JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE
298

Lauri Mikonranta

# Virulence evolution and immune defence

## Pathogen-host interactions between an environmentally transmitted bacterium Serratia marcescens and its insect hosts

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Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella
julkisesti tarkastettavaksi yliopiston Ylistö-rakennuksen salissa YAA303
helmikuun 6. päivänä 2015 kello 12.

Academic dissertation to be publicly discussed, by permission of
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UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2015

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Cover: *Serratia marcescens* bacteria. Picture by Lauri Mikonranta

URN:ISBN:978-951-39-6072-8
ISBN 978-951-39-6072-8 (PDF)

ISBN 978-951-39-6071-1 (nid.)
ISSN 1456-9701



Jyväskylä University Printing House, Jyväskylä 2015

## ABSTRACT

Mikonranta, Lauri
Virulence evolution and immune defence: Pathogen-host interactions between an environmentally transmitted bacterium *Serratia marcescens* and its insect hosts
Jyväskylä: University of Jyväskylä, 2014, 42 p.
(Jyväskylä Studies in Biological and Environmental Science
ISSN 1456-9701;298)
ISBN 978-951-39-6071-1 (nid.)
ISBN 978-951-39-6072-8 (PDF)

Yhteenveto: Virulenssin evoluutio ja immuunipuolustus – Isäntä-loinen -vuorovaikutuksia opportunistisen *Serratia marcescens* -bakteerin ja sen hyönteisisäntien välillä
Diss.

Pathogens are one of the most significant factors limiting population sizes in the wild and major drivers of the evolution of their hosts. Similarly, the antagonistic interactions keep obligatory pathogens co-evolving with their host defences. In opportunistic pathogens that are able to proliferate outside their hosts, the life cycle is however non-dependent from direct host-to-host transmission and more exposed to the outside-hosts environment. In this thesis, factors that affect the successfulness of opportunistic bacterial infection in insects are examined from both pathogens' and hosts' perspectives. Evolutionary experiments reveal that virulence of the entomopathogenic bacterium *Serratia marcescens* is shaped by the interplay of selection pressures in a non-host environment and within hosts. Results show that anti-predatory adaptation outside the host can directly trade off with bacterial virulence traits. Even in a setting where the pathogen is let to evolve within the host, minimizing the between-hosts selection, increased virulence is not necessarily the best strategy for pathogen fitness. Results on host defence show that the insect innate immunity has an acquired aspect where infection with a previously encountered pathogen can be diminished without the antibody-based immune memory akin to vertebrates. Further, Lepidopteran hosts are able to adjust their immunity related gene expression accordingly to the infecting pathogen strain. Together these findings can have implications on epidemiological consequences of host-parasite co-evolution, management of environmentally transmitted opportunistic pathogens, and biological control of insect pests.

Keywords: Experimental evolution; host; immune priming; insect immunity; pathogen; *Serratia marcescens*; virulence.

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## LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-IV.

I Mikonranta L., Friman V.-P. & Laakso J. 2012. Life History Trade-Offs and Relaxed Selection Can Decrease Bacterial Virulence in Environmental Reservoirs. *PLoS ONE* 7: e43801.

II Mikonranta L., Mappes J., Laakso J. & Ketola T. 2014. Within-host evolution decreases virulence in an opportunistic bacterial pathogen. Submitted manuscript.

III Mikonranta L., Mappes J., Kaukoniitty M., & Freitak D. 2014. Insect immunity: oral exposure to a bacterial pathogen elicits free radical response and protects from a recurring infection. *Frontiers in Zoology* 11: 23.

IV Mikonranta L., Dickel F., Mappes J. & Freitak D. 2014 Attack and defence: Diverse life-history strategies between bacterial pathogens and Lepidopteran hosts. Manuscript.

## CONTRIBUTIONS:

| | I | II | III | IV |
|---|---|---|---|---|
| Original idea | LM, VF, JL | LM, JM, JL, TK | LM, JM, MK, DF | LM, FD, JM, DF |
| Experimental work | LM, VF | LM, TK | LM, JM, MK, DF | LM, FD, JM, DF |
| Statistics | LM, VF | LM, TK | LM, DF | LM |
| Manuscript | LM, VF, JL | LM, JM, JL, TK | LM, JM, DF | LM, FD, JM, DF |

LM: Lauri Mikonranta, VF: Ville-Petri Friman, JL: Jouni Laakso, JM: Johanna Mappes, TK: Tarmo Ketola, MK: Minna Kaukoniitty, DF: Dalial Freitak, FD: Franziska Dickel

# 1 INTRODUCTION

## 1.1 What is virulence?

All known multicellular animals have a unique microbial flora that consists of a continuum of beneficial symbionts and harmful parasites. Parasitism has approved to be one of the most successful life-history strategies, considering that at least half of the living species have been estimated to be pathogens (May 1998). Unicellular bacteria have their own parasites and even viruses that are often considered as non-living entities can be infected by other viruses (La Scola *et al.* 2008). Over 1400 different parasites that infect humans have so far been identified (Woolhouse and Gowtage-sequeria 2005). Pathogen-host interactions are a major evolutionary force creating and maintaining diversity on earth when both counterparts of this tug-of-war adapt to maximize their own fitness (Anderson and May 1982).

Pathogens acquire resources from their hosts in order to reproduce and in the process, cause harm to them. The term virulence in an evolutionary context describes the varying degree of these negative fitness effects, which can usually be equated with a severity of the disease (Bull 1994). From a clinical perspective, however, medically severe condition does not necessarily translate into a fitness cost. Thus, the definitions of virulence might not always agree across disciplines and there is considerable amount of discrepancy among virulence literature about the term. Also, quantification of host fitness is not necessarily easy. The most often used and probably the most unambiguous measure of virulence is the host mortality rate (May and Anderson 1983). Obviously, also sub-lethal infections can have fitness consequences and thus different proxies, such as host body mass, pathogen replication rate, pathogen load, and tissue damage have been used to measure fitness reduction in empirical studies (Read 1994).

The traditional formulation of virulence is, however, very pathogen-centric. While pathogens certainly exhibit different degrees of characteristics that contribute to high virulence, it is often neglected that the outcome, fitness reduction, always depends on both, the hosts and the pathogens (Read 1994).

For example, the protective immune reaction can cause costs or direct self-harming effects to the hosts (Sheldon and Verhulst 1996, Lipsitch and Moxon 1997, Sadd and Siva-Jothy 2006). These kind of host-dependent factors demonstrate that virulence is not only a pathogen trait but also a consequence of host-pathogen interaction (Antia *et al.* 1994, Casadevall and Pirofski 2003).

## 1.2 Evolution of virulence

Selection acting on the hosts clearly tends to minimize virulence because, by definition, high virulence equals low host fitness. Contrastingly, it is not self-evident when the traits leading to high virulence are selected for in the pathogens. Pathogens use host resources to reproduce and to transmit forward, creating a possible trade-off between prudent host exploitation that maximizes transmission, and rapid reproduction that can decrease the odds of host-to-host contact (Read 1994, Frank 1996). A classic example of this kind of epidemiological trade-off comes from the attempt to control rabbit populations in Australia with an introduction of rabbit myxoma virus. High virulence first led to reduction of host density and consequently lowered transmission. This in turn selected for virulence attenuation to an optimal, intermediate level (Fenner *et al.* 1956, Fenner and Ratcliffe 1965, Mead-Briggs and Vaughan 1975). Intermediate virulence has been suggested to maximize transmission in sexually transmitted diseases such as HIV (Fraser *et al.* 2007), and vector-borne infections seem to be less dependent on the normal host function allowing high virulence (Ewald 2004, Brown *et al.* 2006). The existence of the virulence-transmission trade-off is further backed up by the observation that environmentally persistent, i.e. less host dependent human respiratory pathogens have a tendency to be more virulent than the less persistent ones (Walther and Ewald 2004). However, the opposite has been shown to be the case with bacteriophages (Taddei & De Paepe 2006, Heinemann & Brown 2012). High virulence can also be costly for the pathogen through narrowed host range or tight local adaptation (Laine and Barrès 2014). The traditional trade-off model has been challenged in its generality and applicability because virulence might not necessarily be costly for the pathogen and in some instances transmission and virulence could evolve independently from each other (Lipsitch and Moxon 1997, Ebert and Bull 2003). In some cases high virulence could be positively correlated with, or even essential for transmission (Wickham *et al.* 2007). For example, some transmission strategies might require host death (Kunttu *et al.* 2009).

Most likely a universal model for evolution of virulence, applicable in every possible pathogen-host pair, does not exist (Alizon *et al.* 2009). However it seems that the selective forces can be roughly categorized to be acting at the between-hosts or within-host levels (Alizon *et al.* 2011). In the context of the trade-off model, the division leads to a dichotomy where these different levels of selection can have opposite fitness peaks. Within the hosts, mutations

conferring fast growth rate are selected for if they provide benefits in competition against the more benign genotypes. On the other hand, such short-sighted evolution of high virulence might be detrimental at the between-hosts level (Levin and Bull 1994). In addition to direct resource competition, evolutionary dynamics within the host can be dependent on other social interactions among the infecting pathogen population. For example co-operation, cheating, kin-selection, and spite can have both negative and positive effects on virulence evolution (Griffin *et al.* 2004, Harrison *et al.* 2006, Buckling and Brockhurst 2008, Brown *et al.* 2009, Racey *et al.* 2010). Thus, within and between-hosts levels of selection inextricably contribute to virulence and the effects reflect from one level to another (Luciani and Alizon 2009). However, evolutionary change does not require natural selection. Stochastic processes such as genetic drift and reduced purifying selection may be more important drivers of pathogen evolution than generally assumed (Hershberg *et al.* 2008, Alizon *et al.* 2011). Nevertheless, in obligatory pathogens evolution of virulence is highly intertwined with what kind of genotype gets transmitted to the next host (Lipsitch and Moxon 1997, Holt and Barfield 2006, Alizon *et al.* 2009).

## 1.3 Opportunistic pathogens

### 1.3.1 Opportunism and epidemiological implications

In medical literature the term opportunistic pathogen is usually associated with an infective agent that causes a disease in somehow immunocompromized individuals but not in normal healthy hosts. These kinds of opportunistic infections include for example *Pseudomonas aeruginosa* in cystic fibrosis patients and *Streptococcus pneumoniae* in the elderly people (Brown *et al.* 2012). However, there are number of opportunists in which the capacity of causing a disease is not solely dependent on hosts' immunological status. For example *Escherichia coli* is a commensal part of human gut flora but the variant, prevalent in cattle, that bears a shiga-toxin encoding prophage causes severe human disease (Steinberg and Levin 2007). Brown *et al.* (2012) have proposed a broader definition of an opportunistic pathogen to cover all non-obligate and/or non-specialist infective agents. In this thesis, opportunistic pathogens are defined by their non-obligatory nature, i.e. their ability to reproduce outside their hosts.

Environmentally proliferating microbes do not conform to the traditional epidemiological framework with obligatory pathogens (Casadevall and Pirofski 2007, Brown *et al.* 2012). Their ability to engage in a free-living lifestyle makes them independent from the transmission-virulence feedback loop. Thus one could postulate that opportunism is an extreme form of generalist life-history strategy where the hosts are just another ecological niche to be occupied. Applying the virulence-transmission trade-off model implicitly to these kinds of infections would lead to infinitely increasing virulence because the opportunists do not pay similar costs of reduced transmission than obligatory

pathogens. Clinical isolates of some opportunistic pathogens have indeed been reported to be more virulent than isolates from free-living environment (Newell *et al.* 1985, Fenner *et al.* 2006). Also, intensive farming conditions seem to favour high virulence in aquaculture (Pulkkinen *et al.* 2010). It is still hard to judge if more virulent genotypes colonise the host more efficiently from the environment or if higher virulence is selected within the host. Although scarce, evidence of the contrary also exists (II). *P.aeruginosa* clones with costly virulence factors lose the intraspecific competition to the more benign clones in chronic infection, suggesting that low virulence could be favoured within the hosts (Smith *et al.* 2006).

Even though environmental opportunists such as *Pseudomonas* spp., *Klebsiella* spp., and *Serratia* spp. do not necessarily require their human hosts for reproduction or survival, they regularly occur in severe outbreaks, and can even cause sporadic epidemics involving several healthcare facilities at the same time (Podschun and Ullman 1998, Harris *et al.* 1999, Mahlen 2011). Thus, it seems unlikely that the within-host environment would be evolutionarily insignificant for their life cycle and virulence merely a coincidental side-product of accidental infection in all cases. However, the ratio between outside-host and within-host replication is likely to differ massively between strains, which makes estimating the significance of within-host selection in general very hard.

### 1.3.2 Coincidental evolution of virulence in opportunists

Some pathogen virulence factors might have originally evolved in response to selection pressures acting outside the parasitic context (Levin 1996). Levin and Svanborg-Eden (1990) suggested that *Escherichia coli* attachment molecules, adhesins, are an adaptation to the intestinal tract where they are associated with the normal commensal life-style of the bacterium that is not harmful to the host. In the urinary tract the trait causes a painful infection and leads to immune response mediated clearance of the bacterium. This suggests that the coincidentally evolved virulence trait is an evolutionary dead-end. However, coincidental does not necessarily mean maladaptive. Another example from *E.coli* shows that a virulence factor, shiga toxin-encoding prophage, increases bacterial survival in the presence of grazing ciliated protozoa. Thus, a trait behind high virulence may have evolved in the free-living environment as an anti-predatory adaptation (Steinberg and Levin 1997). In this case, it is not clear if the toxin production is a fitness benefit in a human host, but it has been shown that grazing amoeba can select for increased bacterial survival against human macrophages because of similar feeding mechanisms (Cirillo *et al.* 1999, Matz and Kjelleberg 2005, Casadevall 2008). It is possible that coincidental selection of virulence factors works also the other way around: If protozoan selection leads to resistance against macrophages, survival in hosts could select for increased predation resistance as well. Occasional passage through hosts might thus, by chance, select for traits that confer advantage in the soil or other kinds of outside-host environments. (Casadevall and Pirofski 2007).

General theory on life-history evolution suggests that adaptation to a particular environment is likely maladaptive in another environment, i.e. there are trade-offs between different survival strategies (Stearns 1989). In this light there are bad odds of free-living environment coincidentally selecting for traits that are beneficial within the host (I). *Vice versa*, within-host adaptation is less likely to lead to better free-living survival than a simple fitness trade-off is to occur between the two very different environments. The environmental trade-off hypothesis is supported by for example the studies showing that in pathogenic *E. coli* and *Francisella tularensis* virulence decreases when they are released from their hosts to environmental reservoirs (Duriez *et al.* 2008, Thelaus *et al.* 2008). However, metagenomic sampling of environmental microbes has revealed that functional virulence genes are surprisingly prevalent in free-living bacteria, suggesting that they could have alternative roles benefitting bacterial survival in the soil (Søborg *et al.* 2014).

## 1.4 Experimental evolution

Thousands of years of artificial selection by humans have led to the diversification of East Asian wolf into the countless races of domestic dogs we see today (Savolainen *et al.* 2002). While this kind of 'unnatural selection' where the breeder chooses the desired traits certainly leads to evolution, it also differs from experimental evolution where the researcher exposes populations to specified conditions and lets the 'struggle for existence' do the work by selecting the fit individuals (Buckling *et al.* 2009). Experimental evolution studies are not restricted to unicellular organisms, but the short generation times of microorganisms make them ideal for observing the evolution in real time. Moreover, many microbes can be cryopreserved allowing the researcher to go back in time to make direct comparisons between the ancestral form and the evolved selection lines (Lenski *et al.* 1991, Lenski and Travisano 1994). Richard Lenski and colleagues are, without a doubt, the modern pioneers in the field. Their work with *E. coli* has inspired a rapid increase in studies using experimental microbial populations to answer a whole spectrum of questions concerning evolutionary processes (Buckling *et al.* 2009). Their *E. coli* populations started to adapt to a novel laboratory environment in 1988. Currently at 60.000$^{th}$ generation they are still adapting although with a diminishing return (Lenski *et al.* 1991, de Visser *et al.* 1999, Buckling *et al.* 2009, Lenski 2014). One major benefit of using experimental microbial systems in the study of evolution is the ability to divide a clonal population into replicates and to apply identical conditions to these genetically identical populations. This way it is possible to study how consistently a certain selection pressures cause convergent or divergent changes, or even what kind of different evolutionary solutions can be found to achieve similar fitness peaks (Lenski *et al.* 1991, Travisano *et al.* 1995).

There are caveats in evolution in a test tube. It has to be kept in mind that when first taken from the wild into a laboratory, organisms are entering a completely novel and unnatural environment. Especially, artificial environments lack much of the complexity found in natural systems. However, the researcher can make a virtue of the simplicity by directly testing the effects of a specific selection pressure rather than trying to guess its relative importance in the wild. One must, of course, be cautious in making generalizations across species limits and admit that not all evolutionary questions can be answered by studying microorganisms (Buckling *et al.* 2009).

Experimental studies on evolution of parasites and hosts have mostly been conducted using bacteria and bacteriophages (e.g. Buckling and Rainey 2002, Brockhurst *et al.* 2005). These systems are especially intriguing, because also the hosts' generation times are short enough for coevolution to be observed in real time. Although bacteria have a primitive immune system against viruses (Horvath and Barrangou 2010), pathogen specific adaptations to more complex immune defence of multicellular organisms require a different approach. Within-host adaptations in infections that usually last multiple pathogen generations have been addressed experimentally with so-called serial passages in higher organisms. In a serial passage pathogens are sequentially transferred from host to host. The within-host evolved lines are then isolated and compared to the ancestral form. Essentially, this approach extracts the within-host level of selection from the pathogen evolution and relaxes the pressures for between-hosts adaptation. Accordingly to the trade-off theory, serial passages usually lead to rapid reproduction in the host and consequently, increased pathogen virulence (Ebert 1998).

## 1.5 Immune defence – minimizing virulence

### 1.5.1 Insect immunity

Given the vast variety and abundance of parasites, all living beings are under a constant threat of infection. Thus, even the simplest life forms have developed ways to recognize and eliminate infectious agents (Horvath and Barrangou 2010). These defences have to keep up with the arms race against the constantly evolving pathogens (Ebert and Hamilton 1996), but they must also be effective in discriminating the self from non-self in order to prevent autoimmune disease (Zipfel 2009).

In addition to the convenience of using insects as model systems, their significance as disease vectors, pollinators, agricultural pests, and biological control agents makes the insect immunology worth of research on its own (Rolff and Reynolds 2009). There are differences between vertebrate and invertebrate immune systems but they also bear resemblance that might, for example, shed light on better treatment of immunocompromised human patients that only possess the innate part of defence machinery (Pham *et al.* 2007). Insect's defence

against an intruder can begin by behaviour that decreases the risk of pathogen encounter, after which the integument serves as a physical barrier. Actual immune system response initiates when a pathogen is able to breach this first line of defence. For an appropriate response, the threat must be first recognized. Several molecules such as gram-negative binding protein (GBP), peptidoglycan recognition proteins (PGRPs) and lectins serve this purpose by binding into pathogen-associated molecular patterns (Gillespie and Kanost 1997, Schmid-Hempel 2005). The binding can trigger various responses such as phagocytosis, encapsulation, melanization, release of reactive oxygen species (ROS), and synthesis of different anti-microbial peptides (AMPs). Two major signalling pathways Toll and Imd mediate and regulate many of these defence mechanisms. For example, immune challenges with gram-negative bacteria can be recognized with GNBP and PGRP-LB, which then activate the Imd pathway. PGRP-SA binds to the surface structure of gram-positive bacteria and activates the Toll cascade. The selective binding of recognition molecules can thus provide a way to mount a defence towards a pathogen with a rough-cut specificity (Michel *et al.* 2001, Lemaitre and Hoffman 2007, Vallet-Gely *et al.* 2008).

Lepidopterans have been in the core of the insect immunity studies from the early days of the field. Starting from the beginning of the 17th century, commercially important silk worm (*Bombyx mori*) inspired germ theory research (Rolff and Reynolds 2009) and in the 1980's the first insect AMP cecropin was isolated from, and named after, the moth *Hyalophora cecropia* (Steiner *et al.* 1981). Currently, *H. cecropia, B. mori, Manduca sexta*, and *Galleria mellonella* are the prime examples of well-known Lepidopteran model species in immunology (Jiang *et al.* 2010).

Life-history theory suggests that similarly to pathogens, the hosts can face trade-offs in the evolutionary arms race against the enemies. The trade-offs resulting from immune defence can occur in terms of pleiotropic constraints or genetic covariance with other fitness related traits (Sheldon and Verhulst 1996, Schmid-Hempel 2005). For example, evolutionarily increased resistance can be traded-off with fecundity or developmental time (Ferdig *et al.* 1993, Yan *et al.* 1997). More immediate resource allocation costs can also occur because maintaining immune defence requires energy allocation (Armitage *et al.* 2003). In addition, mechanisms such as reactive oxygen species that help in pathogen clearance can be destructive for the hosts' own tissue (Sadd and Siva-Jothy 2006, Dowling and Simmons 2009). This interestingly suggests that hosts might be better off with tolerating than resisting infections to minimize virulence in some situations (Behnke *et al.* 1992, Vale *et al.* 2014, Råberg 2014).

### 1.5.2 Immunological priming

Jawed vertebrates have an antibody-based immunological memory that protects them from a recurring infection of a previously encountered pathogen (Criscitiello and de Figuereido 2013). Invertebrates' innate immune system lacks antibodies, but still increasing amount of reports about acquired

immunity in insects are being made (e.g. Moret and Siva-Jothy 2003, Kurtz 2005, Roth *et al.* 2009). It has been shown that this phenomenon, coined as immune priming, can be pathogen specific and even be transmitted from parents to offspring (Sadd *et al.* 2005, Sadd and Schmid-Hempel 2006, Pham *et al.* 2007, Tidbury *et al.* 2011, Freitak *et al.* 2014). Specific immune priming has been suggested to be mediated by the Toll-pathway and to require haemocytes (Pham *et al.* 2007), but relatively little is still known about the mechanistic basis of immune priming. In principle, there is a fundamental difference whether the prophylaxis is due to an immune reaction that stays expressed until the next pathogen encounter, or due to a an enhanced re-upregulation of defences on the secondary exposure. However, both solutions to the same problem are analogous when it comes to fitness consequences, as long as the persistent defences do not bear too high autoimmune costs (III).

Immune priming does not only have implications on the host fitness because the host responses should also reflect to pathogen populations. Although population level evolutionary effects are extremely difficult to study in the wild, modelling the epidemiological consequences has revealed that depending on the reproductive costs of priming it can have effects on stability of host population, demographic structure and disease prevalence (Tate and Rudolf 2011, Tidbury *et al.* 2012). Further, evolution of priming could cause non-equilibrium population dynamics leading to pathogen extinction (Best *et al.* 2012).

## 1.6 Aims of the study

In this thesis, I tackle questions concerning pathogen-host interactions between *Serratia marcescens* and its insect hosts from versatile perspectives. I rely on experimental settings in order to study virulence evolution in the pathogen (I, II) and to explore the defence mechanisms in the hosts (III, IV).

Evolution of an opportunistic pathogen in a non-host context was assessed by exposing *S. marcescens* to predation by a protozoan ciliate (*Tetrahymena thermophila*) in a long-term evolution experiment (I). Protozoan predation is one of the most important selection pressures affecting the evolution of free-living bacteria and predation-induced changes have been connected to virulence related traits in several bacterial species (reviewed in Matz and Kjelleberg 2005). Thus, the first experiment was established to answer the following questions:

- Which bacterial life-history traits do the ciliate predators select for?
- Are the evolutionary changes in these traits connected to virulence in the greater wax moth *Galleria mellonella*?
- Is bacterial evolution parallel between replicate populations?

Pathogen's within-host evolution was studied in a serial passage setup in *Drosophila melanogaster* fruit fly (II). This experiment was aimed to answer the following questions:

- Does virulence of *S. marcescens* evolve due to selection within the host?
- Is virulence connected to bacterial motility, *in vitro* growth, or secretion of extracellular proteases?

Two immune priming experiments were established in order to examine acquired aspects of insect immunity at phenomenological and mechanistic levels (III, IV). The arctiid moth *Parasemia plantaginis* was first used to study the fitness consequences of immune priming (III). Further, two different strains of *S.marcescens* were used to infect *P. plantaginis* and *G. mellonella* hosts to explore the specificity and diversity of Lepidopteran immune defences (IV). The following questions were addressed in these two studies:

- Are Lepidopterans protected against a recurring infection with a previously encountered pathogen?
- What are the possible mechanisms of immune priming?
- Is the acquired protection pathogen strain specific?
- Which genes are upregulated in the hosts during gut borne infection?

# 2 MATERIAL AND METHODS

## 2.1 Study species

### 2.1.1 Microorganisms and growth media

*S. marcescens* is a gram-negative, rod-shaped enterobacterium. It has a global distribution and is able to grow free-living in various freshwater, marine, and soil environments. It is a broad-spectrum generalist also when it comes to the hosts: *S. marcescens* has been reported to be pathogenic in over 70 species of insects but it also infects other invertebrates, plants, corals, fish, birds, and mammals. In humans, it is mostly a nosocomial pathogen causing for example urinary tract infections, pneumonia, meningitis, and endocarditis (Grimont and Grimont 1978, Grimont *et al.* 1979, Sutherland *et al.* 2010, Mahlen 2011).

Two different strains of *S. marcescens* were used in the thesis. *S. marcescens* ssp. *marcescens* Bizio, the type strain of the species was obtained from American Type Culture Collection (ATCC 13880, I, III, IV). It is an environmental isolate from a pond water sample and produces a distinct red-pigment, prodigiosin, as a secondary metabolite (Martinec and Kockur 1961). The physiological role of prodigiosin is not completely clear but it might be connected to inter-specific competition because of its anti-microbial properties (Bennet and Bentley 2000). Prodigiosins also have immunosuppressive qualities, which potentially connect them to virulence although most clinical isolates of the species lack the pigmentation (Williamson *et al.* 2006, Mahlen 2011). *S. marcescens* ssp. *marcescens* db11 is a non-pigmented, phage resistant mutant of db10 strain, which was originally isolated from moribund Drosophila (Flyg *et al.* 1980, II, III, IV). Db11 has been widely used as a model entomopathogen in insect-bacterium research (e.g. Nehme *et al.* 2007). The strain for these studies was kindly provided by Prof. Hinrich Schulenburg. A standard laboratory adapted *Eschericia coli* K-12 strain was used as a non-pathogenic, gram-negative control bacterium (III, IV).

The bacteria were maintained as batch cultures in either plant based (I) or animal protein based (II, III, IV) media. The plant based medium for the evolutionary experiment was prepared by boiling 2 g of cerophyll powder

(Ward, Rochester, NY, US) in 1 l $dH_2O$, filtering, and diluting it to 2.15 mg $l^{-1}$ final concentration. The medium was buffered to pH 7.5 with 1.5724 g $K_2HPO_4 \cdot 3H_2O$, 0.4 g $KH_2PO_4$, 0.5 g $(NH_4)2SO_4$, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.01 g NaCl and 0.0228 g $CaCl_2 \cdot 2H_2O$ in 1 l $dH_2O$ (Friman *et al.* 2008). The animal protein based liquid culturing was done in a simple LB-medium (10 g tryptone, 5 g yeast extract, 10 g NaCl in 1 l $dH_2O$). For the serial passage experiment, LB-medium was mixed 1:1 with 50 mM sucrose solution. Three kinds of agar plates were used for colony isolation, selective plating, and harvesting bacterial mass: (I) NB agar (10 g Difco nutrient broth, 2.5 g yeast extract and 15 g agar in 1 l dH2O); (II, III, IV) LB agar (10 g tryptone, 5 g yeast extract, 10 g NaCl and 15 g agar in 1 l $dH_2O$); and (II) *S. marcescens* selective agar (42 g deoxyribonuclease test agar with methyl green, 10 g L-arabinose, 5 mg phenol red, 4 ml 1% methyl green, 10 mg ampicillin, 10 mg colistimethate, 20 mg cephalothin, and 5 mg amphotericin B in 1 l of water, modified from Grimont and Grimont (1978)). The bacteria were stored in -80 °C with 1:1 mix of glycerol and the appropriate growth medium.

*T. thermophila* is a ciliate protozoan that can feed upon organic matter but is also a well-known predator of free-living bacteria (Hill 1972). The used strain was originally isolated from fresh water sample and obtained from the ATCC (30008). *T. thermophila* is a widely used model organism in microcosm experiments (e.g. Laakso *et al.* 2003, Ketola *et al.* 2004, Meyer and Kassen 2007). The ciliate was maintained in a proteose peptone (10 g $l^{-1}$) and yeast extract (2.5 g $l^{-1}$) medium.

### 2.1.2 The hosts

*Galleria mellonella* (L. 1758), the greater wax moth, is a very widely used host model organism, especially in insect immunology studies. It has also been shown that bacterial virulence in *G. mellonella* correlates with virulence in mammals and mammalian cell cultures, making it a good surrogate host in human pathogen studies (Jander *et al.* 2000, Miyata *et al.* 2003, Seed and Dennis 2008). In the wild *G. mellonella* larvae live as parasites in beehives feeding on the honeycombs. It has a global distribution and it causes substantial damage to commercial honey production as a pest (Smith 1960). The larvae were obtained from Kreca V.O.F (Ermelo, Netherlands) (I) and Suomen Eläintukku (Hyvinkää, Finland) (IV). They were reared on standard artificial diet (oat flakes, honey, milk powder and wheat flour) at 25 °C (I) or 30 °C (IV) in constant darkness.

*Drosophila melanogaster* (Meigen 1830) wild type Oregon R –strain was used as a model host in the serial passage experiment (II). Because of its very well known genetics, *D. melanogaster* is the most common model organism in innate immunity studies (Broderick *et al.* 2009). The flies were maintained on sugar-semolina-yeast-agar medium at 25 °C and population sizes were kept big (>1000) at all times. The strain was a kind donation from Dr. Christina Nokkala.

*Parasemia plantaginis* (L. 1758), the wood tiger moth, is a diurnal moth species. It has a wide distribution over the northern hemisphere and the larvae

are highly polyphagous (Lindstedt 2008). *P. plantaginis* has mostly been studied because of the warning colouration in the adults and the larvae (e.g. Lindstedt *et al.* 2008, 2009, Nokelainen *et al.* 2011), but its immune functions have also been shown to be affected by food quality (Ojala *et al.* 2005, Zhang *et al.* 2012). The larvae for these experiments (III, IV) were obtained from a stock initiated with wild caught adults from southern Finland. The larvae were fed with *Taraxacum* sp. *ad libitum* (for rearing, see: Lindstedt *et al.* 2009).

## 2.2 Experimental design

### 2.2.1 Protozoan predators and bacterial virulence (I)

To assess the effect of protozoans on the evolution of *S. marcescens*, one ancestral clone was isolated and divided in eight identical starting populations. Bacteria were then let to evolve in the presence or absence of the protozoans for 3 months (Friman *et al.* 2008). After 13 weeks, eight clones from four replicate populations per treatment were randomly isolated totalling 64 evolved clones and the ancestor. The clones were then cryopreserved individually for life-history trait measurements (defence against predators, biofilm formation, maximum growth rate, prodigiosin synthesis, motility, and virulence).

### 2.2.2 Within-host evolution (II)

In order to find out the role of within-host selection on virulence evolution, a single ancestral *S. marcescens* clone was isolated and divided in 20 identical starting populations. Ten replicates were fed to *D. melanogaster* flies with sucrose solution. After 60 h the bacteria were isolated by selective plating and fed to the next round of hosts. The infection cycle was repeated ten times. The remaining ten replicates were let to evolve in the sucrose solution in fly vials for the same amount of time. In the end, ten individual clones were isolated from each population and cryopreserved for life-history trait measurements (maximum growth rate, maximum biomass yield, motility, secretion of extracellular proteases, and virulence). Thus, there were altogether 200 clones and the ancestor.

### 2.2.3 Priming of the host immunity (III)

To test if *P. plantaginis* can defend itself against a recurring infection, 416 larvae were first exposed orally with a standardized amount of *S. marcescens* or harmless *E. coli*. After five days, 15 immune measurement samples were taken and the larvae were injected with either of the two bacteria. The survival of the priming-injection pairs was compared to each other.

### 2.2.4 Specificity of defences in two hosts (IV)

To test between hosts and between pathogens variance in immune defences, priming and gene expression, a follow-up study for (III) was established. The survival data on *P. plantaginis* vs. *S. marcescens* ssp. *marcescens* Bizio was included from the previous experiment but one host species (*G. mellonella*) and one pathogen strain (*S. marcescens* db11) were added. Thus, the two hosts were orally exposed and injected with two pathogens and controls resulting in nine priming-injection combinations. Altogether 898 *P. plantaginis* and 584 *G. mellonella* larvae were used. Samples for immune measurements (15 from *P. plantaginis* and 10 from *G. mellonella*) and midgut gene expression (15 from both species) were taken five days after the oral exposure and the survival of the combinations was compared.

## 2.3 Bacterial life-history trait measurements

### 2.3.1 Growth

Two bacterial growth traits were measured: maximum growth rate and maximum biomass yield. This was done by seeding a trace amount of a clonal bacterial population on fresh medium in a 100-well honeycomb spectrophotometer plate. The growth was measured as the change in turbidity of the medium. Optical density (OD) was read in 5 min intervals with a Bioscreen© spectrophotometer (Growth Curves Ltd, Espoo, Finland) at 460-580nm (I) and 600nm (II) wavelengths in 25 °C. From the raw data, a Matlab (Mathworks, MA, US) script was used to determine a maximum slope of the log turbidity (i.e. maximum growth rate) in a 30 time points sliding window (I, II). Similarly, maximum mean OD in a sliding window was used to determine maximum biomass yield (II). The defence against protozoans was also measured with a spectrophotometer as the maximum population size the bacteria could maintain against the grazing predators (I).

### 2.3.2 Biofilm and prodigiosin

Biofilm forming ability was measured as follows: The bacteria were grown to a late log phase on spectrophotometer plates after which the biofilm bound to the well walls was dyed with 100 µl of 1 % crystal violet solution. The wells were rinsed with water and the dye dissolved in 96% ethanol. The relative amount of biofilm was measured as optical density with a spectrophotometer (O'Toole and Kolter 1998). Prodigiosin expression was detected from colonies on agar plates and graded as red or white (I).

### 2.3.3 Motility and protease secretion

Sterile loop (2 µl, VWR) was used to stick clones on a centre of a semi-fluid agar plater (the appropriate medium with 0.7% agar). The plates were photographed after 48 h (I) or 55 h (II) and the colonized area was determined with ImagePro software (Media Cybernetics, Rockville, MD, US). The protease activity was measured on a 1 % skimmed milk agar plate as a diameter of the casein degradation halo after 55h (II) or 48 h (IV) in 31°C (Tran *et al.* 1993).

## 2.4 Infecting the hosts and measuring virulence

Oral introduction of bacteria to the hosts was performed by feeding them food that was contaminated with bacteria. *D. melanogaster* was infected with overnight cultures of *S.marcescens* mixed with sucrose (described in 2.1.1). The bacterial solution was soaked into a dentist cotton roll (Lifco Dental AB, Enköping, Sweden), which was placed in a vial (Sarstedt AG & Co, Nümbrecht, Germany) with ten flies (II). The diet for the moth larvae (described in 2.1.1) was contaminated with 200 µl of 0.5 OD bacterial cultures. After 48 h the larvae were put on normal, uncontaminated diet (III, IV). It has to be noted that the amount of ingested bacteria cannot be controlled in these setups but the odds of infection are nevertheless the same between the individuals of the same host.

Septic injury infections of moth larvae were carried out with Hamilton syringes by injecting the bacteria in buffer (I) or in LB (III, IV) into the body cavity behind the prolegs. Approximately $8.3 \times 10^6$ cells (I) and 100 000 cells (III, IV) were injected. Sterile phosphate buffer (I) and harmless *E. coli* (III, IV) were used as controls. The larvae were kept individually on petri-plates to record the mortality. In all the studies mortality was checked from 3 to 12 h intervals and virulence determined as the host death rate (I-IV).

## 2.5 Isolating *S. marcescens* from hosts

The flies were surface sterilized before isolating the bacteria in order to acquire only the bacteria that had been able to colonize the hosts. The flies were immersed in 1 ml eppendorf tube of $dH_2O$, 70% EtOH, $dH_2O$, 5 % sodium hypochlorite, and finally $3 \times dH_2O$, with rigorous vortexing between every step. The surface sterile flies were then homogenized, diluted in $dH_2O$, and selectively plated in order to get rid of other host associated microbiota. Bacterial mass from the plates was then cryopreserved for the use in the next passage (II).

*P. plantaginis* and *G. mellonella* guts were dissected and cryopreserved. Thawed samples were homogenized and dilution plated on *S. marcescens* –

selective plates in order to quantify the bacterial presence in the gut. Colony forming units (CFU) were counted after 24h of growth in 31°C (IV).

## 2.6 Host immune assays and gene expression

Phenoloxidase activity (PO) and concentration of reactive oxygen species (ROS) were measured from haemolymph samples taken 120 h after the oral priming (III, IV). The samples were diluted in PBS (8g NaCl, 0.2g KCl, 1.44g $Na_2HPO_4$, 0.24g $KH_2PO_4$ in 1 l of $dH_2O$) buffer and cryopreserved. The thawed samples were centrifuged before use. For PO measurment 25 µl of supernatant was mixed with 200 µl of 3 mM L-Dopa (Sigma-Aldrich, Dorset, UK, #333786). Kinetic activity of the enzyme was measured at 30 °C, 490 nm for 90 minutes with Victor X4 2030 plate reader (Perkin Elmer, Waltham, MA, US). The slope of the absorbance curve was used in the analyses. ROS was measured as follows: 5 µl of the supernatant was added to 90 µl of Pierce PeroXOquant (Thermo Scientific, Waltham, MA, US #23280) quantitative peroxide assay's working solution. $H_2O_2$ dilutions were used as standards. The mix was left to stabilize at room temperature for 25 min before reading the absorbance with a Bioscreen spectrophotometer at 580 nm (III, IV). Lytic activity was measured from pure haemolymph samples by pipetting 5 µl of the sample into a 2.2 mm diameter wells punctured on *Micrococcus* (ATCC #4698) agar plate (III). Plates were then incubated overnight in 31°C and photographed. Serial dilutions (0.031 - 2.0 mg/mL) of lysozyme (Sigma-Aldrich, Dorset, UK, #L7651) were used as standards. Lytic activity was determined from the photo as the diameter of a degradation halo around the well (Freitak *et al.* 2007).

To measure the gene expression in *G. mellonella* and *P. plantaginis* gut tissues, RNA was extracted according to manufacturer's protocol (TriSure, Bioline, London, UK). Extracted RNA from five guts was pooled prior the conversion to cDNA with RevertAid RT Kit (Fermentas, Waltham, MA, US). 100 µg of total cDNA was then used with the SensiFAST SYBR No-ROX kit (Bioline, London, UK). Gene expression was measured using real-time PCR with CFX96 rtPCR system (BioRad, Hercules, CA, US). All the samples were run in two technical replicates (IV).

## 2.7 Statistical analyses

Survival analyses were performed with Kaplan-Meier (I), Cox-regression (II), or their combination (III, IV). In the Cox regression, replicate population identity was used as a categorical covariate (II). Evolution of bacterial traits was analysed with ANOVA (I) or Linear Mixed Model with REML (II) including the population identity nested within the evolutionary treatment as a random factor. When comparing the evolutionary treatment means with the ancestor,

Kruskal-Wallis rank ANOVA was used because of unequal sample sizes (II). Also, clone level correlations between traits were analysed in (I): First, simple bivariate Pearson correlations were assessed within the treatments regardless of the population origin. Further, linear regression where change in one trait was explained with another was performed with population origin and prodigiosin synthesis fitted as covariates. PO, ROS, and lytic activity were analysed with t-test and Mann-Whitney U (III) or Kruskal-Wallis rank ANOVA (IV). Multiple comparisons were Holm-Bonferroni corrected. All analyses were performed with SPSS-software (SPSS Inc., Chicago, IL, US.) The $2^{-\Delta\Delta}{}_{CT}$ criterion was used for quantification of differences in gene expression (Livak and Schmittgen 2001).

# 3 RESULTS AND DISCUSSION

## 3.1 Opportunistic pathogen evolves in a non-host environment (I)

Predation by the protozoan ciliate *T. thermophila* selected for increased defensive ability in *S. marcescens*. This happened via evolution of predator resistant biofilm (Matz and Kjelleberg 2005). Improved defence also led to a corresponding decrease in bacterial virulence in *G. mellonella* and maximum *in vitro* growth rate. Thus, the anti-predatory adaptation that provided better outside-host fitness was costly. In addition, predation selected against the synthesis of red pigment, prodigiosin. Being non-pigmented was, however, not sufficient to provide protection against the predator. Rather, evolution of better defence required that the prodigiosin synthesis was lost. Direct positive clone level correlation between virulence and other traits was not found. In other words, although the loss of prodigiosin best explained the reduction in virulence, it is possible that some underlying trait, which is pleiotropically connected with the pigment, is responsible for high virulence (Loriia *et al.* 1977, Coulthurst *et al.* 2004, Fineran *et al.* 2007).

Replicate populations diverged in terms of maximum growth rate in both treatments. Populations were initially identical and all the genetic changes were thus introduced by *de novo* mutations. Although selection on growth rate has been suggested to be strong in batch cultures leading to convergent evolution (Fong *et al.* 2005), the result could be due to the initial mutations leading to different evolutionary trajectories by chance (Travisano *et al.* 1995). Other traits, including virulence, evolved in parallel in the presence of the predator. In the absence of the ciliate all traits except biofilm formation were significantly affected by the population origin. This suggests that strong selection by the predators directed the evolution in replicate populations to similar trajectories. Contrastingly, between-population differences in the absence of predators suggest that relaxed selection allows more random changes in several life-history traits.

These kinds of trade-offs between pathogens' outside-host fitness and virulence related traits could explain why environmental isolates of opportunistic pathogens are seldom highly virulent, and how polymorphism in virulence is maintained in natural populations of bacteria.

## 3.2 Within-host selection attenuates virulence (II)

Passage through 10 *D. melanogaster* flies attenuated the virulence in *S. marcescens* db11. This happened compared to both, ancestral bacteria, and the bacteria that were evolving in the same sucrose medium with which the strains were fed to the flies. The result suggests that within-host selection pressures acted against virulence, which is somewhat counterintuitive and against most of the theoretical expectations (Frank 1996, Ebert 1998). However it is possible that pathogens evolve adaptations that reduce virulence, to avert host immune system (Gooding 1992, Lipsitch and Moxon 1997), or to better compete with conspecifics (Smith *et al.* 2006). The reduction in virulence could also be due to rapidly fluctuating selection, between the "vector" medium and the within-host environment. It has to be noted that the medium itself selected for increased growth rate, decreased maximum yield, decreased protease secretion and decreased motility. However, it could not have directly selected against virulence because the selection lines that were constantly evolving in the medium maintained virulence at the same level as the ancestor. Previous experimental evidence of this kind of host-environment fluctuation affecting pathogen virulence is non-existent. However, compared to constant environment, temperature fluctuations have been shown to cause evolutionary changes in bacterial life history, including reduced virulence (Ketola *et al.* 2013). Interestingly, there could be an analogy between adaptation to environment-host fluctuation and vector-primary host fluctuation (Weaver *et al.* 1999). Also, transmission through environmental reservoir has been suggested to lead to conflicting selection pressures with direct host-to-host transmission in an epidemiological model (Boldin and Kisdi 2012).

Motility or protease production of the bacteria did not explain the change in virulence although both are well-recognized virulence factors (Josenhans and Suerbaum 2002, Lane *et al.* 2007, Nehme *et al.* 2007, Kannan *et al.* 2009, Bidochka and Khachatourians 1990, Kurz *et al.* 2003). Both traits however were selected against by the LB-sucrose culture medium. Decrease in protease production could be explained by tryptone, enzymatic digest of casein, being abundantly available in the medium (Fraser and Powell 1950). When the bacteria had access to readily digested amino acids, selection could have acted against the costly production of extracellular substances that help in nutrient acquisition in normal circumstances (West and Buckling 2003). Because motility decreased in both evolutionary treatments, it seems that being motile did not have benefits in the medium or in the host colonization.

This study demonstrates that within-host evolution can lead to lower parasite virulence, even if transmission is standardized by manipulation. Parasites might improve their performance within the hosts by being more benign, or they might pay an evolutionary cost of adapting to fluctuations between within-host and non-host situations.

## 3.3 Immune priming and Lepidopteran defences against bacteria (III, IV)

Sublethal oral dose of *S. marcescens* ssp. *marcescens* Bizio provided *P. plantaginis* larvae an efficient protection against a recurring, otherwise lethal septic infection with the same pathogen five days later. Similarly gram-negative but harmless enterobacterium *E. coli* failed to elicit immune responses required for the protection. This suggests that sheer presence of gram-negative bacteria in the midgut, potentially recognized with selective binding molecules, is not a sufficient cue for evoking the prophylaxis. Thus, pathogens are recognized in the midgut with a more specific mechanism. The response can then induce a systemic immune reaction that lasts for several days and protects from a recurring infection.

Bacterial isolation and immune response measurements at the time of the septic injury showed that trace amounts of the pathogen are able to persist in *P. plantaginis* gut, possibly maintaining immune defence, including ROS, at a level that protects from the sepsis. *S. marcescens'* known resistance to oxidative stress (Campbell and Dimmick 1966), however, makes it unlikely that solely ROS could diminish the serious immune insult of direct injection of bacteria through the cuticulum. Rather, ROS is more likely to mediate other immune responses that allow the pathogen clearance from the haemocel (Wu *et al.* 2012). Priming with *S. marcescens* ssp. *marcescens* Bizio could not protect against sepsis with the more virulent *S. marcescens* db11 strain. Still, this cannot be interpreted as strain specificity because priming with db11 results in bacterial proliferation in the gut and consequently host mortality. Thus, it is impossible to tell if db11 produces the same immune response but its ability to invade the haemolymph through the gut prevents protection against the more benign strain to occur. For example, the ROS levels were similarly increased with both pathogens in *P. plantaginis*. There were no differences in lytic activity or PO activity between differently primed larvae, suggesting that these traits did not contribute to the observed protection.

Contrary to *P. plantaginis*, *G. mellonella* was resistant to both pathogens orally and the larvae completely cleared the bacteria from their guts within five days. However, the *G. mellonella* larvae could not be primed against the sepsis, either. Thus it is possible that *P. plantaginis* remained protected thanks to its seeming "inability" to clear every bacterial cell, which interestingly appeared to be adaptive in the case of recurring infection. In *G. mellonella* physiological immune measurements, PO and ROS, showed no response to the oral introduction of bacteria at the time of injection.

Db11 secretes caseinolytic extracellular proteases several orders of magnitude more than *S. marcescens* ssp. *marcescens* Bizio. Secretion of proteases is a well-known virulence factor and they are especially important for penetrating the insect gut membrane or even the outer cuticulum of the body (Bidochka and Khachatourians 1990, Abuhatab *et al.* 1995, Kurz *et al.* 2003, Nehme *et al.* 2007). This is a likely explanation for why db11 is able to penetrate the *P. plantaginis* gut epithelium causing a septic infection through the midgut, and why it is more virulent in septic injury for both hosts. The protease secretion ability could also very well reflect db11's evolutionary history as an entomopathogen (Flyg *et al.* 1980).

Gene expression analysis revealed remarkable differences between the hosts and between the pathogens that coincide with the observations on host survival. Firstly, *G. mellonella* had 40 to 80 fold increased CecropinA expression after being orally exposed to the pathogens, suggesting a likely way of eradicating the bacteria from the gut. The reason why CecropinA levels remained elevated after pathogen clearance is not known but it is nevertheless an interesting observation. Secondly, in *P. plantaginis* that succumbed to db11 oral infection and tolerated the more benign strain in the gut, CecropinA was downregulated, which further supports the role of CecropinA in bacterial clearance in the gut (Moore *et al.* 1996). The trend with CecropinB was the opposite in the hosts: when CeropinA was upregulated, CeropinB was downregulated and *vice versa*, suggesting that the two AMPs could have antagonistic regulatory basis.

PGRP2 was upregulated with db11 infection in *P. plantaginis*, which is consistent with the high amount of bacterial cells isolated from the gut at the same time point. Interestingly, *S. marcescens* ssp. *marcescens* Bizio persisted in the *P. plantaginis* gut but the density did not reach a threshold for PGRP2 upregulation anymore after five days. In *G. mellonella*, that was tested negative for the presence of both pathogen strains, PGRP2 levels remained at the same level with the non-pathogenic control bacterium when exposed to db11, while the more bening strain had induced a slightly elevated PGRP2 expression.

Besides ROS, another factor that might explain the prophylaxis in *P. plantaginis* was upregulation of protein 6-Tox. It was down regulated in *G. mellonella* with db11 and at the same level with controls with *S. marcescens* ssp. *marcescens* Bizio. Interestingly, X-Tox family proteins have been shown not to have a direct anti-microbial role and to be connected to haemocytes (Girard *et al.* 2008, Destoumieux-Garzón 2009), which in turn are needed in specific immune priming in Drosophila (Pham *et al.* 2007).

In conclusion, priming of bacterial resistance in *P. plantaginis* could be related to ROS, upregulation of 6-Tox, or their interaction. CecropinA seems to have an imperative role in eradicating gram-negative bacteria from the insect gut. The studies demonstrate that in some situations, despite the possible autoimmune costs, tolerance to invasion can be more adaptive solution than complete bacterial clearance (Råberg 2014). It has been suggested that long lasting immune response could be host's solution in preventing evolution of AMP resistance in the invading pathogens (Haine *et al.* 2008).

# 4 CONCLUSIONS

In this thesis I show that the interplay of non-host and within-host selection drives the evolution of virulence in an opportunistic bacterial pathogen. Both evolutionary experiments with the pathogens, within and outside the hosts, reveal unexpected outcomes (I, II). Contrary to previous studies (Molmeret *et al.* 2005, Matz and Kjelleberg 2005, Steinberg and Levin 2007), protozoans selected against bacterial virulence factors in the free-living environment (I). The ciliate grazing selected for biofilm formation as a defensive trait. The anti-predatory adaptation was however traded off with the secondary metabolite prodigiosin, which in turn lead to attenuated virulence *in vivo*. Selection for predator-induced adaptation was so strong that replicate populations tended to evolve in parallel in presence of the protozoans. The more relaxed selection in absence of the predators, on the other hand, produced between population divergence in several bacterial traits, including virulence (I).

Moreover, most previous theoretical and experimental work suggests that without epidemiological restrictions, high virulence evolves within the host (Frank 1996, Ebert 1998). In contrast, I show that in spite of being independent from host-to-host transmission, opportunistic pathogens do not necessarily evolve high virulence as a consequence of within-host selection. Rather, they can evolve towards a more benign life-history strategy during sequential infections. This could be due to immune evasion, within-host competition, or rapid fluctuations between non-host and within-host situations (Smith *et al.* 2006, Boldin and Kisdi 2012, II).

Virulence itself, however, is not only a pathogen trait but also a consequence of specific interactions between host defences and pathogen characteristics (III, IV). I found that the insect innate immunity has the ability to distinguish pathogens from harmless bacteria and to mount a corresponding systemic defence that protects from a recurring infection (III). However, little is still known about how immune priming works. A very simple solution for staying prepared for a secondary pathogen encounter would be maintaining the right defences upregulated, a phenomenon sometimes referred to as immunological loitering (III, IV). Interestingly, this could be mediated by

tolerating non-symptomatic amounts of the pathogen instead of complete clearance (Haine *et al.* 2008, Vale *et al.* 2014, IV). The thesis also contributes to the field by pointing out mechanistic factors, such as the protein 6-Tox or ROS, being potentially involved in immune priming (IV). The priming phenomenon can have significant evolutionary consequences on both pathogen and host populations (Tate and Rudolf 2012, Tidbury *et al.* 2012, Best *et al.* 2013). The hosts respond differently to the same pathogen, and the pathogen strains elicit different responses within a same host. Thus, caution should be exercised when generalizing results over particular host-pathogen model systems where virulence is highly dependent on multiple host and pathogen specific factors (IV).

Taken together, the work in this thesis reveals a complex web of interactions between pathogens and their hosts. It challenges traditional epidemiological models in fully explaining the evolution of virulence in opportunistic, environmentally transmitted pathogens. The open questions warrant further studies on positive selection and maintenance of virulence factors in opportunistic pathogens, tolerance-resistance relationship, co-evolutionary dynamics, and mechanistic understanding of host defences and pathogen virulence traits.

*Acknowledgements*

The supervision crew, in order of appearance, this work would not have been possible without you. Jone, Jonna, and Tarmo, I'm truly grateful for all your support. Jone and the whole PD-production team, you took me under your wing already in 2006 as an undergraduate, which initially ignited my interest in microbial stuff and much, much more. Jonna, you have an exceptional eye for science as well as a really mysterious skill to make things happen... I don't wonder at all why you have established your position as such a distinguished scientist. Respect. Tarmo, what can I even say? We sat in the same office basically the whole time I was doing the thesis and shared some good and, well, better times. Discussions with you have helped me in myriad ways, not the least with statistics. I'm sure the future will still bring something exciting to our path. None of you have been afraid to get your hands dirty in the lab or to solve practical issues no matter how big or small. Thank you supervisors.

I have had the pleasure to work with a selection of very talented people. Ville, you deserve a big thank you not only for my master's thesis supervision but also all the help and encouragement even after you went into the big world. I've learned a lot from you. Rock on. Dalial, you initially came along a little bit under the radar, but I'm really glad you did. I hope you realize how big of a contribution you've made to my thesis: Unofficially, you are one of the supervisors. I also want to thank Franzi for the extraordinarily diligent work you did with the immunology experiments, and Minna for an excellent master's thesis. The thesis reviewers Anna-Liisa Laine and Sam Brown did a great job and gave critical advice to make the thesis better. Finnish Cultural Foundation funded a major proportion of my work ultimately making the thesis possible. The two Centers of Excellence provided all the resources I could have hoped for and Biological Interactions Graduate School offered a great way to meet academic peers in a thought provoking setting. Teppo and Jari, you were just the support group I needed. Kaisa, Jimi, and all the other greenhouse workers have been invaluable in practical help with things like keeping animals alive, making animals die, pole dancing, and what not.

Not all my friends are colleagues but I'm happy to say that the people I work with are also friends. You make this the best job in the world. I thank the microbial evolution sub-group for intriguing discussions and good company. Plantaginis group, darwin folks, and the whole eco section, it has been a real pleasure. A special mention goes to the notorious coffee and lunch posse, you know who you are and how much it means to have you there.

It has been a long and a winding road. Especially the last year has shown what friends mean. The Royal Society of Whiskey-lovers, Matti, Aapo and Ossi, I know that you're the ones I can always rely on. Islay we're coming. Rabanderi, Long-John, Kuppe, Tume, Touko and all the good old JKL-boys, now mostly scattered all over, you're the best. Me being SOME-hater, friends are friends in real-life. Anni my retard-friend and a colleague, I've got two words for you: kromo and somi. I'd hyphenate that if I only knew how. Teppo you're *the*

suokkari. Tell my greetings to mojito-man when you see him. Torni-setä, our birding trips with other activities have been essential for my mental health. Pöllö-junnut, you have mostly been concentrating on those other activities, thank you for that. Arto, thanks for everything (and also Nina for tolerance and Havumatti for peer support), your sauna has been an asylum. Thanks for Steve + the sauna-folks, Vakkari, Metrin Slerba, and Kalevi Viipale.

I'm privileged to have such a close circle of relatives around me. Cousins and their spouses, you've been my siblings. Thank you. Most of all, I want to thank my parents. It's your fault after all, that I became a biology-freak in the first place. In the process, you have supported me in every possible and impossible way, and I know that I can turn to you whatever happens. Kiitos isä ja äiti.

# YHTEENVETO (RÉSUMÉ IN FINNISH)

## Virulenssin evoluutio ja immuunipuolustus – Isäntä-loinen vuorovaikutuksia opportunistisen *Serratia marcescens* –bakteerin ja sen hyönteisisäntien välillä

Kaikilla tunnetuilla eläimillä on loisia, jotka käyttävät isäntänsä resursseja omaan lisääntymiseensä. Käynnissä on jatkuva kamppailu, kun isäntäeliöt pyrkivät minimoimaan tartunnasta koituvan haitan immuunijärjestelmällään ja taudinaiheuttajat vastavuoroisesti selviytymään näistä puolustusmekanismeista. Koska loisinta on elinkiertostrategiana niin laajalle levinnyt, isäntä-loinen -vuorovaikutuksen katsotaan olevan suurin yksittäinen evolutiivinen voima, joka vaikuttaa elämän monimuotoisuuden syntyyn ja säilymiseen. Väitöskirjassani tarkastelen *Serratia marcescens* –bakteerin virulenssin evoluutiota sekä infektion aikana että isännän ulkopuolella. Toisaalta selvitän mekanismeja, joilla hyönteisisännät puolustautuvat sekä ennestään tuntematonta että uusiutuvaa infektiota vastaan.

Taudinaiheuttajien virulenssilla tarkoitetaan niiden isännälleen aiheuttamaa haittaa, ja useimmiten virulenssin mittana käytetään isännän kuolleisuutta. Joskus virulenssiin viitataan myös sanalla taudinaiheuttamiskyky, mutta termi on hieman harhaanjohtava: Virulenssi ei ole sidoksissa ainoastaan loisen ”kykyihin” vaan toteutuneen haitan määrittävät myös isännän ominaisuudet. Esimerkiksi jotkin immuunipuolustusreaktiot saattavat olla tuhoisia myös isännän omille kudoksille, jolloin virulenssi kasvaa sitä suuremmaksi mitä voimakkaamman immuunivasteen isäntä taudinaiheuttajaan kohdistaa. Lisäksi, loisen edun mukaista ei aina ole aiheuttaa vakavaa vahinkoa. Esimerkiksi siirtyminen uuteen isäntään, transmissio, voi olla suoraan riippuvaista isäntäeliön normaaleista elintoiminnoista. Vallitsevan käsityksen mukaan isännän sisällä tapahtuva kilpailu kuitenkin suosii useimmiten nopeasti kasvavia ja siten korkean virulenssin genotyyppejä. Tällöin kyseessä on niin sanottu evolutiivinen vaihtokauppatilanne, jossa toisistaan riippuvaiset ominaisuudet, virulenssitaso ja transmissiopotentiaali asettuvat patogeenin kelpoisuuden kannalta optimaalisille tasoille. Patogeenit, jotka voivat lisääntyä isäntänsä ulkopuolella (opportunistiset patogeenit), poikkeavat epidemiologialtaan perustavanlaatuisesti patogeeneistä, jotka ovat tiukasti riippuvaisia isännistään (obligatoriset patogeenit). Voisi ajatella, että koska opportunistisen patogeenin ei tarvitse pitää isäntäänsä elossa transmissiota varten, valinta suosisi ainoastaan korkeaa virulenssia. Isännän ulkopuolella patogeeniin voi kuitenkin kohdistua täysin vastakkaisia valintapaineita kuin infektion aikana, jolloin evolutiivista vaihtokauppaa käydään kahdessa erilaisessa elinympäristössä kelpoisuuteen vaikuttavien ominaisuuksien välillä.

Koska luonnonpopulaatioissa tietyn patogeenin uudelleenkohtaaminen on varsin todennäköistä, selkärankaisille eläimille on kehittynyt vasta-aineisiin perustuva immunologinen muisti, joka tunnistaa aiemmin kohdatun uhan ja

puolustautuu sitä vastaan tehokkaammin. Selkärangattomilta eläimiltä vasta-aineisiin perustuvat mekanismit puuttuvat. Viime vuosikymmenellä on alkanut kuitenkin kertyä todistusaineistoa siitä, että selkärangattomat, kuten hyönteiset, voivat puolustautua uudelleen kohtaamaansa taudinaiheuttajaa vastaan paremmin kuin ensimmäisellä kerralla. Adaptiivisen immuunijärjestelmän olemassaolo ei vaikuta ainoastaan isäntäpopulaatioihin, vaan sen ajatellaan muokkaavan koko isäntä-loissuhteen evoluutiodynamiikkaa. Tästä huolimatta fysiologiset mekanismit, jotka mahdollistavat muistinkaltaisen immuunipuolustuksen selkärangattomissa, ovat suuriltaosin vielä tuntemattomia.

Ensimmäisessä osatyössä selvitin miten isännän ulkopuolella, akvaattisessa ympäristössä, elävän opportunistisen patogeenibakteerin virulenssi kehittyy, kun se altistuu alkueläinpedoille. Yli tuhat sukupolvea kestänyt koe paljasti, että *Tetrahymena thermophila* –alkueläimen saalistus aiheuttaa evolutiivisia muutoksia useissa *S. marcescens* –bakteerin ominaisuuksissa: pedon kanssa kehittyneistä bakteereista muodostui parempia puolustautujia tehostuneen biofilminmuodostuskyvyn ansiosta. Tämä sopeuma oli kuitenkin yhteydessä menetettyyn prodigiosiini –pigmentin tuotantoon ja madaltuneeseen virulenssiin vahakoisa–hyönteisisännässä. Tutkimus osoittaa, että isännän ulkopuolella vaikuttavat valintapaineet voivat aiheuttaa evolutiivisen vaihtokauppatilanteen, jossa opportunistisen patogeenin virulenssi laskee. Lisäksi lähes kaikkien bakteerin ominaisuuksien evoluutio oli yhdenmukaisempaa rinnakkaispopulaatioissa pedon läsnäollessa kuin ilman petoa. Tämä tukee teorioita, joiden mukaan voimakkaat valintapaineet voivat vähentää evolutiivisten satunnaisilmiöiden esiintymistä.

Toinen tutkimus tarkasteli *S. marcescens* –bakteerin evoluutiota infektion aikana *Drosophila melanogaster* –mahlakärpäsessä. Kun patogeenien annetaan siirtyä vapaasti isännästä toiseen niin, että isännän ulkopuolinen valinta minimoituu (ja transmissio maksimoituu), useimmat teoriat ennustavat, että virulenssi kasvaa. Tutkimuksessa kuitenkin havaitsin, että isännän sisällä valinnan läpikäyneille bakteereille muodostui matalampi virulenssi verrattuna kokeen alkuun sekä kontrollivalintalinjaan. Tämä voi johtua esimerkiksi siitä, että isännän immuunireaktiot toimivat selektiivisesti aggressiivisimpia genotyyppejä vastaan, jolloin vähemmän haitalliset patogeenit yleistyvät populaatiossa. Vaihtoehtoisesti nopeasti isännän sisältä ulkopuolelle ja takaisin vaihteleva ympäristö voi valikoida vähemmän virulentteja genotyyppejä verrattuna tasaiseen valintaan isännän ulkopuolella.

Kolmas osatyö osoitti, että täpläsiilikkään toukat voivat puolustautua tehokkaasti aiemmin kohtaamaansa *S. marcescens* –kantaa vastaan. Kun toukat saivat pienen annoksen patogeeniä ruoan mukana, viiden päivän kuluttua suoraan ruumiinonteloon annettu saman bakteerin injektio ei ollut enää tappava. Jos toukkien ravinto kontaminoitiin harmittomalla *Eschericia coli* –bakteerilla kuolleisuus *Serratia*–injektion jälkeen oli kuitenkin huomattava. Tämä osoittaa, että hyönteisen immuunijärjestelmä voi tunnistaa harmittoman määrän tautia aiheuttavaa bakteeria ruoansulatuskanavassaan, ja ylläpitää puolustusta sitä vastaan. Suojan uusiutuvaa infektiota vastaan tarjosi mahdollisesti kohonnut

vapaiden happiradikaalien määrä toukkien hemolymfassa. Koska hapettavat yhdisteet rappeuttavat isännän elimistöä, puolustukseksi kohonneista pitoisuuksista voi siis olla huomattava kustannus.

Neljäs osatyö käsitteli perhosten adaptiivisen immuunipuolustuksen mekanismeja tarkemmin, mm. geenien ilmentymisen kautta. Käytin kahta perhoslajia, täpläsiilikästä ja vahakoisaa, sekä kahta läheistä sukua olevaa *S. marcescens* –kantaa tutkiakseni adaptiivisen immuunivasteen spesifisyyttä ja eroja isäntälajien välillä. Patogeenikantaspesifistä adaptiivista immuunivastetta ei löytynyt uudelleen aiheutetussa infektiossa. Havaitsin kuitenkin että toinen, proteaaseja tuottava, bakteerikanta kykenee infektoimaan täpläsiilikkään toukkia jo pieninä määrinä ruoan mukana annettuna. Toinen isäntälaji, vahakoisa, sen sijaan pystyi poistamaan molemmat bakteerikannat täydellisesti ruoansulatusjärjestelmästään, todennäköisesti ilmentämällä CecropinA–geeniä, joka tuottaa mikrobeja tappavaa yhdistettä. Pieniä määriä vähemmän virulentinkin bakteerikannan soluja pystyi selviämään täpläsiilikästoukkien suolistossa aiheuttamatta silti vakavaa haittaa. Havainto on erityisen mielenkiintoinen, koska selvinneiden bakteerien ylläpitämä immuunireaktio, mahdollisesti 6-Tox geenin ilmentymisen välityksellä, saattoi olla syynä pitkäkestoiseen suojaan uudelleen tapahtuvaa septistä infektiota vastaan. Vahakoisa, joka poisti patogeenit suolistostaan kokonaan, ei sen sijaan pystynyt suojautumaan toistuvalta infektiolta kummankaan bakteerikannan kohdalla. On siis mahdollista, että toistuvan infektion tapauksessa täpläsiilikäs voi saavuttaa kelpoisuusetua sietämällä pieniä määriä patogeeniä elimistössään, verrattuna siihen, että immuunijärjestelmä häätäisi ne kokonaan.

Väitöskirjan tutkimukset osoittavat, että isännän ulkopuolisessa ympäristössä ja infektion aikana vallitsevat valintapaineet voivat yhdessä ohjata opportunistisen taudinaiheuttajabakteerin virulenssin evoluutiota: Sopeumat isännän ulkopuoliseen ympäristöön voivat aiheuttaa korkeaan virulenssiin johtavien ominaisuuksien menettämisen. Toisaalta, vaikka isännän ulkopuoliseen valintaan liittyviä rajoitteita virulenssin evoluutiolle laskettaisiin, suurempi vahinko isännälle ei välttämättä ole patogeenille hyödyllistä infektion aikana. Virulenssin taso riippuu myös isännän puolustusmekanismeista. Selkärangattomien ”muistittomassa” immuunijärjestelmässä patogeenien sietämisen ja niiden totaalisen torjumisen välinen suhde voi olla erityisen tärkeä, koska uusiutuvat infektiot ovat luonnonpopulaatioissa ilmeisen yleisiä.

Useimmat niin sanotut sairaalabakteerit, kuten Klebsiella-, Pseudomonas- ja Staphylococcus-lajit ovat *S. marcescens*in tapaan ympäristön kautta leviäviä ja täten niiden evolutiivisen epidemiologian ymmärtäminen on ensiarvoisen tärkeää. Hyönteiset ovat ihmisen kannalta mm. erittäin merkittäviä pölyttäjiä, tuholaisia ja tartuntatautien vektoreita, joten niiden immuunipuolustusmekanismit ovat itsessään kiinnostavia. Isäntä-loissuhteen evoluution ymmärtäminen vaatii kuitenkin molempien osapuolten ottamista huomioon samanaikaisesti ja lisää perustutkimusta useilla eri systeemeillä.

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# ORIGINAL PAPERS

# I

## LIFE HISTORY TRADE-OFFS AND RELAXED SELECTION CAN DECREASE BACTERIAL VIRULENCE IN ENVIRONMENTAL RESERVOIRS

by

Lauri Mikonranta, Ville-Petri Friman & Jouni Laakso 2012

PLoS ONE 7: e43801

OPEN ACCESS Freely available online

PLOS ONE

# Life History Trade-Offs and Relaxed Selection Can Decrease Bacterial Virulence in Environmental Reservoirs

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## Abstract

Pathogen virulence is usually thought to evolve in reciprocal selection with the host. While this might be true for obligate pathogens, the life histories of opportunistic pathogens typically alternate between within-host and outside-host environments during the infection-transmission cycle. As a result, opportunistic pathogens are likely to experience conflicting selection pressures across different environments, and this could affect their virulence through life-history trait correlations. We studied these correlations experimentally by exposing an opportunistic bacterial pathogen *Serratia marcescens* to its natural protist predator *Tetrahymena thermophila* for 13 weeks, after which we measured changes in bacterial traits related to both anti-predator defence and virulence. We found that anti-predator adaptation (producing predator-resistant biofilm) caused a correlative attenuation in virulence. Even though the direct mechanism was not found, reduction in virulence was most clearly connected to a predator-driven loss of a red bacterial pigment, prodigiosin. Moreover, life-history trait evolution was more divergent among replicate populations in the absence of predation, leading also to lowered virulence in some of the 'predator absent' selection lines. Together these findings suggest that the virulence of non-obligatory, opportunistic bacterial pathogens can decrease in environmental reservoirs through life history trade-offs, or random accumulation of mutations that impair virulence traits under relaxed selection.

**Citation:** Mikonranta L, Friman V-P, Laakso J (2012) Life History Trade-Offs and Relaxed Selection Can Decrease Bacterial Virulence in Environmental Reservoirs. PLoS ONE 7(8): e43801. doi:10.1371/journal.pone.0043801

**Editor:** Boris Alexander Vinatzer, Virginia Tech, United States of America

**Received** April 11, 2012; **Accepted** July 26, 2012; **Published** August 24, 2012



**Funding:** The study was funded by the Finnish Academy (http://www.aka.fi/en-GB/A/ Project #1130724, J.L.), the Finnish Cultural Foundation (http://www.skr.fi/default.asp?docId = 12256 L.M.), and Marie Curie Intra European Fellowship within the 7th European Community Framework Programme (V.F.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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## Introduction

Pathogen virulence (measured as the severity of a disease) is often assumed to evolve in a strict co-evolutionary arms race between the pathogen and its host [1–5]. The theory of virulence also commonly assumes that pathogen reproduction, and consequently the evolution of virulence, is entirely dependent on the host species [2]. While this view might hold for obligate pathogens, it seems inaccurate for opportunists that are capable of reproducing outside their hosts [6–7]. Previous studies have shown that opportunists are exposed to many different selective pressures in environmental reservoirs, which could have correlative effects on bacterial virulence ('coincidental selection hypothesis') [8–14]. For example, toxicity and tolerance against degradative enzymes of mammalian macrophages may have evolved originally as defence mechanisms against protist predation [12,15–17]. In addition to this "dual-use" of virulence factors, pathogenicity could be, for example, an evolutionary remnant of adaptation for accidental passage through another organism, or merely an inevitable consequence of within-host persistence in microbes that hitchhike in their hosts to disperse into new locations [6]. Even so, virulence evolution in opportunistic pathogens is usually not considered in a wider ecological context across different environments [7].

Even though virulence and survival in the outside-host environment correlate positively in some pathogens, environmental bacterial isolates are seldom as virulent as clinical isolates of the same species [18–19]. Therefore, it is possible that selection in the outside-host environment conflicts with bacterial pathogenicity: traits needed for survival are traded off with traits connected to virulence so that an investment in one trait leads to a corresponding decrease in another. This 'conflicting selection hypothesis' is seldom directly tested but some studies support it demonstrating that virulence traits can incur fitness costs in environmental reservoirs [8,20–22]. Fitness trade-off between within-host and outside-host environments has also been observed in some [23] but not all plant pathogens [24]. Instead of trading off with survival traits, virulence traits could also be neutral in the external environment. In that case, virulence traits could be lost even without negative selection if they are impaired due to random accumulation of mutations ('relaxed selection hypothesis') [25–26].

Here we tested the 'conflicting selection' and 'relaxed selection' hypotheses by studying how predation by *Tetrahymena thermophila* protist changes the defensive and virulence traits of an opportunistic bacterial pathogen, *Serratia marcescens*. We define an opportunistic bacterium as a pathogen, which does not require a host for reproduction, and which can be transmitted between hosts through the environment [7]. *S. marcescens* is a prime example of such an opportunist: it is able to infect a wide range of plant, invertebrate and vertebrate hosts (including humans) and is commonly found in different environmental reservoirs [27–29]. Previous experiment showed that protozoan predation decreases *S.*

*marcescens* virulence in *Parasemia plantaginis* lepidopteran host, when genetically diverse inocula (i.e. mixture of numerous clones with different characteristics) are used for infections. The experiment suggested that the decrease in virulence was connected to reduced growth rate and motility, and to loss of a red pigment, prodigiosin [13].

Here we study the changes in these traits (pigmentation, biofilm, growth rate and motility) in more detail by using single bacterial clones that have evolved in the absence or presence of a protist predator in a long-term selection experiment. First, we investigate whether we can link changes in these traits into either virulence or anti-predator defence. Second, we examine whether the previously observed pattern of decreased virulence emerges when intraspecific interactions that arise due to diversity (e.g. competition, cooperation and cheating [30–34]) are excluded during infection. For example, competition between different bacterial genotypes can affect the severity of infection [33–35] but the use of single clones should exclude competition. Third, we study whether changes in bacterial life-history traits are similar (parallel evolution) or different (divergent evolution) among the replicate populations within the 'predator present' and 'predator absent' conditions: if replicate selection lines diverge more clearly in the absence of predation, it would indicate relaxed selection in an enemy-free environment.

Our results suggest that when the natural protist enemy is present, survival of *S. marcescens* can correlate negatively with virulence (conflicting selection). At the same time, however, most life history traits (including virulence) are likely to diverge more between replicate selection lines in the absence of a strong selective agent such as predation (relaxed selection).

## Materials and Methods

### The long-term evolutionary experiment and the isolation of clones

We used clones isolated from a prior long-term experiment, where the bacterium (a single ancestral clone from ATCC#13880 strain of *Serratia marcescens* ssp. *marcescens*) was exposed to a predatory protist, *Tetrahymena thermophila* (ATCC #30008), for 13 weeks totalling approximately 1300 bacterial generations (for a detailed description, see: [36]). We used four populations that had evolved in the presence or absence of protists, and randomly isolated eight clones per population (a total of 64 clones, 32 clones per treatment). All populations and the ancestral clone stored in −80°C were first thawed, diluted and plated on agar plates (10 g of Difco$^{TM}$ nutrient broth, 2.5 g of Bacto$^{TM}$ yeast extract and 15 g of Bacto$^{TM}$ agar in 1 L of $dH_2O$). After 48 h of cultivating at 25°C, individual clones were randomly picked and cryopreserved separately in −80°C (mixed with 45% of glycerol and 9% of Nutrient Broth: 10 g of Difco$^{TM}$ nutrient broth, 2.5 g of Bacto$^{TM}$ yeast extract in 1 L of $dH_2O$. Becton, Dickinson and Co., Franklin Lakes, NJ). We recorded the colony colour of every isolated clone (red or white indicating prodigiosin pigment synthesis or lack thereof, respectively). The colony colour frequencies in the populations were also recorded.

### Measuring changes in bacterial life history traits

We measured bacterial ability to sustain biomass in the presence of predators (i.e. defence), ability to form biofilm (cell aggregates attached to surfaces) and maximum growth rate in liquid culture medium with Bioscreen C$^{TM}$ spectrophotometer (optical density measured with wideband option: 420–580 nm, 25°C, 400 µL volume; Growth Curves Ltd, Helsinki, Finland). While optical density (OD) is not an exact measure of bacterial cell numbers, it can be used for reliably comparing differences in bacterial growth [13,37]. We cultivated the bacteria in cereal leaf extract medium, which was also used in the prior long-term experiment [36]: 1 g/L of leaf extract (Cerophyll$^{TM}$, Ward's natural science) was first boiled for 5 min in $dH_2O$, cooled down and filtered through glass fibre filter (CF/C, Whatman) resulting in a final concentration of 2.15 mg plant detritus/L. After autoclaving (121°C, 20 min.), the medium was adjusted to pH 7.5 with sterile phosphate buffer ($K_2HPO_4 \cdot 3H_2O$ 1.5724 g, $KH_2PO_4$ 0.4 g, $(NH_4)2SO_4$ 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, NaCl 0.01 g and $CaCl_2 \cdot 2H_2O$ 0.0228 g in 1 L of $dH_2O$).

Bacterial defence and ability to form biofilm in the presence of predators were measured as follows. The clones were grown individually to similar high densities on microplates (in 370 µL of fresh culture medium; Honeycomb 2 microtitre plates, Thermo Electron Oy, Vantaa, Finland) before adding 30 µL inoculum of protist predators (approximately 100 *T. thermophila* individuals), which then reduced the bacterial biomass by grazing. Defence was measured as bacterial biomass after 93 h of cocultivation with the predator: the higher the OD, the better the defence.

Biofilm formation was measured as follows: 100 µL of 1% crystal violet solution (Sigma-Aldrich) was added into the microplate wells and rinsed off with distilled water after 10 minutes. The remaining crystal violet attached to bacteria was dissolved in 96% ethanol, and the amount of biofilm formed was measured as OD at 420–580 nm (a method modified from [38]).

To measure maximum growth rate ($r_{max}$) we introduced a 10 µL inoculum of bacteria into 400 µL of fresh culture medium. Growth was measured as the change in OD in five-minute intervals as described above.

Motility was assessed by sticking a trace inoculum (~2 µL) of each clone onto the centre of a semi-fluid agar plate (as described above, except 0.7% agar) with a sterile loop (VWR). The plates were photographed after 48 h, and the colonised area was determined with ImagePro Plus 4.5 software (Media Cybernetics).

### Measuring bacterial virulence

Virulence was measured using wax moth larvae (*Galleria mellonella*, Lepidoptera; Pyralidae) as hosts. Bacterial virulence measured in *G. mellonella* correlates with virulence measured in mammals and mammalian cell cultures, making the larvae an ideal model host for general virulence testing [39–41]. Our larvae were randomly selected from four different batches (Kreca V.O.F, Ermelo, Netherlands). We used each of the 64 bacterial clones to infect ten larvae: a total of 320 individuals were infected with 'predator present' clones and 320 individuals with 'predator absent' clones. We also infected 60 larvae with the ancestral clone. Additionally, 40 larvae were injected with distilled water to control for the damage caused by the injection itself (total n = 740 larvae). The larvae in these four treatment groups had comparable mean body mass (ANOVA, $F_{3,\ 796} = 0.022$, $p = 0.996$, water: M = 121.9, SD = 42.4, predator present: M = 120.8, SD = 34.1, predator absent: M = 119.2, SD = 29.8, ancestor: M = 123.1, SD = 41.2

The bacterial clones were first thawed and spread on agar plates at high density. After 48 hours of incubation at 25°C, the bacterial mass was collected by scraping, mixed with phosphate buffer (described above in the context of cereal leaf extract medium) and diluted to OD 2.0 at 420–580 nm. Before infection, all clones were further diluted with the phosphate buffer into density of $16.6 \times 10^8$ CFU $ml^{-1}$ ($+/-1.6 \times 10^8$ CFU $mL^{-1}$). Bacterial densities (in CFU) did not differ between the treatments (ANOVA, $F_{2,\ 69} = 0.829$, $p = 0.441$). The larvae were injected between the abdominal segments six and seven with 5 µL (on average $8.3 \times 10^6$ CFU) of

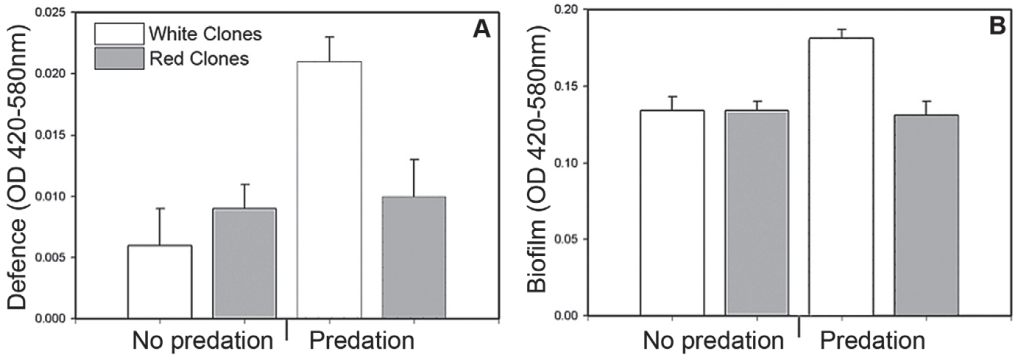


**Figure 1. Effect of predation on bacterial defence and biofilm formation.** (A) The bacterial clones' defence, i.e. ability to sustain biomass, and (B) ability to form biofilm in the presence of predators within the 'predator present' and 'predator absent' treatments. White bars denote white, and grey bars denote red bacterial clones. Error bars denote 2 s.e.m.
doi:10.1371/journal.pone.0043801.g001

the solution using a Hamilton syringe. Infected larvae were placed individually on empty Petri dishes and their survival was monitored at three to twelve-hour intervals for six days at 25°C. The larvae were infected during four consecutive days in constant conditions. Injection day had no effect on survival (Kaplan-Meyer survival analysis, log-rank statistics, $\chi^2 = 0.349$, $p = 0.951$), and all the treatments were injected in random order.

### Statistical analyses

We used ANOVA (GLM) to explain variance in the dependent variables (life-history traits) with predation treatment (predator absent or predator present) and colony colour (red or white) as fixed factors, including predation×colony colour interaction. The effect of population identity was taken into account in the main analysis by nesting replicate selection lines within the predation treatments as random factors. Population divergence was further studied within both predation treatments separately by fitting the replicate selection line as a random factor into the model; significant population effect indicates divergence within the treatment with respect to the given trait [42]. Five outlier data points were excluded from motility analysis because of swarming behaviour [43].

Bacterial virulence was analysed as host survival with Kaplan-Meier survival analysis and log-rank statistics. For the ANOVA (see above) and for a genetic correlation analysis virulence was also compressed to a mean virulence value (per clone) by taking the inverse of the average time (h) that was required for a given clone to kill the host replicates (*Virulence* = 1/[mean time of death]). All the survived larvae were given a maximum survival value of 150 h.

Genetic correlations were first analysed at the level of bivariate correlations (Pearson's correlation coefficient between two traits measured from individual clones) within predation treatments (present or absent) by pooling clones together regardless of their population origin. These results were then contrasted with an analysis of covariance by (1) including the effect of population identity as a cofactor in the linear regression model (one trait explained with another) and (2), including both population identity and colony colour as cofactors.

Statistical analyses were performed with SPSS-software (v.20.0, SPSS Inc., Chicago, IL).

## Results

### Evolutionary changes in bacterial colony colour frequencies and life-history traits

Protist predation decreased the frequency of prodigiosin pigment-synthesizing clones in the experimental populations ($F_{1,\,6} = 43.7$, $p = 0.001$): only 32.4% of all the bacterial colonies were red after evolving in the presence of predators compared to 89.1% in the absence of predators.

White bacterial clones that had evolved in the presence of protists during the previous long-term selection experiment were able to sustain higher biomass in the presence of these predators than white clones that had evolved with the protists absent. However, the biomass of red clones did not depend on predation treatment. In other words, the effect of previous predation on the evolution of bacterial defence interacted with prodigiosin synthesis (Fig. 1A & Table 1). Similar interaction was also found in the formation of predation-resistant biofilm: white clones formed more biofilm than the red only if they had evolved in the presence of predators (Fig. 1B & Table 1). Consequently, the bacterium's ability to sustain biomass and form biofilm in the presence of predators correlated positively (Pearson's $r = 0.875$, $p<0.001$) due to the emergence of highly defensive white clones. Predation treatment, colony colour, or their interaction had no effect on bacterial maximum growth rate. Motility was only affected by the colony colour: red clones were more motile than white clones (Table 1).

Absence of predators led to more divergence between the replicate selection lines. Populations in the 'predator absent' treatment diverged in all measured traits except the formation of predation-resistant biofilm (defence, growth rate, motility and virulence, Table 1). Replicate populations within the 'predator present' treatment differed only in their maximum growth rate (Table 1).

### Evolutionary changes in virulence

Protist predation clearly reduced bacterial virulence, whereas the clones that had evolved in the absence of predators were intermediately virulent compared to the most virulent ancestral strain (Fig. 2A, main effect of selection line: $\chi^2 = 245.66$, $p<0.001$. For pairwise comparisons including the water control treatment,

**Table 1.** Effects of predation, pigment synthesis, predation×pigment synthesis and replicate population on trait means.

| | Predation: | | | Prodigiosin synthesis: | | | Predation×Prodigiosin: | | Pop[1], Predator absent: | | Pop[1], Predator present: | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | test values | significance | direction[2] | test values | significance | direction[2] | test values | significance | test values | significance | test values | significance |
| **Defence** | $F_{1, 6.4} = 8.1$ | **p = 0.027** | increase | $F_{1, 52} = 2.4$ | p = 0.130 | 0 | $F_{1, 52} = 11.5$, | **p = 0.001** | $F_{3, 27} = 3.4$, | **p = 0.032** | $F_{3, 27} = 1.6$ | p = 0.203 |
| **Biofilm** | $F_{1, 6.7} = 4.9$ | p = 0.064 | 0 | $F_{1, 54} = 10.7$ | p = 0.002 | decrease | $F_{1, 54} = 10.3$ | **p = 0.002** | $F_{3, 28} = 0.1$ | p = 0.940 | $F_{3, 28} = 1.0$ | p = 0.389 |
| **rmax** | $F_{1, 6.7} = 0.2$ | p = 0.696 | 0 | $F_{1, 54} = 0.5$ | p = 0.447 | 0 | $F_{1, 54} = 1.2$ | p = 0.272 | $F_{3, 28} = 85.0$ | **p<0.001** | $F_{3, 28} = 35.7$ | **p<0.001** |
| **Motility** | $F_{1, 6.2} = 0.1$ | p = 0.768 | 0 | $F_{1, 49} = 5.4$ | p = 0.025 | increase | $F_{1, 49} = 0.3$ | p = 0.573 | $F_{3, 27} = 14.1$ | **p<0.001** | $F_{1, 49} = 0.3$ | p = 0.573 |
| **Virulence** | $chi^2 = 10.7$ | **p = 0.001** | decrease | $chi^2 = 46.1$ | **p<0.001** | increase | **Table S2** | **Table S2** | $F_{3, 31} = 3.9$ | **p = 0.018** | $F_{3, 28} = 1.22$ | p = 0.318 |

[1]Pop describes the effect of replicate selection lines within the treatments (predator absent or predator present) on the trait means, i.e. the divergence of populations with identical selective environment.
[2]The direction (where applicable) means the direction of mean change of the 'predator present' treatment compared to the 'predator absent' treatment, or the direction of mean change of the red clones compared to the white clones. Null (0) denotes no difference between these treatments.
doi:10.1371/journal.pone.0043801.t001

see Table S1). Red clones were generally more virulent than white ones within both predation treatments ($\chi^2 = 46.120$, $p<0.001$, Table 1). White clones that had evolved in the presence of predators were the least virulent (pairwise comparison for the effect of colony colour within predation treatment: $\chi^2 = 6.80$, $p = 0.009$, Fig. 2B, Table S2).

Replicate selection lines diverged with respect to their virulence only within the 'predator absent' treatment (Table 1, in Fig. 2C&D).

### Life-history trait correlations

None of the bivariate correlations or linear regression models (ANCOVA) of life-history traits were significant within the 'predator absent' treatment (Table 2). In contrast, two significant correlations were found within the 'predator present' treatment: formation of predation-resistant biofilm correlated negatively with both maximum growth rate and virulence (Table 2). Population affected both correlations suggesting that the evolution of biofilm had different effects on maximum growth rate and virulence among replicate selection lines within the predation treatment (Table 2, Fig. 3A&B). However, including population identity in the linear regression model did not turn non-significant covariates significant, or vice versa (Table 2). Colony colour affected only the correlation between virulence and predation-resistant biofilm: adding both population identity and prodigiosin as cofactors into the regression model erased the negative correlation between predation-resistant biofilm and virulence, which suggests that variation in virulence was best explained by differences between the white and the red clones (Table 2, Fig. 3C&D).

## Discussion

We studied experimentally how protist selection affects bacterial defensive adaptations and virulence measured *in vivo*. We also studied which life-history traits are connected to anti-predator defence and virulence, and whether these are genetically correlated. Furthermore, we investigated whether evolutionary changes in life-history traits are consistent (parallel evolution) or different (divergent evolution) among replicate populations: more population divergence would indicate relaxed selection.

Our results show that protist predation increased the frequency of non-pigmented (white) *S. marcescens* clones that were more defensive but less virulent compared to the ancestral-like red clones. Bacterial defence, i.e. the ability to sustain high biomass in the presence of a predator, was mechanistically connected to the formation of predation-resistant biofilm, which protects several bacterial species from various protist predators [11,14,44]. Adaptation to predation with biofilm formation led to negative correlation with both maximum growth rate and virulence. Reduced competitive ability (i.e. lowered growth rate) can lead to less efficient host exploitation and hence decreased virulence [2,33] but we did not find correlation between virulence and growth rate: maximum growth rate did not explain variation in virulence (Table 2). Therefore, even though anti-predator defence incurred a clear fitness cost in terms of reduced growth rate (Fig. 3 A, Table 2), it was not directly related to virulence. One explanation for this is that growth rate in a plant-based culture medium is a poor proxy for *S. marcescens*' growth and virulence within an insect host. Contrary to previous findings that biofilm formation correlates positively with both bacterial virulence and anti-predator defence [44,45], we found a negative genetic correlation between these traits: predator-induced increase in the formation of biofilm decreased virulence (Table 2., Fig. 3).

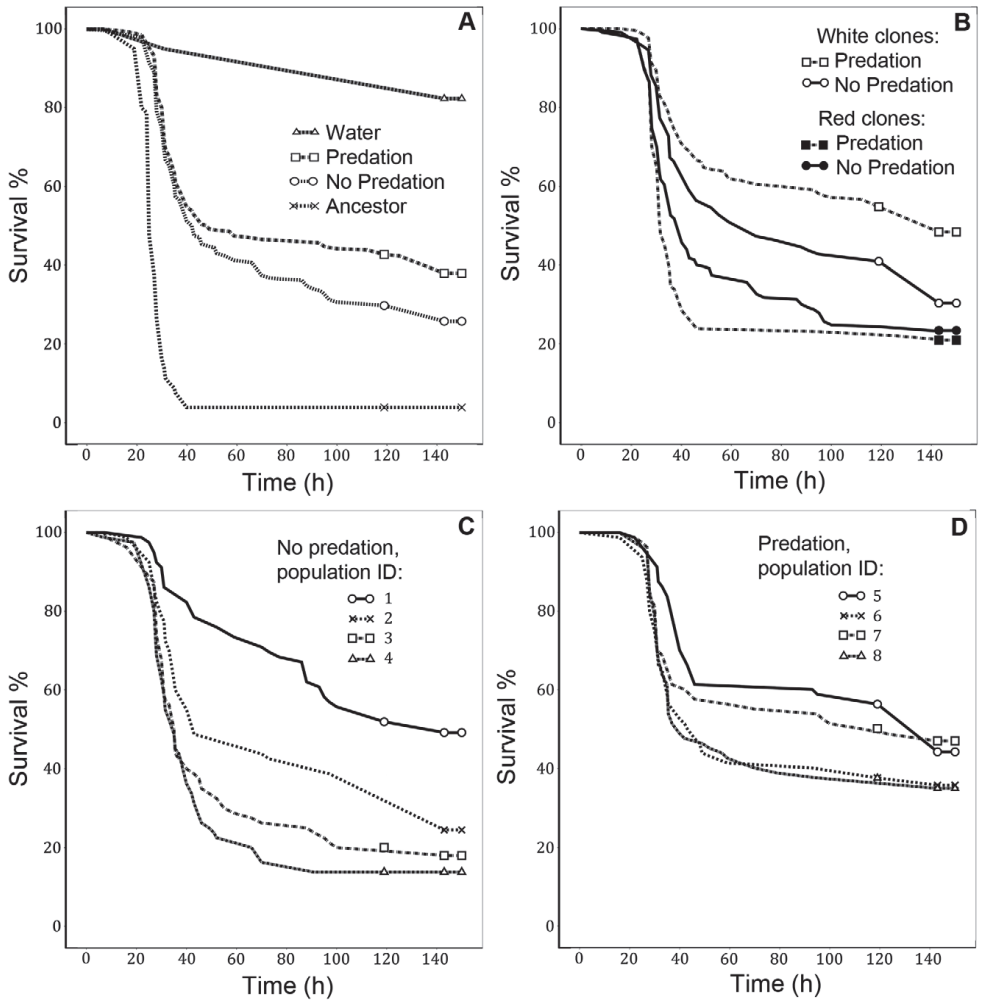


**Figure 2. Survival of *G. mellonella* larvae when infected with different bacterial clones.** Larvae infected with (A) clones from the 'predator present' and 'predator absent' treatments, the ancestral clone, and the water control, with (B) white and red clones from the 'predator present' and 'predator absent' treatments, and with (C & D) the clones from different replicate populations of the 'predator present' and 'predator absent' treatments.
doi:10.1371/journal.pone.0043801.g002

This decrease in virulence was best explained by the loss of prodigiosin synthesis although clinical *S. marcescens* isolates have been primarily found to be non-pigmented [29]: white clones were consistently less virulent regardless of their defensive ability, whereas red clones were consistently poorly defended and more virulent than white ones (Fig. 1 & 2B). Because prodigiosin is toxic for some eukaryotic cells [45], and prodigiosin expression and virulence can be regulated pleiotropically [46–47], it is possible that the loss of pigmentation directly reduced *S. marcescens* virulence in the wax moth host. Alternatively, prodigiosin expression could be tightly linked with some other important virulence factor such as protease production [48].

Motility had no clear effects on virulence (Table 2), even though it has previously been linked to *S. marcescens* virulence [13–14] and bacterial pathogenicity in general [49–52]. Measuring motility at the level of populations (clone mixes) can be confounded by intraspecific interactions between clones (e.g. competition, cooperation and cheating). It is known that bacterial motility can increase when cells cooperate in producing surfactants that turn the microenvironment more suitable for moving [53]. Bacteria

**Table 2.** Pairwise correlations and covariance analyses with population and pigment synthesis fitted as cofactors.

| **Predator absent:** | **Pearson correlation:** | | **Linear regression with population as a covariate:** | | | | **Linear regression with population and prodigiosin as covariates:** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *r* | *P* | *R2* | *P* | *Covariate* | *Pop*[1] | *R2* | *P* | *Covariate* | *Pop*[1] | *Prod*[2] |
| Virulence-Motility | −0.226 | 0.222 | 0.136 | 0.129 | 0.912 | 0.108 | 0.155 | 0.202 | 0.895 | 0.151 | 0.45 |
| Virulence-rmax | −0.334 | 0.062 | 0.157 | 0.083 | 0.954 | 0.219 | 0.18 | 0.129 | 0.995 | 0.207 | 0.382 |
| Virulence-BF | 0.007 | 0.972 | 0.159 | 0.081 | 0.823 | **0.026** | 0.183 | 0.125 | 0.788 | **0.029** | 0.374 |
| rmax-BF | 0.138 | 0.972 | 0.69 | **0.001** | 0.49 | **0.001** | 0.692 | **0.001** | 0.51 | **0.001** | 0.725 |
| Motility-BF | −0.008 | 0.966 | 0.427 | **0.001** | 0.856 | **0.001** | 0.483 | **0.001** | 0.849 | **0.001** | 0.101 |
| **Predator present:** | | | | | | | | | | | |
| | *r* | *P* | *R2* | *P* | *Covariate* | *Pop*[1] | *R2* | *P* | *Covariate* | *Pop*[1] | *Prod*[2] |
| Virulence-Motility | 0.19 | 0.333 | 0.21 | 0.053 | 0.085 | **0.027** | 0.437 | **0.003** | 0.39 | **0.024** | **0.005** |
| Virulence-rmax | −0.243 | 0.18 | 0.116 | 0.167 | 0.842 | 0.182 | 0.373 | **0.004** | 0.901 | 0.223 | **0.002** |
| Virulence-BF | −0.457 | **0.009** | 0.352 | **0.002** | **0.003** | **0.017** | 0.446 | **0.001** | 0.064 | **0.018** | **0.037** |
| rmax-BF | −0.366 | **0.039** | 0.83 | **0.001** | **0.006** | **0.001** | 0.843 | **0.001** | **0.005** | **0.001** | 0.356 |
| Motility-BF | −0.266 | 0.171 | 0.172 | 0.094 | 0.189 | 0.093 | 0.223 | 0.103 | 0.685 | 0.087 | 0.221 |

Pairwise (Pearson) correlations of trait pairs, and covariance analyses (linear regression) with replicate selection line, and replicate selection line with colony colour fitted as covariates. The first trait of the trait pairs is the dependent variable in the regression analyses. Significant values (>0.05) are highlighted.

[1]Effect of replicate selection line, i.e. population identity.

[2]Effect of colony colour, i.e. prodigiosin synthesis.

doi:10.1371/journal.pone.0043801.t002

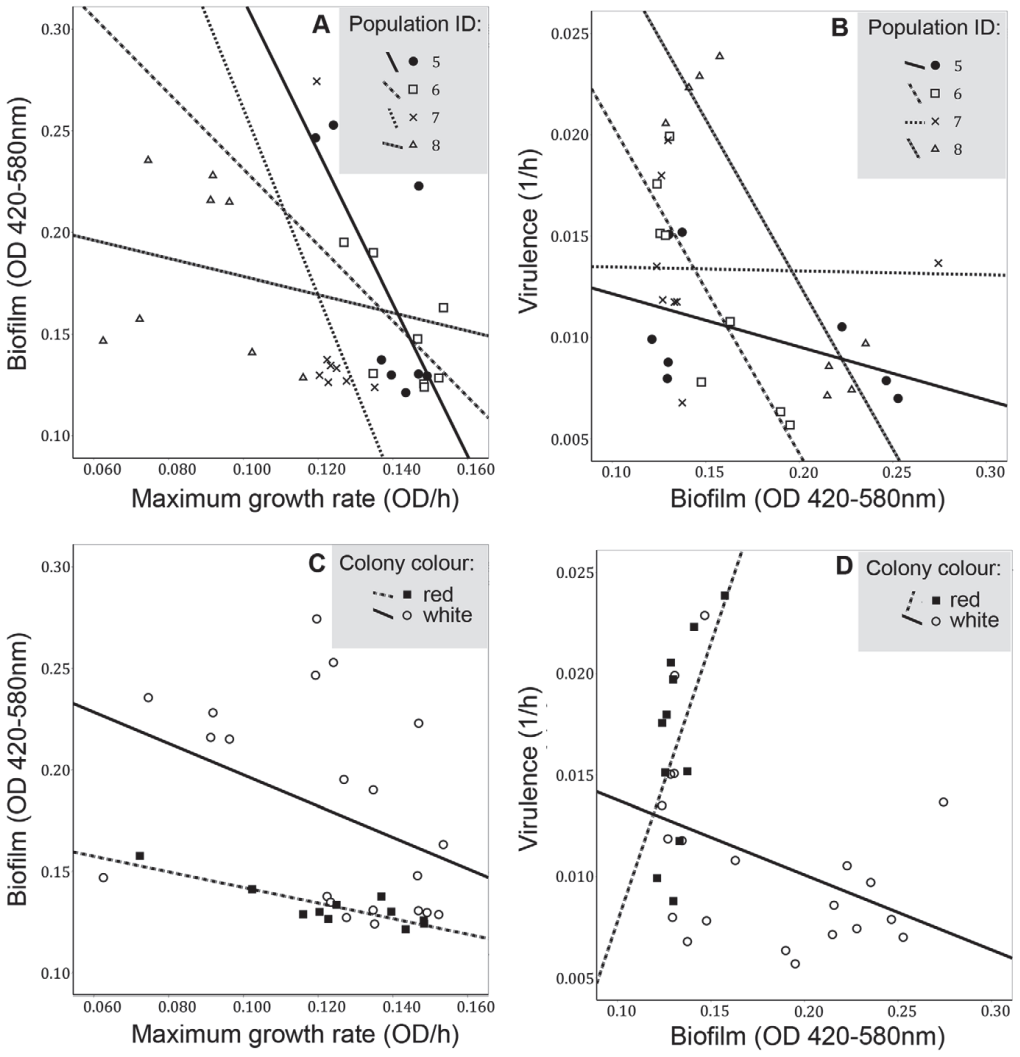


**Figure 3. Bacterial life-history trait correlations within predation treatments.** Population-level (replicate selection lines numbered 5–8) correlations between (A) predation-resistant biofilm and maximum growth rate, and (B) virulence and predation-resistant biofilm. Correlations between (C) predation-resistant biofilm and maximum growth rate by colony colour, and (D) virulence and predation-resistant biofilm by colony colour.
doi:10.1371/journal.pone.0043801.g003

might, however, cooperate less and consequently move less when relatedness of the population decreases, i.e., population becomes more diverse due to the emergence of cheating genotypes [30,54]. This kind of social conflict could explain why motility was observed to decrease with virulence in a previous experiment where bacterial traits were measured at population level (multiple clones interacting) [13], whereas we found no difference in this experiment because relatedness was the highest possible (motility measured at the level of individual genotypes).

Somewhat surprisingly, virulence decreased also in some of the replicate selection lines within the 'predator absent' treatment. This decrease was accompanied with more divergent evolution also in the other life-history traits measured. In contrast, the replicate selection lines evolved in a more parallel manner within the 'predator present' treatment (Table 1, Fig. 2C&B). The

populations within both treatments were originally generated from the same individual, and conditions were kept constant throughout the experiment. Therefore, all genetic variation within and between replicate selection lines arose initially from *de novo* mutations. Mutation accumulation can deteriorate bacterial traits randomly if the traits do not affect fitness in a given environment [25,42,55–59]. Our results are consistent with this, demonstrating that selection for bacterial traits was relaxed in the absence of predation. As a result, also virulence changed in some of the replicate selection lines probably due to random accumulation of mutations affecting unused virulence traits [25–26,42]. While it has been demonstrated that bacterial virulence genes can evolve parallel between different hosts (i.e. replicate within-host populations) [60], our study shows that selection in the outside-host environment can also lead to parallel evolutionary changes in virulence.

In conclusion, our results show that virulence can decrease in an opportunistic bacterial pathogen if it is traded off with anti-predator adaptations, but also if randomly accumulating mutations impair virulence traits under relaxed selection. These results seem to contradict studies according to which protist predation selects for increased virulence in opportunistic bacteria [11–13,61]. However, classifying opportunistic pathogens broadly to one category is an over-simplification [6]. Selection by protists (or by any other agent) is most likely highly case-specific and can thus lead to a positive or a negative correlation with virulence depending on the species and traits under selection (7). For example, amoebal predation can prepare an intracellular pathogen *Legionella* to resist human macrophages, whereas ciliate predation decreases *Serratia* virulence via a trade-off between prodigiosin and biofilm. Negative life-history correlations are likely to be especially important with environmentally transmitted bacteria that regularly encounter conflicting selection pressures in within-host and outside-host environments [8]. For example, two different variants of *Burkholderia ambifaria* cystic fibrosis isolates have superior fitness either in plant rhizosphere or the lungs of cystic fibrosis patients [62]. If these kind of trade-offs between pathogenic and environmental life-history strategies are common, they could partly explain why opportunistic bacteria found from the environment are seldom highly virulent, and how polymorphism in virulence traits is maintained in environmentally transmitted pathogen populations.

## Supporting Information

**Table S1** Pairwise comparisons of host survival when injected with clones from predator absent or predator present treatments, with ancestor clone, or with water.
(PDF)

**Table S2** Pairwise comparisons of host survival when injected with red and white clones from both predator absent and predator present treatments.
(PDF)

## Acknowledgments

The authors thank T. Ketola, C. Lindstedt and three anonymous reviewers for fruitful comments, and K. Viipale for conceptual help.

## Author Contributions

Conceived and designed the experiments: LM VF JL. Performed the experiments: LM VF. Analyzed the data: LM VF. Contributed reagents/materials/analysis tools: JL. Wrote the paper: LM VF JL.

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# II

## WITHIN-HOST EVOLUTION DECREASES VIRULENCE IN AN OPPORTUNISTIC BACTERIAL PATHOGEN

by

Lauri Mikonranta, Johanna Mappes, Jouni Laakso & Tarmo Ketola 2014

Submitted manuscript

# III

## INSECT IMMUNITY: ORAL EXPOSURE TO A BACTERIAL PATHOGEN ELICITS FREE RADICAL RESPONSE AND PROTECTS FROM A RECURRING INFECTION

by

Lauri Mikonranta, Johanna Mappes, Minna Kaukoniitty & Dalial Freitak 2014

Frontiers in Zoology 11: 23

Mikonranta et al. Frontiers in Zoology 2014, 11:23
http://www.frontiersinzoology.com/content/11/1/23

FRONTIERS IN ZOOLOGY

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# Insect immunity: oral exposure to a bacterial pathogen elicits free radical response and protects from a recurring infection

Lauri Mikonranta[1*], Johanna Mappes[1], Minna Kaukoniitty[1] and Dalial Freitak[1,2]

## Abstract

**Background:** Previous exposure to a pathogen can help organisms cope with recurring infection. This is widely recognised in vertebrates, but increasing occasions are also being reported in invertebrates where this phenomenon is referred to as immune priming. However, the mechanisms that allow acquired pathogen resistance in insects remain largely unknown.

**Results:** We studied the priming of bacterial resistance in the larvae of the tiger moth, *Parasemia plantaginis* using two gram-negative bacteria, a pathogenic *Serratia marcescens* and a non-pathogenic control, *Escherichia coli*. A sublethal oral dose of *S. marcescens* provided the larvae with effective protection against an otherwise lethal septic infection with the same pathogen five days later. At the same time, we assessed three anti-bacterial defence mechanisms from the larvae that had been primarily exposed to the bacteria via contaminated host plant. Results showed that *S. marcescens* had induced a higher amount of reactive oxygen species (ROS) in the larval haemolymph, possibly protecting the host from the recurring infection.

**Conclusions:** Our study supports the growing evidence of immune priming in insects. It shows that activation of the protective mechanism requires a specific induction, rather than a sheer exposure to any gram-negative bacteria. The findings indicate that systemic pathogen recognition happens via the gut, and suggest that persistent loitering of immune elicitors or anti-microbial molecules are a possible mechanism for the observed prophylaxis. The self-harming effects of ROS molecules are well known, which indicates a potential cost of increased resistance. Together these findings could have important implications on the ecological and epidemiological processes affecting insect and pathogen populations.

**Keywords:** Bacterial resistance, Gram-negative, Immune priming, Immunological loitering, Insect immunity, Reactive oxygen species, *Parasemia plantaginis*, *Serratia marcescens*

## Introduction

Recurring infections are common in the natural environment. Antibody based immunological memory has evolved in jawed vertebrates to cope with the threat of multiple infections. Invertebrates, being relatively short lived, lack antibodies [1]. However, evidence of insects being protected from pathogens they have previously encountered, has accumulated during the past decade e.g. [2-6]. The phenomenon has been coined as immune priming, and advances in insect immunity have shown that the innate and adaptive systems might be functionally closer to each other than previously thought [7,8].

Development, upregulation, and long-term maintenance of the innate immunity come with fitness costs that can be seen in various life-history traits [2,9-12]. A balance between the costs and the benefits of defences must give a selective advantage to individuals that have the optimal level of protection against the pathogens they are likely to encounter [3,13,14]. The protection could be achieved by a mechanism that allows enhanced reactivation of certain immune defences if the host faces a recurring infection, akin to vertebrate immune memory [4]. Alternatively, it might be beneficial to simply stay prepared for a recurring

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immune insult after the first encounter with immune elicitors or anti-microbial molecules that can remain stably expressed in the haemolymph [1-3]. The first encounter would serve as a cue for a threat of infection and upregulate the appropriate repertoire of defensive molecules [2,3,13]. This kind of 'immunological loitering' [3,15] might be considered as just a coincidental side effect of the primary pathogen detection, but we argue that there are reasons to assume that it is an adaptive trait. If non-infective pathogens can be detected before they become infective, the beneficial effect would be similar to density dependent prophylaxis, [16] where higher density of conspecifics indicates a higher risk of parasite encounter. There is ample evidence that many insects can maintain high levels of various immune molecules in their haemolymph for up to 44 days after immune induction [17-24]. Thus, taking the costs into account, it is hard to believe that this kind of prolonged immune reaction could have evolved without fitness benefits [2].

The anti-microbial mechanisms that insects use immediately when infections occur are relatively well known [25]. The detection of invading bacteria by gram-negative binding protein and peptidoglycan recognition protein leads to the activation of Imd and Toll signalling pathways that induce humoral and cellular responses, providing insects with coarse immunological specificity. These pathways can induce the release of bactericidal reactive oxygen species (ROS), different anti-microbial peptides and specialised haemocytes that also control melanisation and phenoloxidase (PO) activity [7,25-28]. At the same time, both PO and ROS related responses are considered to have high costs as they are accompanied with autoimmune effects [29,30]. Although some good explanations, like phagocytosis, controlled by the Toll pathway [7,31,32] have been proposed, the mechanisms behind priming against an infection occurring later in life, or even in subsequent generations, remain largely unknown [4,31,33].

In this paper we report how midgut mediated immune priming occurs in wood tiger moth *Parasemia plantaginis* (Linnaeus 1758) larvae against an environmental opportunistic bacterial pathogen. We primed the larvae, by exposing them orally to a non-infective dose of pathogenic *Serratia marcescens* and to a similarly gram-negative but non-pathogenic control bacterium *Escherichia coli*. We then assessed the consequences of primary oral encounter with the bacteria in two ways: first, indirectly by measuring the level of immunocompetence (PO, lytic, and ROS activity) from the larval haemolymph five days after the initial oral exposure, and then directly by measuring the survival after a severe secondary septic infection. A sublethal oral dose of *S. marcescens* provided the larvae with resistance against an otherwise lethal septic infection but the non-pathogenic control bacterium failed to confer protection. Priming with *S. marcescens* also induced a higher amount of ROS in the larval haemolymph, an antimicrobial defence that persisted until the secondary infection. This finding offers a potential, novel mechanistic explanation for acquired resistance in insects. Additionally, the activation of the protective mechanism seems to require more specific induction than a sheer exposure to any gram-negative bacteria, suggesting systemic pathogen recognition via midgut.

## Results

Larval survival was significantly affected by the interaction between priming (1st exposure) and injection (2nd exposure) treatments (priming, df = 1 Wald = 1.1, p = 0.290; injection, df = 1, Wald = 64.3, p < 0.001; priming × injection, df = 1, Wald = 15.1, p < 0.001). This indicated that larvae survived the injection differently depending on the previous oral priming. The four priming-injection groups (df = 3) were further compared using pairwise Kaplan-Meier log-rank statistics (Table 1). Larvae injected with the control bacterium showed very low mortality and did not differ from each other regardless of the priming (*Serratia-control*: 13.8% mortality and *control-control*: 9.1% mortality). Larvae injected with the pathogenic *S. marcescens* experienced only moderate mortality if they had been previously primed with it (*Serratia-Serratia*: 37.4%), but very high mortality if primed with the control (*control-Serratia*: 90.4%) (Figure 1). There was altogether less than 5% background mortality among the larvae during the priming and no difference between the groups (data not shown).

Larvae that were primarily exposed to *S. marcescens* had 4.8% higher ROS concentration in their haemolymph compared to priming with the control (df = 21, t = −2.43, p = 0.026; Figure 2a). The lytic activity and PO activity did not differ between the two treatments (Lytic: df = 20, U = 54.50, p = 0.679; PO: df = 21, t = 0.17, p = 0.987; Figure 2b & c).

## Discussion

Here we show that a previous oral exposure to *S. marcescens* protects *P. plantaginis* larvae from an otherwise lethal septic infection with the same pathogen. As a response to the priming with *S. marcescens* the moth larvae also

**Table 1 Pairwise differences in larval mortality between the priming-injection treatments**

| Priming-injection | *Serratia-control* | | *control-Serratia* | | *control-control* | |
|---|---|---|---|---|---|---|
| | $\chi^2$ | Sig. | $\chi^2$ | Sig. | $\chi^2$ | Sig. |
| *Serratia-Serratia* | 12.641 | **<0.001** | 64.92 | **<0.001** | 19.72 | **<0.001** |
| *Serratia-control* | | | 115.38 | **<0.001** | 1.07 | 0.300 |
| *control-Serratia* | | | | | 130.57 | **<0.001** |

The larvae were primed orally with either a non-infective dose of pathogenic *Serratia marcescens* or a non-pathogenic control bacterium *Escherichia coli*, and five days later injected with the same or different bacteria.
Statistically significant pairwise differences are bolded.

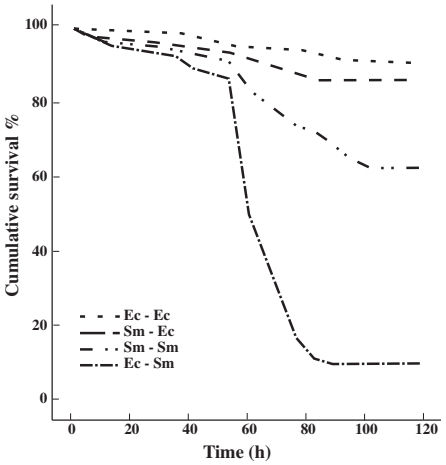


**Figure 1 Larval survival after septic injury.** The four survival curves present different priming-injection groups, where the first term is bacteria used in the priming, and second term bacteria used in septic infection 120 h later. Ec stands for the control bacterium, *E. coli*, and Sm for *S. marcescens*. Treatments from the least to the most virulent combination: Ec-Ec < Sm-Ec < Sm-Sm < Ec-Sm.

showed elevated levels of reactive oxygen species in their haemolymph five days post-treatment, offering a potential explanation for the protection. The elevated ROS levels were measured prior to the secondary immune challenge, which suggests that the mechanism for the prophylaxis could be due to immunological loitering rather than enhanced capacity to re-upregulate immune defences. This is in agreement with earlier studies, which show that different immune molecules can remain in the hemolymph days or even weeks after the immune challenge [22]. It might be that this simple kind of acquired resistance is more prevalent in short lived insects than generally acknowledged, and serves as a natural "vaccination" if pathogens are first detected in sub-lethal doses. The results do not rule out the possibility that insects could, after down regulation, reactivate a stronger immune reaction against a pathogen they have already encountered, which would be functionally more analogous to immunological memory than a simple persistent immune response. It is, for example, possible that immunological loitering and more "memory-like" functions act in concert to fight recurring infections [1,4]. It could also be that some other molecules, such as antimicrobial peptides Cecropins or Gloverin, that we did not measure are up regulated with ROS and that the persistent protection is not solely due to oxidative defence [28].

*S. marcescens* is very common in the environment, e.g. soil, water and plants, and is often isolated from many insect species across various taxa [34,35]. Thus, it is likely that *P. plantaginis* falls within *S. marcescens*' natural host range and real life encounters via contaminated host plants are possible in the wild. It has been shown that a septic infection with *Serratia* in insects can occur in the wild via, for example, an ovipositor of Hymenopteran parasite [36], a nematode vector [37], or a spontaneous gut rupture [34]. It would be of great benefit for the host

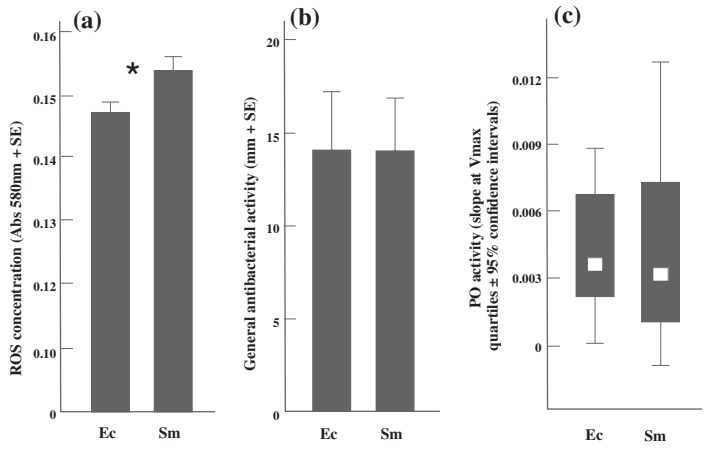


**Figure 2 The immune assays from differently primed larvae 120 h after the initial exposure.** Ec stands for the control bacterium, *E. coli*, and Sm for *S. marcescens*. Asterisk (*) indicates a significant (<0.05) difference between the oral priming treatments. The amount of ROS in the haemolymph **(a)**, the lytic activity **(b)** and the PO activity **(c)**.

to be prepared in advance for such a sudden and intensive immune insult.

We detected higher levels of ROS from the hemolymph of *S. marcescens*-exposed larvae compared to the control group fed with the non-pathogenic *E. coli*. This is a well-known anti-microbial defence mechanism in insects [25], and could have mediated the higher survival when the larvae were infected again with the pathogen. However, the ROS defence alone might not be sufficient to control large doses of *S. marcescens* in septic injury. This is because the bacterium is known to be fairly tolerant against oxidative stress via production of cellular catalases [38]. In addition, ROS are usually thought to control gut microbiota, and might not directly protect against injected pathogens [28,39]. The increased levels of ROS in our study, however, were measured straight from the haemolymph sample. Thus, higher levels of ROS may initially help keep the septic infection under control until other aspects of immunity can diminish it; and/or ROS mediates the regulation of other antimicrobials, such as Diptericin in Drosophila [40].

Interestingly, we did not find correlations between the measured immune traits although several previous studies suggest negative genotypic and phenotypic correlations between different defences [41-43]. For example, encapsulation and lytic activity, which might be targeted against different invaders, have been shown to correlate negatively in a field cricket, *Gryllus bimaculatus* [44]. Two major immunocompetence measures, PO and lytic activity, have been criticized by claiming that these indicators do not predict resistance against a challenge with natural parasites [45,46]. Our findings show ROS being upregulated in the pathogen challenged group, whereas PO and lytic activity show no change. However, the upregulation of different immune pathways are most likely pathogen and host specific. Indeed, it has been shown that *Daphnia magna* with higher induced PO levels are more resistant to their parasite *Pasteuria ramosa* [24]. Our observation of the lack of negative relationships within the immune system traits does not mean that the ROS response would be trade-off free. The major cost of resistance, in this case, could result from the non-specific nature of ROS molecules that are well known to cause self-harm and early senescence [30,40,47]. This could mean that prolonged exposure to the free radicals in the haemolymph requires additional resources to deal with potential tissue damage. Also, pathogen induced persistent immune reactions could have adverse effects on the native gut flora, indirectly contributing to fitness consequences [48].

Given the obvious costs, hosts should avoid unnecessary upregulation of immune responses. Imd-pathway mediated immune defence is often thought to be activated by the presence of peptidoglycan fragments from any gram-negative bacteria [25]. Nehme et al. [27] also proposed this to be the case between Drosophila and *S. marcescens* in septic infection. However, even the highly virulent *S. marcescens* db11-strain did not elicit an immune response via the oral route in that particular system. The defence observed in this paper must have been triggered by a more specific mechanism than a general response to the presence of gram-negative bacteria in the gut because priming with the control bacterium failed to confer the protection. Both bacteria exhibit DAP-type peptidoglycan in their cell wall, which has been shown to activate the Imd-pathway, in contrast to Lys-type found in gram-positives and the Bacillus group [49]. It has been suggested that in Drosophila larvae, haemocytes in the gut can signal pathogen presence to the fat body, via cytokines or by releasing ingested cell wall fragments [50,51]. It is thus possible that *P. plantaginis* haemocytes can recognize potentially harmful *S. marcescens* and ignore benign bacteria. Also, bacterial immune elicitors (e.g. peptidoglycan or lipopolysaccharides) are known to bind to a storage and transport protein vitellogenin in fish [52,53]. It is also a very abundant protein in the insect hemolymph [54] and, interestingly, has antioxidative capabilities protecting organisms against free radical stress [55]. In another lepidopteran, *Manduca sexta*, direct inoculation of *E. coli* in the haemocoel has been shown to offer resistance against *Photorhabdus luminescens* via upregulation of pattern recognition proteins [56]. The seemingly contradictory results with our experiment probably stem from the priming method: if introduced orally, *E. coli* most likely does not penetrate the gut epithelium, nor it is beneficial for the host to actively present antigens from a non-pathogenic bacteria to the fat body or haemolymph [50,51]. In our study, *S. marcescens*, but not *E. coli*, offered the protection and elicited the systemic ROS response in the haemocoel when detected in the gut. The different result with septic first exposure thus provides further support for the intestinal recognition of pathogenic and non-pathogenic bacteria and for the immune systems ability to mount a corresponding systemic defence. Another alternative explanation is that our non-pathogenic control bacterium appears in the gut in quantities that do not exceed the detection threshold of the recognition proteins, compared to *S. marcescens* that might still proliferate in the gut even when being avirulent [27]. Regardless of the mechanism, a harmless encounter with a pathogen via gut transfers into a systemic immune reaction that protects *P. plantaginis* larvae when substantial amount of the same pathogen is introduced straight into haemocoel later in life.

## Conclusions

A lepidopteran species, although having a relatively short life span, remains protected against a previously encountered pathogen, possibly because of persistent

immune responses involving free radicals. The findings evoke interesting questions on the evolutionary and epidemiological consequences that priming might have in insect populations, through increased resistance, and on the other hand, through increased costs due to oxidative stress. Although the ecological and evolutionary effects of priming are very hard to study at the population level in the wild, modelling suggests that it has evident consequences on both pathogen (disease prevalence) and host (demographic structure) population dynamics, as well as on the stability of host-parasite systems [57,58].

## Material and methods

### Study species

*P. plantaginis*, the wood tiger moth, is a day active moth distributed over the northern hemisphere. It has been extensively studied for its warning coloration [59-61]. Also, a few studies describe moth immunocompetence, and interaction between larvae and *S. marcescens*. For example, larval diet has been shown to have a substantial effect on the level of immune defence [62-64]. Larvae used in this experiment were obtained from a population originating from wild individuals caught in southern Finland and kept for three generations in the laboratory (see methods for rearing in [61]).

*S. marcescens* is a cosmopolite opportunistic pathogen that is commonly found in water and soil. It causes nosocomial infections in humans and has been isolated from various insect species [34,35,65]. The strain used in the experiment was obtained from American Type Culture Collection (ATCC# 13880). Laboratory adapted *E. coli* K-12 was used as the non-pathogenic control strain. The bacteria were maintained in standard LB-medium (10 g tryptone, 5 g yeast extract, 10 g NaCl in 1 L of $dH_2O$).

### Priming and injection

416 three-week-old moth larvae were weighed, after which they were randomised to the two primary exposure treatments (*S. marcescens*, N = 207 and *E. coli*, N = 209). *E. coli* was used as a control treatment instead of a completely naïve group because we wanted to see how the sheer presence of gram-negative bacteria in the diet would compare to the actual pathogen. It has been shown previously that in spite of being non-pathogenic, the presence of *E. coli* may induce a general immune reaction in insects e.g. [41, 66]. The larvae were placed individually on Petri dishes and reared at 21°C under a 15 hour light: 9 hour dark cycle. Larval weight did not differ between treatments ($df = 414$, $t = -0.6$, $p = 0.55$). Larvae were first fed with their natural diet, dandelion (*Taraxacum sp*), after which it was supplemented with the priming cultures. The bacterial mass was grown overnight on LB-agar plates in 31°C, scraped off with sterile loops and mixed to liquid LB. To standardise the amount of cells the mass was diluted until 0.50 optical density (OD) at 600 nm was reached. These dilutions were then added to the larval diet by pipetting a 200-μl droplet (approximately $6 \times 10^7$ cells) of the priming solution to each dandelion leaf surface. After 48 h, majority of the larvae had consumed all the contaminated food and they were given normal diet again. 120 h after the primary priming exposure larvae were infected by injecting 2 μl (OD 0.16, 90 000 *S. marcescens* and 110 000 *E. coli* cells) of bacteria (the previously encountered pathogen, or the control bacterium) directly into the body cavity. The injection was given behind the fifth proleg with a 10 μl Hamilton syringe. The larvae were kept under constant conditions with *ad libitum* food and survival was recorded every three hours. We took haemolymph samples from 15 random larvae (not included in the survival analysis) per priming treatment before the injection.

### Immune assays

PO and ROS activities were estimated from samples containing 10 μl of larval haemolymph diluted in 30 μl ice-cold potassium phosphate buffer which was then frozen at −80°C. For measurements, the samples were thawed and centrifuged (9000 g) at 4°C for 10 minutes to obtain the clear supernatant. For PO, 25 μl of supernatant was added to 200 μl of 3 mM L-Dopa (Sigma, #333786). Kinetic activity of the enzyme was measured at 30°C, 490 nm for 90 minutes (1 minute intervals) with Victor X4 2030 plate reader (Perkin Elmer, Waltham, MA, US). The slope of the absorbance curve from 10–80 minutes was used in the analyses [41].

Pierce PeroXOquant™ quantitative peroxide assay kit (Thermo Scientific, Waltham, MA, US #23280) was used to estimate the amount of ROS in the haemolymph: 5 μl of the supernatant was mixed with 90 μl of the manufacturers working solution. Eight dilutions (ranging from 1 to 1000 μM) of $H_2O_2$ were used as standards. The mix was left to stabilize at room temperature for 25 min after which absorbance was read with a Bioscreen™ spectrophotometer (Growth Curves Ltd., Helsinki, Finland) at 580 nm.

Lytic activity was assessed straight from the haemolymph samples by pipetting 5 μl of fresh haemolymph into a 2.2 mm diameter wells punctured on *Micrococcus* (ATCC #4698) agar plate, incubated over night in 31°C and then photographed. 7 serial dilutions (0.031 - 2.0 mg/mL) of lysozyme (Sigma, #L7651) were used as standards. Lytic activity was measured from the photo as the diameter of a degradation halo around the well [41].

### Statistical analyses

Larval survival was first analysed using Cox-regression with priming, injection, and their interaction in the model. The four priming-injection groups (*Serratia-control*, *control-control*, *Serratia-Serratia* and *control-Serratia*) were

then compared with pairwise Kaplan-Meyer survival analysis. PO activity and ROS were analysed with a t-test. Mann–Whitney U-test was used for lytic activity because of non-normal distribution. All analyses were performed with SPSS statistics 21.0.

#### Competing interests
The authors declare that they have no competing interests.

#### Authors' contributions
LM, JM, MK, and DF designed the experiment and carried out the experimental work. LM and DF analyzed the data. LM, JM, and DF wrote the manuscript. All authors read and approved the final manuscript.

#### Acknowledgements
The authors thank Kaisa Suisto & Jimi Kirvesoja for their help with the experiment, Tarmo Ketola, Kalevi Viipale and two anonymous reviewers for comments, and Swanne Gordon & Samuel Waldron for improving the language. Finnish Cultural Foundation (LM), Vanamo Foundation (MK), The Academy of Finland (project 1268, JM), and the Finnish Centre of Excellence in Biological Interactions (DF, MK, JM and LM) funded the study.

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Received: 13 February 2014 Accepted: 3 March 2014
Published: 7 March 2014

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doi:10.1186/1742-9994-11-23
**Cite this article as:** Mikonranta *et al.*: Insect immunity: oral exposure to a bacterial pathogen elicits free radical response and protects from a recurring infection. *Frontiers in Zoology* 2014 **11**:23.

# IV

## ATTACK AND DEFENCE: DIVERSE LIFE-HISTORY STRATEGIES BETWEEN BACTERIAL PATHOGENS AND LEPIDOPTERAN HOSTS

by

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