LRE	Absent	R2
4	M1	LRE	Absent	R2
5	M1	LRE	Absent	R3
*6	M1	LRE	Absent	R3
7	M1	LRE	Absent	R4
8	M1	LRE	Absent	R4
9	M1	LRE	Absent	R5
10	M1	LRE	Absent	R5
11	M2	HRE	Absent	R1
12	M2	HRE	Absent	R1
*13	M2	HRE	Absent	R2
14	M2	HRE	Absent	R2
*15	M2	HRE	Absent	R3
16	M2	HRE	Absent	R3
17	M2	HRE	Absent	R4
18	M2	HRE	Absent	R4
*19	M2	HRE	Absent	R5
20	M2	HRE	Absent	R5
*21	M3	LRE	Present	R2
22	M3	LRE	Present	R2
23	M3	LRE	Present	R2
*24	M3	LRE	Present	R2
25	M3	LRE	Present	R2
26	M3	LRE	Present	R3
27	M3	LRE	Present	R3
*28	M3	LRE	Present	R3
29	M3	LRE	Present	R3
30	M3	LRE	Present	R3
*31	M4	LRE	Present	R1
32	M4	LRE	Present	R2
33	M4	LRE	Present	R3
34	M4	LRE	Present	R4
*35	M4	LRE	Present	R5

36	M4	HRE	Present	R1
37	M4	HRE	Present	R1
38	M4	HRE	Present	R3
39	M4	HRE	Present	R3
*40	M4	HRE	Present	R4
41	M5	HRE	Present	R2
42	M5	HRE	Present	R2
*43	M5	HRE	Present	R2
44	M5	HRE	Present	R4
45	M5	HRE	Present	R4
*46	M5	HRE	Present	R4
47	M5	HRE	Present	R4
*48	M5	HRE	Present	R5
49	M5	HRE	Present	R5
50	M5	HRE	Present	R5
*51	M6	LRE	Present	R1
52	M6	LRE	Present	R1
*53	M6	LRE	Present	R2
54	M6	LRE	Present	R2
55	M6	LRE	Present	R2
56	M6	LRE	Present	R3
57	M6	LRE	Present	R4
58	M6	LRE	Present	R4
*59	M6	LRE	Present	R5
60	M6	LRE	Present	R5

All isolates selected at the end of the evolution experiment (i.e. on day 30).

All isolates used to characterize colony morphology and cell shape.

* Three isolates from each morphotype picked randomly for further assays.

IDENTIFYING TRAIT DIFFERENCES

Cell shape, capsule expression and motility assays

Overnight cultures of cryopreserved isolates were incubated 16 hours in the M9KB medium at 220 rpm and 28°C. 2 µl of overnight cultures were mixed with 4 µl of India ink on the microscope slide and microscopy was done. India ink was used for contrast to ease the visualization of capsulated cells. Photographs were taken to record cell shape. We diluted the dense overnight cultures to be able to observe motility.

Cellulose production assay

We used calcofluor (Fluroescent Brightener 28) for the visualization of cellulose. Calcofluor binds to acetylated form of cellulosic polymers (ACP), and fluoresces blue under 240 nm wavelength. We prepared M9KB agar plates containing 200 µg ml^{-1} calcofluor. Subsequently, 5 µl of overnight cultures of cryopreserved isolates were dropped onto the M9KB-calcoflour plates and allowed to dry and incubated at 28°C for 24 hours. Next day, each drop was scrapped off and suspended in 200 µl dH_2O and vortexed at maximum speed for 45 seconds. 6 µl of the suspension was dropped onto a microscope slide and viewed via fluorescent microscopy.

Biofilm formation and siderophore assays

To test for biofilm formation, we randomly selected three isolates from each morphotype (Table 1). We used wrinkly spreader, fuzzy spreader and ancestral smooth morph as controls. Overnight cultures of each isolate were inoculated into microcosms filled with 6 ml full-strength M9KB medium with three replicates. Microcosms were incubated for five days at 28°C without disturbance. We took photographs of the microcosms after 48 hours showing the presence or absence of biofilm. We also tested for siderophore production after five days of incubation by checking whether bacteria produced bright green colored medium.

Growth inhibition assay

To investigate the effect of secondary metabolites produced by bacteria on ciliate growth, we performed two experiments. We randomly selected three isolates from each morphotype (Table 1). First, we centrifuged overnight cultures of each isolate and the ancestor at 13,000 rpm for 10 minutes. Subsequently, subsamples from the supernatants were inoculated into 24-well plates with two technical replicates. We added 2100 ciliates into each well containing the supernatant (i.e. bacterial broth) that included leftover resources and compounds secreted by the bacteria. We incubated well plates at 28°C for 72 hours. Finally, we counted the number of ciliates with Flow cytometer (Chapter 1 for details), and calculated the growth rates. Since we couldn't control whether or not any bacteria were left alive in the broth after centrifugation, we performed another experiment. In this experiment, we froze the overnight cultures without any protectant (i.e. without glycerol) and kept them at -80°C for 48 hours. We defrosted the samples, and followed the same procedure explained above.

Measuring the defense levels of morphotypes

We measured defense levels of three isolates from each morphotype (Table 1) both in 5% and 20% M9KB media. We inoculated 2 μl from overnight cultures of each isolate into 2 ml of 5% and 20% M9KB media in 24-well plates with three technical replicates. Then, we added ~2100 ciliates from stock cultures into each well. Before adding ciliates, PPY medium was removed by centrifugation (3000 rpm, 8 minutes, 0°C) and the remaining pellet containing the ciliates was re-suspended in the test medium (either 5% or 20% M9KB media). After 48 hours, ciliates were counted using Flow Cytometer, and differences in final ciliate densities were taken as estimates for the bacterial defense levels. This approach gives direct information about the strength of the trophic link between prey and predator. Defense level of morphotypes were calculated relative to ancestor by using the formula of $D=1-(CD_{evolved}/CD_{ancestor})$, where $CD_{evolved}$ is the density of naive ciliates fed on evolved bacteria, and $CD_{ancestor}$ the density of naive ciliates fed on the ancestral bacteria. When D equals to 0, defense level of a morphotype is equal to the defense level of ancestor. D values greater than 1 indicate higher levels of defense relative to the ancestor, and vice versa.

Measuring the growth parameters of morphotypes

We obtained growth curves of three isolates from each morphotype (Table 1) and ancestor both in 5% and 20% M9KB media. We inoculated 2 µl from overnight cultures of each isolate into 198 µl of 5 % and 20 % M9KB media in 96-well plates with three technical replicates. This assay was replicated three times with samples in randomized positions on 96-well plates. Kinetic measurements of OD_{600} were obtained every 5 minutes at 28°C with Epoch2 plate reader (Biotek). Prior to every measurement, the plate was shaken for 5 seconds in orbital mode with a frequency of 282 cpm. We analyzed the growth curves for several parameters such as Area Under Curve (AUC), lag phase, growth rate after log transformation of the OD_{600} values (Chapter 1 for details).

IDENTIFYING GENOTYPES

Designing primers for candidate genes

We designed primers to perform PCR-based Sanger sequencing (Table 2) for genotyping three isolates of M4, M5 and M6 each (Table 1). We prepared primer stocks with final concentrations of 100 pmol μl^{-1} from dried primers (Metabion). We stored primer stocks at –20°C.

Table 2 | Primers designed for PCR and targeted Sanger sequencing

Name	Sequence (5' → 3')	Annealing Temp. (°C)	Target
*PFLU1224_f1	CGGTGATGGTGGTGTCCTAC	58	*wspF*
*PFLU1224_r1	CGGTCTTGATGTCGTCGATCTG	58	*wspF*
PFLU1224_f2	TGGCTATCGGCTCCTCG	58	*wspF*
PFLU1224_f3	CGTGAGCCGGGTGTTC	58	*wspF*
PFLU1224_f4	GGCAGAGCCGGTGAAC	58	*wspF*
*PFLU5329_f1	TGTTCCTGTTCGACTGCCTAC	58	*mwsR*
*PFLU5329_r1	GGATATGCAGGGCTTCGTTG	58	*mwsR*
*PFLU5329_r2	CGAGGAACATCAACACCACC	58	*mwsR*
PFLU5329_f2	GAGTTCTTTGTGACGGCCAG	58	*mwsR*
PFLU5329_f3	TTGCACCAGCACAGCAC	58	*mwsR*
*PFLU5211_f1	TGTTTGAAGAGGCCGTGC	58	*awsX*
*PFLU5211_r1	GTTGTGTTCGGCATACACCC	58	*awsX*
PFLU5211_r2	ATG CGG CAA ACA GGT GG	58	*awsX*
*PFLU1301_f1	CAACATGACTGGCACGACG	58	*nlpD*
*PFLU1301_r1	CCTCAAGCTCAAGCGGC	58	*nlpD*
PFLU1301_f2	GTTGGGGATGGCCTTCAAATG	58	*nlpD*

* These primers used both for PCR and Sanger sequencing, while others used only for Sanger sequencing.

Targeted Sanger sequencing

We randomly selected three isolates from each morphotype (Table 1). To obtain DNA templates, we centrifuged 250 µl of the overnight cultures at 13000x g for 1 minute, then removed 200 µl of the supernatant and re-suspended the pellet in 200 µl dH_2O. Polymerase chain reaction (PCR) of the candidate genes was performed for each morphotype. One reaction with a final volume 25 µl consisted of 11 µl dH_2O, 5 µl 5x Combinatorial Enhancer Solution (original recipe from Dr. Jenna Gallie), 2.5 µl 10x NH_4 reaction buffer (Stratec), 0.75 µl 50mM $MgCl_2$ solution (Stratec), 0.5 µl 50x dNTP mix (Metabion), 2 µl of forward primer with 5 pmol $µl^{-1}$ concentration, 2 µl of reverse primer with 5 pmol $µl^{-1}$ concentration, 0.25 µl Taq Q-DNA polymerase (Stratec) and1 µl of DNA template. We used the following PCRs program; denaturation at 96°C for 2 minutes, amplification cycle, which includes denaturation at 96°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 1 min, repeated 29 times, final extension step at 72˚C for 5 minutes, and samples were cooled to 4 ˚C indefinitely. Obtained PCR products were checked on 1% agarose gel stained with 5 µl SYBR safe (Thermo Fisher). Log-2 DNA ladder (Thermo Fisher) was used to confirm the size of the PCR product. ExoI/TSAP (Thermo Fisher) cleanup was performed to remove left over dNTPS and primers. 5 µl ExoI/TSAP mixture consisted of 4.7 µl dH_2O, 0.2 µl TSAP and 0.1 µl ExoI added into PCR products and incubated at 37°C for 90 minutes, and then 85°C for 15 minutes. Purified PCR products were used as templates for the sequencing reaction and we used three or more primers depending on the fragment size to have overlapping regions (Table 1). Each reaction had the final volume of 10 µl consisting of 0.5 µl Big Dye Terminator (Thermo Fisher), 2 µl 5x Big Dye buffer (Thermo Fisher), 5.5 µl HPLC water, 1 µl primer with 5 pmol $µl^{-1}$ concentration and 1 µl of purified PCR product. Reactions were performed with the following program; denaturation at 96°C for 1 minutes, amplification cycle, which includes denaturation at 96°C for 10 seconds, annealing at 58°C for 15 seconds and extension at 60°C for 4 min, repeated 29 times, and samples were cooled to 4˚C for 5 minutes. Finally, X-Terminator cleanup was performed with a mixture of 47 µl SAM solution (Thermo Fisher) and 10 µl X terminator solution (Thermo Fisher) for one sample. Purification was performed by incubating samples at room temperature at 2000 rpm for 30 minutes. Subsequently, they were centrifuged at 1000x g for 2 minutes. Samples are stored at 4˚C until they were run for

Sanger sequencing. Obtained sequences were aligned and mapped to reference sequence using Geneious.

Statistical analysis

Statistical analyses were performed with R (R Core Team, 2015) in Rstudio Version 1.0.143). To test for differences in defense level and growth parameters (AUC, lag phase, growth rate) among morphotypes in different resource levels we used generalized linear models (GLM) with gamma error distribution and resource level and morphotype as fixed effects. In order to obtain pairwise comparisons among morphotypes and resource levels, we applied Tukey honest significant difference (HSD) *post hoc* test using the multcomp package (Hothorn et al. 2008).

RESULTS

IDENTIFYING TRAIT DIFFERENCES

Colony morphology

We isolated 10 clones from each morphotype across different treatments and replicate populations in order to identify different classes of morphotypes (Table 1). In populations evolved in the absence of predation under both resource levels we identified smooth-edged colonies (Fig. 1A; Morphotype 1: M1 and Morphotype 2: M2), which are similar to the ancestral clone (Fig. 1A; Ancestor). Populations evolved in the presence of predation in the low resource environment included large colonies with smooth-edges (Fig. 1A; Morphotype 3: M3), small colonies with smooth-edges (Fig. 1A; Morphotype 4: M4) and large, filamentous-like colonies (Fig. 1A; Morphotype 6: M6). In populations evolved in the presence of predation in the high resource environment we found small colonies with smooth-edges (M4) and small, wrinkly-edge colonies (Fig. 1A; Morphotype 5: M5).

Cell shape, cellulose production and capsule production

Cell shape and capsule production for each morphotype was assayed with all isolates stained with India ink. We found that all other morphotypes except M6 produced rod-shaped, single bacteria cells similar to the ancestor. M6 produced cell chains (Fig 1B; M6). In addition, all morphotypes produced motile cells, and had similar proportion of capsulated cells compared to the ancestor (not quantified). We found that all morphotypes produced cellulose (not quantified).

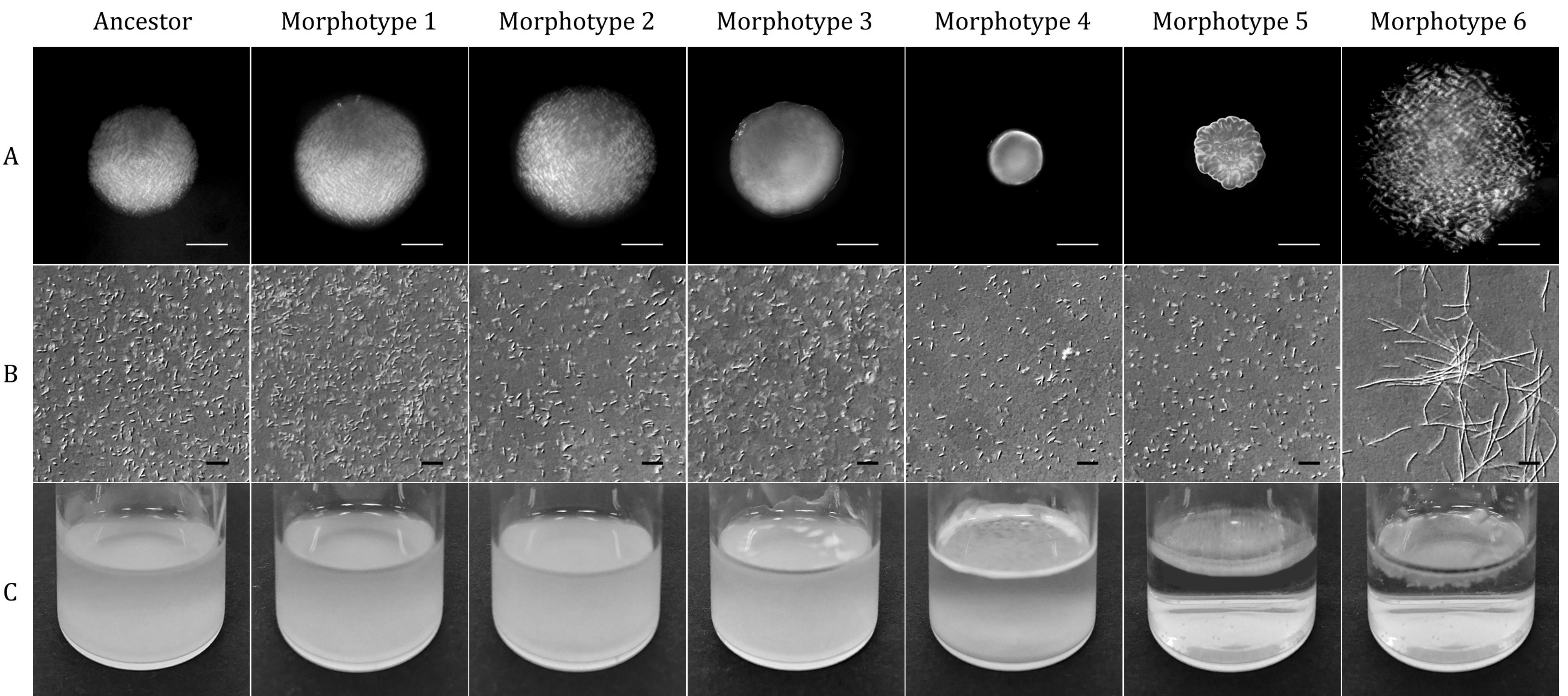

Figure 1 | Trait differences of evolved morphotypes
Morphotypes are characterized based on **A**. colony morphology photographed after 48 hours incubation on M9KB agar plates at 28 °C, **B.** cell shape photographed after staining the cultures, which were incubated 16 hours in full-strength M9KB medium at 200 rpm and at 28 °C, with India ink, and **C.** biofilm formation in the microcosm photographed after 24 hours static incubation in full-strength M9KB medium. Scale bars represent 1 mm on panel **A**, and 10 µm on panel **B**. Contrast and brightness adjusted in Photoshop.

Biofilm formation and siderophore production in full-strength medium

In order to test for biofilm formation, we randomly selected three isolates from each morphotype (Table 1). We found that ancestor, M1 and M2 (evolved in the absence of predation) and M3 (evolved in the presence of predation in the low resource environment) did not form biofilm after 48 hours (Fig. 1C) but only after 4 days of incubation. On the contrary, M4, M5 and M6 (evolved in the presence of predation) formed biofilm already after 24 hours of incubation (Fig. 1C). Also, we observed that biofilms of M4 and M5 were thicker than the biofilm of M6. Moreover, M4 colonized both the broth and on the air-liquid interface, while M5 and M6 colonized only on the air-liquid interface. In addition to biofilm formation, we followed the siderophore production by checking whether the medium became fluorescent over time. After 3 days we observed the production of siderophore for all morphotypes except M6. For M6 production of siderophore could only be observed after 5 days.

Secondary metabolite production

To obtain information about the possible defense strategies, we tested whether any secondary metabolites produced by the evolved bacteria had an adverse effect on ciliate growth (Methods for details). We did not find a considerable difference in ciliate growth rates (ciliate growth rate when grown in the broth of ancestral bacteria=0,049128 ±0,007274; in the broth of M4=0,044052±0,005528; in the broth of M5 =0,045442±0,002591; in the broth of M6=0,053814±0,002836), which showed that bacteria did not evolve to use secondary metabolites as a defense strategy.

Defense level of morphotypes in different resource levels

To test whether the resistance against grazing differs among morphotypes and depending on the resource level of the environment, we measured the defense of each morphotype relative to the ancestor both in the low resource environment (LRE) and in the high resource environment (HRE) (Methods for details). First, for each morphotype we compared its defense level in the LRE with its defense level in the HRE. We found that M3 had a significantly higher defense level in the LRE than in the HRE, and M6 had a significantly lower defense level in the LRE than in the HRE (Fig. 2; Tukey's HSD:

defense level of M3 in LRE vs. HRE, p *adj* < 0.01 and defense level of M6 in LRE vs. HRE, p *adj* = 0.0188).

Both in the low and high resource environment M1, M2 and M3 had significantly lower defense levels compared to the other morphotypes (Fig. 2; Table S1 for statistics). In the LRE, the defense level of M4 did not significantly differ from that of M5 and M6 (Fig. 2; Tukey's HSD: defense level in the LRE: M4 vs. M5, p *adj* = 0.0703 and M4 vs. M6, p *adj* = 1). In the HRE, M4 had a significantly lower defense level than M5 while having similar defense level with M6 (Fig. 2; Tukey's HSD: defense level in the HRE: M4 vs. M5, p *adj* = 0.0397 and M4 vs. M6, p *adj* = 0.6301). In the LRE, M5 had a significantly higher defense level than M6, whereas in the HRE defense levels of M5 and M6 were similar (Fig. 2; Tukey's HSD: defense level in the LRE: M5 vs. M6, p *adj* = 0.0218 and defense level in the HRE: M5 vs. M6, p *adj* = 0.9513).

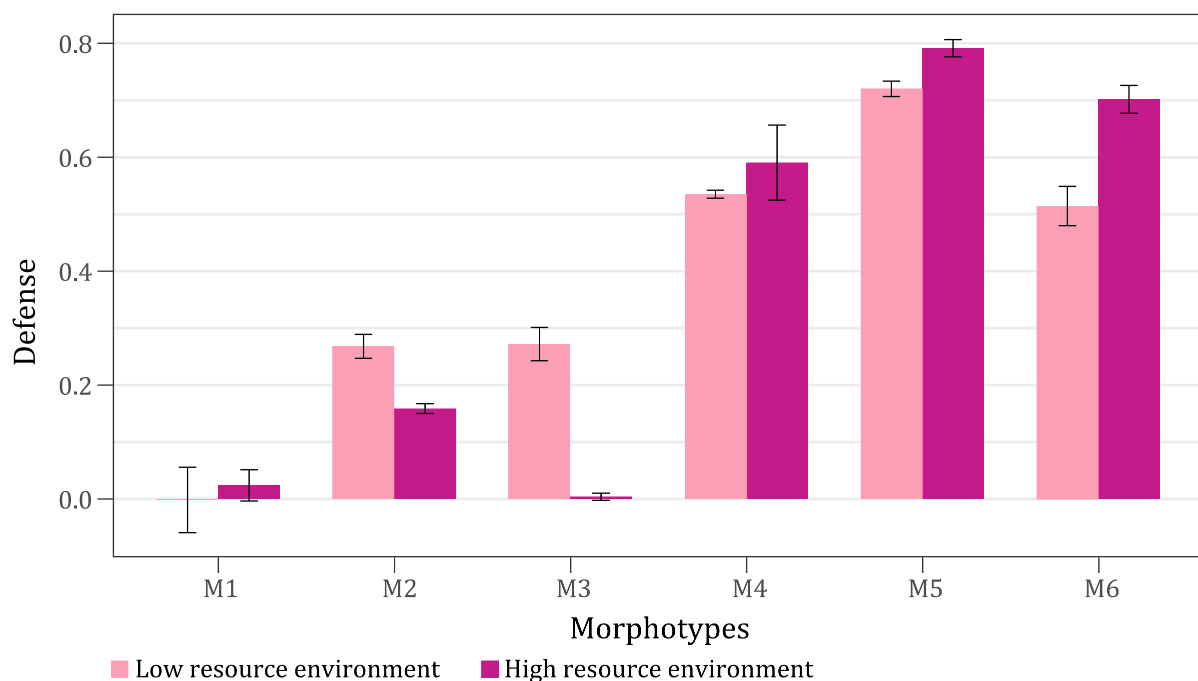

Figure 2 | Defense against predation in different resource levels for each morphotype

Defense shown in pink for low resource environment, and in magenta for high resource environment. Defense = 0 indicates ancestral defense. Bars: mean of three isolates, error bars: standard error of mean, Table S1 for statistics.

Competitive ability of morphotypes in different resource levels

To investigate whether competitive ability differs among morphotypes and depending on the resource level of the environment, we obtained growth curves by kinetic measurement of optical density at 600 nm (Fig. 3), and calculated several growth parameters relative to ancestor (Methods for details).

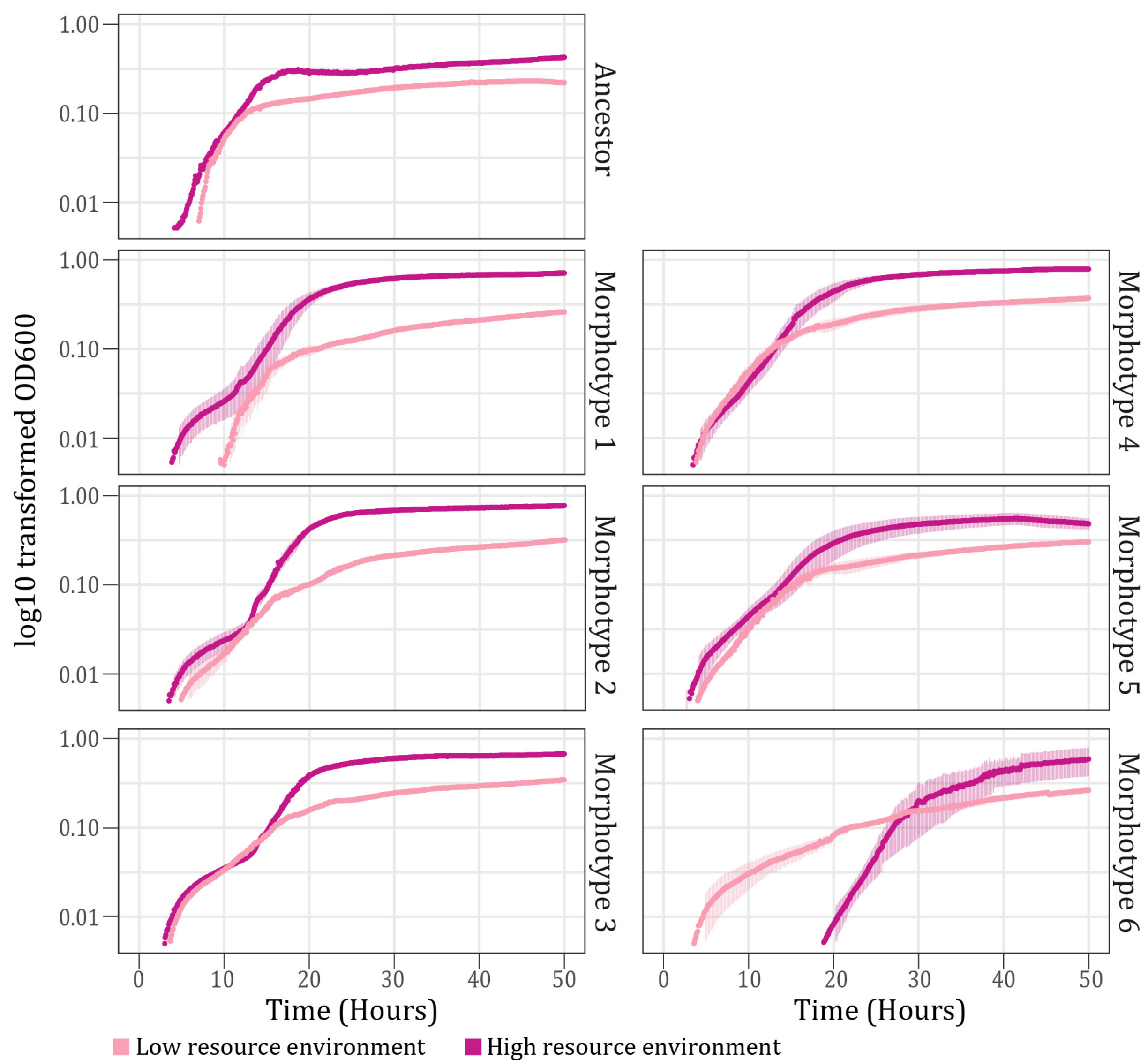

Figure 3 | Growth curves of the morphotypes in different resource levels

Growth curves shown in pink for the low resource environment, and in magenta for the high resource environment. Lag phase, which starts at 0 hour and ends with the start of the exponential phase in each growth curve, is not depicted. Lines: mean of three isolates, error bars: standard error of mean.

We calculated the Area Under Curve (AUC) relative to the ancestor for each morphotype in different resource levels to summarize the growth rate and population density from the growth curves (Methods for details). First, for each morphotype we compared its Area Under Curve (AUC) in the LRE with its AUC in the HRE. We found that M1 had a significantly larger AUC in the HRE compared to its AUC in the LRE (Fig. 4; Tukey's HSD: AUC of M1 in the LRE vs. HRE, p *adj* < 0.01). For other morphotypes there was no significant difference in their AUC between two resource levels. (Fig. 4; Table S2 for statistics). Then, we compared AUC of morphotypes within one resource level. Both in the LRE and HRE M4 and M5 had similar AUC, while M4 had a significantly larger AUC than M6 (Fig. 4; Tukey's HSD: AUC in the LRE: M4 vs. M5, p *adj* = 0.9304; and AUC in the HRE: M4 vs. M5, p *adj* = 0.6249; AUC in the LRE: M4 vs. M6, p *adj* = 0.0221 and AUC in the HRE: M4 vs. M6, p *adj* < 0.01). AUC of M5 did not significantly differ than that of M6 in the LRE, but it was significantly larger than M6 in the HRE (Fig. 4; Tukey's HSD: AUC in the LRE: M5 vs. M6, p *adj* = 0.8882 and AUC in the HRE: M5 vs. M6, p *adj* = 0.0269).

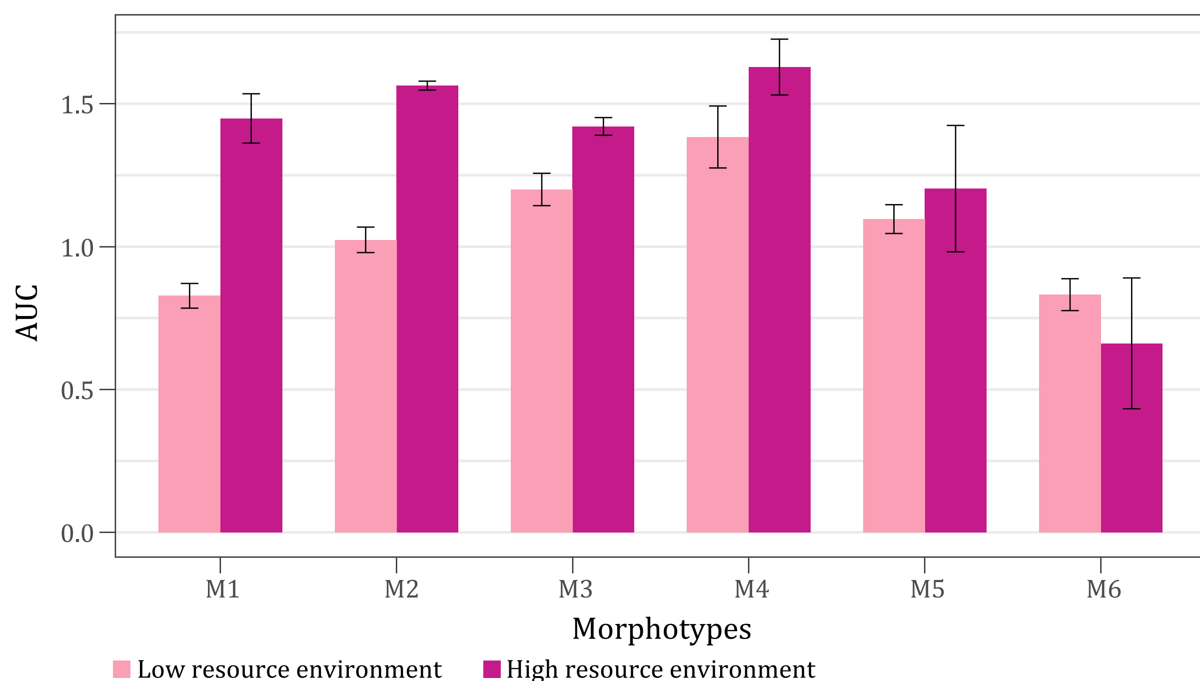

Figure 4 | Growth of morphotypes in different resource levels

AUC shown in pink for the low resource environment, and in magenta for high resource environment. Relative AUC = 1 indicates ancestral AUC. Bars: mean of three isolates, error bars: standard error of mean, Table S2 for statistics.

Lag phase relative to the ancestor obtained from the growth curves demonstrating the duration before the population starts the exponential phase. (Methods for details). For each morphotype we compared its lag phase in the LRE with its lag phase in the HRE. We discovered that M6 had significantly longer lag phase in the HRE compared to its lag phase in the LRE (Fig. 5; Tukey's HSD: Lag phase of M6: LRE vs. HRE, p *adj* < 0.01). For other morphotypes there was no significant difference in their lag phases between two resource levels (Fig. 5; Table S3 for statistics). Then, we compared lag phases of morphotypes within one resource level. Both in the LRE and HRE lag phase of M4 and M5 were not significantly different from each other (Fig. 5; Tukey's HSD: Lag phase in the LRE: M4 vs. M5, p *adj* =0.9981; and Lag phase in the HRE: M4 vs. M5, p *adj* =1). M6 had significantly longer lag phase than M4 and M5 in the HRE, whereas it was not significantly different from M4 and M5 in the LRE (Fig. 5; Tukey's HSD: Lag phase in the LRE: M4 vs. M6, p *adj* = 0.4498; and M5 vs. M6, p *adj* = 0.9935; Lag phase in the HRE: M4 vs. M6, p *adj* < 0.01; and M5 vs. M6, p *adj* < 0.01).

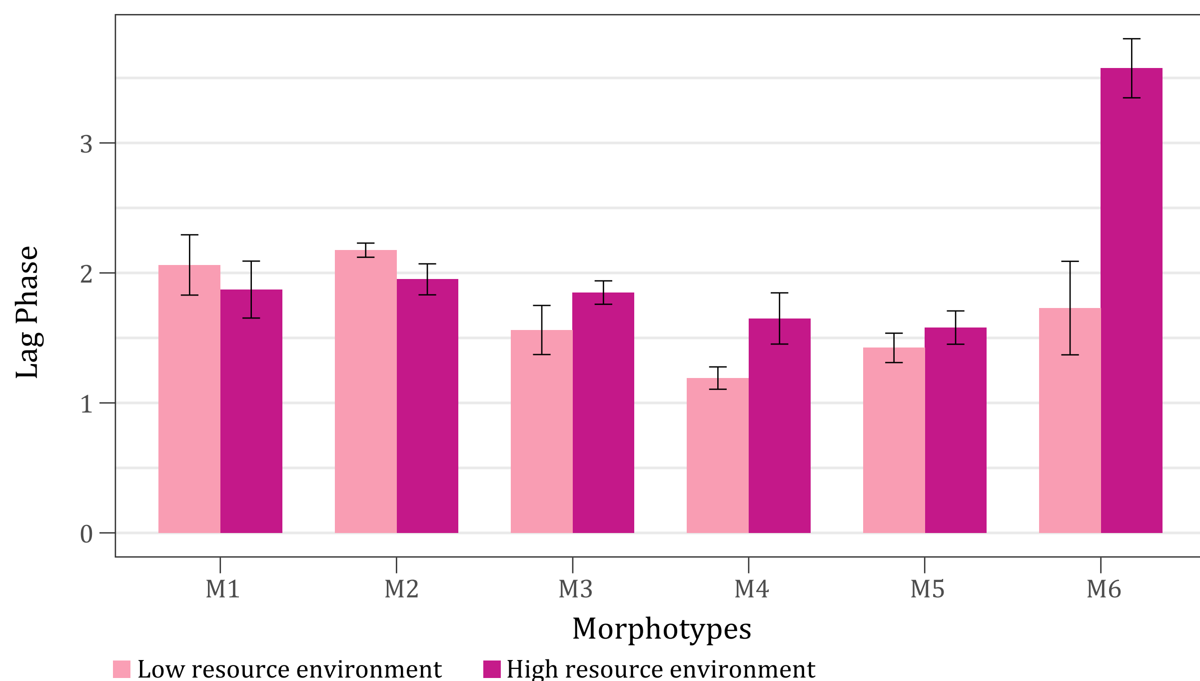

Figure 5 | Lag phase of morphotypes in different resource levels

Lag phase shown in pink for the low resource environment, and in magenta for high resource environment. Lag phase = 1 indicates ancestral lag phase. Bars: mean of three isolates, error bars: standard error of mean, Table S3 for statistics.

Finally, for each morphotype we compared its growth rate in the LRE with its growth rate in the HRE (Methods for more details). We found that growth rate of M1 and M2 were significantly higher in the HRE compared to their growth rates in the LRE (Fig. 6; Tukey's HSD: Growth rate of M1: LRE vs. HRE, p *adj* < 0.01 and M2: LRE vs. HRE, p *adj* < 0.01). For other morphotypes there was no significant difference in their growth rates between two resource levels (Fig. 6; Table S4 for statistics). Then, we compared growth rates of morphotypes within one resource level. We did not find any significant difference among the growth rates of M4, M5 and M6 in neither of the environments (Fig. 6; Table S4 for statistics).

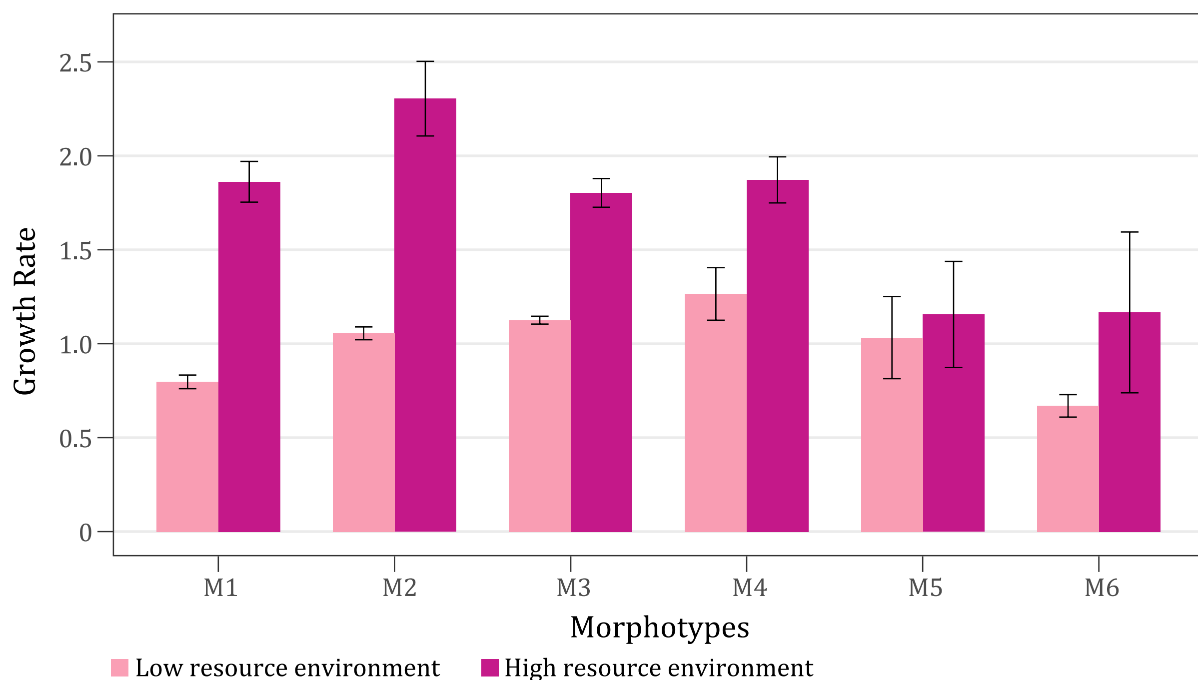

Figure 6 | Growth rate of morphotypes in different resource levels

Growth rate shown in pink for the low resource environment, and in magenta for high resource environment. Growth rate = 1 indicates ancestral growth rate. Bars: mean of three isolates, error bars: standard error of mean, Table S4 for statistics.

IDENTIFYING GENOTYPES

We used three candidate genes to genotype biofilm-forming morphotypes. Specifically, previous studies reported three genes (*wspF*, *awsX* and *mwsR*), which are negative regulators of the di-guanylate cyclases (DGCs) synthesis. DGCs take part in the synthesis of the bis-(3'-5-)-cyclic dimeric guanosine monophosphate (c-di-GMP), and thus responsible for the production of cellulose. Any loss-of-function mutations in these genes increase the production of DGCs, and thereby biofilm-forming phenotype arises. As a result, we were able to identify genotypes of two biofilm-forming morphotypes. Isolates of M4 showed mutation in *mwsR* gene, while isolates of M5 has mutation in *wspF* gene (Table 3). These two morphotypes belong to previously described class of wrinkly spreaders.

The *nlpD* mutant of *Pseudomonas fluorescens* SBW25 produces cell-chaining bacteria (Lind et al. 2016). Sequencing isolates of M6 for *nlpD*, we did not find any mutation in that gene; and we were not able to identify mutation(s) responsible from cell-chaining phenotype.

In our phenotypic assays, we did not find any difference between ancestor and M1, M2 and M3. Therefore, we were not able to decide on candidate genes to genotype those morphotypes.

Table 3 | Identified mutations in M4 and M5

Isolate	Morphotype	Locus	Gene ID	Mutation	Effect
IS31	M4	PFLU5329	*mwsR*	C1219G	Substitution
IS35	M4	PFLU5329	*mwsR*	C1219G	Substitution
IS40	M4	PFLU5329	*mwsR*	A459T	Substitution
IS43	M5	PFLU1224	*wspF*	C479G	Substitution
IS46	M5	PFLU1224	*wspF*	C923T	Substitution
*IS48	M5	PFLU1224	*wspF*	-	-

* No mutation identified in this isolate for neither of the loci.

DISCUSSION

Complex environments create simultaneously acting selection pressures, whose relative strength changes over time (reviewed in Thompson 2013 and Hendry 2016). In our evolution experiment, we found that different colony morphologies evolved in different resource levels in the presence of predation, and we showed that the contribution of resource level to predation differed between the two resource levels (i.e. stronger predation pressure in the high resource environment, Chapter 1). Here, we investigated ecologically relevant traits of evolved morphotypes to understand how differences in the environments might determine diverse trait evolution.

We discovered that the coexisting morphotypes in the low resource environment exhibited different defense strategies. In particular, Morphotype 4 (M4) formed aggregated cells via exopolymer production (i.e. clumping morphotype), whereas Morphotype 6 (M6) produced cell chains (i.e. cell-chaining morphotype). These morphotypes had similar defense levels, but M4 was a better competitor than M6 due to its significantly higher growth when tested in isolation (Fig. 4). These data suggested that differences in the defense traits were not solely selected by predation, but by predation in combination with resource competition. Previous studies showed that when predation and resource competition are acting together, defensive and competitive types might coexist through a growth-defense trade-off (e.g. Bohannan et al. 2002). In our experiment, however, we showed that the coexistence in the low resource environment did not result from a growth-defense trade-off because while having different competitive abilities the coexisting morphotypes had similar defense levels. Instead, we proposed that the relative strengths of predation and resource competition acting simultaneously might differ over time and alter the fitness landscape. Specifically, an increase in bacterial density led to strong resource competition and eventually increased the density of predator. Strong predation decreased the bacterial density, and therefore reduced the strength of resource competition (Fig. 7). Due to such fluctuations in the strength of resource competition, the competitive morphotype could not exclude the other one. Therefore, we suggested that this change in the fitness landscape mediated coexistence of clumping and cell-chaining morphotypes.

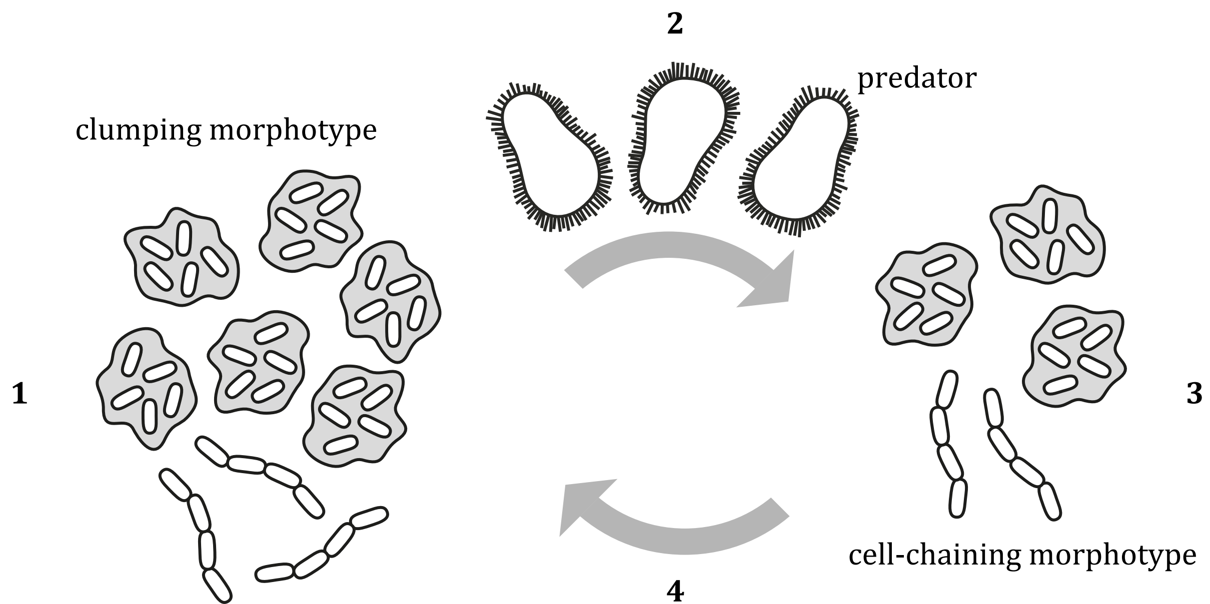

Figure 7 | Hypothesized mechanism acting on clumping and cell-chaining morphotypes in the low resource environment

1. When predators are low in density, bacteria increase in density, and thus the strength of resource competition increases, **2.** over time predator density increases and reduces the overall bacterial density. **3.** Consequently, resource competition decreases, and **4.** cell-chaining morphotype increases in density again.

In the high resource environment either M4 or M5 dominated replicate populations in the presence of predation. We identified their genotypes based on the existing knowledge on the genetics of *Pseudomonas fluorescens* SBW25. After identifying loss-of-function mutations in two different loci of DGC regulatory pathways (i.e. *wspR* and *wspF*), we classified both morphotypes as wrinkly spreaders overproducing cellulose-like exopolymers. We found that they had similar defense levels and growth. Therefore, we proposed that the one that dominated the population might be depending on the order of mutation. Moreover, wrinkly spreaders have been shown to colonize air-liquid interface in the absence of predation when the environment is spatially structured (i.e. static incubation) (Rainey & Travisano 1998). It has been also shown that there is a strong correlation between the level of resources of the environment and the percentage of evolved wrinkly spreaders in a population (Travisano & Rainey 2000). Since we did not observe diversification in the absence of predation during our evolution experiment, we confirmed that wrinkly spreaders were not favored in the high resource environment of the evolution experiment (i.e. four times less resources

relative to full strength medium, Chapter 1 Material and Method). Thus, we concluded that evolution of this trait (i.e. exoploymer overproduction) in the high resource environment was not due to an adaptation to spatially structured environment but to predation.

Furthermore, we showed that the fitness of M6 decreased when tested in the high resource environment due to its longer lag phase and smaller AUC than M4 and M5. This could explain why we did not observe the cell-chaining phenotype in the high resource environment during the evolution experiment (Chapter 1, Fig. 1).

Overall, our data indicate that predation led to a strong directional selection for defense evolution favoring one defensive phenotype (clumping morphotype) in the high resource environment by generating a fitness landscape with a single fitness peak. This is consistent with our findings from Chapter 1, where we showed predation pressure was stronger in the high resource environment than in the low resource environment. Additionally, in the low resource environment, two different defense traits were selected for and differed in their competitiveness. Thus, we suggest that in the low resource environment relative strengths of predation and resource competition were changing over time and created a fitness landscape with multiple fitness peaks. In conclusion, our study draws attention on studying simultaneously acting ecological processes, because such dynamic ecological interactions could provide substantial insight into the mechanisms promoting or constraining trait divergence.

CHAPTER 3

Predation minimizes fitness difference and enables stable coexistence

INTRODUCTION

Understanding the ecological conditions, under which species having similar ecological traits can coexist, has been one of the major challenges in ecology (reviewed in Hille Ris Lambers et al. 2012). The competitive exclusion principle states that two species cannot coexist, if they compete for the same limiting factor (i.e. same niche), because the one having the slightest fitness advantage over the other eventually takes over the population (Gause 1932). Additionally, the modern coexistence theory explains stable coexistence of species with two mechanisms: 1) stabilizing mechanisms that reduce niche overlap between species, and 2) equalizing mechanisms that reduce the average fitness difference between species (Chesson 2000).

Niche differentiation mediates coexistence, if competing species specialize on different resources or inhabit different parts of the environment (Fig. 1A). For instance, different species of *Anolis* lizards coexist by adapting to various habitats differing in vegetation and other environmental factors, which results in non-overlapping niches, and leading to stable coexistence (Losos 1998). Despite overlapping niches, species can coexist through a stabilizing mechanism reducing interspecific competition (Fig. 1B). For example, three bumblebee species in the *Bombus lucorum* complex coexist stably by differing in their forage use and temporal activity patterns (Scriven et al. 2016). When the degree of niche overlap leads to higher interspecific competition relative to intraspecific competition due to the lack of a strong stabilizing mechanism competitive exclusion occurs (Fig. 1C). However, even a weak stabilizing mechanism can mediate coexistence, if competing species have similar fitness levels (Chesson 2000; Adler et al. 2007). Despite the high degree of niche overlap due to the lack of a strong stabilizing mechanism, equalizing mechanisms can contribute to stable coexistence by reducing the fitness difference between species (Fig. 1D). For example, diversity in *Brassica* species increased in the presence of pathogens because inferior species produced more seeds through a compensatory response to pathogen infection and overcame competitive exclusion (Bradley et al. 2008). Overall, stabilizing mechanisms through generating low interspecific competition relative to intraspecific competition and together with equalizing mechanisms through reducing fitness inequalities promote the maintenance of diversity.

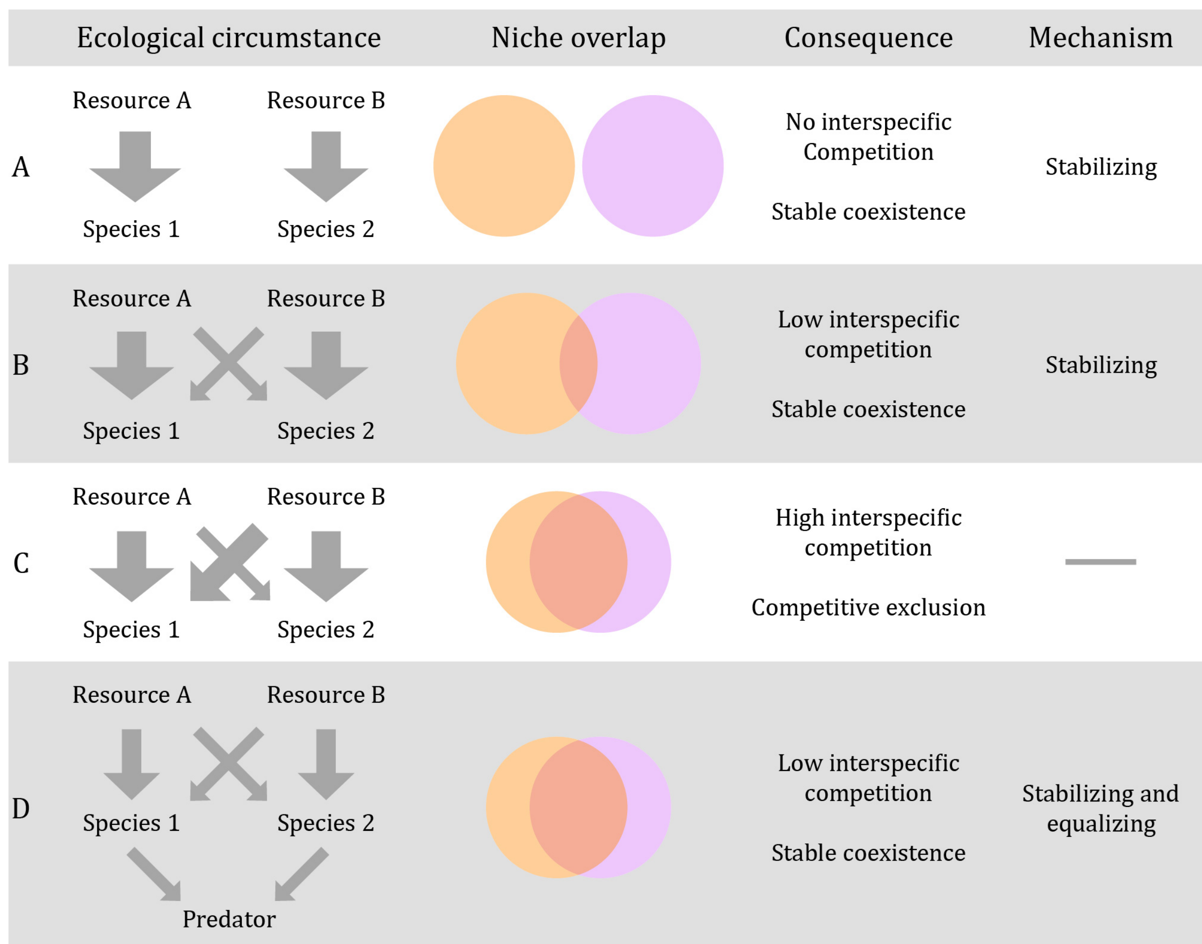

Figure 1 | Possible ecological scenarios leading to stable coexistence

In an environment two species (Species 1 and 2) compete for two different resources (Resource A and B). The degree of utilizing resources differs between the species (i.e. relative thickness of arrows) and determines whether they coexist or not. Orange and pink circles (i.e. niches) represent Resource A and B, respectively, while the degree of overlap determined by the rate of resource utilization of the species. Stable coexistence occurs **A**. when species use different resources, where there is no interspecific competition**,** or **B**. when species use both resources but at different rates, where interspecific competition is lower relative to intraspecific competition. Stable coexistence cannot occur **C**. if one of the species is competitively superior (i.e. Species 1) leading to stronger interspecific competition relative to intraspecific competition. In this case Species 1 outcompetes Species 2. Coexistence can be achieved **D**. when fitness of competing species is equalized through another ecological interaction, despite high niche overlap.

Species interactions might affect both the strength of interspecific competition and the fitness inequalities between coexisting species, and therefore they might act both as stabilizing and equalizing mechanisms. Resource competition between species has been shown to lead resource partitioning as a stabilizing mechanism (e.g. Cody 1991). Besides, predation has been shown to operate as a stabilizing mechanism when it leads to density-dependent mortality through host-specific predators or pathogens (Hille Ris Lambers et al. 2002). Studies on the contributions of simultaneously acting species interactions as stabilizing and equalizing mechanisms have been, however, inadequate. Predation in combination with resource competition could create differences in niche overlap by leading to trade-offs allowing species to coexist stably. For example, it has been shown that herbivores could mediate coexistence of white-sand specialist and clay specialist trees through growth-defense trade-off (Fig. 2; Fine et al. 2006).

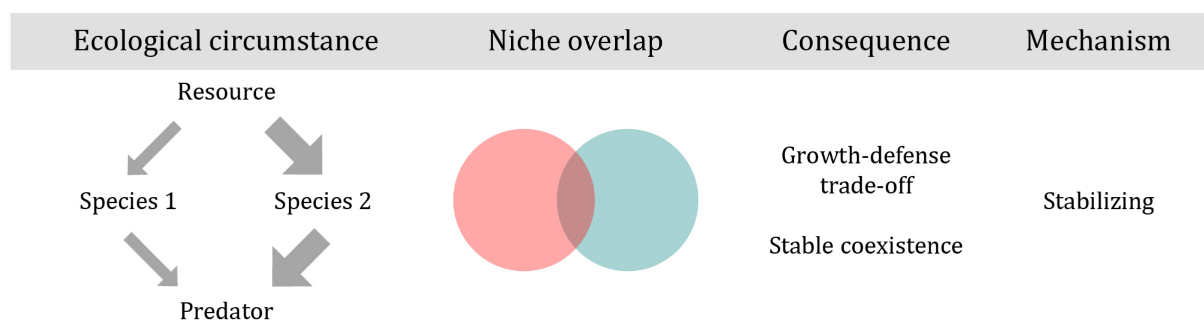

Figure 2 | Growth-defense trade-offs leading to stable coexistence

In an environment two species (Species 1 and 2) compete for a resource in the presence of predation. The degree of specialization on growth and defense differently in comparison to each other (i.e. relative thickness of arrows) determines that they coexist. Red and green circles (i.e. niches) represent defense and growth, respectively.

Nevertheless, the effect of predation in combination with resource competition might have various effects on the stable coexistence resulting from the feedback between them. In our evolution experiment we observed two morphotypes evolved and coexisted in the low resource environment and in the presence of predation. We suggested that coexistence of these morphotypes (Chapter 2, Fig. 7) could be tested based on the modern coexistence theory of species. In chapter 2, we found that clumping morphotype (M4) and cell-chaining morphotype (M6) showed similar defense

levels against predation, while M4 was superior in growth compared to M6. These data suggested that M4 would eventually outcompete M6, yet they coexisted in the presence of predation throughout our evolution experiment (Chapter 1). In fact, inferior M6 evolved and increased in frequency over time while M4 was present in replicate populations.

We proposed that predation and resource competition could interact to operate as both stabilizing and equalizing mechanisms. Therefore, we designed an experiment in which we manipulated environments in terms of resource level and predation to test for stable coexistence of competing clumping and cell-chaining morphotypes. We measured the fitness of each morphotype relative to the ancestor with head-to-head competition experiments, which provides direct and comparable competitive fitness. We showed that when we removed predation from the environment fitness difference between these two morphotypes increased suggesting competitive exclusion of cell-chaining morphotype. When we increased the amount of resources in the absence of predation, we found that fitness difference between two morphotypes decreased confirming that predation equalizes the fitness differences through altering the strength of resource competition. Overall, our data show even if there is a weak stabilizing mechanism, stable coexistence might be facilitated by predation. Our findings highlight the importance of investigating both types of coexistence mechanisms, before inferring that high degree of niche overlap as the main limitation to stable coexistence.

MATERIAL AND METHODS

Bacterial strains used in the competition assays

We used three randomly selected isolates of M4 and M6, which evolved in the presence of predation in the LRE and coexisted until the end of the experiment (Chapter 2; Table 2 for evolutionary history of each isolate) and *Pseudomonas fluorescens* SBW25-*lacZ* (obtained from Dr. Jenna Gallie) to perform head-to-head competition assays between each isolate and the ancestral strain in different environments. SBW25-*lacZ* was generated by integrating *lacZ* gene into a defective prophage locus (Zhang & Rainey 2007). The *lacZ* gene product (beta-galactosidase) catalyzes the hydrolysis of X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside), and produces blue-colored colonies on agar plates containing X-gal. To test whether the *lacZ* marked strain has an equal fitness compared to the ancestor used to start the evolution experiment (Chapter 1), we performed head-to-head competition assays between our ancestral SBW25 and SBW25-*lacZ*. We did not find a considerable difference in the population density of these two genotypes when started 1:1 ratio in the LRE and HRE with or without predators after 72 hours (average CFUs ml^{-1} in the LRE with predators: SBW25=4,3x10^6 and SBW25-*lacZ*=3,8x10^6; in the LRE without predators: SBW25=3,6x10^7 vs. SBW25-*lacZ*=3,3x10^7; in the HRE with predators: SBW25=3,1x10^7 and SBW25-*lacZ*=2,4x10^7 in the HRE without predators: SBW25=4,8x10^8 vs. SBW25-*lacZ*=6x10^8).

Manipulating the ecological conditions

To test for fitness of each morphotype in response to the ecological conditions, we changed the presence and absence of predation and incubation mode as well as the resource level in the environment (Table 1). Environment 1 represents the environment, in which M4 and M6 evolved in and coexisted until the end of the evolution experiment (Chapter 1). In the Environment 2, where we removed predation, we expect that resource competition will be stronger than in the Environment 1 favoring the competitive type. Additionally, to exclude the advantage of biofilm formation in the absence of predation, we test the effect of increased resource competition on morphotypes in Environment 3, where shaken incubation was applied (200 rpm). Moreover, by removing predation and adding more resources in the

Environment 4, where the strength of resource competition was expected to be less than Environment 2 and 3.

Table 1 | Ecological conditions for the head-to-head competitive fitness assays

Ecological factors	Environment 1*	Environment 2	Environment 3	Environment 4
Resource level	Low	Low	Low	High
Predation	Present	Absent	Absent	Absent
Incubation	Static	Static	Shaken	Static

* Environment 1 represents the environment, in which M4 and M6 evolved in and coexisted until the end of the evolution experiment (Chapter 1).

Head-to-head competitive fitness assays in different environments

In order to test for the adaptive advantage of each morphotype across different environments, we performed head-to-head competition assays between each isolate and the ancestor (SBW25-*lacZ*). For each environment we measured fitness of the three isolates of each morphotype with five technical replicates. To perform competition assays, overnight cultures of cryopreserved isolates were incubated 16 hours in the M9KB medium at 220 rpm and 28 °C. Using optical density (600 nm) of overnight cultures, we determined inoculation volume of each genotype to obtain 1:1 ratio of evolved morphotype and the ancestor to start the experiment. Each vial was filled with 6 ml 5% or 20% M9KB media, and we kept the total bacterial density (i.e. ~10^5 cells in 6 ml medium) similar for each replicate and environment. For the environments with predators, we added 6300 ciliates from stock cultures into each test tube. Before adding ciliates, PPY medium was removed by centrifugation (3000 rpm, 8 minutes, 0 °C) and the remaining pellet containing the ciliates was re-suspended in the test medium (i.e. 5% M9KB medium). Each vial was vortexed at maximum speed for 45 seconds and dilutions were spread onto M9KB agar plates containing 60 ug ml^{-1} X-gal (Sigma Aldrich) with three replicates. We incubated these plates for 48 hours at 28 °C, and then performed blue-white screening to determine the initial frequencies of the ancestor (blue) and evolved morphotypes (white) to confirm the population densities at t=0. Vials were incubated at 28 °C for 72 hours either static or shaken (200 rpm). After 72

hours, each vial was vortexed at maximum speed for 45 seconds, and dilutions were spread onto M9KB agar plates containing 60 ug ml^{-1} X-gal. We incubated these plates for 48 hours at 28 °C and then we counted the number of white and blue colonies to obtain the populations densities at t=72.

Head-to-head competition assays allowed us to calculate the malthusian parameters for competing genotypes (Lenski, 1991). Specifically, for each competition assay population densities were determined at times t=0 h and at t=72 h (DA_0 and DA_{72} for population density of ancestor, while DE_0 and DE_{72} for evolved genotypes). Using these population densities, the malthusian parameter was calculated for both competitors (MA for Malthusian parameter of ancestor, while ME for evolved genotype): $MA=\ln[DA_{72}/DA_0]/72$. Malthusian parameters of evolved morphotypes and ancestor were then used for calculating the fitness of each morphotype relative to ancestor in different environments. Relative fitness is calculated as: RF= ME/MA. When RF equals to 1, relative fitness of a morphotype is equal to the fitness level of ancestor. RF values greater than 1 indicate higher levels of fitness relative to the ancestor.

Statistical analyses

Statistical analyses were performed with R (R Core Team 2015) in Rstudio Version 1.0.143). Fitness data were analyzed using generalized linear models (GLM) with gamma error distribution. Specifically, different environments and morphotypes were the explanatory variables for comparing of the mean fitness of morphotypes. Then, Tukey honest significant difference (HSD) *post hoc* test was performed using the multcomp package (Hothorn et al. 2008) to test for pairwise comparisons.

RESULTS

Relative fitness was calculated for each morphotype by measuring the rate of increase in population density (i.e. malthusian parameter) after head-to-head competitions between the morphotype and the ancestor in each environment (Methods for detail). We measured the fitness of M4 and M6 relative to ancestor, and compared their fitness levels across various ecological conditions (Table 1). We found that M4 and M6 had a similar fitness in their evolved environment (Fig. 2; Tukey's HSD: fitness of M4 vs. M6 in E1, p *adj* = 0.09756). M4 had a significantly higher fitness than M6 both in Environment 2, where predation was excluded, and in Environment 3, where predation and environmental heterogeneity were excluded (Fig. 2; Tukey's HSD: fitness of M4 vs. M6 in E2, p *adj* < 0.001 and fitness of M4 vs. M6 in E3, p *adj* < 0.001). Furthermore, we found no significant difference in fitness between M4 and M6 in the Environment 4, where the resource level of the environment was increased in the absence of predation (Fig. 2; Tukey's HSD: fitness of M4 vs. M6 in E4, p *adj* = 0.11197).

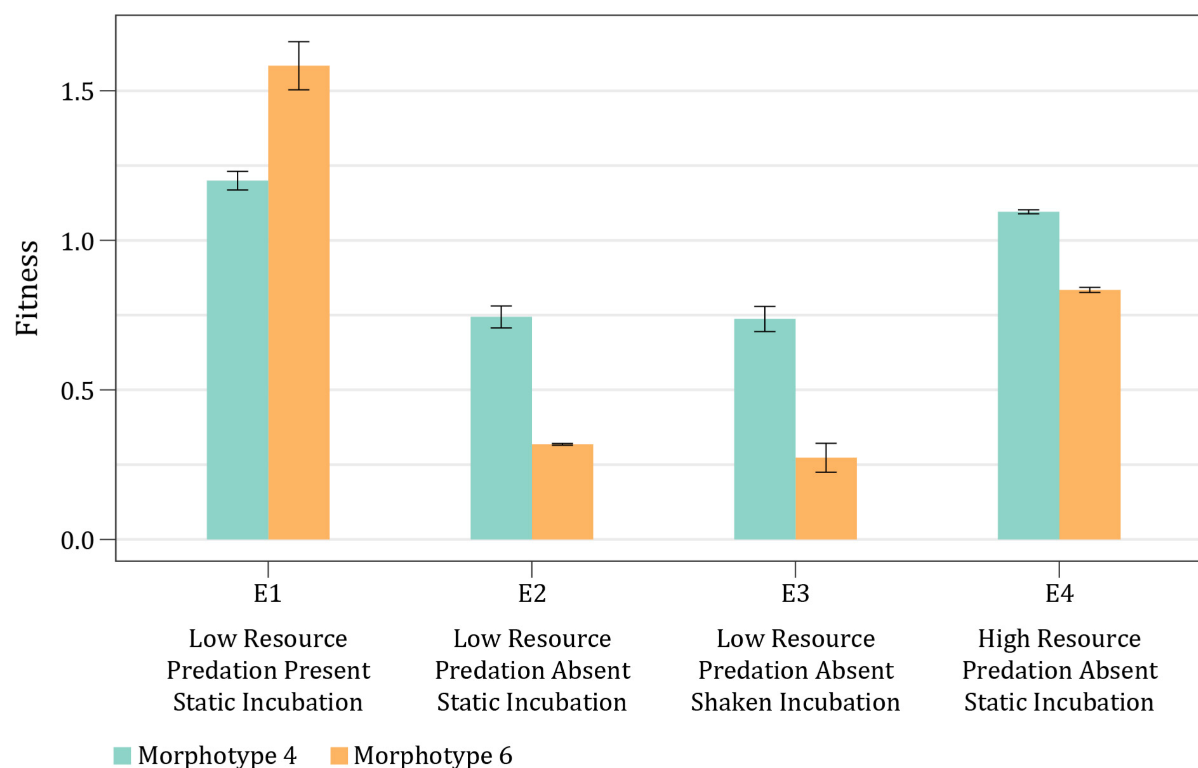

Figure 2 | Relative fitness of Morphotype 4 and 6 in different ecological conditions

Fitness shown in green for M4, and in orange for M6. Fitness = 1 indicates ancestral fitness. Bars: mean of three isolates, error bars: standard error of mean.

DISCUSSION

Interacting ecological processes play an important role in the outcome of coexistence by mediating the degree of overlapping niches and the fitness difference between interacting species (reviewed in Hille Ris Lambers et al. 2012). In this chapter we investigated potential roles of ecological conditions under which clumping morphotype (M4) and cell-chaining morphotype (M6) stably coexist. We showed that predation reduced the strength of resource competition and decreased the average fitness difference between these two morphotypes. Although we did not show a distinct stabilizing mechanism reducing the niche overlap between these competing morphotypes, we provided substantial evidence addressing to a stable coexistence of M4 and M6. We showed that these morphotypes could stably coexist even in the presence of a weak stabilizing mechanism (i.e. high degree of niche overlap), because predation operating as an equalizing mechanism considerably reduced the fitness inequalities between them.

Stabilizing mechanisms might arise through various species interactions, such as resource competition and predation. Specifically, predation might generate frequency-dependent selection (e.g. switching prey depending on their frequency) or might interact with resource competition and have varying effects on different prey types (e.g. growth-defense trade-off, where one type is limited more by resources and the other by predation). Under both conditions predation operates as a stabilizing mechanism leading each prey type to limit its own growth more than the growth of the other type, and thus allows coexistence (Chesson & Kuang 2008). However, if predation does not play a role as a stabilizing mechanism, it can still contribute to coexistence as an equalizing mechanism. Theoretically, equalizing mechanisms might support stabilizing mechanisms by reducing fitness inequalities and facilitate coexistence (Chesson 2000).

By characterizing morphotypes, we discovered that while having similar defense levels, clumping morphotype (M4) was the stronger competitor regarding growth than cell-chaining morphotype (M6) (Chapter 2). Interestingly, they showed similar fitness levels in the Environment 1 that represents the ecological condition where they evolved and coexisted during our evolution experiment (Fig. 2). To unravel how their fitness could

be equalized in the presence of predation in the low resource environment, we investigated the role of predation experimentally. When we tested for fitness differences between M4 and M6 in the absence of predation (Environment 2), M4 had a higher fitness level relative to M6 suggesting that M4 would eventually dominate the population in the absence of predation. This data indicated that predation decreased the fitness difference.

Since the static incubation creates environmental heterogeneity and thus potential niches for M4 to colonize (i.e. biofilm formation on the air-liquid interface), the high fitness of M4 in the absence of predation might also be due to its advantage in spatially structured environment. Therefore, we measured the fitness of morphotypes in the absence of predation and in spatially homogenous environment (i.e. shaken incubation, Environment 3), which excludes the advantage of biofilm-formation. As a result, we found that both with and without environmental heterogeneity M4 was competitively superior in the absence of predation (Fig. 2). Consequently, we confirmed that predation acted as an equalizing mechanism reducing the fitness difference.

Moreover, to investigate whether the equalizing role of predation was through changing the strength of resource competition, we performed another experiment, where we decreased the strength of resource competition by increasing resources in the absence of predation (Environment 4). Here, we predicted that predation and the increase in the resource level of the environment might have similar effects on resource competition by decreasing population density and by increasing availability of resources, respectively. Since we found that increased resources in the absence of predation had a similar impact on the fitness of competing morphotypes as predation, we confirmed that predation might equalize the fitness difference between clumping morphotype (M4) and cell-chaining morphotype (M6) by decreasing the strength of resource competition.

Identifying the degree of niche overlap experimentally in complex ecological conditions is difficult because ecological processes can interact, alter each other's effects and change over time. In our experiment, even though we were not able to show how explicitly and at what extent these morphotypes differed in their niches, we argue that there might be a stabilizing mechanism. Since cell-chaining morphotype (M6) arose

later than clumping morphotype (M4) in the evolution experiment, and increased in frequency in the presence of M4 (Chapter 1), we suggest that niches of these morphotypes did not overlap fully so that M6 could overcome the competitive exclusion during the early stages of diversification. Moreover, we showed that M4 and M6 differ in their cell shape, exopolymer production, lag phase and overall growth (AUC) (Chapter 2). Given these differences, we expect that some degree of resource partitioning is likely to occur between these two morphotypes. In conclusion, our findings highlight that predation in interaction with resource competition promoted the maintenance of diversity.

CONCLUSION

The question of how life became so diverse has puzzled scientists ranging from ecologists to theoretical evolutionary biologists. It has become apparent that we need a better understanding of how environmental complexity translates into selection, which generates diversity. This realization draws attention on microbial evolution experiments, which enable bridging the observations and predictions from these different fields. One gap in our knowledge has been the role of other species interactions than resource competition on diversification. Theory suggests that predation could both constrain and promote diversity. However, up to date, predation has been shown to affect diversification only through modifying the diversity resulted from resource competition or through leading to trade-offs when acting together with resource competition.

In this study we explicitly tested for the role of predation, and also address the combined effects of predation and resource competition on diversification. Our data revealed that (1) predation promotes diversification (Chapter 1), (2) resource level of the environment can contribute to the strength of predator-prey interaction, and therefore leads to differences in the extent of diversity (Chapter 1), (3) predation combined with resource competition can lead to divergence in defense traits (Chapter 2), (4) equalizing mechanisms can have substantial impact on stable coexistence of competing genotypes (Chapter 3), (5) predation, in particular, can have significant implications on the maintenance of prey diversity as an equalizing mechanism (Chapter 3). Overall, our study demonstrated that predation is an important species interaction both for driving diversification and maintaining diversity. Also, the interplay between predation and resource competition can determine the extent of diversity. Therefore, these two important ecological processes should be taken into account symmetrically. Based on this study and growing theoretical predictions, we emphasized the potential roles of other species interactions on diversification, in contrast to placing resource competition at the center of future field and experimental studies on diversification.

Ecological conditions leading to stable coexistence in complex environments are not yet resolved. Our work shed light on the role of predation and resource competition for

stable coexistence of evolved morphotypes, and showed that equalizing mechanisms can have vital consequences on stable coexistence. Thus, results of this work give directions for future experimental and theoretical investigations into the role of interacting ecological processes on the different mechanisms of stable coexistence.

One might argue that it is irrelevant to study diversification with microbial evolution experiments. We should, however, be equally interested in understanding diversification in microbial life, which constitutes a large portion of the tree of life. Specifically, microbes represent the origin of diversification. Therefore, we must learn more about lower levels of organization by disentangling the interacting effects that generate selection. This know-how can be then transferred to our understanding on the evolution of complex traits in higher levels of organization. Moreover, to broaden our knowledge on microbes is of the utmost importance, as we have to tackle the emerging problems such as microbial drug resistance related to excessive use of antibiotics, microbial invasion initiated by climate change and infectious epidemics caused by bacteria.

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APPENDIX

Chapter 1

We followed the changes in densities of predator (ciliates) and prey (bacteria) both in the low and high resource environment over 72 hours (Methods for details). In the short-term experiment, population dynamics revealed that predation significantly decreased the prey density in both resource levels (average density of prey in the absence of predation after 48 hours in the LRE=3,8x10^8 and in the HRE=7x10^8; Table S10 for statistics). Predation decreased the densities of prey populations in the HRE significantly more than it decreased the densities of prey populations in the LRE (Fig. S1; Table S10 for statistics). Predator densities were not significantly affected by different resource levels (FigS1; Table S11 for statistics). Furthermore, in the short-term experiment, we observed the emergence of wrinkly spreader morphotype from 60 hours on both in the LRE (about 0.1% of the population, data not shown) and in the HRE (about 33% of the population, data not shown). Importantly, we did not find wrinkly spreader in the absence of predation during 72-hours in neither resource levels.

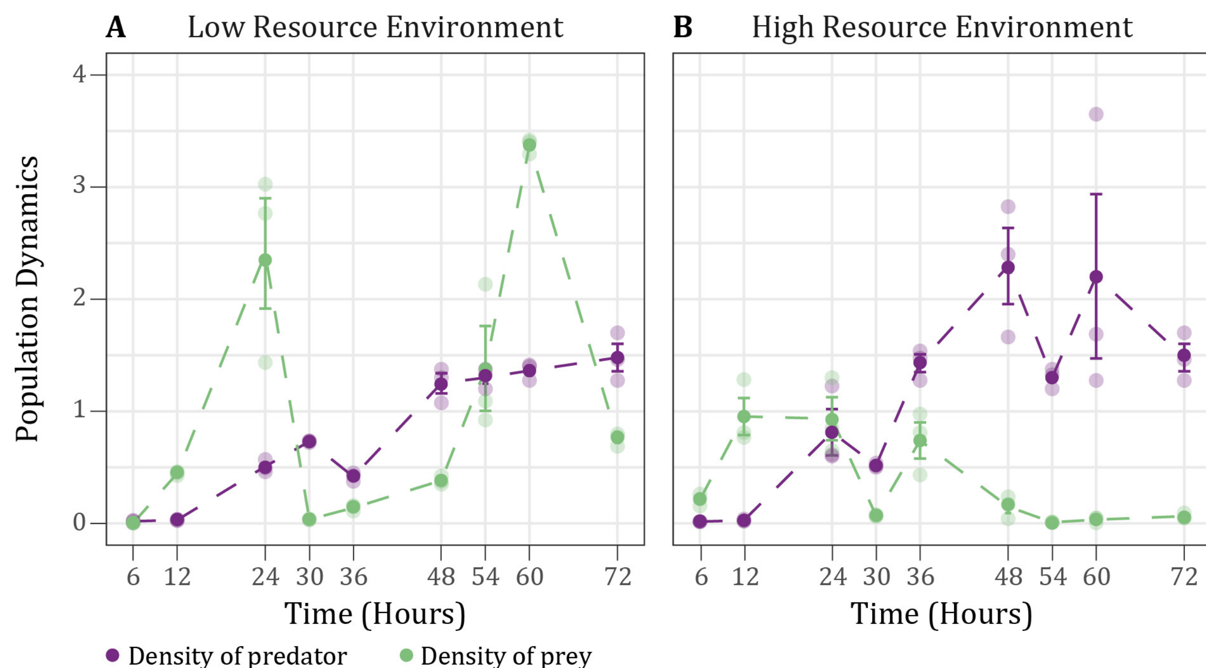

Figure S1 | Predator-prey dynamics during the short-term experiment

Density of predator shown in purple and density of prey shown in green **A.** in the low resource environment, and **B.** in the high resource environment. Densities as 8x10^4 ciliates and 4x10^7 bacterial cells per ml.

Chapter 1

Table S1 | Effect of predation, resource level, time, and their interactions on the diversity of bacterial populations

Model Parameter	D.f.	X^2	P
Predation	**1**	**20.7870**	**5.133e-06**
Resource level	**1**	**11.4644**	**7.094e-04**
Time	**1**	**8.8809**	**2.882e-03**
Predation x Resource level	**1**	**17.5916**	**2.738e-05**
Predation x Time	**1**	**15.4039**	**8.681e-05**
Resource level x Time	**1**	**9.7023**	**1.840e-03**
Predation x Resource level x Time	**1**	**12.6950**	**3.666e-04**

Significant parameters are in bold. Degree of freedom is reported for numerator.

Chapter 1

Table S2 | Effect of predation, resource level, time, and their interactions on the defense of bacterial populations

Model Parameter	D.f.	X^2	P
Predation	**1**	**44.355**	**2.739e-11**
Resource level	1	2.606	1.065e-01
Time	**1**	**42.793**	**6.085e-11**
Predation x Resource level	**1**	**9.768**	**1.776e-03**
Predation x Time	1	4.056	4.402e-02
Resource level x Time	1	5.713	1.684e-02
Predation x Resource level x Time	1	2.656	1.032e-01

Significant parameters are in bold. Degree of freedom is reported for numerator.

Chapter 1

Table S3 | Effect of predation, resource level, time, and their interactions on the AUC of bacterial populations

Model Parameter	D.f.	X^2	P
Predation	**1**	**80.082**	**< 2.2e-16**
Resource level	1	2.411	1.205e-01
Time	**1**	**28.610**	**8.854e-08**
Predation x Resource level	**1**	**7.248**	**7.097e-03**
Predation x Time	**1**	**33.750**	**6.267e-09**
Resource level x Time	1	2.589	1.076e-01
Predation x Resource level x Time	1	2.316	1.280e-01

Significant parameters are in bold. Degree of freedom is reported for numerator.

Chapter 1

Table S4 | Effect of predation, resource level, time, and their interactions on the lag phase of bacterial populations

Model parameters	D.f.	X^2	P
Predation	**1**	**77.100**	**<2.2e-16**
Resource level	**1**	**6.750**	**0.009376**
Time	**1**	**30.551**	**3.253e-08**
Predation x Resource level	1	3.162	0.075384
Predation x Time	**1**	**4.238**	**0.039519**
Resource level x Time	1	2.115	0.145816
Predation x Resource level x Time	1	1.280	0.257982

Significant parameters are in bold. Degree of freedom is reported for numerator.

Chapter 1

Table S5 | Effect of predation, resource level, time, and their interactions on the growth rate of bacterial populations

Model parameters	D.f.	X^2	P
Predation	1	2.130	0.144410
Resource level	**1**	**6.633**	**0.010013**
Time	1	0.027	0.870534
Predation x Resource level	**1**	**34.331**	**4.65e-09**
Predation x Time	1	0.624	0.429692
Resource level x Time	**1**	**9.172**	**0.002458**
Predation x Resource level x Time	**1**	**8.648**	**0.003274**

Significant parameters are in bold. Degree of freedom is reported for numerator.

Chapter 1

Table S6| Effect of predation, resource level, time, and their interactions on the density of prey (bacteria) populations during evolution experiment

Model Parameter	D.f.	X^2	P
Predation	**1**	**13.238**	**0.0002743**
Resource level	**1**	**14.745**	**0.0001231**
Time	**1**	**4.566**	**0.0326151**
Predation x Resource level	**1**	**42.371**	**7.551e-11**
Predation x Time	1	0.571	0.4500017
Resource level x Time	1	1.383	0.2395390
Predation x Resource level x Time	**1**	**4.877**	**0.0272216**

Significant parameters are in bold. Degree of freedom is reported for numerator.

Chapter 1

Table S7 | Effect of resource level, time and their interactions on the density of predator (ciliate) populations during evolution experiment

Model Parameter	D.f.	X^2	P
Resource level	**1**	**8.9482**	**0.002777**
Time	1	3.6569	0.055839
Resource level x Time	1	0.0735	0.786337

Significant parameters are in bold. Degree of freedom is reported for numerator.

Chapter 1

Table S8 | Effect of resource level, time and their interactions on the ratio of predator-prey during evolution experiment

Model Parameter	D.f.	X^2	P
Resource level	**1**	**51.575**	**6.890e-13**
Time	1	2.383	0.1227
Resource level x Time	**1**	**16.581**	**4.661e-05**

Significant parameters are in bold. Degree of freedom is reported for numerator.

Chapter 1

Table S9 | Effect of resource level, time and their interactions on the ratio of predator-prey during short-term experiment

Model Parameter	D.f.	X^2	P
Resource level	1	2.8113	0.0936
Time	**1**	**19.3114**	**1.110e-05**
Resource level x Time	**1**	**22.8528**	**1.749e-06**

Significant parameters are in bold. Degree of freedom is reported for numerator.

Chapter 1

Table S10 | Effect of predation, resource level, time, and their interactions on the density of prey (bacteria) populations during short-term experiment

Model Parameter	D.f.	X^2	P
Predation	**1**	**24.9516**	**5.879e-07**
Resource level	1	0.4519	0.5014562
Time	**1**	**22.6125**	**1.982e-06**
Predation x Resource level	**1**	**7.5039**	**0.0061564**
Predation x Time	1	0.0905	0.7635920
Resource level x Time	**1**	**9.3097**	**0.0022795**
Predation x Resource level x Time	**1**	**11.5380**	**0.0006819**

Significant parameters are in bold. Degree of freedom is reported for numerator.

Chapter 1

Table S11 | Effect of resource level, time and their interactions on the density of predator (ciliate) populations during short-term experiment

Model Parameter	D.f.	X^2	P
Resource level	1	2.9581	0.08545
Time	**1**	**31.5891**	**1.905e-08**
Resource level x Time	1	2.4240	0.11949

Significant parameters are in bold. Degree of freedom is reported for numerator.

Chapter 2

Table S1 | Effect of resource levels on the defense of morphotypes

Comparisons	Estimates	Standard Error	Z Value	P Value
M2.5kb - M1.5kb	**-0.0594984**	**0.0094943**	**-6.267**	**<0.01**
M3.5kb - M1.5kb	**-0.0602718**	**0.0094870**	**-6.353**	**<0.01**
M4.5kb - M1.5kb	**-0.1059523**	**0.0090707**	**-11.681**	**<0.01**
M5.5kb - M1.5kb	**-0.1327805**	**0.0095827**	**-13.856**	**<0.01**
M6.5kb - M1.5kb	**-0.1027030**	**0.0090994**	**-11.287**	**<0.01**
M1.20kb - M1.5kb	-0.0063414	0.0100107	-0.633	1.0000
M2.20kb - M1.5kb	**-0.0371913**	**0.0097071**	**-3.831**	**<0.01**
M3.20kb - M1.5kb	-0.0013974	0.0100602	-0.139	1.0000
M4.20kb - M1.5kb	**-0.1143915**	**0.0089968**	**-12.715**	**<0.01**
M5.20kb - M1.5kb	**-0.1421748**	**0.0094739**	**-15.007**	**<0.01**
M6.20kb - M1.5kb	**-0.1302733**	**0.0088605**	**-14.703**	**<0.01**
M3.5kb - M2.5kb	-0.0007734	0.0088687	-0.087	1.0000
M4.5kb - M2.5kb	**-0.0464538**	**0.0084219**	**-5.516**	**<0.01**
M5.5kb - M2.5kb	**-0.0732820**	**0.0089710**	**-8.169**	**<0.01**
M6.5kb - M2.5kb	**-0.0432046**	**0.0084528**	**-5.111**	**<0.01**
M1.20kb - M2.5kb	**0.0531570**	**0.0094268**	**5.639**	**<0.01**
M2.20kb - M2.5kb	0.0223071	0.0091038	2.450	0.3690
M3.20kb - M2.5kb	**0.0581010**	**0.0094794**	**6.129**	**<0.01**
M4.20kb - M2.5kb	**-0.0548931**	**0.0083422**	**-6.580**	**<0.01**
M5.20kb - M2.5kb	**-0.0826764**	**0.0088547**	**-9.337**	**<0.01**
M6.20kb - M2.5kb	**-0.0707749**	**0.0081951**	**-8.636**	**<0.01**
M4.5kb - M3.5kb	**-0.0456805**	**0.0084137**	**-5.429**	**<0.01**
M5.5kb - M3.5kb	**-0.0725087**	**0.0089633**	**-8.090**	**<0.01**
M6.5kb - M3.5kb	**-0.0424312**	**0.0084446**	**-5.025**	**<0.01**
M1.20kb - M3.5kb	**0.0539304**	**0.0094194**	**5.725**	**<0.01**

M2.20kb - M3.5kb	0.0230804	0.0090962	2.537	0.3143
M3.20kb - M3.5kb	**0.0588743**	**0.0094721**	**6.216**	**<0.01**
M4.20kb - M3.5kb	**-0.0541197**	**0.0083339**	**-6.494**	**<0.01**
M5.20kb - M3.5kb	**-0.0819030**	**0.0088469**	**-9.258**	**<0.01**
M6.20kb - M3.5kb	**-0.0700015**	**0.0081866**	**-8.551**	**<0.01**
M5.5kb - M4.5kb	-0.0268282	0.0085214	-3.148	0.0703
M6.5kb - M4.5kb	0.0032492	0.0079740	0.407	1.0000
M1.20kb - M4.5kb	**0.0996109**	**0.0090000**	**11.068**	**<0.01**
M2.20kb - M4.5kb	**0.0687609**	**0.0086611**	**7.939**	**<0.01**
M3.20kb - M4.5kb	**0.1045548**	**0.0090551**	**11.547**	**<0.01**
M4.20kb - M4.5kb	-0.0084392	0.0078568	-1.074	0.9956
M5.20kb - M4.5kb	**-0.0362225**	**0.0083989**	**-4.313**	**<0.01**
M6.20kb - M4.5kb	-0.0243210	0.0077003	-3.158	0.0694
M6.5kb - M5.5kb	**0.0300774**	**0.0085520**	**3.517**	**0.0218**
M1.20kb - M5.5kb	**0.1264391**	**0.0095158**	**13.287**	**<0.01**
M2.20kb - M5.5kb	**0.0955891**	**0.0091960**	**10.395**	**<0.01**
M3.20kb - M5.5kb	**0.1313830**	**0.0095679**	**13.732**	**<0.01**
M4.20kb - M5.5kb	0.0183890	0.0084427	2.178	0.5629
M5.20kb - M5.5kb	-0.0093943	0.0089494	-1.050	0.9964
M6.20kb - M5.5kb	0.0025072	0.0082973	0.302	1.0000
M1.20kb - M6.5kb	**0.0963616**	**0.0090289**	**10.673**	**<0.01**
M2.20kb - M6.5kb	**0.0655117**	**0.0086912**	**7.538**	**<0.01**
M3.20kb - M6.5kb	**0.1013056**	**0.0090838**	**11.152**	**<0.01**
M4.20kb - M6.5kb	-0.0116885	0.0078899	-1.481	0.9452
M5.20kb - M6.5kb	**-0.0394718**	**0.0084299**	**-4.682**	**<0.01**
M6.20kb - M6.5kb	**-0.0275703**	**0.0077341**	**-3.565**	**0.0188**
M2.20kb - M1.20kb	-0.0308500	0.0096411	-3.200	0.0609
M3.20kb - M1.20kb	0.0049440	0.0099965	0.495	1.0000

M4.20kb - M1.20kb	**-0.1080501**	**0.0089255**	**-12.106**	**<0.01**
M5.20kb - M1.20kb	**-0.1358334**	**0.0094063**	**-14.441**	**<0.01**
M6.20kb - M1.20kb	**-0.1239319**	**0.0087881**	**-14.102**	**<0.01**
M3.20kb - M2.20kb	**0.0357939**	**0.0096926**	**3.693**	**0.0118**
M4.20kb - M2.20kb	**-0.0772001**	**0.0085837**	**-8.994**	**<0.01**
M5.20kb - M2.20kb	**-0.1049834**	**0.0090826**	**-11.559**	**<0.01**
M6.20kb - M2.20kb	**-0.0930820**	**0.0084408**	**-11.028**	**<0.01**
M4.20kb - M3.20kb	**-0.1129941**	**0.0089811**	**-12.581**	**<0.01**
M5.20kb - M3.20kb	**-0.1407773**	**0.0094590**	**-14.883**	**<0.01**
M6.20kb - M3.20kb	**-0.1288759**	**0.0088446**	**-14.571**	**<0.01**
M5.20kb - M4.20kb	**-0.0277833**	**0.0083191**	**-3.340**	**0.0397**
M6.20kb - M4.20kb	-0.0158818	0.0076132	-2.086	0.6301
M6.20kb - M5.20kb	0.0119015	0.0081715	1.456	0.9513

Significant p-values are in bold.

Chapter 2

Table S2 | Effect of resource levels on the AUC of morphotypes

Comparisons	Estimates	Standard Error	Z Value	P Value
M2.5kb - M1.5kb	-0.0529338	0.0385426	-1.373	0.9675
M3.5kb - M1.5kb	-0.0925454	0.0371861	-2.489	0.3397
M4.5kb - M1.5kb	**-0.1275713**	**0.0360433**	**-3.539**	**0.0200**
M5.5kb - M1.5kb	-0.0700469	0.0418461	-1.674	0.8767
M6.5kb - M1.5kb	-0.0011982	0.0404057	-0.030	1.0000
M1.20kb - M1.5kb	**-0.1386859**	**0.0356926**	**-3.886**	**<0.01**
M2.20kb - M1.5kb	**-0.1569910**	**0.0351285**	**-4.469**	**<0.01**
M3.20kb - M1.5kb	**-0.1339752**	**0.0358405**	**-3.738**	**<0.01**
M4.20kb - M1.5kb	**-0.1666179**	**0.0348387**	**-4.783**	**<0.01**
M5.20kb - M1.5kb	-0.0931265	0.0407798	-2.284	0.4808
M6.20kb - M1.5kb	0.0548099	0.0425246	1.289	0.9799
M3.5kb - M2.5kb	-0.0396116	0.0351018	-1.128	0.9932
M4.5kb - M2.5kb	-0.0746375	0.0338888	-2.202	0.5407
M5.5kb - M2.5kb	-0.0171131	0.0400054	-0.428	1.0000
M6.5kb - M2.5kb	0.0517355	0.0384961	1.344	0.9722
M1.20kb - M2.5kb	-0.0857521	0.0335156	-2.559	0.2970
M2.20kb - M2.5kb	-0.1040572	0.0329142	-3.161	0.0669
M3.20kb - M2.5kb	-0.0810415	0.0336731	-2.407	0.3939
M4.20kb - M2.5kb	**-0.1136841**	**0.0326047**	**-3.487**	**0.0240**
M5.20kb - M2.5kb	-0.0401927	0.0388886	-1.034	0.9968
M6.20kb - M2.5kb	0.1077436	0.0407146	2.646	0.2500
M4.5kb - M3.5kb	-0.0350259	0.0323377	-1.083	0.9952
M5.5kb - M3.5kb	0.0224985	0.0387002	0.581	1.0000
M6.5kb - M3.5kb	0.0913471	0.0371379	2.460	0.3587
M1.20kb - M3.5kb	-0.0461405	0.0319464	-1.444	0.9530

M2.20kb - M3.5kb	-0.0644456	0.0313148	-2.058	0.6466
M3.20kb - M3.5kb	-0.0414299	0.0321115	-1.290	0.9797
M4.20kb - M3.5kb	-0.0740725	0.0309893	-2.390	0.4051
M5.20kb - M3.5kb	-0.0005811	0.0375446	-0.015	1.0000
M6.20kb - M3.5kb	**0.1473553**	**0.0394329**	**3.737**	**<0.01**
M5.5kb - M4.5kb	0.0575244	0.0376034	1.530	0.9304
M6.5kb - M4.5kb	**0.1263731**	**0.0359936**	**3.511**	**0.0221**
M1.20kb - M4.5kb	-0.0111146	0.0306086	-0.363	1.0000
M2.20kb - M4.5kb	-0.0294197	0.0299488	-0.982	0.9980
M3.20kb - M4.5kb	-0.0064039	0.0307809	-0.208	1.0000
M4.20kb - M4.5kb	-0.0390466	0.0296083	-1.319	0.9760
M5.20kb - M4.5kb	0.0344448	0.0364131	0.946	0.9986
M6.20kb - M4.5kb	**0.1823812**	**0.0383571**	**4.755**	**<0.01**
M6.5kb - M5.5kb	0.0688487	0.0418033	1.647	0.8882
M1.20kb - M5.5kb	-0.0686390	0.0372675	-1.842	0.7893
M2.20kb - M5.5kb	-0.0869441	0.0367275	-2.367	0.4211
M3.20kb - M5.5kb	-0.0639283	0.0374091	-1.709	0.8607
M4.20kb - M5.5kb	-0.0965710	0.0364504	-2.649	0.2471
M5.20kb - M5.5kb	-0.0230796	0.0421651	-0.547	1.0000
M6.20kb - M5.5kb	0.1248568	0.0438548	2.847	0.1575
M1.20kb - M6.5kb	**-0.1374876**	**0.0356425**	**-3.857**	**<0.01**
M2.20kb - M6.5kb	**-0.1557928**	**0.0350775**	**-4.441**	**<0.01**
M3.20kb - M6.5kb	**-0.1327770**	**0.0357905**	**-3.710**	**0.0108**
M4.20kb - M6.5kb	**-0.1654197**	**0.0347872**	**-4.755**	**<0.01**
M5.20kb - M6.5kb	-0.0919283	0.0407359	-2.257	0.5011
M6.20kb - M6.5kb	0.0560081	0.0424825	1.318	0.9761
M2.20kb - M1.20kb	-0.0183051	0.0295259	-0.620	1.0000
M3.20kb - M1.20kb	0.0047106	0.0303696	0.155	1.0000

M4.20kb - M1.20kb	-0.0279320	0.0291805	-0.957	0.9984
M5.20kb - M1.20kb	0.0455594	0.0360661	1.263	0.9828
M6.20kb - M1.20kb	**0.1934958**	**0.0380278**	**5.088**	**<0.01**
M3.20kb - M2.20kb	0.0230158	0.0297045	0.775	0.9998
M4.20kb - M2.20kb	-0.0096269	0.0284876	-0.338	1.0000
M5.20kb - M2.20kb	0.0638645	0.0355078	1.799	0.8143
M6.20kb - M2.20kb	**0.2118009**	**0.0374988**	**5.648**	**<0.01**
M4.20kb - M3.20kb	-0.0326426	0.0293612	-1.112	0.9940
M5.20kb - M3.20kb	0.0408487	0.0362124	1.128	0.9932
M6.20kb - M3.20kb	**0.1887851**	**0.0381666**	**4.946**	**<0.01**
M5.20kb - M4.20kb	0.0734914	0.0352211	2.087	0.6249
M6.20kb - M4.20kb	**0.2214278**	**0.0372274**	**5.948**	**<0.01**
M6.20kb - M5.20kb	**0.1479364**	**0.0428385**	**3.453**	**0.0269**

Significant p-values are in bold.

Chapter 2

Table S3 | Effect of resource levels on the lag phase of morphotypes

Comparisons	Estimates	Standard Error	Z Value	P Value
M2.5kb - M1.5kb	-0.011688	0.030088	-0.388	1.0000
M3.5kb - M1.5kb	0.063839	0.033757	1.891	0.7552
M4.5kb - M1.5kb	**0.129742**	**0.037215**	**3.486**	**0.0236**
M5.5kb - M1.5kb	0.085998	0.039902	2.155	0.5704
M6.5kb - M1.5kb	0.039703	0.032546	1.220	0.9866
M1.20kb - M1.5kb	0.021542	0.031657	0.680	0.9999
M2.20kb - M1.5kb	0.012262	0.031211	0.393	1.0000
M3.20kb - M1.5kb	0.024353	0.031794	0.766	0.9998
M4.20kb - M1.5kb	0.050752	0.033096	1.533	0.9276
M5.20kb - M1.5kb	0.061019	0.038214	1.597	0.9062
M6.20kb - M1.5kb	**-0.108042**	**0.026062**	**-4.146**	**<0.01**
M3.5kb - M2.5kb	0.075527	0.033265	2.270	0.4865
M4.5kb - M2.5kb	**0.141430**	**0.036769**	**3.846**	**<0.01**
M5.5kb - M2.5kb	0.097686	0.039486	2.474	0.3456
M6.5kb - M2.5kb	0.051390	0.032035	1.604	0.9027
M1.20kb - M2.5kb	0.033230	0.031132	1.067	0.9956
M2.20kb - M2.5kb	0.023950	0.030679	0.781	0.9998
M3.20kb - M2.5kb	0.036041	0.031271	1.153	0.9916
M4.20kb - M2.5kb	0.062440	0.032595	1.916	0.7395
M5.20kb - M2.5kb	0.072706	0.037780	1.924	0.7345
M6.20kb - M2.5kb	**-0.096354**	**0.025422**	**-3.790**	**<0.01**
M4.5kb - M3.5kb	0.065903	0.039828	1.655	0.8828
M5.5kb - M3.5kb	0.022158	0.042349	0.523	1.0000
M6.5kb - M3.5kb	-0.024137	0.035504	-0.680	0.9999
M1.20kb - M3.5kb	-0.042298	0.034691	-1.219	0.9866

M2.20kb - M3.5kb	-0.051578	0.034285	-1.504	0.9361
M3.20kb - M3.5kb	-0.039486	0.034816	-1.134	0.9926
M4.20kb - M3.5kb	-0.013088	0.036009	-0.363	1.0000
M5.20kb - M3.5kb	-0.002821	0.040763	-0.069	1.0000
M6.20kb - M3.5kb	**-0.171882**	**0.029674**	**-5.792**	**<0.01**
M5.5kb - M4.5kb	-0.043745	0.045153	-0.969	0.9981
M6.5kb - M4.5kb	-0.090040	0.038806	-2.320	0.4498
M1.20kb - M4.5kb	-0.108200	0.038064	-2.843	0.1552
M2.20kb - M4.5kb	-0.117480	0.037694	-3.117	0.0739
M3.20kb - M4.5kb	-0.105389	0.038177	-2.761	0.1900
M4.20kb - M4.5kb	-0.078991	0.039269	-2.012	0.6748
M5.20kb - M4.5kb	-0.068724	0.043669	-1.574	0.9138
M6.20kb - M4.5kb	**-0.237785**	**0.033555**	**-7.086**	**<0.01**
M6.5kb - M5.5kb	-0.046295	0.041390	-1.119	0.9935
M1.20kb - M5.5kb	-0.064456	0.040695	-1.584	0.9105
M2.20kb - M5.5kb	-0.073736	0.040349	-1.827	0.7945
M3.20kb - M5.5kb	-0.061644	0.040801	-1.511	0.9342
M4.20kb - M5.5kb	-0.035246	0.041824	-0.843	0.9995
M5.20kb - M5.5kb	-0.024979	0.045980	-0.543	1.0000
M6.20kb - M5.5kb	**-0.194040**	**0.036512**	**-5.314**	**<0.01**
M1.20kb - M6.5kb	-0.018161	0.033514	-0.542	1.0000
M2.20kb - M6.5kb	-0.027441	0.033093	-0.829	0.9996
M3.20kb - M6.5kb	-0.015349	0.033642	-0.456	1.0000
M4.20kb - M6.5kb	0.011049	0.034876	0.317	1.0000
M5.20kb - M6.5kb	0.021316	0.039765	0.536	1.0000
M6.20kb - M6.5kb	**-0.147745**	**0.028288**	**-5.223**	**<0.01**
M2.20kb - M1.20kb	-0.009280	0.032219	-0.288	1.0000
M3.20kb - M1.20kb	0.002812	0.032783	0.086	1.0000

M4.20kb - M1.20kb	0.029210	0.034048	0.858	0.9994
M5.20kb - M1.20kb	0.039477	0.039041	1.011	0.9973
M6.20kb - M1.20kb	**-0.129584**	**0.027261**	**-4.753**	**<0.01**
M3.20kb - M2.20kb	0.012092	0.032353	0.374	1.0000
M4.20kb - M2.20kb	0.038490	0.033634	1.144	0.9921
M5.20kb - M2.20kb	0.048757	0.038680	1.260	0.9826
M6.20kb - M2.20kb	**-0.120304**	**0.026742**	**-4.499**	**<0.01**
M4.20kb - M3.20kb	0.026398	0.034175	0.772	0.9998
M5.20kb - M3.20kb	0.036665	0.039152	0.936	0.9987
M6.20kb - M3.20kb	**-0.132396**	**0.027419**	**-4.829**	**<0.01**
M5.20kb - M4.20kb	0.010267	0.040217	0.255	1.0000
M6.20kb - M4.20kb	**-0.158794**	**0.028920**	**-5.491**	**<0.01**
M6.20kb - M5.20kb	**-0.169061**	**0.034659**	**-4.878**	**<0.01**

Significant p-values are in bold.

Chapter 2

Table S4 | Effect of resource levels on the growth rate of morphotypes

Comparisons	Estimates	Standard Error	Z Value	P Value
M2.5kb - M1.5kb	-0.069883	0.056246	-1.242	0.9841
M3.5kb - M1.5kb	-0.085942	0.055449	-1.550	0.9207
M4.5kb - M1.5kb	-0.114890	0.054053	-2.126	0.5874
M5.5kb - M1.5kb	-0.064417	0.062413	-1.032	0.9967
M6.5kb - M1.5kb	0.063864	0.071659	0.891	0.9991
M1.20kb - M1.5kb	**-0.206930**	**0.050000**	**-4.139**	**<0.01**
M2.20kb - M1.5kb	**-0.253690**	**0.048202**	**-5.263**	**<0.01**
M3.20kb - M1.5kb	**-0.199573**	**0.050300v**	**-3.968**	**<0.01**
M4.20kb - M1.5kb	**-0.208161**	**0.049951**	**-4.167**	**<0.01**
M5.20kb - M1.5kb	-0.092552	0.060513	-1.529	0.9273
M6.20kb - M1.5kb	-0.094876	0.055012	-1.725	0.8471
M3.5kb - M2.5kb	-0.016060	0.051502	-0.312	1.0000
M4.5kb - M2.5kb	-0.045008	0.049995	-0.900	0.9990
M5.5kb - M2.5kb	0.005466	0.058935	0.093	1.0000
M6.5kb - M2.5kb	0.133746	0.068651	1.948	0.7139
M1.20kb - M2.5kb	-0.137048	0.045584	-3.006	0.1005
M2.20kb - M2.5kb	**-0.183808**	**0.043604**	**-4.215**	**<0.01**
M3.20kb - M2.5kb	-0.129691	0.045913	-2.825	0.1591
M4.20kb - M2.5kb	-0.138278	0.045529	-3.037	0.0915
M5.20kb - M2.5kb	-0.022669	0.056918	-0.398	1.0000
M6.20kb - M2.5kb	-0.024993	0.051031	-0.490	1.0000
M4.5kb - M3.5kb	-0.028948	0.049097	-0.590	1.0000
M5.5kb - M3.5kb	0.021526	0.058175	0.370	1.0000
M6.5kb - M3.5kb	0.149806	0.067999	2.203	0.5303
M1.20kb - M3.5kb	-0.120988	0.044597	-2.713	0.2079

M2.20kb - M3.5kb	**-0.167748**	**0.042571**	**-3.940**	**<0.01**
M3.20kb - M3.5kb	-0.113631	0.044933	-2.529	0.3069
M4.20kb - M3.5kb	-0.122219	0.044541	-2.744	0.1937
M5.20kb - M3.5kb	-0.006610	0.056131	-0.118	1.0000
M6.20kb - M3.5kb	-0.008933	0.050151	-0.178	1.0000
M5.5kb - M4.5kb	0.050474	0.056845	0.888	0.9991
M6.5kb - M4.5kb	0.178754	0.066865	2.673	0.2257
M1.20kb - M4.5kb	-0.092040	0.042848	-2.148	0.5707
M2.20kb - M4.5kb	**-0.138800**	**0.040735**	**-3.407**	**0.0305**
M3.20kb - M4.5kb	-0.084683	0.043198	-1.960	0.7058
M4.20kb - M4.5kb	-0.093270	0.042790	-2.180	0.5476
M5.20kb - M4.5kb	0.022339	0.054752	0.408	1.0000
M6.20kb - M4.5kb	0.020015	0.048603	0.412	1.0000
M6.5kb - M5.5kb	0.128280	0.073788	1.738	0.8405
M1.20kb - M5.5kb	-0.142514	0.053007	-2.689	0.2191
M2.20kb - M5.5kb	**-0.189274**	**0.051314**	**-3.689**	**0.0115**
M3.20kb - M5.5kb	-0.135157	0.053290	-2.536	0.3026
M4.20kb - M5.5kb	-0.143744	0.052960	-2.714	0.2072
M5.20kb - M5.5kb	-0.028135	0.063020	-0.446	1.0000
M6.20kb - M5.5kb	-0.030459	0.057758	-0.527	1.0000
M1.20kb - M6.5kb	**-0.270794**	**0.063634**	**-4.255**	**<0.01**
M2.20kb - M6.5kb	**-0.317554**	**0.062231**	**-5.103**	**<0.01**
M3.20kb - M6.5kb	**-0.263437**	**0.063870**	**-4.125**	**<0.01**
M4.20kb - M6.5kb	**-0.272024**	**0.063595**	**-4.277**	**<0.01**
M5.20kb - M6.5kb	-0.156416	0.072188	-2.167	0.5575
M6.20kb - M6.5kb	-0.158739	0.067643	-2.347	0.4263
M2.20kb - M1.20kb	-0.046760	0.035181	-1.329	0.9732
M3.20kb - M1.20kb	0.007357	0.038006	0.194	1.0000

M4.20kb - M1.20kb	-0.001231	0.037542	-0.033	1.0000
M5.20kb - M1.20kb	0.114378	0.050755	2.254	0.4932
M6.20kb - M1.20kb	0.112055	0.044052	2.544	0.2976
M3.20kb - M2.20kb	0.054117	0.035606	1.520	0.9302
M4.20kb - M2.20kb	0.045529	0.035111	1.297	0.9777
M5.20kb - M2.20kb	**0.161138**	**0.048985**	**3.290**	**0.0436**
M6.20kb - M2.20kb	**0.158815**	**0.042000**	**3.781**	**<0.01**
M4.20kb - M3.20kb	-0.008588	0.037940	-0.226	1.0000
M5.20kb - M3.20kb	0.107021	0.051051	2.096	0.6081
M6.20kb - M3.20kb	0.104698	0.044393	2.358	0.4175
M5.20kb - M4.20kb	0.115609	0.050706	2.280	0.4737
M6.20kb - M4.20kb	0.113285	0.043996	2.575	0.2799
M6.20kb - M5.20kb	-0.002324	0.055699	-0.042	1.0000

Significant p-values are in bold.

Chapter 3

Table S1 | Effect of environment on the relative fitness of morphotypes

Comparisons	Estimates	Standard Error	Z Value	P Value
M6.E1 - M4.E1	-0.20243	0.07445	-2.719	0.09756
M4.E2 - M4.E1	**0.51061**	**0.11262**	**4.534**	**< 0.001**
M6.E2 - M4.E1	**2.31359**	**0.23180**	**9.981**	**< 0.001**
M4.E3 - M4.E1	**0.52298**	**0.11337**	**4.613**	**< 0.001**
M6.E3 - M4.E1	**2.82967**	**0.26748**	**10.579**	**< 0.001**
M4.E4 - M4.E1	0.07915	0.08802	0.899	0.98276
M6.E4 - M4.E1	**0.36523**	**0.10397**	**3.513**	**< 0.01**
M4.E2 - M6.E1	**0.71304**	**0.10574**	**6.744**	**< 0.001**
M6.E2 - M6.E1	**2.51602**	**0.22853**	**11.009**	**< 0.001**
M4.E3 - M6.E1	**0.72541**	**0.10653**	**6.809**	**< 0.001**
M6.E3 - M6.E1	**3.03211**	**0.26465**	**11.457**	**< 0.001**
M4.E4 - M6.E1	**0.28158**	**0.07902**	**3.564**	**< 0.01**
M6.E4 - M6.E1	0.56766	0.09647	5.884	**< 0.001**
M6.E2 - M4.E2	**1.80298**	**0.24365**	**7.400**	**< 0.001**
M4.E3 - M4.E2	0.01237	0.13598	0.091	1.00000
M6.E3 - M4.E2	**2.31906**	**0.27782**	**8.348**	**< 0.001**
M4.E4 - M4.E2	**-0.43146**	**0.11569**	**-3.729**	**< 0.01**
M6.E4 - M4.E2	-0.14538	0.12824	-1.134	0.93871
M4.E3 - M6.E2	**-1.79061**	**0.24400**	**-7.339**	**< 0.001**
M6.E3 - M6.E2	0.51608	0.34384	1.501	0.77695
M4.E4 - M6.E2	**-2.23444**	**0.23330**	**-9.577**	**< 0.001**
M6.E4 - M6.E2	**-1.94836**	**0.23978**	**-8.126**	**< 0.001**
M6.E3 - M4.E3	**2.30670**	**0.27812**	**8.294**	**< 0.001**
M4.E4 - M4.E3	**-0.44383**	**0.11642**	**-3.812**	**< 0.01**
M6.E4 - M4.E3	-0.15775	0.12890	-1.224	0.91014

M4.E4 - M6.E3	**-2.75052**	**0.26878**	**-10.233**	**< 0.001**
M6.E4 - M6.E3	**-2.46444**	**0.27442**	**-8.980**	**< 0.001**
M6.E4 - M4.E4	0.28608	0.10729	2.667	0.11197

Significant p-values are in bold.

AUTHOR CONTRIBUTIONS

Chapter 1

Predation promotes diversification of prey

Ayan, G.B. and Becks, L. designed the study. Ayan, G.B. performed the experiments. Ayan, G.B. and Becks, L. analysed the data. Ayan, G.B. wrote the chapter.

Chapter 2

Predation in interaction with resource competition leads to divergence in defense traits

Ayan, G.B. and Gallie, J. designed the study. Ayan, G.B. performed the experiments. Ayan, G.B. and Becks, L. analysed the data. Ayan, G.B. wrote the chapter.

Chapter 3

Predation minimizes fitness difference and enables stable coexistence

Ayan, G.B. designed the study and performed the experiments. Ayan, G.B. and Becks, L. analysed the data. Ayan, G.B. wrote the chapter.

PUBLICATION

During the time of my doctoral research, I have authored the following publication, which is not included in my thesis:

Hiltunen, T., Ayan, G.B. & Becks, L., 2015. Environmental fluctuations restrict eco-evolutionary dynamics in predator-prey system. *Proceedings of the Royal Society B*, 282.

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DECLARATION

Hereby I declare that

i. apart from my supervisor's guidance, the content and design of this dissertation is the product of my own work. The coauthors' contributions to specific chapters are listed in the Author Contributions section of this thesis;

ii. this thesis has not already been submitted either partially or wholly as part of a doctoral degree to another examination body, and no other materials are published or submitted for publication than indicated in the Publication section of this thesis;

iii. the preparation of the thesis has been subjected to the Rules of Good Scientific Practice of the German Research Foundation.

Plön, 27 September 2017

Gökçe B. Ayan