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Lepidopteran species have a variety of defence strategies against bacterial infections

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ABSTRACT

The insect immune system has versatile ways of coping with microbial insults. Currently, innate immune priming has been described in several invertebrates, and the first insights into its mechanistic basis have been described. Here we studied infections with two different strains of *Serratia marcescens* bacteria in two different Lepidopteran hosts. The results reveal fundamental differences between the two hosts, a well-known model organism *Galleria mellonella* and a non-model species *Arctia plantaginis.* They differ in their strategies for resisting oral infections; priming their defences against a recurring sepsis; and upregulating immunity related genes as a response to the specific pathogen strains. The two bacterial strains (an environmental isolate and an entomopathogenic isolate) differ in their virulence, use of extracellular proteases, survival in the larval gut, and in the immune response they evoke in the hosts. This study explores the potential mechanistic explanations for both host and pathogen specific characters that significantly affect the outcome of Gram-negative bacterial infection in Lepidopteran larvae. The results highlight the need to pay greater attention to the differences between model and non-model hosts, and closely related pathogen strains, in immunological studies.

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Keywords: 6-Tox; bacterial infection; Cecropin; Defensin; extracellular protease; *Galleria mellonella*; gene expression; immune priming; immunity; *Arctia plantaginis*; resistance; *Serratia marcescens*; tolerance; virulence

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Abbreviations[1]

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[1] AMPs = Antimicrobial peptides, PGRPs = Peptidoglycan recognition proteins, PO = Phenoloxidase, OD = Optical density, ROS = Reactive oxygen species, *ENV* = *S. marcescens* ssp. *marcescens* Bizio, *ENT* = *S. marcescens* ssp. *marcescens* db11

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1. INTRODUCTION

Pathogens are one of the most significant factors causing mortality in the wild, and hosts have evolved a range of defence mechanisms against them. Insects and entomopathogenic bacteria have been widely studied in this context, mostly due to their importance for agriculture and medicine (Rolff & Reynolds 2009). Recent research shows that invertebrate defences against pathogens are more complex and sophisticated than previously thought (Little & Kraaijeveld 2004, Criscitiello & de Figueiredo 2013). The innate immune system, consisting of humoral and cellular subdivisions, has also an adaptive aspect, which allows a more efficient response in the case of a recurring infection (Moret & Siva-Jothy 2003, Kurtz 2005, Sadd & Schmid-Hempel 2006). Mechanistic studies usually concentrate on model species such as *Drosophila melanogaster* and have been proven extremely valuable for understanding the regulation of host immunity in these species. Most studies, however, have utilized the same general pathogens as used in the well-known model host systems (Keebaugh & Schlenke 2014). Often laboratory-adapted hosts fed with artificial diet are also infected in a non-natural way, for example by direct injection of bacteria into the body cavity instead of oral infection (Rolff & Reynolds 2009). While these approaches have yielded tremendous benefits, alone it may bias our understanding of innate immunity. It may be that different strategies of pathogen resistance and tolerance have evolved (Råberg 2014), and that these differences have been overlooked.

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Lepidopterans, like other insects, activate their immune defence against microorganisms by recognizing the pathogen surface structure with an array of binding proteins. Antigen recognition then triggers a variety of responses such as antimicrobial peptide (AMP) production, activation of phagocytosis, phenoloxidase (PO) cascade, and release of reactive oxygen species (ROS) (Kanost *et al.* 2004). Entomopathogenic bacteria, on the other hand, have developed a variety of strategies that help them to counteract insect defences. Virulence factors such as proteases, lipases, haemolysins, and many toxins can help bacteria to persist in the gut, evade or suppress the immune system, or cause tissue damage in order to facilitate colonization of the host body (Ffrench-Constant *et al.* 2003, Vallet-Gely *et al.* 2008). Although invertebrates lack an anti-body based immunological memory, adaptive-like immune functions have been reported in several cases (Moret & Siva-Jothy 2003, Criscitiello & de Figueiredo 2013). The innate immune system seems to have an inducible dimension, immune priming, functionally analogous to the adaptive immune defence in vertebrates (Cristiciello & de Figueiredo 2013). Whether the observations of protection against a recurring infection are mainly due to a prolonged, persistent immune reaction that stays up regulated (Moret & Siva-Jothy 2003, Mikonranta *et al.* 2014), or an enhanced re-up regulation of defences during the secondary pathogen encounter still remains somewhat controversial (Sadd & Schmid-Hempel 2006). This makes studying a larger variety of hosts and pathogens even more important. Because some of the immune priming effects have been reported to be remarkably specific, it seems unlikely that simple "immunological

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loitering" alone could explain all these observations (Pham *et al.* 2007, Sun *et al.* 2011, McTaggart *et al.* 2012).

The majority of the studies in insect immunology use heat-killed, not necessarily pathogenic, bacterial cells or cell wall fragments injected directly into the haemolymph to elicit an immune reaction (Rolff & Reynolds 2009, but see e.g.: Freitak *et al.* 2007, 2009a, 2009b). The injection method is useful in studying certain aspects of immunity, for example the related signalling pathways and the synthesis of different AMPs, or pathogens that normally enter the host through wounding. However, this approach might miss aspects relating to gut borne infections, which is a serious drawback because invasion through the midgut is a prevalent route for bacterial infections in the wild (Rolff & Reynolds 2009). Also, the use of non-entomopathogenic bacteria does not take into account the specific adaptations that both insect hosts and their pathogens might have evolved (Rolff & Reynolds 2009). The current comprehensive knowledge on immune and stress-inducible genes in model hosts provides the possibility to study these responses in a more natural setting and identify the similarities, and differences, between several hosts and pathogens (Vogel *et al.* 2011).

We compared the immune reactions and survival of two Lepidopteran hosts: a well-established model species *Galleria mellonella* and a non-model species *Arctia plantaginis* in oral (1st challenge, i.e. priming) and septic (2nd challenge) bacterial infections. Both host species were infected with two different strains of *Serratia marcescens*: an entomopathogenic (*ENT*) and an environmental (*ENV*) isolate, after which bacterial persistence and proliferation was investigated in the larval gut. Our

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previous work on *A. plantaginis* revealed an immune priming phenomenon with *Serratia marcescens* (Mikonranta *et al.* 2014). For this study we added another strain of the pathogen and another host species, to further understand Lepidopteran immune defence against Gram-negative bacterial infection in general. Importantly, we also assessed the expression of several immunity related genes after the oral infections to shed light on the mechanistic basis of differences between the two hosts and the two pathogens. The hosts are genetically and ecologically distinct and thus likely to have developed divergent ways to cope with pathogens. We show how the two host species respond to the introduction of live bacteria through the midgut, and, that the differences between the pathogen strains reflect on the host responses, from gene expression level to direct fitness consequences. The study thus demonstrates significant differences between the two strains of *S. marcescens* in host exploitation capability and ability to overcome local and systemic immune defences. Furthermore, we show that the two hosts cope with the same type of infection differently. Differences in gene expression, between the hosts, and between the pathogen strains are discussed in relation to the survival of specific host-pathogen pairs. The results indicate that the variety of defence strategies between model and non-model hosts, and contrasting host responses to pathogen strains that are similar, cannot be neglected when generalizing findings in immunological studies.

2. MATERIALS AND METHODS

2.1. Study organisms

2.1.1. Hosts

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Wood tiger moth *A. plantaginis* is a day active Arctiid moth (formerly *Parasemia plantaginis,* Rönkä *et al.* 2016). Its larvae are generalists, and the species has a wide distribution range over the northern hemisphere (Leraut 2006). The larvae used in the experiment were from a stock initiated with wild caught adults from southern Finland. They were kept in the laboratory for three generations and fed with dandelion, plantago and lettuce leaves *ad libitum* (see further methods for rearing from Lindstedt *et al.* 2009). The greater wax moth (*G. mellonella*) is a specialist pest species parasitizing beehives, where the larvae feed on honeycombs (Smith 1960). The larvae were obtained from Suomen Eläintukku (Hyvinkää, Finland) and reared on a standard wax moth diet consisting of oat flakes, honey, milk powder and wheat flour (not autoclaved but made of standard grocery-grade ingredients). Because the diet was not sterile it is possible that differences in diets influenced the disease dynamics of the species. Both host species were in their penultimate larval instar during the experiment, *A. plantaginis* (N=900) weighing on average 30 mg and *G. mellonella* (N=800) 120 mg, respectively.

2.1.2 Pathogens

We used two strains of *Serratia marcescens*, which is a Gram-negative enterobacterium. It is an opportunistic pathogen that is transmitted via environment (e.g. soil or water) and is able to infect a large variety of organisms, for example plants, insects, fish, and mammals, including humans (Grimont and Grimont, 1978; Tan, 2002; Mahlen, 2011). *S. marcescens* ssp. *marcescens* db11 (from now on *ENT*) was isolated from *Drosophila melanogaster* (Flyg *et al*. 1980) and is a widely used model

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entomopathogen and was kindly donated by H. Schulenburg. The *S. marcescens* ssp. *marcescens* Bizio type strain (from now on *ENV* due to its environmental origin) is an environmental isolate from a fresh water source. The *ENV* strain (ATCC 13880) produces a distinctive red pigment, prodigiosin (Martinec & Kockur 1961). The non-pigmented *ENT* strain was originally isolated from a moribund fruit fly (Flyg *et al.*, 1980) and has been widely used as a model entomopathogen (e.g. Nehme *et al* 2007). Different strains of *S. marcescens* have been reported to be pathogenic in over 70 species of insects, including many Lepidopterans (Grimont *et al.* 1979). 52 shotgun sequences from *ENV* were identified as highly similar to *ENT* by NCBI's BLAST (BLASTN 2.2.30, megablast, Zhang *et al.* 2000). Aligned with the *ENT* whole genome (HG326223.1) they returned sequence similarities ranging from 93-99% with an average of 97% demonstrating the high relatedness of the strains. As a non-pathogenic ram-negative control bacterium, we used a standard laboratory-adapted *Eschericia coli* K-12 kindly donated by M. Jalasvuori. All the bacteria were cultivated in LB-medium (10 g tryptone, 5 g yeast extract, 10 g NaCl in 1 L of dH_2O) batch cultures and LB-agar (1%) in 31 °C.

2.2 Priming and septic injury experiments

The priming and injection methods used for *A. plantaginis* are described in Mikonranta *et al.* (2014), and survival data with the *ENV* strain and this host is originally from that experiment. Briefly, 898 penultimate instar larvae were reared individually in Petri dishes with the diet (4x4 cm Taraxacum leaf) mixed with 200 µl of 0.5 OD (optical density at 600 nm, c.a. 5×10^7 cfu/mL) bacterial cultures (*ENV*,

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ENT, or *E. coli*) for the 1st immune challenge. The amount of food not consumed by the larvae was considered negligible. The larvae were switched to uncontaminated food after 48 h. 72 h after that (i.e. 120h from the start), the larvae were injected (the 2nd immune challenge) with 2 µl of 0.16 OD (c.a. 2.75×10^8 cfu/ml) bacterial cultures, resulting in all nine possible priming/injection combinations. The survival was recorded at three-hour intervals. The sample sizes for the injected individuals (priming-injection) were as follows: *ENT-ENT*: 57; *ENT-ENV*: 56; *ENT-CTRL*: 57; *ENV-ENT*: 93; *ENV-ENV*: 91; *ENV-CTRL*: 94; *CTRL-ENT*: 91; *CTRL-ENV*: 94; *CTRL-CTRL*: 88. *G. mellonella* (N=584) was primed (200 µl of 0.5 OD bacterial solution mixed with 0.5g diet) and injected similarly, with the exception that the control priming solution was sterile LB-medium instead of *E. coli* culture (i.e. *ENV*, *ENT*, or LB for the 1st immune challenge). The sample sizes for the injected individuals (priming-injection) were as follows: *ENT-ENT*: 132; *ENT-ENV*: 65; *ENT-CTRL*: 50; *ENV-ENT*: 82; *ENV-ENV*: 38; *ENV-CTRL*: 47; *CTRL-ENT*: 52; *CTRL-ENV*: 28; *CTRL-CTRL*: 45. Note, however, that the control injection was still executed with *E. coli* and that the RNA and other immune measurement control samples were taken from *E. coli* primed individuals, making the immune measurements from the two hosts comparable. One reason for doing first oral introduction and then injection is that according to our previous experiments (Anttila *et al.* 2016) there is no sublethal septic dose for db11 in *G. mellonella*, and just a couple of cells seem to be enough for mortality to occur in both species. Also, even with high oral doses, the SMM strain does not seem to cause significant mortality in practical and purposeful timescales in *A. plantaginis* (Zhang *et al.* 2012). This enforces us to use injection as a secondary

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infection method. It is also supported by our earlier experiments (Mikonranta et al., 2014), where injection approved to be a good way to test the efficacy of oral priming.

2.3. Sampling

All the immune trait samples were taken from primed larvae (both species with *E. coli* as a control) at the time of the injections (i.e. 120 h after the start of the experiment). Haemolymph was taken aseptically with a pipette during dissection from 15 individuals per priming treatment from *A. plantaginis* and 10 individuals from *G. mellonella*. For the immune assays, 10 µl of haemolymph was diluted in 30 µl ice cold 1 × PBS buffer and 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 midgut RNA samples, 15 larvae per treatment were dissected and the midgut was preserved at −80 °C in 200 µl of TriSure (Bioline).

2.4. Within-host bacterial growth

To quantify the bacterial persistence in the hosts' midguts, ten guts per treatment were dissected, homogenised, and frozen in 200 µl of sterile glycerol. Serial dilutions of the gut homogenate were cultivated on *S. marcescens* –selective agar (42g deoxyribonuclease test agar with methyl green, 10g 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 1L of water, medium modified from Grimont & Grimont (1978)), and colony-forming units (CFU) were counted.

2.5. qRT-PCR for gene expression

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We determined the gene expression from the larval gut samples for known immune related signalling (e.g. PGRP) and effector (e.g. Cecropins, Defensin) molecules for which we had primers available in both host species. For RNA extraction the gut samples were thawed on ice, homogenized and the manufacturers (TriSure, Bioline, London, UK) protocol was followed. The RNA pellet was dissolved in 30 µl of RNA-storage solution (Ambion, Life Technologies, NY, US). Concentration of the samples was determined photospectrometically using NanoDrop (PeqLab, Erlangen, Germany), adjusted to 100 µg/µl and five of these samples were pooled together resulting in three pools per treatment. To avoid contamination with gDNA, DNA digest was performed using DNAse according to the manufactures protocol (Invitrogen, Waltham, MA, US). After the digest, RNA was further purified using RNA CleanUP Kit (Qiagen, Hilden, Germany) and the concentration was again determined using NanoDrop. 500 ng of DNA-free total RNA was converted into single-stranded DNA using oligo-dT20 primers using the RevertAid RT Kit (Fermentas, Waltham, MA, US). The qRT-PCR primers (Supplementary Table 1) were designed using online Primer3, an Internet based interface (http://www.ncbi.nlm.nih.gov/tools/primer-blast/, Untergasser *et al.* 2012). Primers were designed by the rules of highest maximum efficiency and sensitivity to avoid formation of self- and hetero-dimers, hairpins and self-complementary strands. Primers for *A. plantaginis* were designed based on the sequences from in-house EST library and RPS18 was added to serve as endogenous control (normalizer). *G. mellonella* primer sequences were taken from previously published papers or designed based on the EST sequences available in public databases (Freitak *et al.*

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2014; Vogel *et al.* 2011). Gene expression was determined using real-time PCR with a CFX96 rtPCR system (BioRad, Hercules, CA, US). 100 µg of total cDNA was used with the SensiFAST SYBR No-ROX kit (Bioline). The following real-time PCR conditions were used: 95°C for 15 min (denaturation), 40 times repeated cycle of 94 °C for 15 s, 55 °C for 30 s, and 70 °C for 30 s (amplification and quantification). All the samples were run as two technical replicates.

2.6. Phenoloxidase

15 µl of supernatant (see: 2.3.) was added to 100 µl of 3 mM L-Dopa (Sigma, #333786) on 96-well plates on ice. 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.

2.7. Reactive oxygen species

5 µl of the supernatant (see: 2.3.) was mixed with 90 µl of Pierce PeroXOquant™ (Thermo Scientific, Waltham, MA, US #23280) quantitative peroxide assay's working solution. 8 (from 1 to 1000 µM) H_2O_2 dilutions were used as standards. The mix was left to stabilize at room temperature for 25 min and absorbance was read with a Bioscreen™ spectrophotometer (Growth Curves Ltd., Helsinki, Finland) at 580 nm. Some samples were lost in one treatment due to which ROS data is missing from naive *A. plantaginis* larvae.

2.8. Lytic activity

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Lytic activity was assessed straight from the haemolymph samples by pipetting 5 µl of fresh haemolymph into 2.2 mm diameter wells punctured on Micrococcus (ATCC #4698) and *ENV* agar plates, incubated over night in 31°C and then photographed for growth inhibition halos. 7 serial dilutions (0.031 - 2.0 mg/mL) of lysozyme (Sigma, #L7651) were used as standards (Freitak *et al*. 2007).

2.9. Secretion of bacterial extracellular proteases

The bacterial strains (three clones from each of the strains) were grown overnight in individual cultures of LB (31 °C) after which the ODs were adjusted to 0.15 (c.a. 4.7×10^7 cfu/ml). A sterile loop (2 µl, VWR) was used to inoculate the cultures on the centre of a 1 % skimmed milk agar plates. The amount of proteases was quantified as a diameter (measured from photographs with a ruler tool in Adobe Photoshop CS5) of the casein degradation halo after 48 h in 31°C (Tran *et al.* 1993). Three independent replicates were used per bacterial strain.

2.10 Statistical analyses

Survival of the larvae of both host species was analyzed first with Cox regression, with oral exposure, injection, and their interaction in the model. In addition, pairwise comparisons between all the oral exposure/injection combinations were analyzed with Kaplan-Meier survival analysis. Differences in PO, ROS, and bacterial persistence in the gut were assessed with Kruskal-Wallis rank ANOVA. Bacterial protease activity was analyzed with ANOVA. Multiple comparisons were corrected for with the Holm-Bonferroni method. The $2^{-\Delta\Delta_{cT}}$ method was used for quantification of differences in gene expression (Livak & Schmittgen 2001), where the pathogen

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treatments were compared to the treatment with harmless control bacteria (*E. coli*). All the analyses were performed with SPSS statistics v. 21.0.

3. RESULTS

3.1. Host survival

A. plantaginis survival was significantly affected by the oral exposure (N=853, df=2, W=99.1, p<0.001), injection (df=2, W=110.9, p<0.001), and oral exposure × injection interaction (df=4, W=100.3 p<0.001). The *ENT* was already virulent in oral infection (Fig. 1 a., pairwise comparisons in Table 1.), and regardless of the priming killed the larvae faster than *ENV* in a septic injury. *E. coli* injection caused only minor mortality, excluding the effect by the previous *ENT* exposure (Fig. 1 b.). The interaction indicates that mortality after septic injury was significantly affected by the previous oral exposure. Oral *ENV* exposure primed the larvae to better withstand the *ENV* injection compared to the priming with the control *E.coli* or the virulent *ENT* (Fig. 1 b., pairwise comparisons between exposure / injection groups in Table 2.).

G. mellonella was completely resistant to the oral exposure with both pathogen strains, and the survival differences were solely due to the injection: *ENT* killed the larvae faster than the *ENV* strain regardless of priming (N=539, Oral exposure: df=2, W=0.2, p =0.9; injection: df=2, W=23.9, p<0.001; oral exposure × injection: df=4, W=2.4, p =0.7). *E. coli* was avirulent in septic injury. Survival curves are presented in Fig. 2 and pairwise comparisons between oral exposure / injection groups in Table 3.

3.2. Bacterial extracellular proteases

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The *ENT* strain produces more caseinolytic proteases compared to SSM and *E. coli* ($F_{2, 8}$=39, p<0.001; Multiple comparisons: *E. coli-ENV*: p =0.575; *E. coli-ENT*: p =0.001; *ENT-ENV*: p =0.001; Fig. 3 a).

3.3. Bacterial persistence in the gut

The *ENT* was able to persist and proliferate in *A. plantaginis* gut five days after the oral exposure, and there were also trace amounts of *ENV* present. There were at most 12 colonies of *ENV* in the gut homogenate, whereas *ENT* reached up to 1800 CFU (N=19, U=0.00, p<0.001, Fig. 3 b). There were no detectable amounts of either *S. marcescens* strain in the *G. mellonella* midgut (data not shown).

3.4. Immune assays

The ROS levels in *A. plantaginis* were elevated after oral exposure to *ENT* and *ENV* compared to *E. coli* (N=34, df=2, H=8.14, p =0.017, Fig. 4 a). Pairwise comparisons: *E. coli-ENV*: H=9.37, p =0.048; *E. coli-ENT*: H=11.27, p =0.024; *ENT-ENV*: H=1.90, p =0.647. In *G. mellonella*, the oral exposure with bacteria did not have an effect on ROS (N=60, df=3, H=3.46, p =0.326, Fig. 4 c). The treatment (*ENT*, *ENV*, or *E. coli* oral exposure and naïve larvae) had no effect on PO activity in either host species (*A. plantaginis*: N=40, df=3, H=1.14, p =0.77; *G. mellonella*: N=60, df=3, H=6.45, p =0.09; Fig. 4 b & d). We also tested general bactericidal activity of the haemolymph in all treatments on both *S. marcescens* (*ENV*), and *Micrococcus luteus* bacterial lawns, but failed to find any lytic effects (data not shown).

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3.5. Gene expression

In *A. plantaginis*, oral exposure to both pathogens led to upregulation of 6-Tox and downregulation of CecropinA in the midgut, compared to exposure to the harmless bacterium *E. coli*. Defensin was differently expressed depending on the bacterial strain: *ENV* caused upregulation and *ENT* downregulation of Defensin. Also, Peptidoglycan recognition protein was upregulated with *ENT*, whereas *ENV* exposure did not cause it to exceed the 2-fold threshold (Fig. 5 a). In *G. mellonella* both pathogen strains induced upregulation of CecropinA, and down regulation of CecropinB. PGRP2 was slightly elevated with *ENV* and 6-Tox down regulated with *ENT* (Fig. 5 b).

4. DISCUSSION

The pathogen-host arms race has a major evolutionary role in creating and maintaining diversity in both of the participants (Anderson & May 1982). Lepidopterans can protect themselves against bacterial pathogens with a vast variety of immunological mechanisms, while entomopathogens have evolved means to overcome these host defences. We show here that host species' are responding in variable ways to the same pathogen, and that defence mechanisms act differently against the two closely related pathogen strains in the same host. The two strains, environmental (*ENV*) and entomopathogenic (*ENT*) isolates, of the bacterium have

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different capabilities in resisting the local and systemic immune responses of the two hosts. *A. plantaginis* larvae gain protection against otherwise lethal sepsis if they have been feeding on the same *S. marcescens* strain earlier (Mikonranta *et al.* 2014). This may occur via tolerating trace amounts of the pathogen in the midgut and thus maintaining the immune response. *G. mellonella* larvae effectively clear the bacteria first but then succumb rapidly to the recurring septic infection.

The two strains of the pathogen differ in the induced prophylaxis against septic infection. In *A. plantaginis*, the *ENV* priming is not able to protect the larvae against *ENT*, nor is the *ENT* oral exposure offering resistance against *ENV*. It has to be noted, that *ENT* efficiently overcomes *A. plantaginis* defences after oral infection, making it impossible to tell if the lack of protection is due to sheer virulence of *ENT* or due to specificity of *ENV* induced protection. Also, the mortality in oral challenge inevitably leads to a situation where only a subset of survivors is injected in the second challenge. It is however remarkable, that *ENV* priming does not even slow down the septic infection with *ENT* compared to control priming. Moreover, *E. coli* priming fails to protect *A. plantaginis* from *ENV*, demonstrating that the presence of Gram-negative bacterial cells alone is not a sufficient cue for eliciting the prophylaxis. In *A. plantaginis,* ROS was increased after oral exposure to both pathogens. It is possible that the elevated ROS levels were able to tackle the *ENV* septic injury directly (Mikonranta *et al.* 2014) but insufficient against the generally more virulent *ENT*. Alternatively, ROS could act as a signal for up regulating systemic immune response (Wu *et al.* 2012). We cannot show, however, whether the *ENT*-induced immune responses would work against septic infection with *ENV* due to the high virulence of

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ENT and the resulting larval mortality. There were no differences between the bacterial treatments in phenoloxidase activity. This is in concordance with the notions that phenoloxidase is mostly effective in infections that are related to a breaching of insect cuticle (Ashida & Brey 1995), and that unnecessary induction of the defence in gut-borne infection could be costly (Sadd & Siva-Jothy 2006).

Contrary to *A. plantaginis*, *G. mellonella* could not be colonized by either of the pathogens orally, as there were no bacteria in the larval guts five days after infection. The *G. mellonella* larvae are larger and thus may have received a smaller amount of bacteria relative to the body size. It has to be noted that despite of this they succumb much more rapidly to the septic infection. The within species variance in size should not cause systematic bias due to the high replication, randomization, and the use of larvae of similar age. Within host species, we can be confident that the amount of ingested bacteria is on average even between host individuals regardless of the bacterial species. It is possible that the two hosts on average ingested different amounts because of different diets. However, both host species were inevitably exposed to the bacteria, and the bacteria do persist in the diets for the study period. It might be that the efficiency of *G. mellonella* in clearing the ingested pathogens from its midgut also prevents the priming against *ENV* to occur. For example, the native gut flora in *G. mellonella* could be composed in a way that never allows the invading pathogens to reach a density required for their detection (Dillon & Dillon 2004), or the biochemical milieu could be otherwise unfavourable for *S. marcescens*. The PO and ROS defences were not elevated, but the significant up regulation of AMP CecropinA, known to degrade bacterial cell walls (Steiner et al. 1981, Hetru et al.

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2003), suggests that both pathogens were indeed first detected and then successfully cleared from the *G. mellonella* midgut. Thus, the results demonstrate that the two host species had different resistance/tolerance solutions for coping with both the initial oral infection and the recurring sepsis. Having bacterial density measurements from the guts at multiple time points after the oral infection would shed more light on the dynamics of the infection/defence processes, but unfortunately it has to be the scope for future studies.

Notably, we did not find any immunity related genes that would have been similarly up or down regulated in both hosts after pathogen exposure. Peptidoglycan recognition proteins (PGRPs) are a class of recognition proteins that specifically bind to Gram-negative or Gram-positive bacteria (Michel *et al.* 2001, Vallet-Gely *et al.* 2009). The expression of PGRP2 (Pauchet *et al.* 2010) in both host species was somewhat consistently correlated with the findings of bacterial colonies in the midgut: In *A. plantaginis*, *ENT* was isolated in high quantities and PGRP2 was expressed three-fold higher than the control. On the other hand only trace amounts of *ENV* were able to persist in *A. plantaginis* gut and the PGRP2 level was not increased. In *G. mellonella* the PGRP2 levels remained at the same level with the controls after *ENT* introduction and there was no bacterial growth in the midgut. Why the generally more benign *ENV* lead to slightly increased PGRP2 levels in *G. mellonella* remains unclear but it is possible that *ENT* has means of suppressing the *G. mellonella* immune system. For example, *Pseudomonas aeruginosa* can limit insect AMP expression with a yet unknown mechanism (Vallet-Gely 2008).

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CecropinA, which is synergistically affected by the Imd and Toll pathways (Tanji *et al.* 2007), was highly upregulated in *G. mellonella* and downregulated in *A. plantaginis* with both pathogens. Where CecropinA was upregulated, CecropinB was consistently downregulated and *vice versa*, suggesting an antagonistic co-regulation of these two AMPs in both hosts. The generally more virulent and protease-equipped *ENT* strain induced two-fold higher CecropinA expression in *G. mellonella* gut than *ENV*. Whether the high upregulation of CecropinA in *G. mellonella* was the reason for its ability to diminish oral infection with both pathogens cannot be confirmed, but it is noteworthy that the levels remained elevated after complete bacterial clearance. AMPs including Cecropins are susceptible to degradation by proteases (Akuffo *et al.* 1998, Liehl *et al.* 2006), but CecropinB has been reported to resist this degradation in the gut (De Lucca *et al.* 2000).

Our *in vitro* assay revealed that *ENT* secreted more extracellular proteases compared to *ENV*. Proteases are a known virulence factor in *S. marcescens* as well as in other pathogen species (e.g. Bidochka & Khachatourians 1990, Kurz *et al.* 2003, Liehl *et al.* 2006). They are especially important for parasites in penetrating the insect gut membrane or even the outer cuticle of the body (Bidochka & Khachatourians 1990, Abuhatab *et al.* 1995, Ffrench-Constant *et al.* 2003). This offers a likely explanation why *ENV* is avirulent to *A. plantaginis* in oral infection whereas *ENT* is able to penetrate the epithelium causing a septic infection through the midgut, and also why *ENT* is generally more virulent in septic injury in both hosts.

Differences between hosts in pathogen-induced 6-Tox and Defensin upregulation could potentially explain the ability for immunological priming in *A.*

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plantaginis and the lack of it in *G. mellonella*. X-Tox proteins are a Lepidopteran specific Defensin-like molecule family (Girard *et al.* 2008). Insect defensins have been reported to act mainly on Gram-positive bacteria (Broderick *et al.* 2009), and at least in *D. melanogaster*, suggested not to have a role in specific immune priming (Pham *et al.* 2007). However, in *Spodoptera littoralis*, X-Tox family protein 11-Tox was shown to be upregulated in both Gram-negative and Gram-positive challenges. Interestingly, the translated protein did not have a direct anti-microbial activity *in vitro* suggesting that it has some other, yet unknown, function with the systemic immune response in the haemocytes (Girard *et al.* 2008, Destoumieux-Garzón 2009). Thus, 6-Tox up regulation could have a role in the observed immune priming.

The persistence of a non-symptomatic density of *ENV* in the *A. plantaginis* gut may raise questions about the relationship between a host's pathogen tolerance and it's pathogen resistance (Schneider & Ayres 2008, Vale *et al.* 2014, Råberg 2014). A host's ability to withstand a certain pathogen density level (tolerance) could be a more adaptive solution compared to a complete clearance (resistance), if the clearance-associated costs outweigh the benefits (Vale *et al.* 2014). Interestingly, harbouring small and sublethal amounts of the pathogen in the gut could in this case offer a fitness benefit for the host via protection against a recurring infection. Red-pigmented *Serratia*, like *ENV*, have been reported to transmit via the ovipositors of hymenopteran parasites in *G. mellonella* and other insects (Bucher 1963, Jackson *et al.* 2004). This suggests that because *ENV* lacked the ability to infect through oral route, the host would have gained a fitness benefit in a natural septic injury by ingesting

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bacteria from the environment, which would then serve as a cue for the immune priming.

In conclusion, both *A. plantaginis* and *G. mellonella* appear to adjust their defences accordingly to the properties of the infecting pathogen strain. The gained protection against septic *ENV* infection in *A. plantaginis* could potentially be related to the antimicrobial protein 6-Tox, which might be expressed due to the larvae having minute amounts of the pathogen in their midguts already at the time of the sepsis. *G. mellonella* larvae cleared their oral infections completely, likely involving the up regulation of CecropinA, but succumbed rapidly to the septic infections with both pathogens regardless of previous exposure. It seems that two host-dependent mechanisms, resistance and tolerance, act in concert to minimize negative fitness effects in *A. plantaginis*. The interplay of tolerance and resistance could have profound effects on the pathogen-host coevolution: Whereas increased resistance should favour an antagonistic arms race, the evolution of increased tolerance has been suggested to lead to neutral or even positive effects on the pathogen (Roy & Kirchner 2000, Schneider & Ayres 2008). Major differences between the hosts' responses suggest that caution should be taken when generalizing results from particular host-pathogen model systems. In this case, two Lepidopteran hosts exhibit different strategies to fight the same pathogens, and two closely related Gram-negative bacteria elicit distinct virulence factor dependent defences in the same hosts.

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5. ACKNOWLEDGEMENTS

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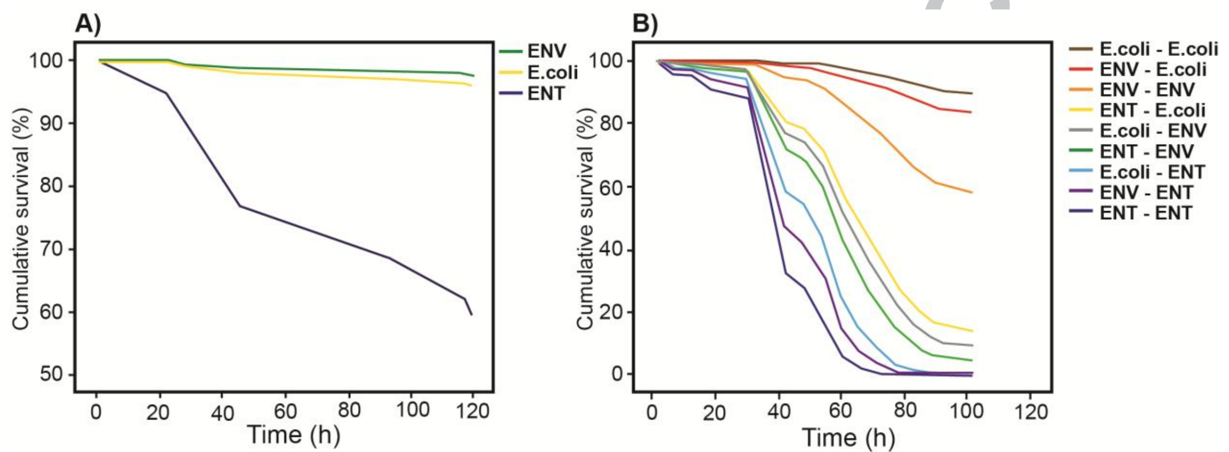

FIGURE 1 *A. plantaginis* survival after bacterial exposure. 48 h oral introduction with the three bacteria plus 72 h on uncontaminated diet (A), and the same priming groups injected with all the bacterial strains (totalling to nine priming – injection combinations) observed 100 h after the injections (B). *ENV* denotes *Serratia marcescens* ssp. *marcescens, ENT* denotes *S. marcescens ENT* and E. coli denotes *Escherichia coli*. The first term in the legend denotes priming and the second denotes the injection.

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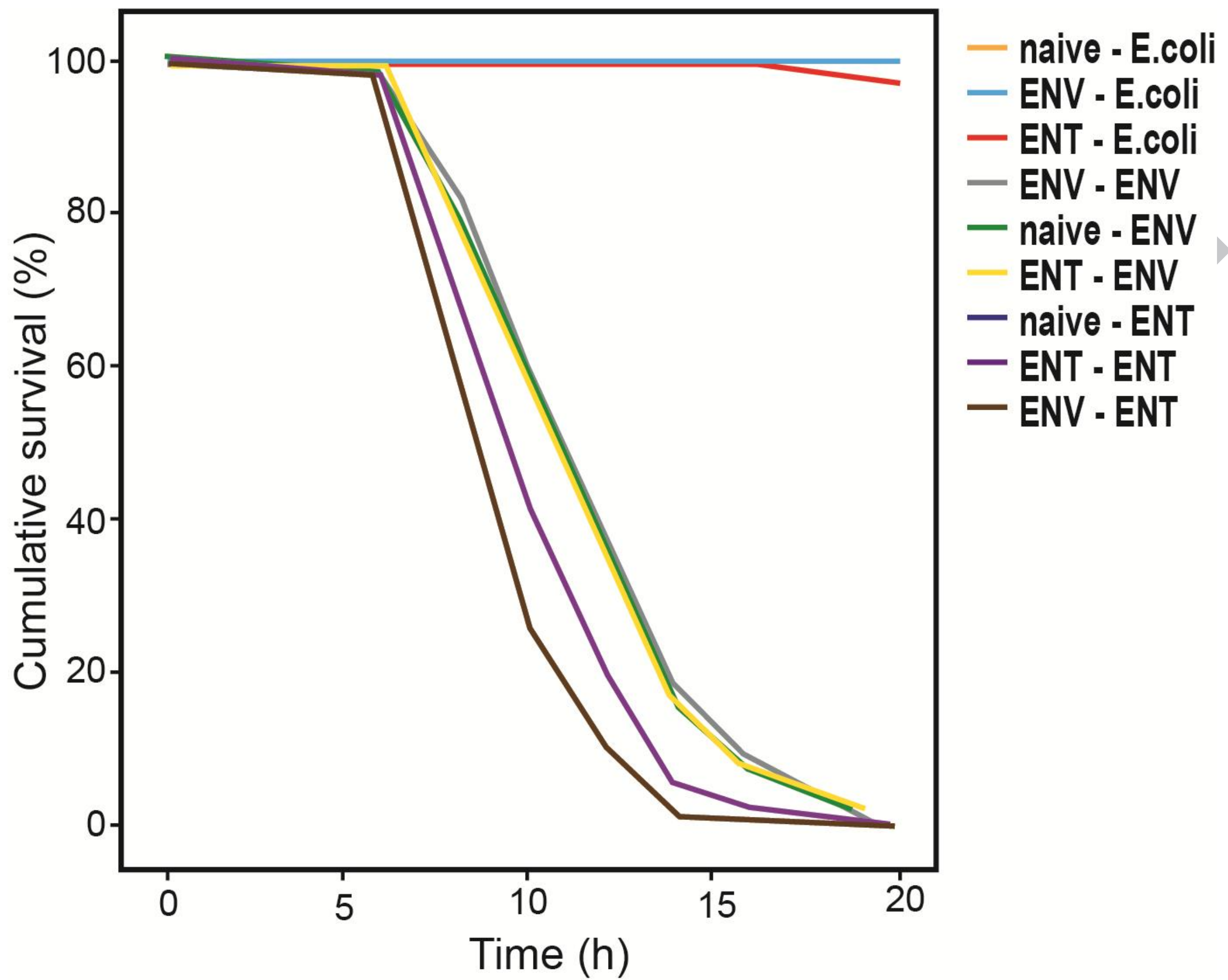

FIGURE 2 *Galleria mellonella* survival after bacterial exposure. All the oral introduction (priming) – injection combinations. *ENV* denotes *Serratia marcescens* ssp. *marcescens, ENT* denotes *S. marcescens* db11, naive denotes non-primed group and E. coli denotes *Escherichia coli*. The first term in the legend denotes priming and the second denotes the injection.

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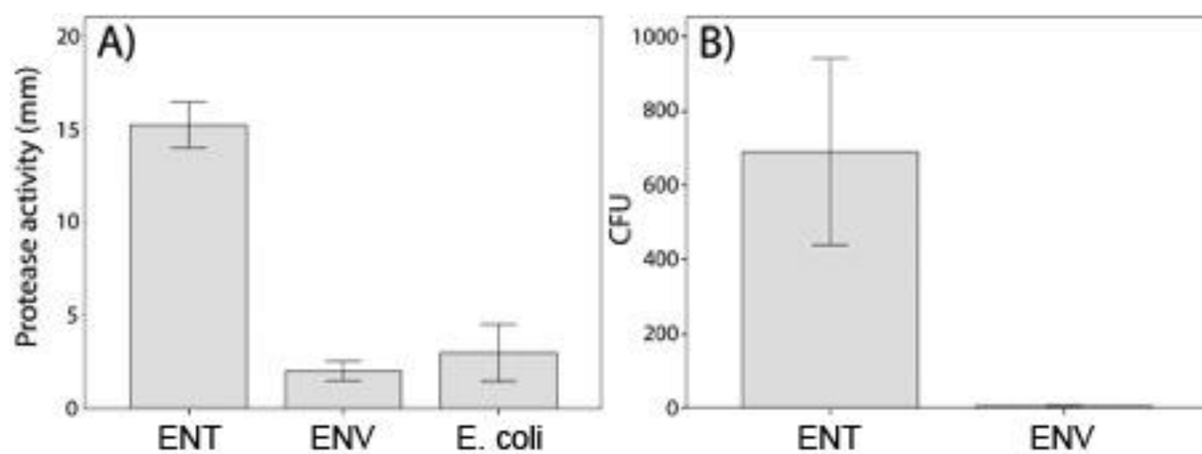

FIGURE 3 Bacterial extracellular protease activity measured as the diameter of protein degradation halo on skim milk agar. *ENV* denotes *Serratia marcescens* ssp. *marcescens, ENT* denotes *S. marcescens* db11, and E. coli denotes *Escherichia coli*. (A). Error bars are +/- SE. Bacterial colony forming units (CFU) isolated from *A. plantaginis* midgut 120h after oral exposure (B). Error bars are +/- SD.

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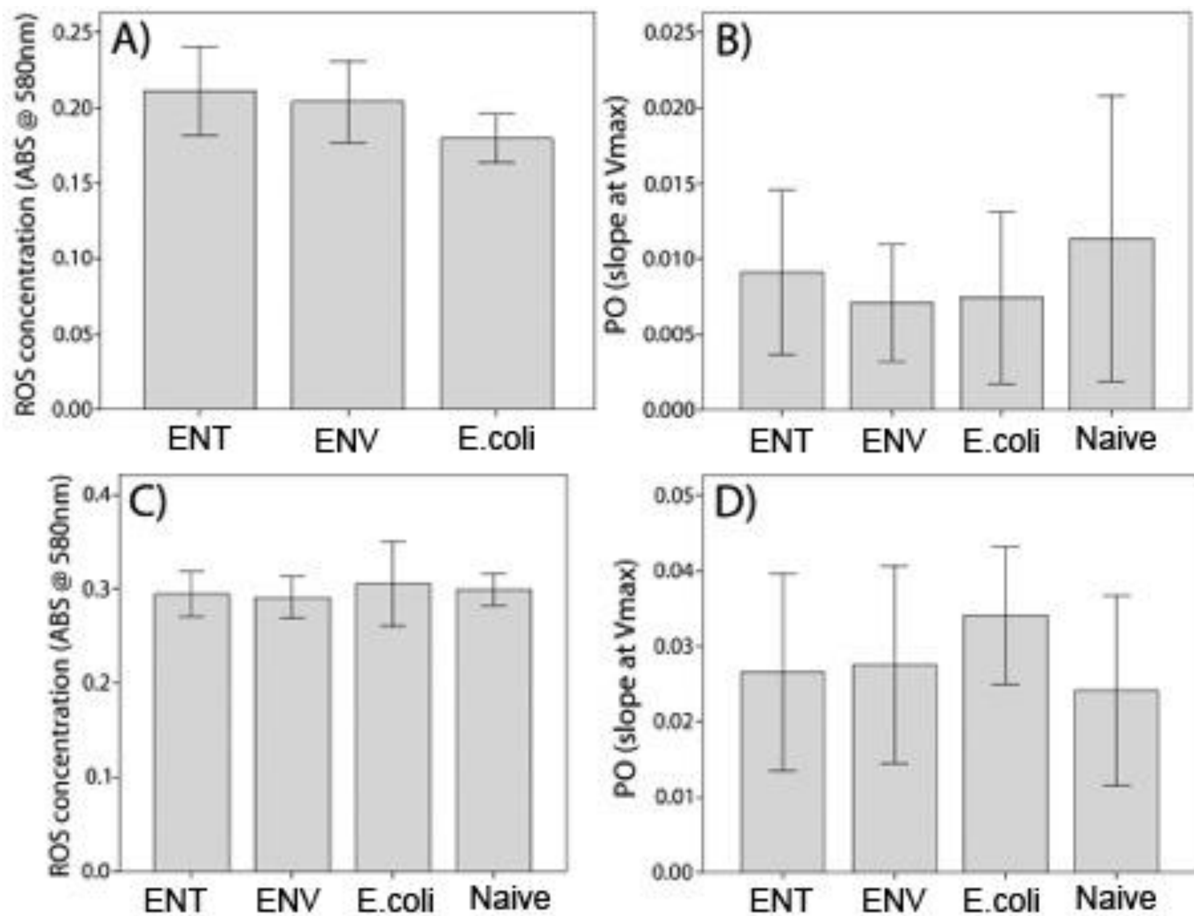

FIGURE 4 ROS concentration and PO activity in the haemolymph 120h after the pathogen exposure in *A. plantaginis* (A & B), and *G. mellonella* (C & D). *ENV* denotes *Serratia marcescens* ssp. *marcescens, ENT* denotes *S. marcescens* db11, naive denotes non-primed group and E. coli denotes *Escherichia coli*. Error bars are +/- SD.

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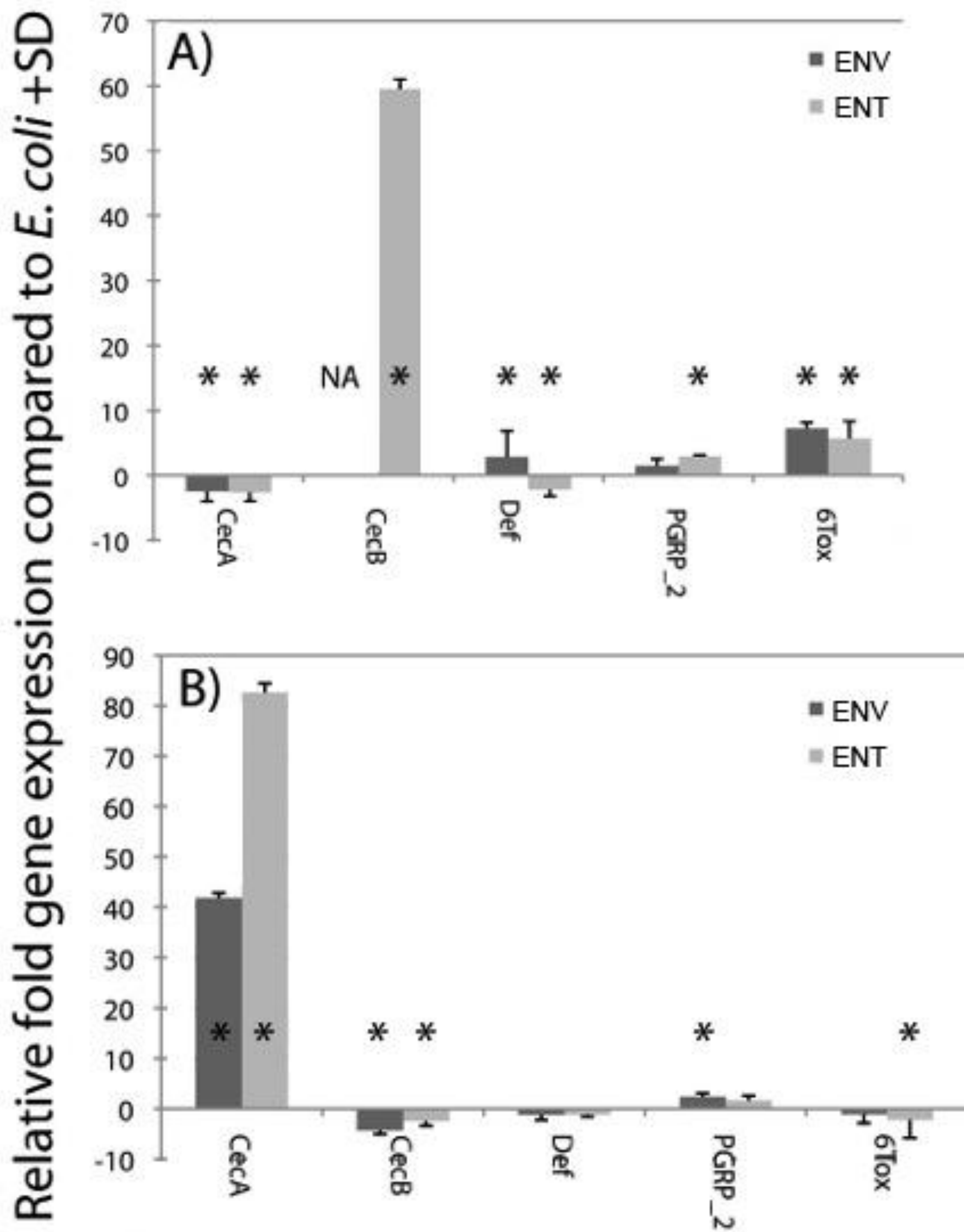

FIGURE 5 Gene expression in *A. plantaginis* (A) and *G. mellonella* (B) midgut 120h after bacterial exposure with *ENV* (dark grey bars) and *ENT* (light grey bars). *ENV* denotes *Serratia marcescens* ssp. *marcescens* and *ENT* denotes *S. marcescens* db11. Asterisks (*)

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denote significant, two-fold differences compared to the control bacterium *E. coli* according to the $2^{-\Delta\Delta_{cT}}$ method. Error bars are SD. NA means no data.

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TABLE 1. Pairwise differences in *P. plantaginis* mortality rate when exposed orally with *S. marcescens* ssp. *marcescens db11* (*ENT*), *S. marcescens* ssp. *marcescens* Bizio (*ENV*), or *E. coli* K-12. *ENV* denotes Serratia marcescens ssp. marcescens, *ENT* denotes S. marcescens db11, and E. coli denotes Escherichia coli.

Oral exposure	*ENT*		*ENV*		E. coli	
	X^2	p =	X^2	p =	X^2	p =
ENT			123.36	**<0.001**	110.09	**<0.001**
ENV	123.36	**<0.001**				
E.coli	110.09	**<0.001**	0.97	0.325		

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TABLE 2. Pairwise differences in *P. plantaginis* mortality rate when exposed orally with *S. marcescens* ssp. *marcescens ENT* (*ENT*), *S. marcescens* ssp. *marcescens* Bizio (*ENV*), or *E. coli* K-12, and five days later injected with the same bacteria. The first term denotes priming and the second denotes the injection.

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Oral - injection	*ENT-ENV*		*ENT*-E. coli		*ENV-ENT*		*ENV-ENV*		*ENV*-E. coli		E. coli-*ENT*		E. coli-*ENV*		E. coli-E. coli	
	X^2	p =	X^2	p =	X^2	p =	X^2	p =	X^2	p =	X^2	p =	X^2	p =	X^2	p =
ENT-ENT	51.303	**<0.001**	77.824	**<0.001**	4.973	**0.026**	134.105	**<0.001**	151.644	**<0.001**	11.364	**0.001**	91.916	**<0.001**	168.235	**<0.001**
ENT-ENV			9.692	**0.002**	29.151	**<0.001**	68.727	**<0.001**	113.973	**<0.001**	8.390	**0.004**	3.068	0.080	129.945	**<0.001**
ENT-E. coli					67.149	**<0.001**	48.294	**<0.001**	99.409	**<0.001**	38.471	**<0.001**	2.896	0.089	121.172	**<0.001**
ENV-ENT							144.359	**<0.001**	178.512	**<0.001**	4.367	**0.037**	64.971	**<0.001**	193.480	**<0.001**
ENV-ENV									12.641	**<0.001**	119.865	**<0.001**	64.923	**<0.001**	19.719	**<0.001**
ENV-E. coli											163.203	**<0.001**	115.375	**<0.001**	1.074	0.300
E. coli-*ENT*													27.506	**<0.001**	179.903	**<0.001**
E. coli-*ENV*															130.567	**<0.001**

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TABLE 3. Pairwise differences in *G. mellonella* mortality rate when exposed orally with *S. marcescens* ssp. *marcescens* db11 (*ENT*), *S. marcescens* ssp. *marcescens* Bizio (*ENV*), or sterile growth medium (naive), and five days later injected with the same bacteria or *E. coli* K-12. The first term denotes priming and the second denotes the injection.

Oral -	naive-*ENV*		naive-E. coli		*ENT*-*ENT*		*ENT*-*ENV*		*ENT*-E. coli		*ENV*-*ENT*		*ENV*-*ENV*		*ENV*-E. coli	
injection	X^2	p =	X^2	p =	X^2	p =	X^2	p =	X^2	p =	X^2	p =	X^2	p =	X^2	p =
naive-*ENT*	12.934	**<0.001**	98.640	**<0.001**	7.853	0.005	12.609	**<0.001**	106.996	**<0.001**	7.673	**0.006**	16.019	**<0.001**	101.996	**<0.001**
naive-*ENV*			77.997	**<0.001**	30.437	**<0.001**	0.194	0.660	85.215	**<0.001**	25.368	**<0.001**	0.840	0.360	80.888	**<0.001**
naive-E. coli					134.249	**<0.001**	98.462	**<0.001**	1.819	0.177	120.067	**<0.001**	82.870	**<0.001**	.	**.**
ENT-*ENT*							41.524	**<0.001**	144.450	**<0.001**	.248	0.618	37.793	**<0.001**	138.372	**<0.001**

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ENT-*ENV*	106.998	**<0.001**	34.146	**<0.001**	1.985	0.159	101.891	**<0.001**
ENV-E. coli			129.121	**<0.001**	90.675	**<0.001**	1.900	0.168
ENV-*ENT*					31.933	**<0.001**	123.719	**<0.001**
ENV-*ENV*							85.999	**<0.001**

Table S1. Primer sequences used in the PCR

Parasemia plantaginis

Gene	**Forward primer**	**Reverse primer**
Cecropin A	CGTCGGCCATAGGAAAATAA	GCTATGGTAACCCGACTACT
Cecropin B	GTGTTCGTTTTCGCTTGTTT	ATGTGCTGTCCAAGTTTCTC
Defensin	AAATCCGTCCATGTGCTATG	GGGAAATTCGATTGGTGTCA
6-Tox	GGTCGCATAAAGTTCTGGTT	AGATACAGTTATGGACGGCA
PGRP2	AAGTTTTCTAGTCGTTGCCC	GAGTCGGACGAATTGTGTAG

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Galleria mellonella

Gene	Forward primer	Reverse primer
Cecropin A	ATATCCTAGCTTGCAGACGA	AATGACCACGGTCTACATCT
Cecropin B	GCCATGTTCTTCACCACGAC	TCAGTCACCGCCTTTAATGAT
Defensin	ATTCCTAATCGTGTTCCGTG	AGAATATCCTTTCGGCAAGC
6-Tox	GAACGCGTTGTTAGTTAACGG	CGTTGGTGTCTGTATCTATCG
PGRP2	GCGTTTCACC TGATCATTGT T	TAGTCGGTGG TAATGGCAAA G

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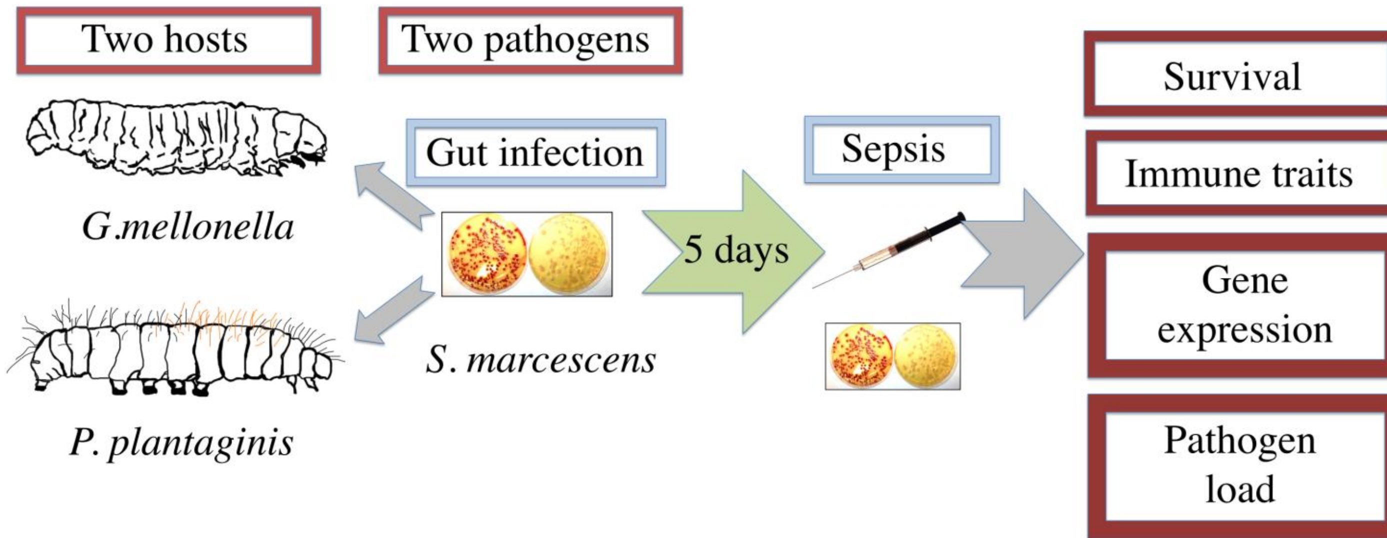
Graphical abstract



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- Lepidopteran species differ greatly in their defences against bacterial pathogens
- Host responses also vary between closely related pathogens
- Care should be taken when generalizing results obtained from model systems

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