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260

Ji Zhang

Impact of Biotic and Abiotic Factors on Bacterial Virulence

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Sit down before fact as a little child, be prepared to give up every preconceived notion, follow humbly wherever and to whatever abysses nature leads, or you shall learn nothing

Thomas Henry Huxley

ABSTRACT

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The characteristics of host and pathogen can vary depending on the interplay of the biotic and abiotic factors in the outside-host environment. I hypothesize these factors can affect both current disease dynamics and pathogen virulence. To study the current infection, the genetic background and diet of the moth larvae was manipulated and orally infected with two bacterial strains. I also investigated the impact of thermal fluctuations, predatory and parasitic enemies on the evolution of virulence. The pathogen *Serratia marcescens* evolved under these challenges in a simulated pond water environment in microcosms. In another setting, the association of colony morphotype on virulence, growth and protozoan resistance in *Flavobacterium columnare* was studied to find out the possible correlation between protozoan predation and virulence. We found the disease dynamics depended on interactive effects between environmental factors and genotypes of host and pathogen. The thermal fluctuations affected both species' invasiveness and virulence. There was no correlation between virulence and protozoan resistance in *F. columnare*. Virulence evolution in the antagonistic environment was in contrast to the hypothesis and evidence suggesting that free-living protozoan predation could select for high virulence. The evolved virulence was attenuated and insensitive to composition of bacterial enemy community. Taken together, the results suggest that biotic and abiotic factors outside host can not only change the current dynamics of infectious disease, but also cause evolutionary changes in bacterial virulence.

Keywords: Bacteriophage; coincidental evolution; genotype-environment interaction; protozoan predation; virulence; virulence evolution.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers:

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II Ketola, T., Mikonranta, L., Zhang, J., Saarinen, K, Örmälä, A.-M., Mappes, J. and Laakso J. 2013. Fluctuating temperature leads to evolution of thermal generalism and pre-adaptation to novel environments. *Evolution;* Accepted.

III Zhang, J., Laakso J., Mappes, J., Laanto, E., Ketola, T., Bamford, J.K.H., Kunttu, H. and Sundberg L.-R. 2013. Association of colony morphotype on virulence, growth and resistance against protozoan predation in the fish pathogen *Flavobacterium columnare*. Manuscript.

IV Zhang, J., Örmälä, A.-M., Mappes, J. and Laakso J. 2013. Relative impact of bacteriophage and protozoan predators on the top-down regulation of bacterial population. Manuscript.

V Zhang, J., Örmälä, A.-M., Ketola, T., Mappes, J. and Laakso J. 2013. Coincidental loss of bacterial virulence with amoeba, ciliate and bacteriophage. Manuscript.

The table shows the contributions to the original papers. Ji Zhang: JZ, Ville-Petri Friman: VP, Jouni Laakso: JL, Johanna Mappes: JM, Lauri Mikonranta: LM, Kati Saarinen: KS, Anni-Maria Örmälä: AO, Elina Laanto: EL, Tarmo Ketola: TK, Jaana K.H Bamford: JB, Heidi Kunttu: HK, Lotta-Riina Sundberg: LS.

	I	II	III	IV	V
Original idea	JZ, VF, JL & JM	TK & JL	JZ, JL, JM, JB & LS	JZ, AO, JM & JL	JZ, AO, JM & JL
Experimental work	JZ, VF, & JM	TK, LM, JZ, KS, & AO	JZ, EL, HK & LS	JZ & AO	JZ & AO
Statistical analysis	JZ, VF, & JL	TK	JZ, JL, JM, TK & LS	JZ & JL	JZ, TK &JL
Manuscript	JZ, VF, JL & JM	TK, LM, JZ, KS, AO, JM & JL	JZ, JL, JM, TK, HK & LS	JZ, JM, AO & JL	JZ, JM, TK & JL

1 INTRODUCTION

1.1 Bacterial virulence embedded in a web of interactions

Virulence has been a controversial concept conflicting among researchers of various disciplines in history (Casadevall & Pirofski 1999, Casadevall & Pirofski 2003, Casadevall 2008, Sacristan & Garcia-Arenal 2008). One of the recent accepted definitions defines virulence as the relative capacity of a microbe to cause damage in a host (Casadevall & Pirofski 2003). It is usually measured as host damage (or host mortality) caused by the pathogen (Casadevall & Pirofski 1999, Sacristan & Garcia-Arenal 2008).

In the late twentieth-century, researchers begin to notice microorganisms that can cause serious infectious disease to some individuals but harmless to the others. These pathogens are opportunists that are only able to infect host with impaired immunity (von Graevenitz 1977). Later many other opportunistic pathogens were discovered. This led the 'pathogen-centered' view gradually transforming to the 'host-pathogen interaction' view of virulence and pathogenicity. The 'damage-response' framework of microbial pathogenesis proposed that virulence should be defined as 'the relative capacity of a microorganism to cause damage in a host', and the host damage is the result of host-pathogen interaction. Thus a pathogen or its virulence cannot be defined without also considering the host (Casadevall & Pirofski 2003, Casadevall 2008).

Both host and pathogen are not invariants and they are embedded in a complex web of interactions with other biotic and abiotic factors. These factors can act as selection pressures maintaining or reshaping the phenotypic traits of both host and pathogen. In addition, these factors can affect the current physiological conditions and immunocompetence of the host, as well as the expression of bacterial virulence factors (Smirnova et al. 2001, Ojala et al. 2005, Zaborin et al. 2009). Thus, degree of host damage can vary depending on the interplay of these factors. As a result, there are likely to be circumstances where virulence will increase or decrease due to interactive effects between environmental factors and genotypes of host and pathogen. The understanding

on the relative importance of these factors on virulence would have important implications in controlling and preventing infectious diseases.

1.2 Lifestyles of facultative bacterial pathogen and obligate bacterial pathogen

Traditionally, depending on the capability of living inside or outside the host cells, pathogenic bacteria can be generally divided into three categories: extracellular bacterial pathogen, facultative intracellular bacterial pathogen, and obligate intracellular bacterial pathogen (Mietzner 1999, Engelkirk et al. 2011). Although this classification is extensively used, it is more from 'host perspective' and does not fully reflect the lifestyles of the bacterial pathogen.

Pathogenic bacteria can also be divided into facultative and obligate pathogens depending on the capability of free-living existence (Engelkirk et al. 2011, Busby et al. 2013). This classification is less used, but it nevertheless reflects the important distinction in the lifestyles of the pathogens and thus it is more 'pathogen perspective'.

Obligate bacterial pathogen must rely on the host to survive but does not need to be an obligate intracellular pathogen. For example *Mycobacterium tuberculosis* is a facultative intracellular bacterial pathogen, but is incapable of a free-living existence, being thus an obligate bacterial pathogen (Casadevall 2008). Facultative bacterial pathogen can have two distinctive lifestyles 1) as a free-living microorganism in environmental reservoirs outside the hosts, and 2) as parasite capable of infecting one or more host types (Casadevall 2008, Engelkirk et al. 2011).

Since obligate bacterial pathogens are completely dependent on hosts for survival and replication, their virulence is more dependent on the host-pathogen interaction (Toft & Andersson 2010). However facultative bacterial pathogens usually have to directly deal with different environmental factors in the outside host environment during their free-living life stage. For this reason the impacts of biotic and abiotic factors on virulence are especially important for facultative pathogens. In this thesis, I will mainly focus on facultative bacterial pathogens.

1.3 Hypotheses of virulence evolution

Only very few bacteria are pathogenic to multicellular organisms (Hacker et al. 2003). Compared to the vast knowledge on the prevention and treatment of the existing bacterial infectious disease, relatively less is known about why and how some of the vastly abundant and diverse benign environmental bacteria evolve virulence and become parasitic pathogens. We may also ask why there are so few parasitic bacteria utilizing the super-rich resource of multicellular

organisms? Is it solely because of the efficient immune system or due to some other constraints that the environmental bacteria face in the outside-host environment?

Evolution of virulence has been commonly seen as a coevolutionary arms race between the host and the parasite (May & Anderson 1983, Levin & Svanborg Eden 1990, Levin 1996, Holt & Barfield 2006, Alizon & Baalen 2008, Sacristan & Garcia-Arenal 2008). There are hypotheses that have been proposed to describe the evolution of virulence, which mainly focus on transmission and within host competition. For example, the 'trade-off hypothesis' suggested that virulence would tend to be balanced with the transmission success to new host (Ebert 1994, Day 2001, Ebert & Bull 2003, Alizon et al. 2009, Barrick et al. 2009). The 'shortsighted evolution hypothesis' on the other hand suggested that the competition within the host would select for population with high reproduction rate and high transmission rate (Levin & Bull 1994, Mosquera & Adler 1998).

Importantly however, none of above hypotheses could answer how some harmless environmental bacteria evolved to dangerous pathogens in the first place. Contrary to the 'host-pathogen interaction' perspective of virulence evolution, the 'coincidental evolution of virulence hypothesis' proposed that virulence sometimes is not a direct target of selection, but a by-product of bacterial evolution in response to selection forces caused by biotic and abiotic factors in the outside-host environment (Levin & Svanborg Eden 1990, Levin 1996, Steinberg & Levin 2007, Adiba et al. 2010, Friman et al. 2011a).

Compared to the life in the within-host environment, facultative pathogen bacteria are directly exposed to the complex web of environmental, biotic and abiotic interactions during their free-living life stage. The interplay of these factors could drive evolution of bacterial virulence to different directions. For example, abiotic fluctuating environmental factors have been suggested to select for genotypes that are capable of performing well across a wide range of environments (Levins 1968, Lynch & Gabriel 1987, Gomulkiewicz & Kirkpatrick 1992, Scheiner 1993, Kassen 2002). Fluctuating environments are suggested to select for individuals with good tolerance over the most frequently experienced environments, as their tolerance curve width evolves to match the width of the environmental variation (Levins 1968, Lynch & Gabriel 1987, Gomulkiewicz & Kirkpatrick 1992, Scheiner 1993, Kassen 2002). This is also supported by most of the experimental studies to date demonstrating that generalists often emerge when populations are evolving in fluctuating environments (reviewed in (Kassen 2002, Buckling et al. 2007), but see:(Jasmin & Kassen 2007)). This evolved tolerance could also decrease or increase the ability to inhabit other, novel, environments (Huey & Hertz 1984, Hoffmann & Parsons 1993, Cullum et al. 2001); in the latter case pre-adaptation to a novel environment could play a key role in species invasions and in the emergence of new pathogens (Arnold et al. 2007, Lee & Gelembiuk 2008).

The nutritional conditions of the bacterial growth environment can also significantly affect bacterial metabolism and expression of virulence factors (Friedman & Kautter 1962, Heckly & Blank 1980, Midelet-Bourdin et al. 2006). For example it has been found that the virulence of the pathogenic fungi was

highly correlated to C/N ratio of the culturing medium (Safavi et al. 2007, Ali et al. 2009, Wu et al. 2010). Evolutionary experiments also provide evidence that low nutrient environment could be connected to attenuated bacterial virulence (Friman et al. 2009, Gomez & Buckling 2011).

In recent years, the role of biotic factors, such as protozoan predation and bacteriophage parasitism in virulence has attracted more attention (Greub & Raoult 2004, Brüssow et al. 2004, Brüssow 2007, Friman et al. 2011a, Mikonranta et al. 2012). The 'top-down control' by protozoan predators and bacteriophages is the major biotic cause for bacterial mortality, thus an important shaping force not only for bacterial community structure, but also phenotypic and genotypic traits of bacteria including virulence (Jürgens & Matz 2002).

There is accumulating evidence over the past two decades indicating that biotic interactions in the outside host environment like predation by free-living protozoans and parasitic bacteriophages can play a vital role in the evolution of bacterial pathogens (Brüssow et al. 2004, Greub & Raoult 2004, Brüssow 2007, Steinberg & Levin 2007, Adiba et al. 2010). For example, defending against enemies may be very costly for growth and virulence related traits, thus trade-offs between bacterial anti-predatory traits and bacterial virulence could result in decreased virulence (Friman et al. 2009). On the other hand, defense against enemies may be positively correlated with defense against host immune system and thus promote the evolution of virulence (Brüssow 2007, Cosson & Soldati 2008, Adiba et al. 2010, Al-Quadan et al. 2012).

1.4 Role of protozoan predators and bacteriophages in the evolution of bacterial virulence

Bacteriophages and protozoa are the two major biotic causes of bacterial mortality in the natural environment (Jürgens & Matz 2002, Suttle 2005). Unsurprisingly many bacterial traits were selected primarily by predation and parasitism (Matz & Kjelleberg 2005, Brüssow 2007, Abedon 2008). Predation by free-living protozoa can also positively contribute to the evolution of the bacterial virulence (Greub & Raoult 2004, Steinberg & Levin 2007, Lainhart et al. 2009). Presumably because of the common, conserved eukaryotic machineries shared by protozoan cells and the multicellular host, the defence function selected by protozoan predation can be relatively easily adapted to cope with eukaryotic cells like macrophages in the immune system (Gao et al. 1997, Al-Quadan et al. 2012). For this reason the free-living amoeba are also called the 'evolutionary crib' of virulence (Greub & Raoult 2004).

Perhaps a most typical example would be the evolution of *Legionella pneumophila*. Many virulence traits of *L. pneumophila*, such as resistance of lysosomal degradation did not evolve from human-bacteria interaction, but 'coincidentally' evolved from amoeba-bacteria interaction (Levin 1996, Greub & Raoult 2004, Ensminger et al. 2012). Originally human being and *L. pneumophila* are two ecologically irrelevant species. *L. pneumophila* is mainly found as a

parasite of free-living amoeba (Rowbotham 1980, Abu Kwaik et al. 1998, Ohno et al. 2008). Extensive applications of air-condition and water systems in where amoeba lives created such an overlap in their biogeographic niche, so that *L. pneumophila* could 'spillover' and invade into the human population (Holt & Hochberg 2002, Sokurenko et al. 2006, Keesing et al. 2010).

Bacteriophages could positively contribute to bacterial virulence too by generating great genetic diversity in bacterial populations through introduction of new genes. Bacteriophages are known to carry important bacterial virulence genes (Hacker & Kaper 2000, Hacker et al. 2003, Brüssow et al. 2004). For example, they have been found to contain genes encoding exotoxins and other virulence factors that can be horizontally transferred into the bacterial genome (Casas & Maloy 2011, Boyd 2012). Bacteriophage transduction serves as important source of bacterial virulence genes and vector for horizontal gene transfer (HGT) (Brüssow et al. 2004, Busby et al. 2013). In fact, many virulence genes on the bacterial genomes were found to be bacteriophage origins (Brüssow et al. 2004, Schmidt & Hensel 2004). Pathogenicity islands in bacterial genomes were frequently found to originate from ancient bacteriophage integration events (Hacker & Kaper 2000, Hacker et al. 2003). Moreover, the new genes carried by the bacteriophages such as Insertion Sequence (IS) can cause reshuffle/rearrangement of the bacterial genomes resulting changes on genetic and phenotypic traits. Moreover, bacteria can alter their cell surface antigens to evade phage adsorption (Labrie et al. 2010), and on the other hand, host immune system identifies bacterial invaders through bacterial surface antigens. Thus, in principle bacteriophage selected bacterial surface antigen variation could indirectly facilitate host entry.

On the other hand, although the bacterial enemies are common and potentially capable of driving virulence to a higher degree, defense against predators and parasites can be costly and other traits related, for example to host entry and growth in the host can therefore be traded off (Levin & Bull 1994). Thus it is reasonable to hypothesize that there are scenarios where the virulence is subject to negative selection. Indeed, there is evidence indicating that both protozoan predation and bacteriophage parasitism do not select for higher virulence. For example bacteria mobility can trade off with anti-predator traits when facing the protozoan predation selection pressure, resulting in attenuated virulence (Friman et al. 2009). Also it has been shown that higher temperature can select for higher virulence in *S. marcescens*, while bacteriophages counteract this effect (Friman et al. 2011a). Facultative pathogens have two distinctive life styles, either as free-living organism or as a parasite. The machineries required for outside-host life can be either similar or different, and the associated allocation cost to these traits may also differ. Thus, in more general terms, the environmental lifestyle can attenuate or strengthen virulence depending on the underlying biology of the system.

1.5 Top-down regulation of bacterial communities by the protozoan predators and bacteriophages in the outside host environment

Bacterial communities in the natural environment are typically regulated in two ways: by the availability of resources ('bottom-up control'), and by organisms in higher trophic levels ('top-down control'). The prevailing view is that bacteriophages and protozoan are the two major biotic causes of bacterial mortality, though at a given time point and place, one or the other enemy group may dominate (Fuhrman 1999, Suttle 2005).

These bacterial enemies use quite different strategies to consume bacterial biomass. For example, most flagellates and ciliates are specialized in preying on suspended bacteria, whereas amoebae are thought to almost exclusively feed on biofilm (Rodriguez-Zaragoza 1994, Molmeret et al. 2005). Bacteriophages are parasites that attack bacteria in the free water as well as those hidden in biofilms (Sillankorva et al. 2008, Azeredo & Sutherland 2008). The capability of a bacteriophage to infect biofilms (formed by an otherwise susceptible bacterial host) depends on viral access on bacterial surface, which in turn is determined by the structure of biofilm and the capability of bacteriophage to degrade the extracellular polymers forming the matrix of the biofilm (Sutherland et al. 2004).

Top-down control by protozoan predators and parasitic bacteriophages can have a huge impact on the bacterial communities and cause profound changes in their pheno- and genotypes (Fuhrman 1999, Jürgens & Matz 2002). However, little is known about relative roles of different enemies on the temporal and spatial dynamics of bacterial population, or how these connect to the virulence potential of bacteria in the environmental reservoirs.

1.6 Controlled evolutionary experiments in microbial ecology and evolution studies: advantages and disadvantages

Pure culture techniques of the microorganisms give a unique opportunity to study the species interactions in a controllable environment. There are many advantages in such experimental settings, including direct measurement of the bacterial biomass, easy manipulation of environmental variables, ability to store and revive the experimental strains that allows direct comparison of ancestral and evolved strains. Experiments also allow large population sizes and number of generations (Elena & Lenski 2003, Buckling et al. 2009).

Despite these advantages there are some limitations. The natural conditions can be much more complicated than the simplified laboratory settings. For example, the natural environment is typically not as 'clean' as in the bottles in the laboratory. It is almost always filled with all kinds of foreign

genetic material that could potentially be horizontally transferred into the bacterial genome. Unlike multicellular organisms, one striking feature of the pathogenic bacteria is that most bacterial virulence determinants are predominantly located on pathogenicity islands (PAIs), and uptake of large fragments of DNA containing virulence genes are common among most of the bacterial pathogens (Schmidt & Hensel 2004), especially those who come from niches that are colonized by diverse and large numbers of bacteria (Dobrindt et al. 2004). Moreover, comparative genomics study on bacterial whole genome sequences revealed that there are significant part of the genes in the bacterial genome are strain-specific rather than species-specific, presumably acquired through horizontal gene transfer (HGT) (Medini et al. 2005, Tettelin et al. 2008, Deng et al. 2010, Muzzi & Donati 2011, Jacobsen et al. 2011). Thus to fully understand the evolution of the bacteria, the role of HGT should not be ignored.

In this thesis, microbial microcosm and experimental evolution methodology was used to uncover the role of outside host interactions on microbial community dynamics and trait evolution, with special focus on virulence traits (paper III, IV and V). Although the possibility of HGT is eliminated in such simplified experimental settings, still it could serve as an important step toward the comprehensive understanding of bacterial virulence evolution, and hopefully provide useful information for the future studies.

1.7 Two 'gears' of the bacterial evolution

Unlike most multicellular eukaryotes, bacteria as asexual organisms cannot combine the genetic material through sexual reproduction and thereby generate descendant with new genotype. Instead, bacteria can generate new genotypes by point mutations and genetic rearrangements. This can be seen as the 'slow gear' of bacteria to evolve new genes and acquire virulence potential. In this way, it would take much longer time for bacteria to evolve new genes and novel traits such as antibiotic resistance and virulence factor (Barrick et al. 2009, Wielgoss et al. 2011, Wielgoss et al. 2013).

The other way bacteria generate new genotypes is by taking in foreign genetic material and adapted to its genome, namely horizontal gene transfer (HGT). This is usually a much faster way for bacteria to acquire new genes and generate new genetic variants in their descendants, thus can be seen as a 'fast gear' of bacterial evolution (Hacker & Kaper 2000). Through HGT bacteria do not need to 'invent' new gene or gene cluster. By taking in and adapting foreign genetic material for the closely related strains, sometime even from other species, bacteria can quickly gain necessary gene set to invade host, to survive in new environment, or gain resistance to antibiotics (Hacker & Kaper 2000, Lopez-Pascua & Buckling 2008, Morgan et al. 2009, Juhas et al. 2009). Horizontal gene transfer plays a significant role in the evolution of pathogenic bacteria. It allows bacteria to acquire new genes and new phenotypic traits in a short time, thus also called 'quantum leaps' (Groisman & Ochman 1996, Hacker & Carniel

2001, Schmidt & Hensel 2004) or evolutionary 'fast route' (Medini et al. 2005), which enable bacteria to adapt to new environments at astonishing speed. There are many examples of avirulent bacterial strains that seem to have acquired the ability to cause disease in the first place or increase virulence dramatically in this one step manner (Knapp et al. 1986, Johnson et al. 1986, Karaolis et al. 1999, Blum-Oehler et al. 2000, Hensel 2004).

Although this thesis do not concentrate the discussion of HGT or 'fast gear' of bacterial evolution, a complete understanding of how bacteria evolve in the natural conditions would be helpful to interpret the results and see things in a broader perspective.

1.8 Aims of the study

In this thesis I study how abiotic and biotic factors affect bacterial virulence in environmentally growing opportunistic pathogens. I view bacterial virulence not as an invariant trait of a microorganism ('pathogen-centered' view), but put it in a 'damage-response' framework and see it as an outcome of host-microorganism interaction, and as a dynamic variable that responds to different biotic and abiotic factors.

Firstly I study how environmental conditions like host diet would interact with different host and pathogen genotypes and change the outcome of infection (paper I). Secondly I investigate how abiotic factors such as fluctuating environmental temperature affects the evolution of virulence (paper II).

Parasitic bacteriophages and predatory protozoans are the main biotic sources of bacterial mortality in nature and top-down control by these enemies can have huge impact on the bacterial communities and cause profound changes in their pheno- and genotypes, including bacterial virulence (Fuhrman 1999, Jürgens & Matz 2002). If these biotic interactions have played a role in the evolution of pathogenic bacteria, chances are that there is still evidence of such interaction in the existing pathogens. I studied 13 strains of fish pathogen *Flavobacterium columnare*, searching for correlations of past exposure to bacteriophages or starvation to bacterial virulence, biofilm forming ability, growth and performance against protozoan predators.

In the paper IV and V, I investigated that 1) how the bacterial population dynamics changes under the top-down control by common bacterial predators and parasitic bacteriophages (paper IV), and 2) how the bacterial virulence evolves in the outside-host environment when the bacteria were exposed to their enemies in different combinations (paper V). Lastly, we set up an evolutionary experiment where facultative pathogen *Serratia marcescens* strain DB11 grew alone or with its lytic bacteriophage Semad11 and two typical protozoan predators: particle feeding ciliate and surface feeding amoeba, either alone or in different combinations. In this experiment the population dynamics, gross biomass production, biofilm production of bacteria and population dynamics of predators were followed closely. After the experiment, bacterial

strains were isolated from the microcosms and studied in separate experiments to detect the possible evolutional changes, e.g. in growth, biofilm-forming ability, motility, predator defense and virulence.

2 MATERIAL AND METHODS

2.1 The study species

2.1.1 Bacterial strains

In paper I, we used two *S. marcescens* strains (ATCC 13880 and DB11) as pathogens with different genotypes in the animal oral infection model. In paper II, IV and V, these *S. marcescens* strains were used in the evolutionary experiments.

S. marcescens is a gram negative facultative pathogenic bacterium that can either grow in the environment or opportunistically infect a broad spectrum of hosts, including plants, corals, nematodes, insects, fish and mammals (Grimont & Grimont 1978, Flyg et al. 1980). *S. marcescens* is commonly present as a free-living form in soil, freshwater, and marine ecosystems (Sutherland et al. 2010, Mahlen 2011) and frequently encounters parasitic and predatory enemies.

Bacterium *S. marcescens* strain ATCC 13880 was used as a medium virulent pathogen in wood tiger moth larvae infection experiment (paper I). Bacterium *S. marcescens* strain DB11 was used as prey the in evolutionary experiments (paper IV - V) and as a high virulence pathogen in wood tiger moth larvae infection experiment (I). *S. marcescens* strain DB11 was kindly provided by Prof. Hinrich Schulenburg and it was initially isolated from dead *Drosophila* fruit fly (Flyg et al. 1980). The *S. marcescens* strain ATCC 13880 was originally isolated from pond water and was obtained from the American Type Culture Collection.

The bacterium investigated in the paper III was *F. columnare* (Bacteroidetes), an opportunistic environmentally growing fish pathogen that can causes disease outbreaks and large economic losses in freshwater fish farming worldwide (Wagner et al. 2002, Suomalainen et al. 2006, Kubilay et al. 2008, Pulkkinen et al. 2010). When isolated from diseased fish, free water or from the environment, *F. columnare* exhibits rhizoid (Rz) colony phenotype. In laboratory conditions rough (R) and soft (S) type colony variants rapidly appear in response to serial culture and starvation with consequent loss of virulence

(Kunttu et al. 2009, Kunttu et al. 2011). Similarly, if cultured with lytic bacteriophages the bacteria typically lose their rhizoid type colony morphology in parallel with gaining phage resistance and loss of virulence (Laanto et al. 2012). In total 13 *F. columnare* strains that were isolated from water tanks in fish farms (Fig. 1) and from natural environment outside fish farming. All the strains have two morphotypes: 1) ancestral rhizoid type and 2) rough type (Fig. 2), which was either induced by bacteriophage infection or formed spontaneously during the serial cultures in famine condition in laboratory.

FIGURE 1 Water tank in the fish farm from where the *F. columnare* strains were isolated.

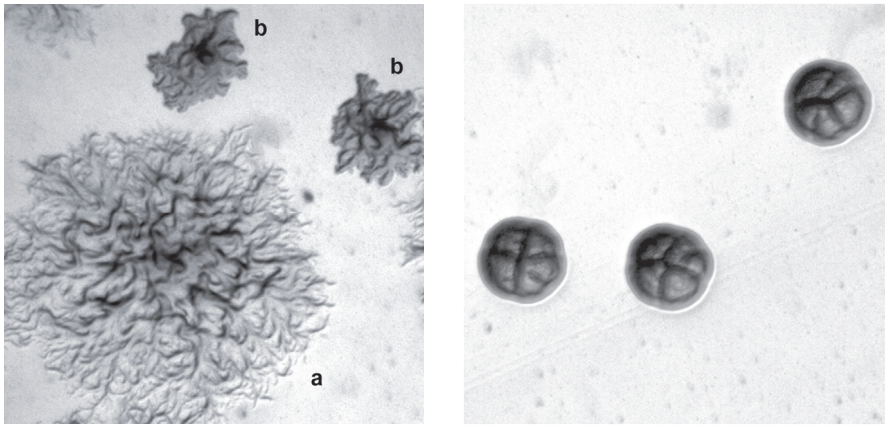

FIGURE 2 The ancestral rhizoid colony of the *F. columnare* strain B245 (left, a) was flat and spreading. In the famine conditions rough type colonies would appear (left b). On the right: after exposure to phage strain B392 lost all the root-like protrusions and formed rough colonies with round and solid edges.

The predatory particle feeding ciliate *Tetrahymena thermophila* strain ATCC 30008 (minimum generation time about 2 hours) (Kiy & Tiedtke 1992) was obtained from American Type Culture Collection and is routinely maintained in PPY (Proteose Peptone Yeast Medium) (Friman et al. 2008).

Free-living amoeba *Acanthamoeba castellanii* strain CCAP 1501/10 (generation time about 7 hours (Kennedy et al. 2012)) was obtained from Culture Collection of Algae and Protozoa (Freshwater Biological Association, The Ferry House, Ambleside, United Kingdom) and routinely maintained in PPG (Proteose Peptone Glucose Medium (Page 1976)).

2.1.2 The bacteriophages

Obligatory lytic bacteriophage Semad11 infecting *S. marcescens* DB11 was isolated from a sewage treatment plant in Jyväskylä, Finland in 2009. Semad11 is a T7-like bacteriophage belonging to *Podoviridae* (A-M. Örmälä, unpublished data).

The *F. columnare* bacteriophages used in the paper III were characterized previously (Laanto et al. 2011) and were used to induce phage resistance of the rhizoid type strains, which led to changes in colony morphotype to bacteriophage resistant rough type colonies as described previously (Laanto et al. 2012).

2.1.3 The hosts used in the infection experiment

The host used in larvae infection experiment (paper I) was wood tiger moth *Parasemia plantaginis* (Arctiidae) larvae. The larvae have an orange patch on the dorsal side of an otherwise black body (Fig. 3). The larvae are aposematic: the patch is used as a warning signal, and the size of the patch is heritable (Lindstedt et al. 2009). Bigger patches are more effective warning signal for

avian predators (Lindstedt C. 2008). On the other hand, investing in a large orange warning signal decreases the amount of cuticular melanin: the amount of melanin correlates positively with immune responses in many taxa including *P. plantaginis* (Armitage & Siva-Jothy 2005, Siva-Jothy et al. 2005, Friman et al. 2009, Laurentz et al. 2012). In this study we used *P. plantaginis* larvae from two selection lines for low or high amount of cuticular melanin. Although detailed immunological mechanisms are not understood, the previous experiments have shown that 'Low melanin' individuals (with larger orange patches) have weaker pathogen resistance than 'high melanin' individuals (with small patches) (Friman et al. 2009). Selection lines for 'high melanin' (small orange patch) and 'low melanin' (large orange patch) in the *P. plantaginis* larvae were established in 2004; fifty-one families were used to set up the selection lines by applying a truncated family selection protocol (Lindstedt et al. 2009).

FIGURE 3 Wood tiger moth *P. plantaginis* larvae have orange patch on the dorsal side of an otherwise black body.

In the paper III, we used adult disease-free wild type zebra fish (*Danio rerio*) as a host to test virulence of *F. columnare*. Zebra fish were unsexed and they were obtained from Core Facilities (COFA) and research services of Tampere University, Finland.

To examine the virulence of the evolved bacteria strains in the evolutionary experiments II, IV and V, 2-3 days old *Drosophila melanogaster* adults from our large laboratory colony (kindly provided by Christina Nokkala from the University of Turku) were fed with a mixture of bacteria suspension and 100 mM sucrose solution.

2.2 The culture media

In paper II, phosphate buffered cereal leaf extract medium (SPL) (Friman et al. 2008) was used to culture the bacteria. In experiments (IV – V) a similar liquid was used. However to satisfy the growth requirements of amoeba, these experiments used pH 7 buffered PAS (Page's modified Neff's amoebae saline) (Page 1988, La Scola et al. 2001, Greub et al. 2004). The nutrition level of this medium was so low that it can be considered a simulation of the nutritional conditions occurring in natural pond water. Since the protozoan predator *A. castellanii* and *T. thermophila* cannot significantly feed on the very low concentration medium, they were forced to feed on the prey bacteria *S. marcescens*, and thus occupied a separate trophic levels when cultured together.

In paper III, Shieh medium (Shieh 1980) or Shieh medium supplemented with tobramycin (Decostere et al. 1997) was used to isolate *F. columnare* strains were isolated from diseased fish or natural freshwater bodies. However in the measurements of bacterial traits such as growth ability, biofilm forming ability, protozoan predation resistance, and food preference test, the nutrition level of the original Shieh medium was reduced to 1/10 of its original concentration (yeast extract 0.05g/l and peptone 0.5g/l). The reason to do so is that although *F. columnare* strains grow fast in original Shieh medium, however for unknown reason after 72 hours no live bacterium can be isolated from the liquid culture. With this modified Shieh liquid medium, live bacteria can still be isolated from it after 2 weeks, which was essential for the one-week long experiment.

2.3 Experimental evolution of bacterium *S. marcescens*

In the evolutionary experiment of paper III, ten replicate populations per treatment were established on two 100-well spectrophotometer plates that were assigned to either stable or fluctuating temperatures. We used two temperature controlled spectrophotometers (Bioscreen C®, Growth curves Ltd, Helsinki, Finland) to create temperature selection regimes: constant 31 °C and daily fluctuating (from 24 to 38 °C, mean 31 °C). Every 24 hours (± 1h) part of the populations were transferred to new wells containing fresh SPL, and returned to the spectrophotometer. Every fourth day we changed the plates between the two machines and set temperatures to match experimental treatments to exclude confounding effects of individual spectrophotometer identity. After 3 weeks 12 clones were isolated from each replicate population by serial dilution and plating.

The experiment in paper IV was initiated in 25 cm^2 polystyrene flasks with non-wettable hydrophobic filter membrane caps (Sarstedt). Each flask was inoculated with 1 ml of microorganism(s) depending on the community composition and the total volume was adjusted to 15 ml with NAS medium (Fig. 4). There were five community composition treatments: 1) bacteria, 2)

bacteria + ciliates, 3) bacteria + amoebae, 4) bacteria + bacteriophages, and 5) bacteria + all three enemies. Each treatment was replicated in 36 flasks. The static liquid cultures were incubated at 25 °C and every 7 days half of the liquid medium was replaced with fresh NAS medium, making the system a pulsed resource type (Friman & Laakso 2011, Friman et al. 2011b). Before every renewal, 4 flasks from each treatment were randomly chosen for destructive sampling. The co-culture experiment lasted for eight weeks.

FIGURE 4 Microcosm design used in the evolutionary experiments.

In evolutionary experiment in paper V, the bacteria *S. marcescens* was either cultured alone or co-cultured with the enemies: 1) bacteria, 2) bacteria + ciliates, 3) bacteria + amoebae, 4) bacteria + bacteriophages, 5) bacteria + ciliates + amoebae, 6) bacteria + ciliates + bacteriophages, 7) bacteria + amoebae + bacteriophages and 8) bacteria + all three enemies. The experiment lasted for 8 weeks (approximately 1300 bacterial generations). After the evolutionary experiment a clone library containing evolved strains was extracted to detect the changes in virulence and other phenotypic traits in separate experiments.

2.4 Bacterial population dynamics and growth

To monitor the bacteria growth, the bacterial culture was seeded into 100-well Honeycomb plates (Oy Growth Curves Ab Ltd) containing fresh culture medium. The choice of the medium depended on the bacterial species and purpose of the experiment. The amount of biomass was measured as optical

density (OD) at 460-580 nm wavelength using Bioscreen C® spectrophotometer (Oy Growth Curves Ab Ltd) at 25 °C without shaking. The measurements typically were repeated at 5 minutes intervals for several days. Then the growth curves were analysed with a Matlab script (J. Laakso, unpublished) developed in our laboratory to estimate the maximum growth rates, and maximum and minimum population size.

2.5 Assay for measuring bacterial biofilm forming ability

Bacteria were usually grown overnight on 100-well Honeycomb plates (Oy Growth Curves Ab Ltd) containing fresh bacterial liquid culture medium. Crystal violet solution (Sigma-Aldrich) was be injected each well to stain the biofilm on the well wall. After 10 minutes, the plates were rinsed with distilled water for three times and then ethanol was added to each well to dissolve crystal violet from the walls for 24 hours (O'Toole & Kolter 1998). The amount of biofilm was quantified with the OD of crystal violet-ethanol solution at 460-580 nm with Bioscreen C® spectrophotometer (Friman & Laakso 2011).

2.6 Protozoan predator population dynamics

To determine ciliate density, the sample was mixed with Lugol's solution and injected into a glass cuvette race (depth 2.34 mm). The ciliate cells were instantly killed, fixed and stained by Lugol's solution. For each sample 8 randomly placed images (total area 18 mm^2) were digitized with an Olympus SZX microscope (32 × magnification). The cell numbers in each image were counted with an Image Pro Plus script (Laakso et al. 2003).

In the experiment of paper IV, to follow the population dynamics of the amoeba, the flasks were carefully flipped upside down and 8 randomly placed images (total area 18 mm^2) from the microcosm bottom wall were digitized with an Olympus SZX microscope (32 × magnification) in the dark-field mode. The cell numbers in each image were counted with a script developed in our lab for the Image Pro Plus software (v. 7.0) (Zhang J., unpublished).

Although dark-field microscope was suitable for observing normal amoeba cells, we realized that it might not good for observing abnormal amoeba cells, e.g. starved amoeba cells. So in the experiment of paper V, we used light-field mode to count the attached amoeba cells directly. First the flasks were carefully flipped and images (total area 5.23 mm^2) of the flask wall were digitized with an Olympus SZX microscope (32× magnification). The amoeba cells attached to the flask wall were counted with a script as described above.

2.7 Measurement of bacterial virulence

In many studies, the virulence of the bacteria is measured in animal infection models (Bohannan & Lenski 2000, Buckling et al. 2006, Friman et al. 2011a). Usually bacteria are injected into the body cavity of the host with a needle (septic injury model; Vodovar et al. 2004). In order to simulate natural infection more realistically, all the pathogens were given either orally (to insect, paper I, II, IV and V) or by bacterial suspension bath immersion (to fish, paper III).

In the tiger moth larvae oral infection model, the larvae were treated with water containing bacteria (DB11 or ATCC 13880). The larvae were monitored until they had drunk the whole inoculum. Then larval survival was recorded twice a day for the following 4 weeks.

In experiment of paper III, the fish were individually challenged in 50 ml of borehole water with overnight grown bacteria for 30 minutes at room temperature. Each infection was done in 6 replicates, and fish exposed to sterile Shieh medium served as negative control. After the bacterial immersion, the fish were transferred into separate plastic aquaria (one fish per aquarium) with borehole water, and monitored for 2 days for disease symptoms and mortality. After this, the fish that had survived were further monitored for another 2 days. From all dead and moribund fish a bacterial culture sample from gills was taken to ensure the cause of death to be columnaris disease.

The *Drosophila* oral infection model used in the experiments II, IV and V was modified from Nehme (2007). First the bacteria were cultured in Luria-Bertani (LB) medium. After 24 hours the bacterial culture was mixed with the same amount of 100 mM sucrose solution. The mixture was absorbed to cotton dental roll (Top Dent, Lifco Dental, Enköping, Sweden) folded on the bottom of standard fly vial (Sarstedt, Nümbrecht, Germany). Ten 2-3 days old *D. melanogaster* adults from large laboratory colony (obtained from University of Turku) were transferred to each vial plugged with cotton plug. This was done for all the bacterial clones and the death rate of flies were monitored over next 4 days, at ca. 6 hours intervals.

2.8 Ciliate predation resistance

The ciliate predator resistance of the bacterial strain was measured by comparing the minimum population size of with or without predator treatments 3 days after adding the predators, namely minimum OD without predator subtracted from minimum OD with predator.

In paper III the ciliate cells were first harvested and washed twice in modified Shieh liquid medium with centrifugation at 1,200×g for 15 min to pellet the cells. After the centrifugation, cells were suspended in modified Shieh liquid medium and adjusted to final concentration. After ca. 50 hours growth on 100-well Honeycomb plates (Oy Growth Curves Ab Ltd), washed ciliate

suspensions (same amount of fresh modified Shieh liquid medium for predator-free wells) were added to the bacterial culture. Then the plates were put back to Bioscreen C® spectrophotometer to follow the changes in growth curves with method described above. Each *F. columnare* colony morphotype had at least 3 replicates on the plates for every treatment.

2.9 Amoeba predation resistance

There are two ways used in my studies to measure the amoeba predation resistance of the bacterial strains. The first way was similar to the measurement of ciliate predation resistance (Friman & Laakso 2011). The amoeba cells were first harvested and washed twice in PAS (Page's modified Neff's amoebae saline) (Page 1976, La Scola et al. 2001, Greub et al. 2004) with centrifugation at 1,200 × g for 15 minutes to pellet the cells. After the centrifugation, cells were suspended in PAS and adjusted to final concentration. After added the amoeba cells to overnight bacterial culture growing on 100-well Honeycomb plates (Oy Growth Curves Ab Ltd), the plates were put back to Bioscreen C® spectrophotometer to follow the changes in growth curves.

The second way was amoeba plaque test that was adapted from previous method (Wildschutte et al. 2004). A sterile paper disk filled with amoeba cells was placed in the center of a bacteria lawn. The amoeba will immediately start to consume bacteria lawn in all directions and create a visible plaque on the surface of the agar plate. A bigger plaque size indicates a smaller amoeba predation resistance of the bacterial strain (Fig. 5).

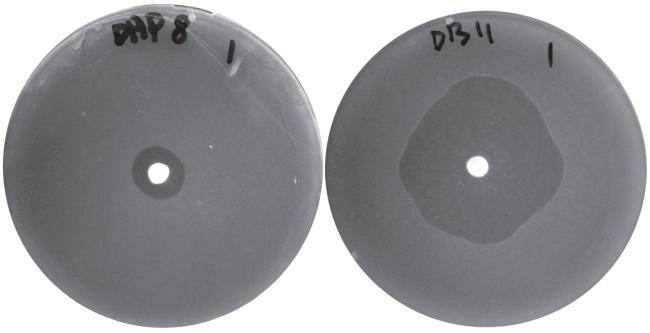

FIGURE 5 Different sizes of amoeba plaque on agar plates.

After the 8-week evolutionary experiment (V), the flasks were first shaken vigorously before 1 ml of the culture was taken and transferred to a new tube containing ddH_2O. After thorough mixing, the tubes were centrifuged to settle down the floating protozoan cells. Then the supernatant was smeared evenly to LN agar plates (PAS supplied with 0.2 % peptone, 0.2 % glucose and 1 % agar).

A total of 10^5 of amoeba cells (washed twice in PAS to remove the nutrients) suspended in PAS solution were added to a sterile paper disk, and then placed in the middle of the plate. All the plates were incubated at 25 °C for 8 days and then photographed. The images of the plates were used to measure plaque sizes with Image Pro Plus software (v. 7.0).

3 RESULTS AND DISCUSSION

3.1 Interactive effects between diet and genotypes of host and pathogen define the severity of infection

In the oral infection experiment with tiger moth larvae, larval cuticular melanin did not enhance survival when the larvae were infected with high virulence bacteria. Survival was strongly dependent on family origin even within the melanin selection lines. *S. marcescens* strain DB11 caused higher host mortality compared to strain ATCC 13880. This result is in accordance with our expectations due to the strains' different origin; bacteria isolated from aquatic environment are likely to be less virulent because they share no close evolutionary history with insect hosts. At the mechanistic level, higher DB11 virulence was connected to more efficient growth and motility measured in vitro. Bacterial growth rate is an indicator of efficient host exploitation rate (Johnson 1991, Meyer et al. 1996, Harrison et al. 2006, Friman et al. 2009). DB11 had both higher maximum growth rate and maximum density in all resource concentrations we used in the in vitro measurements.

Anti-infection diet (plantain) enhanced survival of the 'high melanin' but not the 'low melanin' hosts. This is surprising because plantain extracts have antibacterial effects against both Gram-positive and Gram-negative bacteria (Gomez-Flores et al. 2000). Moreover, plantain diet helps mice to fight systemic infection of *Streptococcus pneumoniae* by stimulating the innate immune system (Hetland et al. 2000), while plantain extracts can also activate macrophages and affect the lymphocyte proliferation (Gomez-Flores et al. 2000). In addition to direct immunological effects, plantain leaves can contain as much as 15 % of protein (Mohamed et al. 2011), which is approximately ten times the concentration in *Lactuca sativa* leaves (USDA Nutrient Database; http://www.nal.usda.gov/fnic/foodcomp/search/). High protein diet can induce cuticular melanin production, which is correlated with anti-infection activity (Midelet-Bourdin et al. 2006, Cotter & Kilner 2010). Since only 'high melanin' larvae benefited from plantain diet it seems that instead of increasing

survival by improving larval condition in general, the plantain diet interacts with the host immune system. If this was due to plantain diet's positive effect on host resistance or tolerance, or direct negative effect on the parasite, remains still unclear. It is notable that larval survival in the water treatment group was quite poor compared to previous studies performed under similar conditions (Friman et al. 2009, Friman et al. 2011a). One explanation could be the abrupt diet switch from dandelion to plantain or lettuce after the first three weeks of larval development. Since individuals in all treatments switched diet from dandelion to lettuce or plantain, the switch itself unlikely biased our results. Most importantly, larval survival was clearly higher in bacterial treatments compared to the water control, which shows that bacterial infection increased larval mortality.

Recent findings suggest that insect hosts can change their diet towards medicating plants after infection (Singer et al. 2009). The tiger moth larvae had an intrinsic preference for lettuce, but no evidence of induced diet shift towards lettuce, and thus signature of self-medication was found. One potential explanation could be starvation-induced dehydration, which might lead preference for lettuce that has relatively high water concentration. Self-medication can also be trans-generational, and directed towards offspring instead of the infected parent (Lefevre et al. 2010, Lefevre et al. 2012). Thus, it is possible that the three-day interval after the infection was too short to observe potential food preference in *P. plantaginis*. Alternatively, complementary diet where larvae consume both medicating and normal growth-enhancing plants could result in highest survival (Ojala et al. 2005).

These results demonstrate that the relative benefit of host cuticular melanin can depend on both diet and pathogen virulence: plantain diet only boosted the immunity of already resistant 'high melanin' hosts, and cuticular melanin increased host survival only when infected with moderately virulent pathogen. Moreover, there was considerable variation in host survival between families within both melanin lines suggesting genetic basis for resistance. These results indicate that although melanin is an important predictor of insect immunity, its effect on disease outcomes greatly depend on other interacting factors such as host genetic background and diet.

3.2 Evolution of virulence in a fluctuating environments

We found fluctuating environmental temperature increased growth rate across measured novel environments. However, the clones adapted to fluctuating temperature had attenuated virulence in *D. melanogaster* insect host. Obviously, this could be seen as an example of cost of generalism (Gilchrist 1995) if less virulent genotypes loses the within-host competition to their more virulent rivals (Frank 1996) and also as evidence against general environmental tolerance. Higher growth and lower virulence in the clones adapted to temperature fluctuations complement a recent study reporting a cost of high

virulence via lower growth rate in *Salmonella typhimurium* strains (Sturm et al. 2011). Mechanistically, this was due to loss of important virulence factor, type III secretion systems, in non-virulent strains, which allowed more efficient growth. Previously it was found that protozoan predation, virus, or temperature could also lead to attenuated virulence in *S. marcescens* (Friman et al. 2009, Friman et al. 2011a), being thus well in line with our findings. Since strains from fluctuating environments had lowered virulence, our result contradicts the idea that environmental harshness would select for increased virulence (Arnold et al. 2007), and that truly general environmental tolerance exists.

3.3 Correlations of virulence with bacteria colony morphotypes, growth ability, biofilm-forming ability, bacteriophage and protozoan resistance

In the experiment with fish pathogen *F. columnare*, rhizoid type bacteria were significantly more virulent than the rough type. This result is similar to previous findings in infection experiments with rainbow trout (*Oncorhynchus mykiss*, Walbaum) (Kunttu et al. 2009) and zebra fish (Laanto et al. 2012). The virulent rhizoid type bacteria also grew faster and had higher population size than rough type variants when cultured in the modified Shieh medium. Rhizoid type also had higher growth rate and maximum population size. The growth parameters seem to fit the general predictions of virulence and bacterial growth rate: fast growth correlates with high virulence (Chesbro et al. 1969, West & Buckling 2003). High population size is associated with high efficiency in transforming the nutrients to bacterial biomass, although in some pathogens also negative correlation can be found (Sturm et al. 2011).

The biofilm-forming ability and the protozoan resistance of the bacteria were related to strain identity but not to the colony type. The tested strains were generally resistant to amoeba predation, while the ciliates strongly reduced bacterial populations both in aqueous phase and in biofilms. We selected two strains, whose rough type variants were induced by bacteriophages, to test the palatability of different colony type to protozoan. Food preference test of the two strains showed that the rough type were able to support higher ciliate population sizes than their rhizoid type ancestors, which is confirmative to the finding in the protozoan resistance test. We did not find correlation between virulence and protozoan resistance in *F. columnare*. There are unidentified selection pressures involved in maintaining the rhizoid type in fish farms and in nature. The relaxation of these pressures in laboratory conditions might explain the colony type polymorphism seen in the laboratory conditions.

3.4 Bacterial enemies and the temporal and spatial dynamics of bacteria

In the paper IV we found that particle-feeding ciliates were most efficient in reducing bacterial biomass in the open water, but least efficient in reducing the biofilm biomass. Ciliates use oral groove to engulf suspended bacteria and select the prey mostly based on size (Gonzalez et al. 1990, Christaki et al. 1998). It has been shown that *Tetrahymena spp.* ciliates can also readily consume biofilms formed by *Pseudomonas spp.* or *Serratia spp.* (Weitere et al. 2005), and change the composition and abundance of bacteria in the biofilm (Parry et al. 2007, Dopheide et al. 2011). However, we did not find that the bacterial biofilm biomass dynamics in the ciliate-bacteria or bacteriophage-bacteria treatments differed from bacteria cultured alone during the eight-week experiment. One explanation could be that when the ciliates feed the open water biomass, it frees the common bacterial resources that can increase the growth of the biofilm biomass and thus compensate for the potential feeding of the biofilm biomass by ciliates.

Contrary to ciliates, amoebae are thought to be specialized surface grazers (Rodriguez-Zaragoza 1994, Molmeret et al. 2005). This effect was also visible on biofilm biomass in our experiment as the treatments with amoeba had the least biofilm in the long term. However, we found amoeba was also able to significantly reduce the bacteria biomass in the free water phase, presumably by prey of suspending bacteria through pinocytosis (Bowers 1977, Vogel et al. 1980, Rodriguez-Zaragoza 1994, Molmeret et al. 2005). This overlapping in niche with ciliates is also consistent with the observed ciliate population dynamics: The ciliate population size was on average higher in the single enemy treatment than in the treatment mixed with bacteriophage and amoeba, and within the multi-enemy treatment the ciliate densities transiently increased right after the decline of amoeba populations

In contrast to the predators, the parasitic bacteriophages generally had the least long-term effect on the bacteria in the open water and had no effect on the amount of the attached biofilm. These results were in contrast with the general opinion that bacteriophages are efficient parasites causing strong mortality effects on bacteria (Abedon 2008). However, high mortality risk also creates strong selection pressure on the host defense and bacteria are also frequently involved in tight co-evolutionary arms-race dynamics with bacteriophages where the fitness of a particular host and parasite genotype depends on time (Buckling & Rainey 2002, Friman et al. 2008).

Our separate short-term experiments with bacteria and bacteriophage clearly demonstrated that the ancestor bacteriophage was transiently capable of infecting and lysing more than 93 % of the bacterial population in 12 hours. However, the effect was only temporary as the defensive host mutants were capable of restoring the populations close to the previous size within 100 hours. The rapid evolution of defense against bacteriophages could rise from various

ways and limited cost due to long history of co-evolution (Labrie et al. 2010). For example *Escherichia coli* B can escape infection of bacteriophage T5 by losing or modifying a single receptor molecule that cost little (Lenski & Levin 1985). However there is evidence suggesting the cost of phage resistance depends on several factors, such as the genetic background of the phage, the number of the phage strain and the environmental conditions (Bohannan & Lenski 2000, Lennon et al. 2007, Lopez-Pascua & Buckling 2008, Koskella et al. 2012). For example in low nutrient environment such as soil and pond water, the costs for phage resistance are likely to be high (Gomez & Buckling 2011). Thus the fitness costs could to explain the persistent long-term negative effect on free-water phase biomass of the bacteriophages in our experiment.

To conclude, the top-down effects on bacterial prey can depend heavily on the type of the enemy. Ciliates were very efficient open water predators, whereas the amoeba was able to reduce bacterial biofilm and also the open water biomass. This could lead to partial and asymmetric overlap in resource use and possibly to the decline of amoeba and to the observed transients in predator dynamics. We also found that while the bacteriophages were initially super-efficient parasites in the short term, the long-term effects were negligible. This result arises from extremely rapid evolution of defense against the bacteriophages and weak ability of the bacteriophages to counteract this. Our results highlight that although data from the single-enemy experiments are valuable, they may not be able to reflect the processes occurring in multi-enemy food webs. Moreover, the rapid evolutionary dynamics between the enemy and the host can radically affect the view on the relative importance of predatory protozoan vs. parasitic phages as regulators of the bacterial abundance and spatial distribution.

3.5 Experimental evolution of bacterial virulence in a multi-enemy microbial communities

Evolutionary experiment of bacteria with three types of common bacterial enemies (amoeba, ciliate and bacteriophages, in all combinations) in a simulated pond water system revealed that compared to the ancestor strain, virulence was generally attenuated, also in the enemy-free treatments. This result is in contrast to the view that the protozoan predators and bacteriophages could potentially promote the evolution of bacterial virulence (Greub & Raoult 2004, Brüssow et al. 2004, Brüssow 2007).

By far the strongest decrease in virulence was found in clones that had evolved with phage-ciliate community. In the analysis where each predator's effect on virulence was tested across the whole dataset amoebae seemed to slow down the loss of virulence. However, this result was solely caused by the fact that the group with the strongest virulence attenuation (i.e. ciliate with phage) does not contain amoebae. When analysis was run without ciliate-phage-treatment the weak amoebae effect completely disappeared.

Moreover, two deviating populations solely caused ciliate-phage community induced attenuation in virulence. Since the changes in virulence occurred only in two populations the strongest loss of virulence in ciliate-phage-treatment could have been caused by the random mutation accumulation or drift, i.e. being caused by the decay of unused traits (Hall & Colegrave 2008, Mikonranta et al. 2012).

Several experimental evolution studies have found that virulence decreases during the experiment (Friman et al. 2009, Gomez & Buckling 2011). One reason for this finding could be the history of the bacterial strain and growth conditions during the experiment. The virulence of pathogenic fungi has been found highly correlated to C/N ratio of the culturing medium (Safavi et al. 2007, Ali et al. 2009, Wu et al. 2010). The strain *S. marcescens* DB11 was isolated from a dead fly over thirty years ago. It has been grown in LB medium and maintained its virulence from generation to generation (Flyg et al. 1980, Nehme et al. 2007). It could be that the protein-enriched environment (low in C/N ratio), like in LB medium (containing 10 g/l tryptone and 5 g/l yeast extract), is crucial for *S. marcescens* to maintain its virulence. Indeed, most of the natural environmental conditions are poor in protein except some rare locations like animal body and animal feces. Our experimental conditions with simulated pond water with low concentration of plant detritus (high C/N ratio) mimicked the natural conditions pathogenic bacteria might experience outside the host.

The treatments had no effect on the growth of the evolved bacteria. However if we compared the evolved bacteria to the ancestor, we found that the evolved bacteria had lower maximum growth rates, but not maximum population sizes. The general prediction is that high growth rate is positively correlated to high virulence (Chesbro et al. 1969, West & Buckling 2003). In low nutrient environment such as soil and pond water, the bacteria might evolve lowered growth rates (Gomez & Buckling 2011). Therefore, the lowered growth rate could partially explain the general decrease in virulence in our experiment (but see: Sturm et al. (2011) for opposite prediction).

Horizontal gene transfer plays a significant role in the evolution of pathogenic bacteria (Hacker & Kaper 2000, Hacker et al. 2003). It allows bacteria to evolve new genes and new phenotypic traits quickly (Groisman & Ochman 1996, Hacker & Carniel 2001, Schmidt & Hensel 2004, Medini et al. 2005). However, in our experimental settings, such possibility of HGT was minimized, and this might be one of the reasons that we did not find increase in virulence. Moreover, bacteria have been evolving on Earth for billions of years. Our 8-week evolutionary experiment might not be able to reflect the evolutionary consequences over a longer time scale.

To summarize we found that microbial communities containing ciliates and/or amoeba caused strong reductions of bacterial biomass and also caused evolutionary changes in traits that confer resistance against predators. However, contrary to the prevailing view suggesting that amoeba, ciliate and bacteriophage can contribute to the evolutionary increase of bacterial virulence (Greub & Raoult 2004, Matz & Kjelleberg 2005, Molmeret et al. 2005, Steinberg & Levin 2007, Cosson & Soldati 2008, Adiba et al. 2010) we found that

experimental conditions lowered the virulence regardless of the predators. The evolved bacteria suffered equally large reduction in virulence and growth compared to ancestor, which could be caused by the fitness costs, such as allocation constraints imposed by nutrient acquisition and costly adaptations imposed by resistance evolution.

4 CONCLUSIONS

In recent years great progress has been made on the genomics and molecular biology of pathogenic bacteria. More and more studies have been published on 'how' the pathogen causes disease, namely the molecular mechanisms of the pathogenicity. Still relatively little is known on 'why' the bacteria cause disease and what influence the degree of bacterial pathogenicity. Besides, most of the studies in this area view virulence as an invariant trait of a microorganism, i.e. a 'pathogen-centered' view.

In this thesis I view virulence as a subject that is embedded in a complex web of interactions with other biotic and abiotic agents. I hypothesize that in such web of interactions, any changes in the relevant biotic and abiotic agents could affect virulence.

The paper I of the thesis demonstrate that although the outcome of infection depends on the genetic background of both host and pathogen, other interacting factors can still significantly affect bacterial virulence.

In the paper II we found that evolved thermal generalism was correlated with increased tolerance of several novel environments. Moreover, generalism can also be costly in terms of reduced virulence. Together these results suggest that thermal fluctuations driven by the climate change could affect both species' invasiveness and virulence.

In the third paper we found that *F. columnare* strains and their colony morphotypes use different strategies for survival. The trade-off between virulence, growth, protozoan predator resistance, and phage resistance cannot explain the evolution and maintenance of the colony type in *F. columnare* strains. However, our study is a first step towards understanding of complex trophic interactions between *F. columnare* and its predators outside the fish host environment.

The treatment and prevention of columnaris disease in the fish farm is still heavily dependent on the extensive usage of antibiotics (Pulkkinen et al. 2010). It comes with the costs of drug resistance and environmental contamination. In our experiment, we showed that all test strains were generally vulnerable to ciliate predation regardless of their colony morphotypes and virulence. This finding could potentially be used as an alternative method to suppress the

density of *F. columnare* in the aquatic environment and stop the outbreaks caused by *F. columnare* in the fish industry.

In the evolutionary experiment with bacteria and their enemies (IV), we showed that the top-down effects on bacterial prey depend heavily on the type of the enemy. Ciliated protozoa were very efficient open water predators, whereas the amoeba was able to reduce bacterial biofilm and also the open water biomass. Presumably when the food supply on the surface was decreased due to consumption, amoeba increased the rate of intake of suspended bacteria through pinocytosis (Bowers 1977, Vogel et al. 1980). We also found that while the bacteriophages were super-efficient parasites in the short term, the long-term effects were negligible. This result arises from extremely rapid evolution of defense against the bacteriophages and weak ability of the bacteriophages to counteract this. Thus the enemy type determined the top-down effects on bacteria.

Results from another evolutionary experiment further strengthen the view that bacterial enemies with different feeding mode have different impact in the life of bacteria. For example we found amoeba resistance of the evolved bacteria had treatment specific changes but did not correlate with the changes in virulence, although in many studies, the resistance of amoeba predation was used as an indicator of bacterial virulence (Cosson et al. 2002, Greub et al. 2004, Smith et al. 2007, Froquet et al. 2009, Bonifait et al. 2011, Lelong et al. 2011, Hasselbring et al. 2011). Also contrary to the accumulating evidence suggesting that amoeba, ciliate and bacteriophage could contribute to the evolutionary increase of bacterial virulence (Greub & Raoult 2004, Brüssow et al. 2004, Molmeret et al. 2005, Steinberg & Levin 2007, Lainhart et al. 2009), the virulence of the bacteria, measured in *Drosophila* oral infection model, attenuated in all treatments regardless of the existence of these bacteria enemies. However the virulence seemed to be insensitive to the composition of bacterial enemy community. Our results thus testify against the common perception that high bacterial virulence could be selected coincidentally as a consequence of protozoan predation and bacteriophage infection.

Taken together, we found the interactive effects of host diet and genotypes of host and pathogen determined the severity of the current infection. Biotic and abiotic factors outside the host environment had significant effects on the evolution of bacterial traits. Under the thermal fluctuating environment, evolved thermal generalism was found paired with tolerance to several novel environments, but at the cost of reduced virulence. Although we found bacterial enemies with different feed mode played different role in the top-down regulation of bacteria population, no correlation was found between colony types and virulence to protozoan resistance in *F. columnare*. In the evolutionary experiments the evolved virulence was attenuated but insensitive to different composition of bacterial enemy community. Our results thus testify against the view that high bacterial virulence is selected coincidentally as a consequence of natural bacterial enemies. The selection pressures imposed by both low nutrition environment and natural enemies led to reduction in

bacterial virulence possibly due to allocation constraints imposed by nutrient acquisition and costly adaptations imposed by resistance evolution.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Bioottisten ja abioottisten tekijöiden vaikutus bakteerien virulenssiin

Bakteerien virulenssilla eli taudinaiheutuskyvyllä tarkoitetaan perinteisesti taudinaiheuttajien haitallisuutta isäntälajille. Tämä määritelmä on kuitenkin riittämätön, sillä tartunnan vakavuus riippuu myös isäntäeliön ominaisuuksista – sama tauti voi esimerkiksi olla harmiton aikuiselle mutta vakava lapselle tai vanhukselle. Isäntälajit ja taudinaiheuttajat eivät myöskään ole muuttumattomia vaan kuten kaikki elävät organismit, ne ovat jatkuvassa vuorovaikutuksessa elottoman ja elollisen ympäristönsä kanssa. Nämä vuorovaikutukset aiheuttavat bakteereille valintapaineita, jotka voivat johtaa evolutiivisiin muutoksiin virulenssissa. Jotta ymmärtäisimme virulenssia, meidän tulee siis ottaa huomioon myös bakteerin elinympäristö ja isäntäeliön geneettinen tausta ja kunto. Virulenssitutkimus on perinteisesti keskittynyt lähinnä ymmärtämään, miten bakteeri tartuttaa ja aiheuttaa vahinkoa isännässä, mutta ei ole juuri ottanut kantaa siihen, miten ja miksi bakteerista tulee taudinaiheuttaja. Väitöskirjatutkimuksessani pyrin ymmärtämään niitä olosuhteita, jotka vaikuttavat bakteerien taudinaiheuttamiskykyyn.

Perinteisessä lääketieteellisessä mikrobiologiassa luonnonvalinnan on ajateltu kohdistuvan lähinnä taudinaiheuttajan siirtymiseen isännästä toiseen (tarttumiskyky) ja sen lisääntymisnopeuteen. Tällainen näkemys sopii kuitenkin vain ns. oblikatorisiin taudinaiheuttajiin, jotka eivät pysty lisääntymään isäntänsä ulkopuolella. Fakultatiivisella taudinaiheuttajalla puolestaan on mahdollisuus elää sekä isäntäeliössä että itsenäisesti isännän ulkopuolella. Tämä taudinaiheuttajien ”ulkopuolinen elämä” on unohdettu lähes täysin, vaikka tämä elämänvaihe on usein bakteerilla vallitseva ja valtaosa taudinaiheuttajista kuuluu tähän ryhmään. Eläessään isäntäeliön ulkopuolella fakultatiiviset taudinaiheuttajat kohtaavat myös luonnossa esiintyviä valintapaineita, joista merkittävimpiä ovat kilpailu ravintoresursseista, pedot ja loiset. Kiinnostavan uuden teorian mukaan bakteerin virulenssi ei ole välttämättä seurausta isäntään sopeutumisesta vaan kyvystä sietää isännän ulkopuolisia valintapaineita. Esimerkiksi petopuolustus, puolustus bakteerien viruksia vastaan, lämpötila ja ravintoresurssien määrä voivat vaikuttaa virulenssiin epäsuorasti. Tutkin väitöskirjassani näiden valintatekijöiden ja niiden yhdysvaikutusten merkitystä bakteerien virulenssiin. Ensimmäisessä osatyössäni manipuloin myös ravinnon laadun ja isännän geneettisen taustan vaikutusta virulenssiin.

Ensimmäisessä tutkimuksessani tutkin geneettisen taustan ja ravinnon merkitystä infektioriskiin. Kokeessa käytin kahta *Serratia marcescens* -bakteerikantaa infektoimaan täpläsiilikkään toukkia (*Parasemia plantaginis*, Arctiidae). *S. marcescens* on fakultatiivinen taudinaiheuttaja, jota tavataan kaikilla eläinryhmillä, myös ihmisillä. Kokeessa käytetyt perhostoukat olivat peräisin paljon ja vähän melaniinia tuottavista valintalinjoista. Melaniini on tärkeä osa hyönteisten immuunipuolustusta, joten kokeessa oli kaksi

geneettiseltä taustaltaan erilaista ryhmää: immuunivasteeltaan heikko ja vahva ryhmä. Geneettisen taustan lisäksi manipuloin myös täpläsiilikkään ravintoa. Immuunivasteeltaan sekä heikot että vahvat ryhmät saivat ravinnokseen joko ratamoa tai salaattia. Ratamolla on arvioitu olevan enemmän antibakteerisia ominaisuuksia kuin salaatilla, ja siksi sen oletettiin olevan erityisen hyvää ravintoa infektion aikana.

Kokeessa havaitsin, että geneettisellä taustalla ja ravinnolla oli yhteisvaikutus siihen, kuinka hyvin isäntäeliö sieti erilaisia infektioita. Jos bakteerin virulenssi oli korkea, sekä isännän geneettinen tausta että ravinto olivat merkityksettömiä niiden selviytymiselle infektiosta. Mikäli bakteerin virulenssi oli matala, toukat, joilla oli korkea immuunivaste selvisivät paremmin kuin heikon immuunivasteen toukat ja antibakteerinen ravinto tehosti infektiosta selviämistä. Kiinnostava havainto oli, että jotkut perhosperheet selvisivät infektioista hyvin riippumatta siitä, olivatko ne peräisin korkean tai matalan immuunivasteen valintalinjoista. Tämä osoittaa, että kyky sietää infektioita on perinnöllinen, mutta ei riipu suoraviivaisesti vain isännän melanisaatiokyvystä. Tuloksissa näkyi myös selviä viitteitä siitä, että ravinnon vaikutus infektioon riippuu isännän geneettisestä taustasta: jotkut genotyypit hyötyvät ravinnon parantavista ominaisuuksista enemmän kuin toiset.

Tutkin lämpötilan vaihtelun, petojen ja viruksen vaikutusta virulenssin evoluutioon. *S. marcescens* –bakteeria altistettiin näille valintatekijöille tuhansien sukupolvien ajan, minkä jälkeen evoluution läpikäyneiden bakteerikloonien virulenssi mitattiin banaanikärpäsillä (*Drosophila melanogaster*). Banaanikärpänen on yleisesti virulenssiominaisuuden mittauksessa käytetty mallieliö. Havaitsin että vaihtelevissa ympäristöissä eläneet bakteerit kehittyivät sietämään paremmin paitsi eri lämpötiloja myös viruksia ja petoja kuin tasaisessa ympäristössä eläneet bakteerit. Parantunut vaihtelevien lämpötilojen sieto johti kuitenkin virulenssin heikentymiseen. Vastaavanlaisessa kokeessa, jossa valintatekijänä oli lämpötilan sijaan petojen tai viruksen läsnäolo, bakteerien virulenssi laski kaikissa käsittelyissä. Tulokset ovat hyvin merkittäviä, sillä aiemmin petojen aiheuttaman valinnan on päinvastoin esitetty johtavan virulenssin kasvuun. Myös tasaisen korkean lämpötilan tiedetään nostavan virulenssia. Havaittu virulenssin heikentyminen voi johtua siitä, että isännän ulkopuolella selviäminen vaatii evolutiivisia muutoksia, jotka ovat ristiriidassa isännässä kasvamiseen tarvittavien ominaisuuksien kanssa.

Viimeisessä osatyössä tutkin petojen merkitystä kolumnaaritautia aiheuttavan kalapatogeenin *Flavobacterium columnaren* virulenssiin. *F. columnare* on maailmanlaajuisesti kalanviljelylle erittäin merkittäviä tappioita aiheuttava taudinaiheuttaja. Tästä bakteerista on löydetty monia erilaisia kantoja: osa kannoista muodostaa pesäketyyppejä, joita kutsutaan ”Rhizoid”-tyypeiksi, osa tuottaa pesäkkeitä, joita kutsutaan nimellä ”Rough”. Vain Rhizoid-tyyppisten kantojen on todettu aiheuttavan kalojen kuolemaan johtavaa kolumnaaritautia. Tavoitteenani oli testata, liittyykö pesäketyyppi virulenssin lisäksi myös petopuolustukseen. Petopuolustuksella ei kuitenkaan havaittu yhteyttä *F. columnare* –bakteerin virulenssiin eikä sillä ollut yhteyttä pesäketyyppeihin.

Väitöskirjassani havaitsin virulenssin riippuvan sekä isännän että taudinaiheuttajan ominaisuuksista. Molempien evoluutio, geneettinen tausta ja ravinto muokkasivat taudinaiheuttajan haitallisuutta isännälle. Tutkimuksieni perusteella isännän ulkopuoliset olosuhteet eivät vaikuta ainoastaan sairauksien dynamiikkaan vaan voimakkaasti myös virulenssin evoluutioon.

Koska ympäristössä ilman isäntääkin kasvavia fakultatiivisia taudinaiheuttajia ei voida eliminoida hoitamalla isäntiä, voi tutkimustuloksiani käyttää mahdollisesti hyväksi biologisen kontrollin kehittämisessä. Muuttamalla ympäristön ekologisia olosuhteita fakultatiivisille taudinaiheuttajille epäsuotuisaksi on ehkä mahdollista ehkäistä uusien taudinaiheuttajien syntyä ja epidemioita. Esimerkiksi kalanviljelylaitoksissa käytetään suuria määriä antibiootteja opportunisti-infektioiden hoidossa, ja se aiheuttaa suurta kuormitusta ympäristöön ja lisää bakteerien antibioottiresistenssiä.

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ORIGINAL PAPERS

I

INTERACTIVE EFFECTS BETWEEN DIET AND GENOTYPES OF HOST AND PATHOGEN DEFINE THE SEVERITY OF INFECTION

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Interactive effects between diet and genotypes of host and pathogen define the severity of infection

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Abstract

Host resistance and parasite virulence are influenced by multiple interacting factors in complex natural communities. Yet, these interactive effects are seldom studied concurrently, resulting in poor understanding of host-pathogen-environment dynamics. Here, we investigated how the level of opportunist pathogen virulence, strength of host immunity and the host condition manipulated via diet affect the survival of wood tiger moth *Parasemia plantaginis* (Arctidae). Larvae from "low cuticular melanin" and "high cuticular melanin" (considered as low and high pathogen resistance, respectively) selection lines were infected with moderately and highly virulent bacteria strains of *Serratia marcescens*, while simultaneously manipulating host diet (with or without antibacterial compounds). We measured host survival and food preference before and after infection to test whether the larvae "self-medicate" by choosing an anti-infection diet (*Plantago major*, i.e., plantain leaf) over lettuce (*Lactuca sativa*). "High melanin" larvae were more resistant than "low melanin" larvae to the less virulent strain that had slower growth and colonization rate compared with the more virulent strain. Cuticular melanin did not enhance survival when the larvae were infected with the highly virulent strain. Anti-infection diet enhanced survival of the "high melanin" but not the "low melanin" hosts. Survival was dependent on family origin even within the melanin selection lines. Despite the intrinsic preference for lettuce, no evidence of self-medication was found. These results demonstrate that the relative benefit of host cuticular melanin depends on both diet and pathogen virulence: plantain diet only boosted the immunity of already resistant "high melanin" hosts, and cuticular melanin increased host survival only when infected with moderately virulent pathogen. Moreover, there was considerable variation in host survival between families within both melanin lines suggesting genetic basis for resistance. These results indicate that although melanin is an important predictor of insect immunity, its effect on disease outcomes greatly depends on other interacting factors.

Introduction

Both the resistance of hosts and the infectivity of pathogens vary considerably in nature (Beldomenico and Begon 2010). Therefore, the severity of infection will crucially depend on the interaction between host and pathogen genotypes (Scholthof 2007; Beldomenico and Begon 2010). In addition to genetic factors (Cotter and Wilson 2002), resistance of the victim depends on its condition, which in turn can depend on the environment. For example, high-quality food can improve the condition and immune defense of individuals (Ojala et al. 2005; Lee et al. 2008; Alaux et al. 2010; Ponton et al. 2011). Moreover, some insects can sequester plant chemicals and directly use them as defensive substances against invading pathogens (Nieminen et al. 2003; Hartmann et al. 2005;

Harvey et al. 2005; Baden and Dobler 2009; Singer et al. 2009). On the other hand, environmental factors can also affect pathogen virulence (Johnson 1991; Mekalanos 1992; Friman et al. 2011). Elevated temperature and environmental productivity, for example, can affect the expression of many important bacterial virulence factors (Meyer et al. 1996; Smirnova et al. 2001; Friman et al. 2011).

While some environmental factors are likely to affect only the host or the pathogen, others might have an additive or interactive effect on both sides. For example, diet can influence both host immunity and bacterial infectivity (Ojala et al. 2005; Frost et al. 2008; Lee et al. 2008; Zaborin et al. 2009): simply changing the phosphate concentration of the medium, the death rate of the host can range from 0% to 60% in the nematode-bacteria infection model (Zaborin et al. 2009). Given the complexity of natural communities, it is likely that a range of interactions is important to epidemiological outcomes (Mitchell et al. 2005; Allen and Little 2011; Vale et al. 2011). Most studies to date, however, have manipulated only one variable (host or pathogen genotype or environmental conditions) at a time; as a result, we understand poorly how environmental conditions interact with different host and pathogen genotypes during the infection.

Here, we studied these interactions experimentally with the herbivorous *Parasemia plantaginis* (Arctidae) tiger moth larvae and *Serratia marcescens* bacteria (Friman et al. 2009). The larvae have an orange patch on the dorsal side of an otherwise black body. The larvae are aposematic: the patch is used as a warning signal, and the size of the patch is heritable (Lindstedt et al. 2009). Bigger patches are more effective warning signal for avian predators (Lindstedt et al. 2008). On the other hand, investing in a large orange warning signal decreases the amount of cuticular melanin: the amount of melanin correlates positively with immune responses in many taxa including *P. plantaginis* (Armitage and Siva-Jothy 2005; Siva-Jothy et al. 2005; Friman et al. 2009; Laurentz et al. 2012). In this study, we used *P. plantaginis* larvae from two selection lines for low or high amount of cuticular melanin. Although detailed immunological mechanisms are not understood, the previous experiments have shown that "Low melanin" individuals (with larger orange patches) have weaker pathogen resistance than "high melanin" individuals (with small patches) (Friman et al. 2009).

Larvae from both selection lines were infected with one of two *S. marcescens* strains, either DB11 or ATCC 13880. *S. marcescens*is a natural pathogen of many insect species including larval Lepidoptera (Grimont and Grimont 1978; Sikorowski and Lawrence 1998; Inglis and Lawrence 2001). To simulate natural infection more realistically, all larvae were infected with the bacteria orally instead of injection (Vodovar et al. 2004, 2005). The strain DB11 is highly virulent to nematodes (Pujol et al. 2001) and *Drosophila* (Nehme et al. 2007) and was originally isolated from a dead fruit fly (Flyg et al. 1980). The strain ATCC 13880 was originally isolated from pond water. Thus, we expected it to be less virulent in *P. plantaginis* host compared with strain DB11 because it has no known close evolutionary history with insect pathogens. We also measured the growth and motility of these two bacterial strains to study potential mechanisms of virulence.

To potentially manipulate host condition via diet, the larvae were fed either plantain (*Plantago major*) or lettuce (*Lactuca sativa*) ad libitum. Plantain leaves is used to heal wounds and infections in traditional medicine in many countries (Hetland et al. 2000; Samuelsen 2000). In addition, it has antiinflammatory, antimicrobial, and antitumor effects (Gomez-Flores et al. 2000). Lettuce, on the other hand, has been shown to decrease larval immune response (Ojala et al. 2005).Thus, it is possible that these plant diets have opposing effects on larval immunity, which could result in difference in survival during infection. Alternatively, diets could have different effects on larval growth, development, and other life-history traits (Ojala et al. 2005; Laurentz et al. 2012), which could also affect larval survival during infection.

Parasites or pathogens can induce infected individuals to adopt a diet that helps them to fight the infection (Clark and Russell Mason 1988; Huffman et al. 1996; Christe et al. 2003; Milan et al. 2012). Self-medication has been recently demonstrated also among caterpillars (Krischik et al. 1988; Lee et al. 2006; Povey et al. 2009; Singer et al. 2009). Therefore, we also tested whether *P. plantaginis* larvae changed their feeding preference after the bacterial infection.

We hypothesized that "high melanin" individuals feeding on plantain would have the lowest infection mortality, if diet and host resistance additively boost host immunity. However, this effect could depend on the level of pathogen virulence, and similarly, diet could have different effects on larval survival depending on the level of host resistance (amount of melanin) and host genetic background. As a result, the relative importance of each experimental manipulation could depend on how it interacts with other factors.

Material and Methods

Host rearing, bacterial infection, and food preference tests

Selection lines for "high melanin" (small orange patch) and "low melanin" (large orange patch) in the *P. plantaginis* larvae were established in 2004; 51 families were used to set up the selection lines by applying a truncated family

selection protocol (Lindstedt et al. 2009). In this experiment, we used a total of 335 individuals originating from 22 different families (Table 1). We used 10 families from high melanin selection line (6–27 individuals per family, 147 larvae in total), and 12 families from low melanin selection line (13–19 individuals per families, 188 larvae in total). Larvae were isolated from a laboratory stock after they were reared on dandelion (*Taraxacum* sp.) in constant laboratory conditions (Friman et al. 2009).

The *S. marcescens* strain ATCC 13880 was obtained from American Type Culture Collection, while the *S. marcescens* strain DB11 was kindly provided by Prof. Hinrich Schulenburg. We prepared the bacterial inoculums by first growing both strains overnight on LB agar plates. After 24-h growth at 25°C, sterile loops (VWR) were used to streak and dilute bacterial cells to sterile water to optical density of 1.0 (Bioscreen C spectrophotometer (Oy Growth Curves Ab Ltd, Helsinki, Finland), OD 420–580 nm, wide band option) equalling approximately 5.4×10^8 (ATCC 13880) and 5.8×10^8 (DB11) bacterial cells/mL. Sterile water was used as a negative control.

When the larvae were 3 weeks old, we separated them individually to 9 cm Petri dishes and permanently changed their diet to either lettuce or plantain for the rest of the experiment. It is possible that mere switch from one food plant to another could be stressful, making it difficult to separate stress effects from effects of food per se. However, in this experiment, we wanted to study the short-term effects of different plant species on larval survival and thus wanted to exclude the long-term developmental effects of different plant diets. As a result, we reared larvae before infection on dandelion (to ensure similar handling for all treatments). To avoid a confounding effect of body mass on survival, we distributed the "high melanin" and "low melanin" larvae to the treatments evenly according to the body mass (Two-way ANOVA [analysis of variance], $P > 0.353$ for all pair-wise comparisons between all treatments). After 2 days on the new diet, we performed a pre-infection diet preference test. All the larvae were food-deprived for 24 h before offering fresh leaves (cut to 2×2cm squares) of both lettuce and plantain. The leaves were put on a moisturized filter paper to prevent drying. We measured the proportion of both leaves consumed after 24 h. The post-infection food preference test was performed twice, 24 and 72 h after the infection as described above (excluding food-deprivation).

Table 1. Summary of the wood tiger moth larvae used in the experiment

Selection line	Diet	Bacterial treatment	*n*
High melanin (*n* = 147)	Plantain (*n* = 71)	NC	21
		ATCC 13880	24
		DB11	26
	Lettuce (*n* = 76)	NC	24
		ATCC 13880	25
		DB11	27
Low melanin (*n* = 188)	Plantain (*n* = 88)	NC	26
		ATCC 13880	32
		DB11	30
	Lettuce (*n* = 100)	NC	31
		ATCC 13880	35
		DB11	34

NC, negative control.

In most bacterial infection studies, bacteria are injected with a needle into the body cavity of the host (septic injury model; Vodovar et al. 2004). In order to simulate natural infection more realistically, all larvae in the present experiment were infected with the bacteria orally (Vodovar et al. 2004, 2005). After the pre-infection food preference test, the larvae were presented with 20 μL of either bacterial inoculum (DB11 or ATCC 13880) or sterile water. The larvae were monitored until they had drunk the whole inoculum. After the infection and the post-infection food preference test, fresh lettuce or plantain leaves were added on to the Petri dishes ad libitum, according to the diet treatment. We recorded larval survival twice a day for the following 4 weeks. The larvae were reared at 25°C during the infection experiment.

Bacterial growth ability and motility measurements

Bacterial growth indicates how efficiently bacteria can turn resources into biomass, which probably correlates with the ability to reproduce within the hosts (host exploitation rate; Harrison et al. 2006). We assessed resource use ability of the *S. marcescens* strains as short-term (24 h) maximum growth rate and maximum density in vitro as follows: small bacterial inoculums (<0.0002% of the maximum population size) were added to fresh bacterial culture medium (hay extract) at a low initial density (Friman et al. 2008). Maximum growth rates and population sizes were determined from biomass growth data recorded for 96 h at 10 min intervals (Bioscreen C spectrophotometer, 420–580 nm optical density). Three different resource concentrations were used to estimate the growth parameters: low-, intermediate- and high-resource concentrations (containing 0.53, 1.07- and 2.15 mg L^{-1} final concentration of plant detritus, respectively). Ten replicate measurements were used for both strains in all resource concentrations.

High motility can increase bacterial virulence through enhanced host colonization ability (Johnson 1991; Josenhans and Suerbaum 2002; Lane et al. 2007). Bacterial motility assays were performed in vitro by stabbing trace amount (2 μL) of each bacterial strain with sterile loops

(VWR) on the center of semi-fluid NB agar plates containing 0.7% of agar (Friman et al. 2009). The motility of strains was determined as the area (mm^2) bacteria were able to colonize within 24 h ($N = 10$ for both bacterial strains). All bacterial trait measurements were conducted at 25°C.

Statistical analyses

We analyzed larval survival with the Cox regression survival analysis and Log-rank statistics. Initially, we used a full Cox regression model where larval survival was explained with following variables: diet, bacterial treatment (bacterial strain type and water), initial body mass, and melanin selection line of the larvae. This model showed a significant difference in survival between control and both bacterial strains (see Results). In the next analysis, we excluded the larvae from water control treatment to directly compare the effect of bacterial strains on larval survival. By adding and subtracting all possible interactions one by one, we found that only the initial body mass × melanin selection line, and initial body mass × diet interactions improved the model significantly ($\chi^2 = 4.577$, $P = 0.032$, Table S1). As a result, the final model included the main effects of diet, bacterial strains, initial body mass, melanin selection line of the larvae, and initial body mass × melanin selection line and initial body mass × diet interactions as explaining factors. To separate the effect of family from the effect of melanin selection line, we also analyzed the effect of above-mentioned factors separately within both selection lines.

We compared the relative consumption of lettuce versus plantain in two ways. First, we analyzed the consumption of different diets before and after the infection separately in each time point (0, 24, and 72 h from infection). In these models, larval food consumption was explained by consumption of the given diet (lettuce or plantain), melanin selection line, diet type, infection treatment, and their interactions. Larval weight was used as covariate and family was nested under the selection line and included in the model as a random factor. Second, we analyzed if the food preference changed through time (proportion of lettuce consumption – proportion of plantain consumption) between pre- and post-infection food preference tests using repeated-measures ANOVA. Two-way ANOVA was used to compare the growth and the motility of *S. marcescens* bacterial strains. All analyses were performed with SPSS v. 20 (IBM, International Business Machines Corp., Armonk, New York).

Results

We found that both *S. marcescens* strains decreased larval survival compared with water control group (DB11: $\beta = 2.138$, $P < 0.001$; ATCC 13880: $\beta = 1.668$, $P = 0.015$, Fig. 1A), Moreover, strain DB11 killed the larvae faster compared with strain ATCC 13880 ($\beta = 0.663$, $P = 0.006$). At the bacterial trait level, the higher virulence of strain DB11 was connected to more efficient growth (maximum population size: $F_{1,\ 53} = 564.3$, $P < 0.001$, maximum growth rate: $F_{1,\ 53} = 199.8$, $P < 0.001$; differences significant in all tested resource concentrations, $P < 0.001$ in all pair-wise comparisons, Fig. 1B) and higher motility ($F_{1,\ 9} = 451.8$, $P < 0.001$, Fig. 1C).

Larvae from the "high melanin" selection line survived better than larvae from the "low melanin" selection line ($\beta = -1.654$, $P = 0.010$), but this was only true when they were infected with the low virulence strain ATCC 13880 ($\beta = -0.634$, $P = 0.007$, Fig. 2A).The difference between the melanin selection lines (host genotypes) vanished when the more virulent strain DB11 was used for infection ($\beta = -0.200$, $P = 0.363$, Fig. 2B). Larger larvae had better survival in general ($\beta = -0.045$, $P < 0.0001$). The effect of larval weight was the same in all treatments.

The main effect of diet in the water control group was close to significant ($\beta = 0.103$, $P = 0.067$), suggesting that plantain diet could increase larval survival in general. However, feeding on common plantain enhanced larval survival only within the "high melanin" selection line ($\beta = -0.513$, $P = 0.013$, Fig. 3B); diet had no effect on larval survival within the "low melanin" selection line ($\beta = -0.126$, $P = 0.467$, Fig. 3A).

Interestingly, the family origin strongly affected larval survival (Wald = 48.633, $P < 0.0001$). However, no interaction between family and bacterial strain was found (Wald = 16.738, $P = 0.541$). This indicates that the effect of family on host survival was similar within both selection lines, and was not affected by the type of bacterial strain.

We did not find evidence of self-medication. Larvae consumed more lettuce compared with plantain before ($F_{1,\ 640} = 5.7$, $P = 0.017$) and after 24 h ($F_{1,\ 635} = 19.1$, $P < 0.001$) of bacterial infection, while no difference was observed after 72 h of infection ($F_{1,\ 633.8} = 0$, $P = 0.9$). However, food preference was similar regardless of bacterial infection treatment (no other significant main effects or interactions were found; all $P > 0.05$). Moreover, the larval food preference did not change (consumption of lettuce relative to plantain) before or after infection (RANOVA: the effect of time and all its interactions were non-significant, $P > 0.2$ in all cases; the effects of diet, bacterial strain, melanin signal line, and all their interactions non-significant, $P > 0.15$ in all cases, Fig. 4).

Discussion

Our goal was to study comprehensively how the level of host resistance, pathogen virulence, and diet interact to

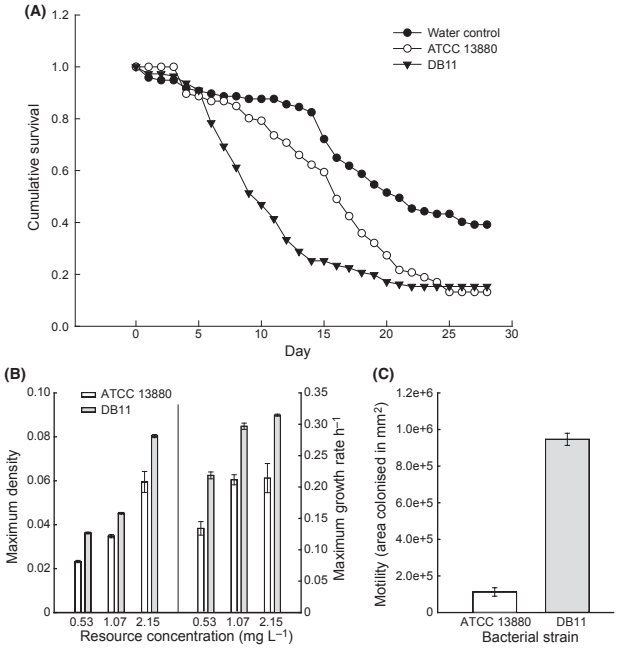

Figure 1. (A) Survival curve for larvae infected with water (filled circles), strain ATCC 13880 (open circles) and strain DB11 (filled triangles). (B) maximum population sizes and growth rates of strain ATCC 13880 (white bars) and DB11 (gray bars) measured in low-, intermediate- and high-resource concentrations. (C) The motility of ATCC 13880 (white bars) and DB11 (gray bars). Error bars in (B) and (C) denote ±1 SEM.

determine the outcome of a bacterial infection. As expected, larvae with high level of cuticular melanin level survived best from infection. Even though larvae preferred lettuce to plantain for a short period after diet switch (~24 h), no evidence for self-medication was found. However, plantain diet boosted the survival of larvae from the high, but not from the low, melanin selection lines. Interestingly, the host genotype (selection line) and diet only had an effect on larval survival when the less virulent strain ATCC 13880 was used for the infection. Together, these results suggest that both the quality of the host's environment and host genotype can decrease the success of a moderately virulent, but not a highly virulent, bacterial pathogen. As a result, self-medication behavior and genetically mediated host resistance are more likely to evolve in the presence of less virulent pathogens.

Serratia marcescens strain DB11 caused higher host mortality compared with strain ATCC 13880 (Fig. 1A). This result is in accordance with our expectations due to the strains' different origin; bacteria isolated from aquatic environment are likely to be less virulent because they share no close evolutionary history with insect hosts. At the mechanistic level, higher DB11 virulence was connected to more efficient growth and motility measured in vitro (Figs. 1B, C). Bacterial growth rate is an indicator of efficient host exploitation rate (Johnson 1991; Meyer et al. 1996; Harrison et al. 2006; Friman et al. 2009). DB11 had both higher maximum growth rate and maximum density in all resource concentrations we used in the in vitro measurements. Together, these results suggest that high bacterial competitive ability could be important for bacterial fitness in both external natural reservoirs and within the host (Walther and Ewald 2004). Growth ability also correlated positively with motility, which can increase virulence by improving host colonization efficiency (Johnson 1991; Pujol et al. 2001; Josenhans and Suerbaum 2002; Lane et al. 2007; Malik-Kale et al. 2007). For example, according to previous studies, motility is needed for successful infection in both *Drosophila* (Nehme et al. 2007)and *Nematode* (Pujol et al. 2001) hosts. Moreover, the non-motile and closely related DB140 mutant strain is non-virulent in nematodes (Pujol et al. 2001), while less motile ATCC

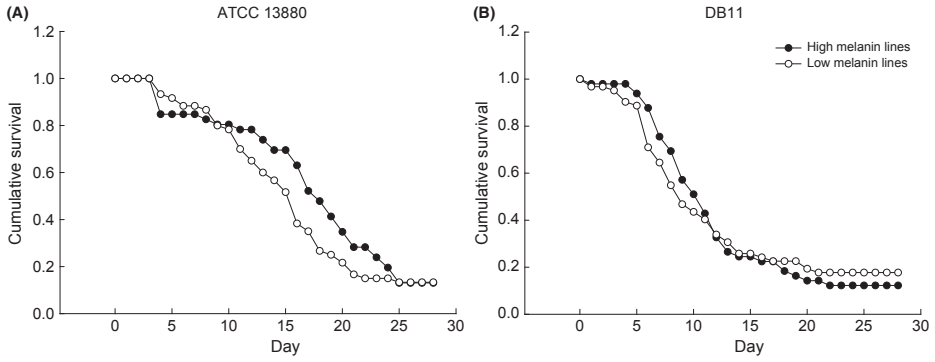

Figure 2. Survival curve of "high melanin" larvae (filled circles) and "low melanin" larvae (open circles) when infected with *Serratia marcescens* strain ATCC 13880 (A) or DB11 (B).

13880 genotypes are less virulent in *P. plantaginis* (Friman et al. 2009, 2011). Unfortunately, our experimental setting is not adequate to estimate the relative importance of these two potential virulence mechanisms. However, in more general perspective, these results suggest that bacterial virulence can correlate positively across different host organisms regardless of its evolutionary origin, i.e., bacterium isolated from Diptera host is also virulent in Lepidoptera host (Jander et al. 2000).

Parasemia plantaginis larvae from the "high melanin" selection line were more resistant than the "low melanin" individuals against the less virulent ATCC 13880 bacterial strain (Fig. 2). Melanization and phagocytosis play a major role in eliminating bacterial infection in insects (Hillyer et al. 2003; Nappi and Christensen 2005) by killing most of the invading bacteria (Haine et al. 2008; Schneider and Chambers 2008). High cuticular melanin content often correlates positively with insects' ability to resist parasites and pathogens (Nappi et al. 1995; Wilson et al. 2001; Cotter et al. 2004) because it is connected to high phenoloxidase (PO) activity (Rowley and Brooy 1986): a major humoral immune defense cascade in insects, which is expressed and regulated in response to the presence of non-self in the haemocoel (Soderhall 1982). Interestingly, there was no benefit of "high melanin" against the more virulent strain suggesting that PO activity-based defense has its limits. For example, it is possible that when the pathogens reproduce fast (which is the case with strain DB11), hosts might not be fast enough in mounting immune responses that require an activation period. Diet quality can also greatly affect the physiological condition and immunocompetence of hosts (Fellous and Lazzaro 2010; Laurentz et al. 2012). We found that plantain diet increased larval survival only within more resistant, "high melanin" selection line (Fig. 3). This is surprising because plantain extracts have antibacterial effects against both Gram-positive and Gram-negative bacteria (Gomez-Flores et al. 2000). Moreover, Plantain diet helps mice to fight systemic infection of *Streptococcus pneumoniae* by stimulating the innate immune system (Hetland et al. 2000), while Plantain extracts can also activate macrophages and affect the lymphocyte proliferation (Gomez-Flores et al. 2000). In addition to direct immunological effects, Plantain leaves can contain as much as 15% of protein (Mohamed et al. 2011), which is approximately 10 times the concentration in *Lactuca sativa* leaves (USDA Nutrient Database; http://www.nal.usda.gov/fnic/foodcomp/search/). High protein diet can induce cuticular melanin production, which is correlated with anti-infection activity (Lee et al. 2008; Cotter and Kilner 2010). As only "high melanin" larvae benefited from plantain diet, it seems that instead of increasing survival by improving larval condition in general, the plantain diet interacts with the host immune system. If this was due to plantain diet's positive effect on host resistance or tolerance, or direct negative effect on the parasite, remains still unclear. It is notable that larval survival in the water treatment group was quite poor compared with previous studies (Friman et al. 2009, 2011). One explanation could be the abrupt diet switch from dandelion to plantain or lettuce after the first 3 weeks of larval development. As individuals in all treatments switched diet from dandelion to lettuce or plantain, the results were unlikely biased by the diet-switching. Most importantly, larval survival was clearly higher in bacterial treatments compared with the water

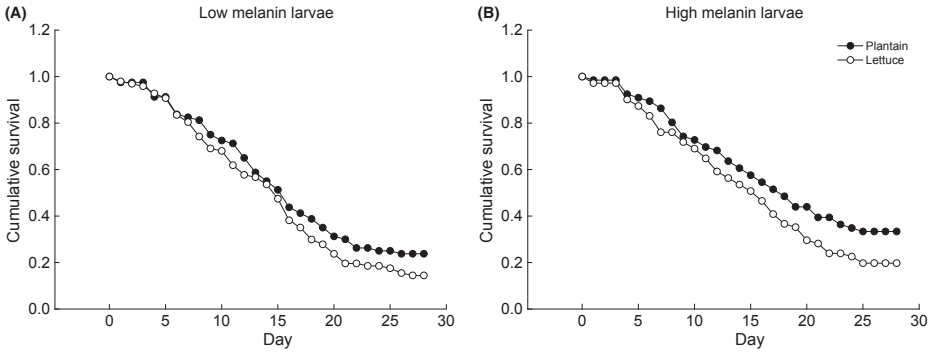

Figure 3. Survival curve of larvae from low melanin (A) and high melanin (B) selection lines when fed with plantain (filled circles) or lettuce (open circles).

control, which shows that bacterial infection increased larval mortality.

Recent findings suggest that insect hosts can change their diet toward medicating plants after infection (Singer et al. 2009).We found intrinsic preference for lettuce, which, however, vanished soon after the diet switch (no difference after 72 h of infection; Fig. 4). Furthermore, all larvae favored lettuce over plantain regardless of whether they were infected with bacteria or not. As a result, this short-term diet preference was unlikely to be connected to self-medication. One potential explanation could be starvation-induced dehydration, which might lead preference for lettuce that has relatively high water concentration. Self-medication can also be trans-generational, and directed toward offspring instead of the infected parent (Lefevre et al. 2010, 2012). Thus, it is possible that the 3-day interval after the infection was too short to observe potential food preference in *P. plantaginis*. Alternatively, complementary diet where larvae consume both medicating and normal growth-enhancing plants could result in highest survival (Ojala et al. 2005). For example, for polyphagous insects, it is often energetically costly to sequester plant chemicals (Berenbaum and Zangerl 1993; Despres et al. 2007; Lindstedt et al. 2010). Thus, while medicating plants could provide direct benefits against pathogens, excessive ingestion of defensive chemicals could reduce larval survival (Singer et al. 2009).

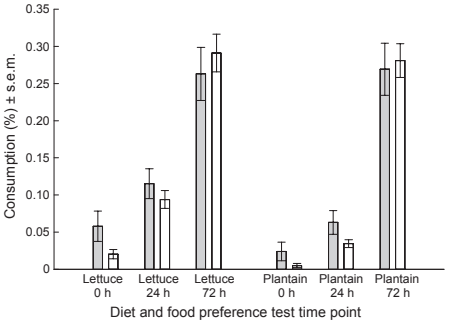

Figure 4. Food preferences of the larvae before and after bacterial infection. The plantain consumption (gray bars) and lettuce consumption (white bars) are shown separately for each bacterial strain. Error bars denote ±1 SEM.

Interestingly, family was one of the most significant determinants for larval survival during the infection, which may be of evolutionary importance. First, it shows that despite artificial selection for more cuticular melanin, within-line genetic variation was still considerably high. Second, it shows that some families resist infection better as a whole, or that some host genotypes cope better with certain bacterial strains. Because we did not find any evidence of family by strain interactions, our results support the first hypothesis of "generally superior genotypes". This finding, however, leads to a new puzzle: why are weak host-genotypes not wiped out by natural selection? One likely explanation could be a trade-off between host resistance and other fitness traits (Kraaijeveld and Godfray 1997) that favored weakly resistant genotypes in the absence of pathogens.

In conclusion, our study shows that both host resistance and pathogen virulence, and the diet, are all important in determining the outcome of bacterial infection. More specifically, our results demonstrate that the high amount of cuticular melanin increases the survival

of *P. plantaginis* moth larvae, and that the medicating plantain diet enhances only the survival of already more resistant, melanic larvae. These results suggest that although melanin is an important predictor of insect immunity, its effect on disease outcome will greatly depend on the three-way interactions between diet and genotypes of both host and pathogen.

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Conflict of interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Cox regression model fitting.

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II

FLUCTUATING TEMPERATURE LEADS TO EVOLUTION OF THERMAL GENERALISM AND PRE-ADAPTATION TO NOVEL ENVIRONMENTS

by

Tarmo Ketola, Lauri Mikonranta, Ji Zhang, Kati Saarinen, Ville-Petri Friman, Anni-Maria Örmälä, Johanna Mappes & Jouni Laakso 2013

Evolution Accepted.

III

ASSOCIATION OF COLONY MORPHOTYPE ON VIRULENCE, GROWTH AND RESISTANCE AGAINST PROTOZOAN PREDATION IN THE FISH PATHOGEN *FLAVOBACTERIUM COLUMNARE*

by

Ji Zhang, Jouni Laakso, Johanna Mappes, Elina Laanto, Tarmo Ketola, Jaana K.H Bamford, Heidi Kunttu & Lotta-Riina Sundberg 2013

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IV

RELATIVE IMPACT OF BACTERIOPHAGE AND PROTOZOAN PREDATORS ON THE TOP-DOWN REGULATION OF BACTERIAL POPULATION

by

Ji Zhang, Anni-Maria Örmälä, Johanna Mappes & Jouni Laakso 2013

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V

COINCIDENTAL LOSS OF BACTERIAL VIRULENCE IN MULTI-ENEMY MICROBIAL COMMUNITIES

by

Ji Zhang, Anni-Maria Örmälä, Tarmo Ketola, Johanna Mappes & Jouni Laakso

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