LIVING WITH THE ENEMY:

UNDERSTANDING THE DYNAMICS OF HOST DEFENCES AGAINST PERSISTENT INFECTIONS

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To women.

Those who fought and pushed through,

Those that history left behind,

Women who keep inspiring me today,

And those who hold the power to change tomorrow.

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LIST OF ABBREVIATIONS

AMP	Antimicrobial peptide
BLUD	Bacterial load upon death
CFU	Colony-forming unit
DPI	Day post-infection
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
FDR	False discovery rate
GNBP	Gram-negative bacteria-binding protein
LB	Lysogeny broth
LC-MS	Liquid chromatography-mass spectrometry
MS	Mass spectrometry
NK-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OD	Optical density
PAMP	Pathogen associated molecular pattern
PGN	Peptidoglycan
PGRP	Peptidoglycan-recognition protein
PPP	Per-parasite pathogenicity
PRP	Pattern-recognition protein
RT-PCR	Real-time polymerase chain reaction
S2	Schneider 2 (*Drosophila melanogaster* cells)
SPBL	Set point bacterial load
SYA	Standard sugar yeast agar

SUMMARY

Because of their clinical and epidemiological consequences, persistent infections play an important role in shaping the selective pressures acting on host-microbe interactions. We can gain more about the contribution of persistent infections to the evolution of host-pathogen interactions by uncovering the dynamics of host defences. The present work takes a multi-angled approach to investigate the dynamics of host defences against persistent bacterial infections in *Drosophila melanogaster*. Firstly, in **Chapter 1**, by looking at the long-term dynamics of infection of various bacterial species, we investigated the conditions under which a pathogen persists or is cleared across various four bacterial species. All bacterial species could be cleared by the host, but the dynamics of clearance depended on the ability of the pathogen to exploit the host resources and reproduce. The most persistent bacteria (*Lactococcus lactis* and *Providencia burhodogranariea*) were those better at exploiting the host resources for their growth. Moreover, we could retrieve bacteria from hosts up to 78-days post-infection, marking an unprecedented estimation for the duration of persistence in insects. Besides virulence, based on the literature we had reason to believe that a previous exposure with a pathogen may enhance the ability of the host to limit or clear a persistent infection. In **Chapter 2**, we tested this hypothesis by using various methods to inactivate the pathogen for the primary encounter and exposing flies to *L. lactis* and *P. burhodogranariea*. Under the conditions tested, there was no advantage of a previous exposure in the face of a chronic infection. Hosts that are predicted to survive the infection while carrying a persistent infection show increased resistance in the early chronic phase, i.e., a lower bacterial load, compared to those predicted to succumb to an uncontrolled growth. To determine whether hosts predicted to have different infection outcomes vary in their tolerance, in **Chapter 3**, we measured fecundity-tolerance during a chronic infection with *L. lactis* and *P. burhodogranariea*. These two bacterial species caused a more pronounced decrease in fecundity over time in flies carrying a high load. However, only flies infected with *L. lactis* experienced a decrease in fecundity-tolerance, indicating that they are less able to counterbalance the fecundity costs associated to the infection. Chronically infected hosts sustain persistent antimicrobial peptide responses. In **Chapter 4**, we confirmed the presence of this response in hosts carrying a persistent *P. burhodogranariea* infection by measuring their protein expression in the chronic phase. In addition, we found that hosts may combine this antimicrobial response with nutritional immunity and a downregulation of other energetically costly branches of the immune system to fight the infection. The present work highlights the importance of considering infections as time- and context-dependent processes where both host and pathogen contribute to shape the outcome of infection.

ZUSAMMENFASSUNG

Aufgrund ihrer klinischen und epidemiologischen Konsequenzen spielen persistierende Infektionen eine wichtige Rolle bei der Gestaltung des Selektionsdrucks, der auf Wirt-Mikroben-Interaktionen wirkt. Wir können mehr über den Beitrag von persistierenden Infektionen zur Evolution von Wirt-Pathogen-Interaktionen erfahren, indem wir die Dynamik der Wirtsabwehr aufdecken. Die vorliegende Arbeit verfolgt einen mehrstufigen Ansatz, um die Dynamik der Wirtsabwehr gegen persistierende bakterielle Infektionen in *Drosophila melanogaster* zu untersuchen. Zunächst haben wir in **Kapitel 1** durch die Betrachtung der Langzeitdynamik der Infektion verschiedener Bakterienspezies die Bedingungen untersucht, unter denen ein Erreger persistiert oder über verschiedene vier Bakterienspezies hinweg beseitigt wird. Alle Bakterienarten konnten vom Wirt beseitigt werden, aber die Dynamik der Beseitigung hing von der Fähigkeit des Erregers ab, die Ressourcen des Wirts auszunutzen und sich zu vermehren. Die persistentesten Bakterien (*Lactococcus lactis* und *Providencia burhodogranariea*) waren diejenigen, die die Wirtsressourcen besser für ihr Wachstum ausnutzen konnten. Darüber hinaus konnten wir die Bakterien bis zu 78 Tage nach der Infektion aus den Wirten zurückholen, was eine noch nie dagewesene Schätzung für die Dauer der Persistenz in Insekten darstellt. Neben der Virulenz hatten wir aufgrund der Literatur Grund zu der Annahme, dass eine vorherige Exposition mit einem Pathogen die Fähigkeit des Wirts, eine persistierende Infektion zu begrenzen oder zu beseitigen, verbessern kann. In **Kapitel 2** testeten wir diese Hypothese, indem wir verschiedene Methoden einsetzten, um den Erreger für die erste Begegnung zu inaktivieren und Fliegen mit *L. lactis* und *P. burhodogranariea* zu exponieren. Unter den getesteten Bedingungen zeigte sich kein Vorteil einer vorherigen Exposition gegenüber einer chronischen Infektion. Wirte, für die vorhergesagt wird, dass sie die Infektion überleben, während sie eine persistente Infektion tragen, zeigen eine erhöhte Resistenz in der frühen chronischen Phase, d. h. eine geringere bakterielle Belastung, im Vergleich zu denen, für die vorhergesagt wird, dass sie einem unkontrollierten Wachstum erliegen. Um festzustellen, ob sich die Wirte, für die unterschiedliche Infektionsergebnisse vorhergesagt wurden, in ihrer Toleranz unterscheiden, haben wir in **Kapitel 3** die Fruchtbarkeitstoleranz während einer chronischen Infektion mit *L. lactis* und *P. burhodogranariea* gemessen. Diese beiden Bakterienarten verursachten bei Fliegen mit einer hohen Belastung eine stärkere Abnahme der Fekundität im Laufe der Zeit. Jedoch erlebten nur Fliegen, die mit *L. lactis* infiziert waren, eine Abnahme der Fekunditätstoleranz, was darauf hindeutet, dass sie weniger in der Lage sind, die mit der Infektion verbundenen Fekunditätskosten auszugleichen. Chronisch infizierte Wirte erhalten persistente antimikrobielle Peptidantworten. In **Kapitel 4** bestätigten wir das Vorhandensein dieser Reaktion bei Wirten mit einer persistierenden *P. burhodogranariea*-Infektion, indem wir ihre Proteinexpression in der chronischen Phase maßen. Darüber hinaus fanden wir heraus, dass die Wirte diese antimikrobielle Antwort mit einer Nahrungsimmunität und einer Herunterregulierung anderer energetisch kostspieliger Zweige des Immunsystems kombinieren können, um die Infektion zu bekämpfen. Die vorliegende Arbeit unterstreicht, wie wichtig es ist, Infektionen als zeit- und kontextabhängige Prozesse zu betrachten, bei denen sowohl der Wirt als auch der Erreger dazu beitragen, den Ausgang der Infektion zu gestalten.

GENERAL INTRODUCTION

1. Insects as model organisms for eco-immunology

With a global diversity estimated in the millions of species, insects are one of the most diverse taxonomic groups on Earth (Stork et al. 2015; Stork 2018). They play key roles in various trophic cascades and biomass cycles, making them important drivers of many ecosystem processes (Yang and Gratton 2014). As such, they occupy a wide range of niches and engage in various biological interactions with other organisms (Scudder 2009). Throughout their evolutionary history, they have co-evolved with a variety of pathogenic organisms, leading to the evolution of an array of defence strategies against infections (Lundgren and Jurat-Fuentes 2012; Hillyer 2015).

The study of insect defences against pathogens has been paramount in our fundamental understanding of invertebrate and vertebrate immunity alike. Particularly, the fruit fly *Drosophila melanogaster* has been the basis of many breakthroughs as a model organism. For instance, the Toll signalling pathway was first discovered to participate in the maternal control of dorsal-ventral pattern formation pathway of flies (Anderson, Bokla, and Nüsslein-Volhard 1985; Morisalo and Anderson 1995). Subsequent work showed that this pathway is also involved in triggering the production of antimicrobial peptides (AMPs) controlled by the NF-κB transcription factors, induced upon fungal infections to kill the pathogen (Rosetto et al. 1995; Lemaitre et al. 1996). These milestones made possible the identification of the Toll-like receptors in humans and proved that the Toll/NF-κB host defence pathway is preserved from invertebrates to vertebrates (Medzhitov, Preston-Hurlburt, and Janeway 1997). Because of its short life cycle and easy rearing conditions (e.g., 10 days at 25 °C), *D. melanogaster* has remained a recurrent model species for immunology studies leading to the extensive characterisation of its immune defence mechanisms (Lemaitre and Hoffmann 2007; Buchon, Silverman, and Cherry 2014). Research over the years in this and other species has shown that insect immunity encompasses complex responses with varying mechanisms at play, which have diversified across insect taxa over the course of their evolutionary history (Zdobnov et al. 2002; Christophides, Vlachou, and Kafatos 2004; Dionne and Schneider 2008)

2. Insect defence strategies against infection

2.1. Avoidance: limiting exposure to the infection

As a first line of defence against pathogens, insects can prevent the establishment of an infection by engaging in prophylactic behaviours that reduce the probability of becoming infected (de Roode and Lefèvre 2012). These behaviours include grooming (Gaugler, Wang, and Campbell 1994; Yanagawa and Shimizu 2007), self-medication (Chapuisat et al. 2007; Simone-Finstrom and Spivak 2010), and avoiding contact exposure with contaminated food or conspecifics (Heinze and Walter 2010; Parker, Elderd, and Dwyer 2010). Moreover, insects possess several physical and chemical barriers that limit pathogen invasion. Insect cuticles act as an external barrier against environmental pathogens (Hajek and St. Leger 1994), and internal barriers, like the peritrophic matrix of the gut offer protection to regions constantly exposed to microbes (Lehane 1997). These protections are doubled down by the gland secretion of antimicrobial substances against pathogens present in the environment (Otti, Tragust, and Feldhaar 2014).

2.2. Within-host pathogen recognition

Pathogens that circumvent this first line of defence and enter the host are recognised by the immune system via molecular interactions between pathogen-associated molecular patterns (PAMPs) and the host pattern-recognition proteins (PRPs) (Medzhitov and Janeway 2002). These receptor proteins can be found at the surface of cells and in the extracellular environment. They bind to molecules produced by or located on the cell walls of pathogens, e.g., lipopolysaccharides of Gram-negative bacteria and peptidoglycans (PGNs) of Gram-positive and Gram-negative bacteria (Akira, Uematsu, and Takeuchi 2006). PAMP-recognition by these receptors triggers a downstream cascade of transduction processes that activates the immune response (Akira, Uematsu, and Takeuchi 2006; Lemaitre and Hoffmann 2007). *D. melanogaster* possesses various PRPs capable of recognising different types of pathogens and subsequently activate the appropriate downstream response. For example, PGN-receptor proteins (PGRPs), distinguish between bacteria based on structural differences of PGNs on the wall of the bacterial cells (Takehana et al. 2002; Leulier et al. 2003; Kurata 2014).

2.3. Resistance: Limiting pathogen replication and the insect immune response

Upon recognition of the pathogen, an immune response is deployed via a multi-layered system of mechanisms that act together to fight the infection (Box 1) (Dionne and Schneider 2008). As part of a **resistance** strategy, these mechanisms are aimed to stop, or limit, pathogen replication (see Box 2 on how to measure resistance) (Restif and Koella 2004; Råberg, Graham, and Read 2009).

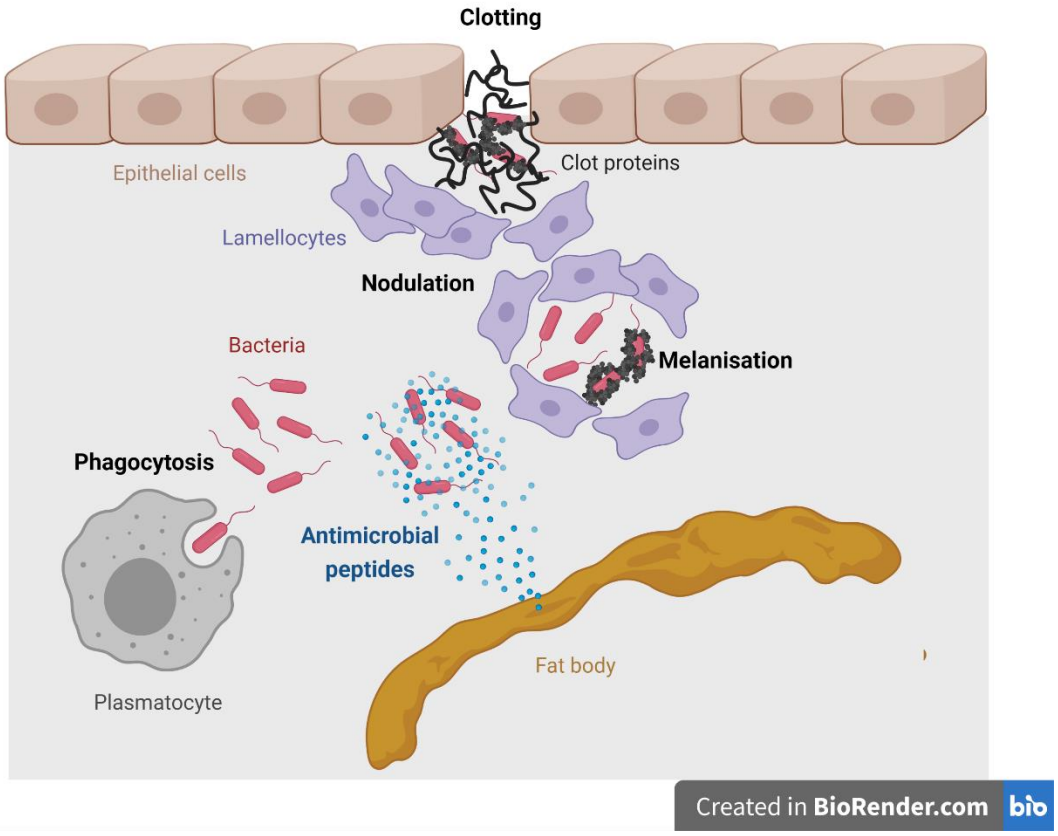
The insect immune response can be functionally classified into humoral and cellular immunity (Lundgren and Jurat-Fuentes 2012). The humoral response mainly depends on AMPs. These proteins are mainly secreted by the fat body upon recognition of PAMPs and downstream activation of two major NF-κB pathways: the Toll pathway, which is mobilized against Gram-positive and fungal infections, and the Imd pathway, which mainly targets Gram-negative infections, and some Gram-positive bacteria (Lemaitre, Reichhart, and Hoffmann 1997; J. A. Hoffmann and Reichhart 2002; J. A. Hoffmann 2003; Ferrandon et al. 2007; Lemaitre and Hoffmann 2007; Govind 2008). In *D. melanogaster*, seven families of AMPs have been identified to date: Cecropins, Attacins, Defensins, Diptericin and Drosocin aimed at bacterial infections, and Metchnikowin and Drosomycin aimed at fungal pathogens (Lemaitre and Hoffmann 2007). *Drosophila* AMPs can be specific in the type of pathogen that they target, acting synergistically or additively against specific bacterial and fungal pathogens (J. A. Hoffmann 2003; Govind 2008; Hanson et al. 2019). Cellular immunity is driven by circulating cells in the haemolymph called haemocytes, which in *D. melanogaster* can be classified as plasmatocytes (90 % of haemocytes), lamellocytes and crystal cells (Lavine and Strand 2002). Haemocytes are produced via differentiation from prohemocytes (i.e., hemocyte stem cells) in the lymph glands and proliferation in the host haemolymph, regulated via various genes and signalling pathways, including the Toll pathway (Carton, Poirié, and Nappi 2008; Strand 2008). Upon infection, plasmatocytes adhere and engulf pathogens through a mechanism known as phagocytosis (Elrod-Erickson, Mishra, and Schneider 2000; Stuart and Ezekowitz 2008). Lamellocytes are responsible for forming an overlapping sheath of cells around an aggregation of bacterial cells, a process known as nodulation; or encapsulation in the case of larger targets (Nappi and Vass 1993).

The line between cellular and humoral immunity is sometimes blurred, as these two categories can contribute to the same processes. This is the case for melanisation, whereby pathogens are

killed by the combination of nodulation or encapsulation by lamellocytes, and the production of melanin pigments (Carton, Poirié, and Nappi 2008). This production is triggered upon infection by the activation of the phenoloxidase cascade, where prophenoloxidase produced by haemocytes and readily available in the haemolymph is cleaved into phenoloxidase to produce melanin (Strand 2008; Kanost and Gorman 2008; González-Santoyo and Córdoba-Aguilar 2012). This chemical reaction produces other components, such as reactive oxygen species, which are toxic to the pathogen (González-Santoyo and Córdoba-Aguilar 2012). Clotting is another process which requires both cellular and humoral immunity, whereby a haemolymph clot is formed around wounds on the epithelium via the secretion of various proteins by the haemocytes and fat body (Vlisidou and Wood 2015).

Box 1. *Drosophila melanogaster* immune response against a bacterial infection

Upon entrance of the pathogen via a wound on the epithelium, different mechanisms of the immune response are triggered to control the infection. Illustration modified from Dionne and Schneider (2008).



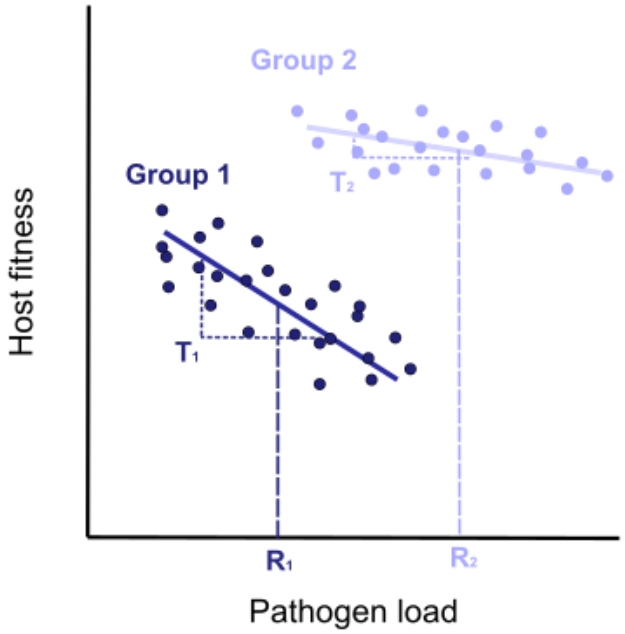
2.4. Tolerance: controlling damage due to the infection

In addition to resistance, hosts can aim to limit the negative effects of an infection on fitness, a strategy known as **tolerance** (see Box 2 on how to measure tolerance) (Råberg, Sim, and Read 2007; Råberg 2014). Infected hosts can sustain fitness costs due to direct damage by the pathogen, but these costs can also arise from the host response itself (reviewed in Medzhitov, Schneider, and Soares 2012). Immune responses can potentially inflict damage to the host tissues and cells, a phenomenon known as immunopathology (Graham, Allen, and Read 2005; Medzhitov, Schneider, and Soares 2012). For example, phenoloxidases are responsible for inflammation-based tissue damage in *Tenebrio molitor* (Khan, Prakash, and Agashe 2017). Hosts are also forced to divert resources from fitness-related traits, such as reproduction or development, into mounting an immune response (Boots and Begon 1993; Bajgar et al. 2015; Bajgar and Dolezal 2018; Nystrand and Dowling 2020).

To deal with these fitness costs, hosts can invest in repairing the damage caused by the pathogen and the immune response, by protecting or repairing damaged tissues (Medzhitov, Schneider, and Soares 2012). While the full spectrum of mechanisms has not been uncovered yet, tolerance has been attributed to physiological processes linked to homeostasis maintenance, rather than specific effector proteins (Medzhitov, Schneider, and Soares 2012; Louie et al. 2016; Lissner and Schneider 2018). Among these processes, metabolic changes and stress reduction mechanisms have been shown to increase tolerance to infection in insects (Dionne et al. 2006; Ayres and Schneider 2009; 2012; Chambers, Song, and Schneider 2012; Troha et al. 2018). Nevertheless, some immune mechanisms involved in resistance may also be involved in disease tolerance. For example, in *D. melanogaster* phagocytosis increases tolerance to infection by *Salmonella typhimurium*, potentially by isolating the pathogen to avoid direct damage to the host tissues (Shinzawa et al. 2009).

Box 2. Measuring resistance and tolerance

Resistance is the ability of a host to reduce its pathogen load, measured by the inverse of the pathogen load (Restif and Koella 2004; Råberg, Graham, and Read 2009; Graham et al. 2011). Tolerance is the ability of a host to reduce the fitness costs of a given infection: it can be described by the slope of the reaction norm, or "range tolerance", of host fitness against pathogen load (Simms 2000; Råberg, Sim, and Read 2007; Råberg, Graham, and Read 2009; Little et al. 2010). Host fitness can be measured through several proxies of health (e.g., weight or cell density), but for short-lived organisms like insects, the most common measures are survival and fecundity (Kutzer and Armitage 2016a). The illustration below represents the reaction norms of two hypothetical host groups, each data point representing one individual. Here, Group 1 is more resistant than Group 2 because it has a lower pathogen load ($R_1 < R_2$). Group 2 has a shallower slope than Group 1 ($T_2 > T_1$), therefore Group 1 has a higher tolerance to the infection, i.e., it sustains a lower fitness reduction for a given pathogen load. Illustration modified from Råberg et al. (2007), Regoes et al. (2014) and Kutzer & Armitage (2016a).



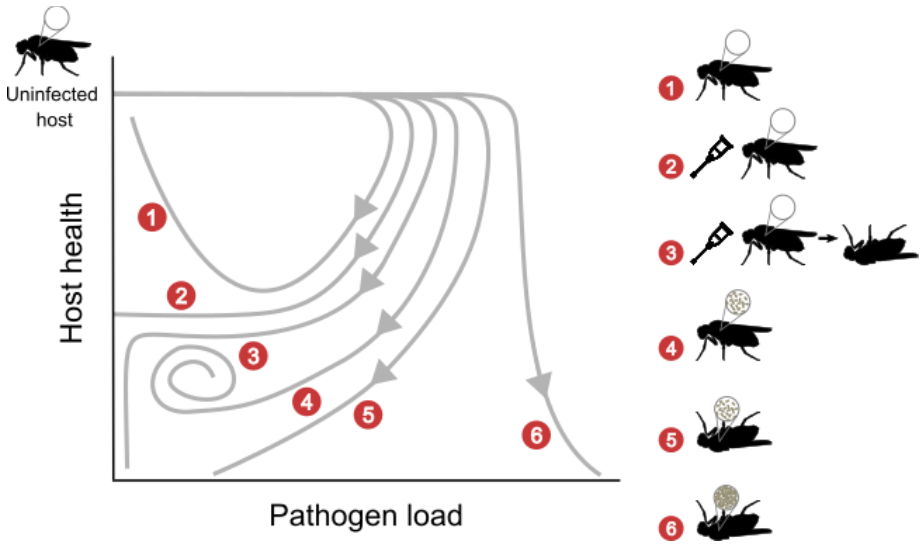
3. Living with the enemy

3.1. What determines the outcome of an infection?

Infections can result in variable outcomes which will be determined by (i) whether the host survives the infection, and (ii) whether the pathogen is cleared or not. Thus, these outcomes can be placed on a gradient ranging from survival of the host with complete clearance of the pathogen, to uncontrolled growth and host death (see Box 3) (Schneider 2011; Duneau et al. 2017). The outcome of an infection is the result of the complex interplay between several host and pathogen processes, which can be variable across different environments (Casadevall and Pirofski 2000; Lazzaro and Little 2009; Kutzer and Armitage 2016a). The role of the environment will not be explored here, but some of the factors relevant for infections include host diet (Howick and Lazzaro 2014; Zeller and Koella 2017; Kutzer and Armitage 2016b; Kutzer, Kurtz, and Armitage 2018; Manzi et al. 2020), temperature (Debes, Gross, and Vasemägi 2017; Manzi et al. 2020; Wang et al. 2020) and interactions with other organisms (Thomas, Watson, and Valverde-Garcia 2003; Libertucci and Young 2019). At the level of the host and pathogen, the processes at play on each side of the interaction are aimed towards gaining fitness (Little et al. 2010). On one hand, the pathogen will increase its fitness by exploiting the host resources while withstanding or evading the immune response (Vallet-Gely, Lemaitre, and Boccard 2008; Råberg and Stjernman 2012). On the other hand, the host will maximise its fitness by defending against the infection through resistance and tolerance (Råberg, Graham, and Read 2009; Råberg 2014). These host- and pathogen-specific factors combine to determine the virulence of the infection (Casadevall and Pirofski 1999; 2003; Råberg and Stjernman 2012). Virulence is the disease severity as assessed by a reduction in host fitness and will reflect the outcome of infection in terms of host survival (Read 1994; Råberg and Stjernman 2012).

Box 3. The outcome of an infection

By plotting host health against pathogen load over time, one can visualise how the infection unravels for an individual host (Schneider 2011; Lough et al. 2015; Torres et al. 2016). Below are illustrated the hypothetical routes and outcomes of infection. Each data point per curve represents one timepoint at which health and pathogen load are assessed for one individual. The possible scenarios are the following: the host clears the infection and survives (1) with a complete recovery, i.e., health returns to uninfected levels; (2) with permanent stable damage, i.e., health is decreased but does not vary over time; (3) with permanent unstable damage, i.e., health decreases over time; (4) the host survives with a persistent infection; (5) the host dies while defeating the pathogen; (6) uncontrolled growth and host death. Illustration modified from Schneider (2011).



3.2. Persistent bacterial infections

Some infections result in an intermediate scenario where the host does not clear the pathogen but survives with a persistent infection. When it comes to bacterial infections, diverse bacterial pathogens can cause persistent infections in various host taxa: in humans (reviewed in Grant and Hung 2013), but also in various insect species, including *D. melanogaster* (Boman, Nilsson, and Rasmuson 1972, 2; Hotson and Schneider 2015; Kutzer, Kurtz, and Armitage 2019), *T.*

molitor (Haine et al. 2008; McGonigle, Purves, and Rolff 2016) and the mosquito *Anopheles gambiae* (Gorman and Paskewitz 2000).

Persistence represents a challenge when it comes to the control of infectious diseases. Eliminating chronic bacteria is difficult, often necessitating prolonged and high-dosed antibiotic treatments, which may lose efficiency due to the emergence of antibiotic resistant bacteria (Brown and Frank 2003; Lyczak, Cannon, and Pier 2002). Chronically infected hosts maintain the pathogen prevalent in a population, spreading the infection as they experience relapses (Marzel et al. 2016). Even when presenting no active symptoms, hosts can become carriers of the disease (Gal-Mor 2018). A prominent example is that of Mary Mallon (a.k.a. Typhoid Mary), an asymptomatic *Salmonella* Typhi carrier who infected 120 people with typhoid fever over the course of 15 years in the late 1800s (Marineli et al. 2013). Asymptomatic carriers are not a rare phenomenon; during typhoid fever epidemics, around 10 % of infected patients become short-term carriers (up to three months) and 1-4 % continue shedding bacteria for more than 12 months (Vogelsang and Bøe 1948; Levine, Black, and Lanata 1982; Buchwald and Blaser 1984). Uncovering under which conditions persistence is established and maintained in a host is without doubt essential to determine the best course of action to treat a patient or handle an epidemic (Young, Stark, and Kirschner 2008; Alizon, Luciani, and Regoes 2011; Schneider 2011). Nevertheless, persistent infections are also interesting for the evolutionary ecology of host-pathogen interactions. Asymptomatic carriers represent instances where the pathogen can infect the host without causing harm, reinforcing the idea that the line between pathogen and commensal may be blurred for some microbes (Casadevall and Pirofski 2000). Understanding how host and pathogen interact over the course of a persistent infection can inform us on the selective pressures that lead to the evolution of mutually benign associations.

3.3. The dynamics of host defences against persistent infections

3.3.1. Host resistance and the onset of persistence

Infections are dynamic because the processes that contribute to virulence do not remain static over the course of an infection (Schneider 2011; Lough et al. 2015; Torres et al. 2016). Louie *et al*. (2016) showed that *D. melanogaster* infected with *Listeria monocytogenes* goes through several stages of infection, characterised by a variation in bacterial load, and the differential

expression of traits associated with its health (e.g., morbidity-correlated genes) and immune response (e.g., expression of AMPs) (Louie et al. 2016). Accordingly, chronic bacterial infections do not automatically switch to persistence upon hosts becoming infected. Rather, persistent infections have distinct phases: an early phase characterised by rapid growth of the pathogen, followed by a resolution phase where bacterial load stabilises, and a chronic phase where hosts carry a constant bacterial load (Howick and Lazzaro 2014; Duneau et al. 2017). However, under identical experimental settings (i.e., host genotype), Duneau *et al*. (2017) showed that in fruit flies not all infections result in persistence: during the resolution phase, bacterial load continues to increase in some hosts, eventually leading to their death. Whether hosts survive the early stages of infection seems to be due to the ability of the host to control the pathogen growth during the resolution phase. It is the time it takes the host to mount an antimicrobial response against the infection that plays a key role in the outcome of the disease (Duneau et al. 2017). Thus, hosts with low levels of resistance in the resolution phase are more likely to die, while hosts that survive and become chronically infected are those able to limit the pathogen growth early in the infection.

3.3.2. How do hosts handle a persistent infection?

Chronically infected hosts sustain constant pathogen loads up to one-week post-infection, each bacterial species being characterised by a specific bacterial load, i.e., the set point bacterial load, or SPBL (Fraser et al. 2014; Duneau et al. 2017). This suggests that the infection has reached one of two scenarios: the host is killing the bacteria at the same rate at which they are growing, or the bacteria are not replicating (Howick and Lazzaro 2014). Howick and Lazzaro (2014) suggest the second scenario to be more likely because they observed that survival and fecundity of infected flies return to uninfected levels after the onset of persistence (Howick and Lazzaro 2014). This scenario would be consistent with the observation of asymptomatic persistent infections by certain human bacterial pathogens (Grant and Hung 2013; Fisher, Gollan, and Helaine 2017), e.g., 95 % of tuberculosis patients contain the growth of *Mycobacterium tuberculosis* in the early phases of infection resulting in an asymptomatic chronic infection (Gomez and McKinney 2004).

Hosts that become chronically infected may limit pathogen growth in the beginning of the infection, then switch to a tolerance strategy whereby they manage a persistent infection via

damage-control (Lazzaro and Rolff 2011). During the chronic infection, hosts will have to deal with previous damage due to the initial pathogen growth and immunopathology (Graham, Allen, and Read 2005; Medzhitov, Schneider, and Soares 2012), on top of potential additional costs due to the presence of the pathogen. For example, fecundity decreases in flies chronically infected flies with *S. typhimurium* due to degeneration of the ovaries during acute infection (Brandt and Schneider 2007). At this point in the infection, a tolerance response may be advantageous to protect the host against these costs. Thus, one would expect to observe an increase in tolerance in hosts carrying a persistent infection. Like resistance, tolerance can vary over the course of an infection. For instance, Kutzer and Armitage (2016b) found that the fecundity-tolerance of infected flies increased between 24- and 72-hours post-infection, albeit this effect was weak and restrained to only one of the two bacterial species tested, *Escherichia coli* (Kutzer and Armitage 2016b). However, whether measures of tolerance vary between hosts that survive with a chronic infection and those that succumb to an uncontrolled bacterial growth, is much less understood for insect hosts. Lough *et al*. (2015) measured the health-tolerance of individual mice infected with *L. monocytogenes* over time using body weight as a measure for fitness. They found that health-tolerance initially decreases upon infection in all hosts, and then increases after the resolution phase, but only in mice that ultimately control and survive the infection (Lough et al. 2015). This suggests that increased tolerance may play a role in the ability of hosts to survive the infection after the resolution phase.

Interestingly, it has been observed that chronically infected hosts sustain persistent AMP responses, suggesting that hosts may continue investing in resistance during the chronic phase (Haine et al. 2008; Chambers et al. 2019). This response is only switched on in the first couple of hours post-infection yet remains active for several weeks (Korner and Schmid-Hempel 2004; Haine et al. 2008; Makarova et al. 2016). In *T. molitor*, most of the bacteria are cleared before the activation of this response, suggesting that it may serve the purpose of "mopping up" the bacteria that have survived the constitutive immune response (Haine et al. 2008). However, the infection dynamics of *D. melanogaster* are considerably different. In flies that survive the early stages of infection, the bacterial load is reduced in the first hours, but the immune system does not kill most of the bacteria (Duneau et al. 2017). Past Duneau and colleagues' measures of SPBL at one-week post-infection (Duneau et al. 2017), Kutzer et al. (2019) observed that bacterial load stayed around the same levels between one day and four weeks after the inoculation, although this was found for only one of the two bacterial species tested (Kutzer,

Kurtz, and Armitage 2019). Whether this long-term pattern is generalisable is not clear because additional estimates of bacterial burden over the course of the infection up to this and later timepoints in *D. melanogaster* are not available.

D. melanogaster sustains a persistent AMP response at seven-days post-infection, which comes with energetic costs expressed as a lower resistance to starvation (Chambers et al. 2019). If bacteria are not "mopped up" by this sustained antimicrobial response, what is then the adaptive value of expressing this costly response on the long term? It could be that AMPs serve another purpose than killing the pathogen. For example, coleoptericin, one AMP expressed in infected *T. molitor* contributes to host survival without affecting the bacterial load (Zanchi, Johnston, and Rolff 2017). Moreover, AMPs constitute only one of the components of immunity. To observe the full extent of the host response, it would be helpful to measure the contribution of other aspects of the immune response (e.g., Korner and Schmid-Hempel 2004), but also of tolerance mechanisms that may be coming into play to maintain host health (Schneider 2011). Furthermore, if we want to assess the adaptive value of the observed immune response, we need to understand what this response is pushing against (Schneider 2011). Bacteria possess many strategies by which they can overcome host immunity and persist (reviewed in Grant and Hung 2013). Some of these strategies have been studied in insect hosts. For example, *Staphylococcus aureus* can persist via intracellular infections in both humans and *T. molitor* (Clement et al. 2005; McGonigle, Purves, and Rolff 2016). However, further research that includes the perspective of the pathogen is yet to be done to fully understand how entomopathogenic microbes achieve persistence.

3.3.3. Virulence and the costs of eliminating a persistent infection

There are clear benefits to eliminating a pathogen, but immune responses can be costly to produce and maintain for the host (Armitage et al. 2003; Schmid-Hempel 2003). While an inefficient immune response will fail to protect the host against uncontrolled pathogen growth, an excessive response can cause host-mediated damage through immunopathogenesis (Casadevall and Pirofski 1999; Graham, Allen, and Read 2005; Medzhitov, Schneider, and Soares 2012). Therefore, an immune response strong enough to clear a pathogen may only be advantageous when the benefits of this response out weight the costs. The ability of hosts to evaluate these costs may stem from danger signals, which alert the host about damage sustained

by its tissues and cells (Matzinger 1994; 2002; Vance, Isberg, and Portnoy 2009). In combination with the identification of the pathogen as non-self via recognition of PAMPs (Medzhitov and Janeway 2002), these danger signals could be used by the host to evaluate the pathogenicity of the invader, and determine the appropriate response (Fontana and Vance 2011; Lazzaro and Rolff 2011). Thus, clearing the pathogen may only be advantageous above a certain threshold of damage (Moreno-García et al. 2014).

Accordingly, Duneau *et al.* (2017) showed that clearance occurred for bacterial species causing low mortality infections, such as *E. coli* and *Erwinia carotovora*, but it was not a recurrent scenario as most hosts became chronically infected (Duneau et al. 2017). Other studies using various bacterial species found that hosts do not clear the infection (Brandt et al. 2004; Dionne et al. 2006; Haine et al. 2008; Kutzer and Armitage 2016b; Kutzer, Kurtz, and Armitage 2019). Nevertheless, Kutzer *et al*. (2019) observed that in contrast with *Lactococcus lactis* which was only cleared in one out of 29 flies four-weeks post- infection, *Pseudomonas entomophila* was cleared by all flies alive at that timepoint (Kutzer, Kurtz, and Armitage 2019). This bacterial species causes significantly higher mortality compared to *L. lactis* (Kutzer, Kurtz, and Armitage 2019), which could be attributed to the presence of the toxin Monalysin shown to mediate lethality during gut infections by this bacterium (Opota et al. 2011). Damage by this toxin, or other mechanisms, may trigger enough danger signals to elicit an immune response aimed to eliminate the pathogen, making persistence more costly than clearance (Moreno-García et al. 2014). In contrast with *Ps. entomophila*, other bacterial species may not cause sufficient damage for clearance to be more advantageous than sustaining a persistent infection (Lazzaro and Rolff 2011). Different bacterial species may differentially contribute to virulence via variation in the damage they induce through a combination of (i) exploitation, which is the pathogen ability to use the host resources for its own growth, and (ii) per-parasite pathogenicity (PPP), constituted by the damage-inducing mechanisms which are independent of pathogen load (Råberg and Stjernman 2012). Microbes can cause density-independent pathogenicity through mechanisms such as toxins (e.g., anthrax toxins secreted by *Bacillus anthracis*, Liu, Moayeri, and Leppla 2014) and immune suppression (e.g., killing of CD4+ T cells by HIV, Regoes et al. 2014; Bertels et al. 2018). Nevertheless, the role of pathogen-mediated damage in the onset of bacterial persistence remains largely understudied.

Other factors may come into play in determining bacterial clearance through their effect on immunity. The initial exposure dose may be relevant because it can determine the strength of

the immune response, as it was shown for the expression of AMPs in *Tribolium* spp. (Jent et al. 2019). The initial microbe density has been shown to affect host survival in a dose-dependent manner, and thus could also contribute to shaping the virulence of the infection (Louie et al. 2016; Miller and Cotter 2017; Chambers et al. 2019). Furthermore, clearance may occur if the host immune response is enhanced, e.g., due to immune priming (Little and Kraaijeveld 2004). While insects do not possess the adaptive immunity mechanisms of vertebrates, they may be capable of using information on a previously encountered pathogen against a secondary exposure (Kurtz 2005; Milutinović and Kurtz 2016; Pradeu and Du Pasquier 2018). Some studies have shown that immune priming induces a stronger immune response upon the secondary encounter (Pham et al. 2007; Lin et al. 2013; Castro-Vargas et al. 2017; Dhinaut, Chogne, and Moret 2018), leading to increased resistance (Boman, Nilsson, and Rasmuson 1972; Pham et al. 2007; Miyashita et al. 2014). In *D. melanogaster*, one study showed that hosts previously exposed to *Streptococcus pneumoniae* had an improved clearance of the infection mediated by phagocytosis (Pham et al. 2007). However, not all studies have found evidence for enhanced resistance (e.g., Kutzer, Kurtz, and Armitage 2019), and some studies show that pathogens are not always eliminated in primed hosts (Rodrigues et al. 2010; Contreras-Garduño et al. 2015). Thus, the contribution of immune priming to the outcome of infection, and clearance, remains unclear (reviewed in Contreras-Garduño et al. 2016; Milutinović and Kurtz 2016).

4. Aims and overview of the thesis

Infections are dynamic interactions whereby the host- and pathogen-specific processes that contribute to virulence can change. Thus, the most appropriate defence strategy at one point in the infection may not be the same at a later point (Schneider 2011; Lough et al. 2015; Torres et al. 2016). For example, a strong immune response may be advantageous in the beginning of the infection to control pathogen growth, but costly to sustain over long periods of time (Duneau et al. 2017; Chambers et al. 2019). Therefore, to understand how persistence arises and is maintained, it is essential to measure these processes at various timepoints (Boughton, Joop, and Armitage 2011; Ayres and Schneider 2012). Following these premises, the present work takes on a dynamic approach to persistence by exploring host defences and their contribution to the outcome of disease over the time course of infection.

Much of the long-term dynamics of persistent infections is not well understood. When it comes to clearing the infection, it seems that dynamics can vary substantially depending on the virulence among (e.g., *L. lactis* and *Ps. entomophila*, Kutzer, Kurtz, and Armitage 2019). In **Chapter 1**, we aimed to uncover the long-term dynamics of infection across different bacterial species, from the onset of persistence to the death of the host. We injected *D. melanogaster* with a gradient of infectious doses of one of four bacterial species: *Enterobacter cloacae*, *L. lactis*, *Providencia burhodogranariea*, and *Ps. entomophila*. Then we measured the host survival and bacterial load over the course of the infection. We found that these different bacterial species could be placed on a gradient of virulence based on host mortality (ranging from lowest to highest: *E. cloacae*, *Pr. burhodogranariea*, *L. lactis* and *Ps. entomophila*), and that they presented substantially different dynamics of infection. All bacterial species could persist or be cleared, although at varying degrees. Clearance most often happened in species at the ends of the virulence spectrum, i.e., *E. cloacae* and *Ps. entomophila*, while the other two bacteria persisted more often. To explain how pathogen virulence shapes the clearance patterns observed, we decomposed virulence into two pathogen factors, exploitation and PPP (Råberg and Stjernman 2012; Bertels et al. 2018). Exploitation, but not PPP, was a good predictor for clearance, indicating that the pathogens that successfully achieve high burdens are cleared less often by the host immune system. Chapter 1 allowed us to identify and characterise the infection dynamics for two highly persistent bacteria, *L. lactis* and *Pr. burhodogranariea*, marking a solid baseline for the following chapters.

Amongst the factors that affect the outcome of infection, whether the host has already encountered the pathogen may play a role in its ability to reduce and clear pathogen load, a phenomenon termed immune priming (Little and Kraaijeveld 2004). While a previous encounter has been shown to increase clearance (e.g., Pham et al. 2007), it seems that the pathogen is not always eliminated (Rodrigues et al. 2010; Contreras-Garduño et al. 2015). Moreover, studies on immune priming have found conflicting evidence on the effects of a previous encounter, which could potentially be explained by the widely different methods across studies (e.g., the antigenic preparation used for the primary exposure, reviewed in Milutinović and Kurtz 2016; Contreras-Garduño et al. 2016). Therefore, **Chapter 2** explores the effect of a previous exposure with different antigenic preparations, namely different inactivation methods, on the outcome of a persistent infection by *L. lactis* or *Pr. burhodogranariea*. We exposed flies to inactivated bacteria, infected them with live bacteria

and assayed survival and resistance in the early and chronic stages of infection (days one and seven). While we did not find an advantage of a previous exposure to neither bacterial species across the different antigenic treatments, we observed that resistance varies over the course of the infection.

In Chapters 1-2, we observed that the bacterial load of flies seems to branch into two populations of hosts with different pathogen loads at one-day post-infection. Duneau *et al*. (2017) showed that these populations with different bacterial loads are predicted to have different infection outcomes: hosts presenting high loads will succumb to an uncontrolled bacterial growth, while those with low loads will survive with a chronic infection (Duneau et al. 2017). In **Chapter 3**, we aimed to test whether these host populations which vary in resistance, also vary in their tolerance to the infection. We infected flies with *L. lactis* or *Pr. burhodogranariea* and measured their fecundity and bacterial load at two timepoints during the early chronic phase (days two and four). By plotting fecundity against bacterial load, we estimated the range fecundity-tolerance for the flies and compared them across host populations (high vs. low load) and timepoints (day two vs. day four). We found that both bacterial species caused a decrease in fecundity over time, and that this decrease was more pronounced in flies carrying a high load, compared to those with low loads. The cost of the infection on fecundity was more pronounced in flies predicted to succumb to the infection. However, only flies infected with *L. lactis* experienced a decrease in fecundity-tolerance over time, and lower tolerance in the flies with high loads vs. low loads. This indicated that for this bacterium, flies with higher resistance also express higher fecundity-tolerance, and that the costs of sustaining a persistent *L. lactis* infection increase over time, while those caused by *Pr. burhodogranariea* may potentially appear later in the infection.

Hosts that carry persistent infections also sustain upregulated AMP responses (Haine et al. 2008; Chambers et al. 2019). Because this response is costly, one would expect that it would confer an advantage to the host in managing the infection, e.g., by reducing pathogen load. Yet in *D. melanogaster*, the bacterial load seems to stay constant over time (Duneau et al. 2017; Kutzer, Kurtz, and Armitage 2019). Beyond this sustained antimicrobial response, it is unclear how the host and pathogen are interacting during the chronic infection. To gain hindsight on what is happening inside the host, in **Chapter 4** we measured the protein expression of the host at seven-days post-infection with *Pr. burhodogranariea*. We aimed to understand the full scope of how the host is responding to the persistent infection. We found that while the most

upregulated proteins in the host were AMPs, the overall host response was complex and involved various branches of the immune system. For instance, we observed upregulation of transferrin, a protein that has been shown to be involved in iron sequestration as a strategy to control pathogen growth, indicating chronically infected hosts may potentially uphold resources from the pathogen (Barber and Elde 2014; Dudzic et al. 2019). As part of Chapter 4, we aimed to understand whether the pathogen is in a non-replicating stage inside the host, which would explain the constant bacterial load observed in *D. melanogaster*. While we did not detect enough pathogen proteins inside the host to determine if it is replicating, we obtained the protein expression patterns of our in vitro dormant and replicating bacterial controls, which may be helpful for future studies to target specific proteins when studying persistent bacteria.

Through the four chapters presented here, this thesis provides a multi-angled investigation on the dynamics of host defences against persistent infections. Our work allowed us to characterise the long-term dynamics of persistence, leading us to find unprecedented estimates for the duration of chronic infections: we could retrieve bacteria from flies up to **75 days** post-infection. All throughout Chapters 1-3, we observed that both resistance and tolerance can vary over the course of the infection, but also across different bacterial pathogens, highlighting the importance of considering virulence and the outcome of infection as resulting from a combination of both host- and pathogen-specific and time-dependent factors. Finally, Chapter 4 underlines the necessity of measuring various aspects of host defences, as different mechanisms linked to both resistance and tolerance may contribute to creating the most suitable strategy to overcome the infection.

CHAPTER 1:

DECOMPOSING VIRULENCE TO UNDERSTAND BACTERIAL CLEARANCE IN PERSISTENT INFECTIONS

Decomposing virulence to understand bacterial clearance in persistent infections

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SA conceived the overall idea. BAH, LS and SA designed the experiments and collected the data. BAH, LS, MF and SA wrote the manuscript. MF & RRR conceived the virulence decomposition and clearance analyses and MF, RRR & SA analysed the data. All authors contributed critically to the drafts.

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Abstract

Hosts are not always successful at controlling and eliminating a pathogen. Insects can sustain persistent bacterial infections, but the conditions under which clearance occurs are not well understood. Here we asked what role pathogen virulence and infection dose play in bacterial persistence and clearance in both live and dead flies. We also sought to understand the basis of variation in virulence, by asking if it is due to differences in exploitation, i.e., how well bacteria can replicate inside the host, or due to differences in the amount of damage per parasite inflicted on the host, i.e., per parasite pathogenicity (PPP), and how exploitation and PPP relate to clearance probability. We injected *Drosophila melanogaster* with one of four bacterial species, which we hypothesised should cover a spectrum of virulence: *Enterobacter cloacae*, *Providencia burhodogranariea*, *Lactococcus lactis* and *Pseudomonas entomophila*. The injection doses spanned four orders of magnitude, and survival was followed to estimate virulence. Bacterial load was quantified in live flies during the acute (1-4 days) and chronic (7-35 days) phases of infection, and we assayed infection status of flies that had died up to ten weeks post infection. We show that sustained persistent infection and clearance are both possible outcomes for bacterial species across a range of virulence. Bacteria of all species could persist inside the host for at least 75 days, and injection dose partly predicts within species variation in clearance. Our decomposition of virulence showed that species differences in bacterial virulence could be explained by a combination of variation in both exploitation and PPP, and that higher exploitation leads to lower bacterial clearance. These results indicate that bacterial infections in insects persist for considerably longer than previously thought, and that decomposing virulence into exploitation and PPP will help us to understand more about the factors affecting infection clearance.

1. Introduction

Once a host has become infected, the immune system will potentially limit pathogen growth, a response termed host resistance (Best, White et al. 2008, Råberg, Graham et al. 2009, Schmid-Hempel 2011). Resistance can therefore be quantified as the inverse of pathogen load (Råberg, Graham et al. 2009). Although there are clear benefits to the host of being able to mount an immune response that suppresses pathogen growth, resistance can come with evolutionary (Boots and Begon 1993, Kraaijeveld and Godfray 1997) and usage costs (Armitage, Thompson et al. 2003) for the host (reviewed in Schmid-Hempel 2003). During infection, hosts may re-allocate resources from other life history traits, such as reproduction (Nystrand and Dowling 2020) or development (Bajgar, Kucerova et al. 2015), into mounting an immune response. Furthermore, immune responses can lead to self-inflicted damage to the host, namely immunopathology (Graham, Allen et al. 2005, Sadd and Siva-Jothy 2006, Khan, Agashe et al. 2017). Therefore, whether a pathogen is eliminated or not, *i.e.*, persists, is likely to depend upon the costs of infection *versus* the costs and effectiveness of the immune response against the infection, in addition to how well the pathogen can survive and replicate in the host environment.

Across host taxa, there is ample evidence of persistent bacterial infections. For example bacterial infections caused by *Escherichia coli* and *Staphylococcus aureus* can evade the human immune system and persist inside the host (Grant and Hung 2013). After injection with bacteria, insects have also been shown to sustain persistent systemic infections, for example in the mosquito *Anopheles gambiae* (Gorman and Paskewitz 2000), the fruit fly *D. melanogaster* (Boman, Ingrid et al. 1972, Hotson and Schneider 2015) and the yellow mealworm beetle *Tenebrio molitor* (Haine, Moret et al. 2008). These experimentally-induced infections can persist for at least 28 days in both *T. molitor* (Haine, Moret et al. 2008) and *D. melanogaster* (Kutzer, Kurtz et al. 2019), although longer term estimates are lacking.

Disparate bacterial species have been shown to be able to chronically infect (here defined as a minimum of seven days) the host species used in this study, *D. melanogaster* (Boman, Ingrid et al. 1972, Brandt, Dionne et al. 2004, Dionne, Pham et al. 2006, Hotson and Schneider 2015, Kutzer and Armitage 2016, Duneau, Ferdy et al. 2017, Chambers, Jacobson et al. 2019, Kutzer, Kurtz et al. 2019). Persistent infections could be influential because the inability to clear an

infection will result in more infected individuals in a population, thereby potentially increase the probability of pathogen transmission. In the chronic infection phase in *D. melanogaster*, the bacterial load has been shown to stabilise around a relatively constant pathogen load over time (Hotson and Schneider 2015, Duneau, Ferdy et al. 2017), which has been termed the set point bacterial load (SPBL; Duneau, Ferdy et al. 2017), after the set point viral load (e.g., Regoes, McLaren et al. 2014). However a stable infection load over time is not necessarily always the case, as the load for some bacterial species can gradually reduce in the days following infection, for example, *D. melanogaster* injected with *E. coli* (Kutzer and Armitage 2016) and *T. molitor* injected with *S. aureus* (Haine, Moret et al. 2008, Zanchi, Johnston et al. 2017). Alternatively, after an initial decline the infection load can start to increase again, as seen in the burying beetle, *Nicrophorus vespilloides*, injected with *Photorhabdus luminescens* (Miller and Cotter 2017).

Bacterial clearance during the chronic infection phase has not been commonly reported in insects and it may be related to the costs and benefits of immune system activation, and the degree of pathogen virulence. Virulence can be defined as disease severity, given as the decrease in host fitness caused by a pathogen (Read 1994), and which we here measure as reduced host survival. Virulence will be influenced by both host and parasite traits, *i.e.*, it depends on resistance and tolerance from the host side, and the ability of the parasite to replicate and cause damage to the host (Råberg and Stjernman 2012). From the pathogen perspective, variation in virulence across parasite strains could be due to differences in exploitation, that is an increase in virulence is a side effect of an increase in pathogen load (Råberg and Stjernman 2012, Råberg 2014). However, variation in virulence could also be due to differences in per-parasite pathogenicity (PPP), *i.e.*, the damage inflicted by each individual pathogen. PPP can be quantified by the slope of the reaction norm linking infection intensity (pathogen load) and host fitness (Råberg and Stjernman 2012, Råberg 2014). A parasite genotype causing a steeper negative slope across a range of infection intensities, suggests higher PPP compared to a parasite genotype infection resulting in a shallower slope. Here we use the concepts of exploitation and PPP to disentangle the causes of variation in virulence caused by infection with different bacterial species. We then use this information to uncover whether exploitation and PPP link to bacterial clearance probability.

Exploitation and PPP are conceptually and mechanistically distinct ways in which parasites may harm their hosts. Accordingly, there could be distinct evolutionary consequences – both

for pathogen and host traits. In the pathogen, reduced PPP and thus reduced virulence may be selected for, because this would result in a longer host infectious period without lowering the transmission rate (Råberg and Stjernman 2012). On the other hand, higher exploitation might be selected for given that higher infection intensities are predicted to increase transmission rates and to take longer to clear (described as the recovery rate). However, increased exploitation and longer clearance will also mean increased virulence and hence trade-offs are expected between virulence, transmission, and clearance (Frank and Schmid-Hempel 2008, Råberg and Stjernman 2012).

Here we focus on how exploitation and PPP affect host traits, specifically the efforts of the host to clear an infection. We hypothesise that exploitation and PPP can affect clearance in different ways, which could result in different, and potentially even opposing, patterns of how variation in virulence is related to clearance. In the following we consider how hosts might be predicted to react to infections with different levels of exploitation, or different levels of PPP. Our argumentation focusses on benefits and costs of clearance. When the costs of mounting an immune response exceed the benefits of clearing an infection, one might predict a host to manage a persistent infection (Lazzaro and Rolff 2011), and vice versa for clearing an infection. Therefore, in general, we assume that hosts are more likely to clear an infection when the ratio of benefits to costs of clearance increases. In addition, as will be explained in more detail below, we assume that exploitation and PPP have different effects on the costs and benefits of clearance.

We assume that PPP mainly affects the benefits of clearance. Higher PPP directly results in a higher host death rate, which makes it more beneficial for the host to clear an infection. Accordingly, we expect that an increase in PPP leads to an increased effort by the host to clear the infection, which should result in a higher clearance rate. In this context it is important to consider that an increased clearance effort could also lead to increased immunopathological effects, *i.e.*, increased clearance costs in the form of increased virulence. Accordingly, such increased virulence would result in an increase in the measured PPP. Thus, the measured PPP could reflect both the pathogen contribution to virulence and also the resulting host contribution to virulence. However, this potentially circular effect does not change our expectation for how PPP should be qualitatively related to clearance. Accordingly, we predict that an increase in measured PPP will be related to an increase in measured clearance.

In contrast to PPP, we assume that exploitation, *i.e.*, infection intensity, affects both the benefits and also the costs of clearance. Similar to the case of PPP, increasing exploitation results in increased virulence, which increases clearance benefits. However, increasing exploitation additionally increases the costs of clearance if we assume that it is more difficult, and thus costlier, to clear infections with higher pathogen loads. Due to this dual increase in benefits and costs, two opposing predictions can be derived. First, if costs increase faster than benefits, we predict that increasing exploitation results in increasing clearance. This prediction is consistent with observations of viral infections in humans and non-human primates where larger viral loads led to a faster decline in viral load or in shorter durations of viremia (reviewed in Althouse, Durbin et al. 2014; , e.g., Ben-Shachar and Koelle 2018). Second, if benefits increase faster than costs, then we predict that increasing exploitation results in decreasing clearance.

The initial exposure dose will also determine the outcome of infection, partly because microbe density at the beginning of an infection can determine the strength of the immune response (Jent, Perry et al. 2019). Not only is dose-dependent survival frequently reported in response to bacterial infections (Louie, Song et al. 2016, Miller and Cotter 2017, Chambers, Jacobson et al. 2019), but bacterial load later in the infection has been demonstrated to correlate with the initial injection dose (Duneau, Ferdy et al. 2017, Chambers, Jacobson et al. 2019). We here test the generality of the latter finding. Furthermore, we predict that lower injection doses are more likely to be cleared; because a smaller population would be more susceptible to being killed by the host immune defences. The latter has been referred to as the inoculum effect in the context of *in vitro* antimicrobial activity studies (Brook 1989, Chin, Rybak et al. 2007, Savini, Luca et al. 2017). However, the extent to which this kind of pattern is generalisable to bacterial infections *in vivo* is poorly understood. Given the argumentation that we present above regarding virulence and clearance, we expect that the relationship between injection dose and clearance to vary according to bacterial virulence.

Here we first injected flies with four candidate bacterial species at a range of infection doses and tested whether they varied in virulence, which was measured via survival after bacterial injection. We then decomposed virulence into its two constituent parts, to ask whether the species-level differences in bacterial virulence that we observed, were due to variation in parasite exploitation (infection intensity) or due to variation in PPP. Thirdly we asked whether all four bacterial species establish a persistent infection by assessing infection status up to 35

days post injection, which is longer than any studies that we are aware of. Fourth we address whether injection dose, bacterial species, or exploitation and PPP affect the likelihood of bacterial clearance from the host. Lastly, by assessing the infection status of flies that had died up to ten weeks post-injection, it allowed us to test whether flies clear the infection before death, and if not, to give a long-term assessment of the duration of persistent bacterial infections in an insect.

2. Materials and Methods

2.1. Fly population and maintenance

We used an outbred population of *Drosophila melanogaster* established from 160 *Wolbachia*-infected fertilised females collected in Azeitão, Portugal (Martins, Faria et al. 2013), and given to us by Élio Sucena. For at least 13 generations prior to the start of the experiments the flies were maintained on standard sugar yeast agar medium (SYA medium: 970 ml water, 100 g brewer's yeast, 50 g sugar, 15 g agar, 30 ml 10 % Nipagin solution and 3 ml propionic acid; Bass, Grandison et al. 2007), in a population cage containing at least 5,000 flies, with non-overlapping generations of 15 days. They were maintained at 24.3 ± 0.2°C, on a 12:12 hours light-dark cycle, at 60-80 % relative humidity. The experimental flies were kept under the same conditions.

2.2. Bacterial species

We used the Gram-positive *Lactococcus lactis* (gift from Brian Lazzaro), Gram negative *Enterobacter cloacae subsp. dissolvens* (hereafter called *E. cloacae*; German collection of microorganisms and cell cultures, DSMZ; type strain: DSM-16657), *Providencia burhodogranariea* strain B (gift from Brian Lazzaro, DSMZ; type strain: DSM-19968) and *Pseudomonas entomophila* (gift from Bruno Lemaitre). *L. lactis* (Lazzaro 2002), *Pr. burhodogranariea* (Juneja and Lazzaro 2009) and *Ps. entomophila* (Vodovar, Vinals et al. 2005) were isolated from wild-collected *D. melanogaster* and can be considered as opportunistic pathogens. *E. cloacae* was isolated from a maize plant, but has been detected in the microbiota of *D. melanogaster* (Cox and Gilmore 2007). These bacterial species were

chosen based on various studies, which together suggest that they may be expected to show a range of virulence (Galac and Lazzaro 2011, Kutzer and Armitage 2016, Duneau, Ferdy et al. 2017, Kutzer, Kurtz et al. 2018, Hanson, Dostálová et al. 2019, Kutzer, Kurtz et al. 2019).

2.3. Experimental design

For each bacterial species, flies were exposed to one of seven treatments: no injection (naïve), injection with *Drosophila* Ringer's (injection control) or injection with one of five concentrations of bacteria ranging from 5 x 10^6 to 5 x 10^9 colony forming units (CFUs)/mL, corresponding to doses of approximately 92, 920, 1,840, 9200 and 92,000 CFUs per fly. The injections were done in a randomised block design by two people. Each bacterial species was tested in three independent experimental replicates. Per experimental replicate we treated 252 flies, giving a total of 756 flies per bacterium (including naïve and Ringer's injection control flies). Per experimental replicate and treatment, 36 flies were checked daily for survival until all of the flies were dead. A sub-set of the dead flies were homogenised upon death to test whether the infection had been cleared before death or not. To evaluate bacterial load in living flies, per experimental replicate, four of the flies were homogenised per treatment, for each of nine time points: one, two, three, four, seven, 14, 21, 28- and 35-days post-injection.

2.4. Infection assay

Bacterial preparation was performed as in Kutzer *et al.* (Kutzer, Kurtz et al. 2019), except that we grew two overnight liquid cultures of bacteria per species, which were incubated overnight for approximately 15 hours at 30 ºC and 200 rpm. The overnight cultures were centrifuged at 2880 rcf at 4 ºC for 10 minutes and the supernatant removed. The bacteria were washed twice in 45 mL sterile *Drosophila* Ringer's solution (182 mmol·L-1 KCl; 46 mol·L-1 NaCl; 3 mmol·L-1 CaCl2; 10 mmol·L-1 Tris·HCl; Werner, Liu et al. 2000) by centrifugation at 2880 rcf at 4°C for 10 minutes. The cultures from the two flasks were combined into a single bacterial solution and the optical density (OD) of 500 µL of the solution was measured in a Ultrospec 10 classic (Amersham) at 600 nm. The concentration of the solution was adjusted to that required for each injection dose, based on preliminary experiments where a range of ODs between 0.1 and 0.7 were serially diluted and plated to estimate the number of CFUs. Additionally, to

confirm *post hoc* the concentration estimated by the OD, we serially diluted to $1:10^7$ and plated the bacterial solution three times and counted the number of CFUs.

The experimental flies were reared at constant larval density for one generation prior to the start of the experiments. Grape juice agar plates (50 g agar, 600 mL red grape juice, 42 mL Nipagin [10 % w/v solution] and 1.1 L water) were smeared with a thin layer of active yeast paste and placed inside the population cage for egg laying and removed 24 hours later. The plates were incubated overnight then first instar larvae were collected and placed into plastic vials (95 x 25 mm) containing 7 ml of SYA medium. Each vial contained 100 larvae to maintain a constant density during development. One day after the start of adult eclosion, the flies were placed in fresh food vials in groups of five males and five females, after four days the females were randomly allocated to treatment groups.

Before injection, females were anesthetised with CO_2 for a maximum of five minutes and injected in the lateral side of the thorax using a fine glass capillary (Ø 0.5 mm, Drummond), pulled to a fine tip with a Narishige PC-10, and then connected to a Nanoject II™ injector (Drummond). A volume of 18.4 nL of bacterial solution, or *Drosophila* Ringer's solution as a control, was injected into each fly. Full controls, *i.e.*, naïve flies, underwent the same procedure but without any injection. After being treated, flies were placed in groups of six into new vials containing SYA medium, and then transferred into new vials every 2-5 days. At the end of each experimental replicate, 50 µL of the aliquots of bacteria that had been used for injections were plated on LB agar to check for potential contamination. No bacteria grew from the Ringer's solution and there was no evidence of contamination in any of the bacterial replicates. In addition, to confirm the concentration of the injected bacteria, serial dilutions were prepared and plated before and after the injections for each experimental replicate, and CFUs counted the following day.

2.5. Bacterial load of living flies

Flies were randomly allocated to the day at which they would be homogenised. Prior to homogenisation, the flies were briefly anesthetised with CO_2 and removed from their vial. Each individual was placed in a 1.5 mL microcentrifuge tube containing 100 µL of pre-chilled LB media and one stainless steel bead (Ø 3 mm, Retsch) on ice. The microcentrifuge tubes were

placed in a holder that had previously been chilled in the fridge at 4 °C for at least 30 minutes to reduce further growth of the bacteria. The holders were placed in a Retsch Mill (MM300) and the flies homogenised at a frequency of 20 Hz for 45 seconds. Then, the tubes were centrifuged at 420 rcf for one minute at 4 ºC. After resuspending the solution, 80 µL of the homogenate from each fly was pipetted into a 96-well plate and then serially diluted 1:10 until $1{:}10^5$. Per fly, three droplets of 5 µL of every dilution were plated onto LB agar. Our lower detection limit with this method was around seven colony-forming units per fly. We consider bacterial clearance by the host to be when no CFUs were visible in any of the droplets. Although we note that clearance is indistinguishable from an infection that is below the detection limit. The plates were incubated at 28 ºC and the numbers of CFUs were counted after ~20 hours. Individual bacterial loads per fly were backcalculated using the average of the three droplets from the lowest countable dilution in the plate, which was usually between 10 and 60 CFUs per droplet.

D. melanogaster microbiota does not easily grow under the above culturing conditions (e.g., Hanson, Dostálová et al. 2019 and personal observation). Nonetheless we homogenised control flies (Ringer's injected and naïve) as a control. We rarely retrieved foreign CFUs after homogenising Ringer's injected or naïve flies (23 out of 642 cases, *i.e.*, 3.6 %). We also rarely observed contamination in the bacteria-injected flies: except for homogenates from 27 out of 1223 flies (2.2 %), colony morphology and colour were always consistent with the injected bacteria (see methods of Lazzaro, Sackton et al. 2006). Twenty one of these 27 flies were excluded from further analyses given that the contamination made counts of the injected bacteria unreliable; the remaining six flies had only one or two foreign CFUs in the most concentrated homogenate dilution, therefore these flies were included in further analyses. For *L. lactis* (70 out of 321 flies), *P. burhodogranaeria* (7 out of 381 flies) and *Ps. entomophila* (1 out of 71 flies) there were too many CFUs to count at the highest dilution. For these cases, we denoted the flies as having the highest countable number of CFUs found in any fly for that bacterium and at the highest dilution (Kutzer and Armitage 2016). This will lead to an underestimate of the bacterial load in these flies.

2.6. Bacterial load of dead flies

For two periods of time in the chronic infection phase, *i.e.*, between 14 and 35 days and 56 to 78 days post injection, dead flies were retrieved from their vial at the daily survival checks and homogenised in order to test whether they died whilst being infected, or had cleared the infection before death. The fly homogenate was produced in the same way as for live flies, but we increased the dilution of the homogenate (1:1 to $1:10^{12}$) because we anticipated higher bacterial loads in the dead compared to the live flies. The higher dilution allowed us more easily to determine whether there was any obvious contamination from foreign CFUs or not. Because the flies may have died at any point in the 24 hours preceding the survival check, and the bacteria can potentially continue replicating after host death, we evaluated the infection status (yes/no) of dead flies instead of the number of CFUs. Dead flies were evaluated for two experimental replicates per bacteria, and 160 flies across the whole experiment. Similar to homogenisation of live flies, we rarely observed contamination from foreign CFUs in the homogenate of dead bacteria-injected flies (3 out of 160; 1.9 %); of these three flies, one fly had only one foreign CFU, so it was included in the analyses. Dead Ringer's injected and naïve flies were also homogenised and plated as controls, with 6 out of 68 flies (8.8 %) resulting in the growth of unidentified CFUs.

2.7. Statistical analyses

Statistical analyses were performed in RStudio version 1.3.1073 (R Core Team 2020). The following packages were used for plotting the data: "ggpubr" (Kassambara 2020), "grid", "gridExtra" (Baptiste 2017), "ggplot2" (Wickham 2016), "scales" (Wickham and Seidel 2020), "survival" (Therneau and Grambsch 2000, Therneau 2020) and "viridis" (Garnier 2018). To include a factor as a random factor in a model it has been suggested that there should be more than five to six random-effect levels per random effect (Bolker, Brooks et al. 2008), so that there are sufficient levels to base an estimate of the variance of the population of effects (Crawley 2007). In our experimental designs, the low numbers of levels within the factors 'experimental replicate' (two to three levels) and 'person' (two levels), meant that we therefore fitted them as fixed, rather than random factors (Crawley 2007). However, for the analysis of

clearance (see 2.7.7) we included species as a random effect because it was not possible to include it as a fixed effect due to the fact that PPP is already a species-level predictor.

2.7.1. Do the bacterial species differ in virulence?

To test whether the bacterial species differed in virulence, we performed a linear model with the natural log of the maximum hazard as the dependent variable and bacterial species as a factor. Post-hoc multiple comparisons were performed using "emmeans". The hazard function in survival analyses gives the instantaneous failure rate, and the maximum hazard gives the hazard at the point at which this rate is highest. We extracted maximum hazard values from time of death data for each bacterial species/dose/experimental replicate. Each maximum hazard per species/dose/experimental replicate was estimated from an average of 33 flies (a few flies were lost whilst being moved between vials etc.). To extract maximum hazard values we defined a function that used the "muhaz" package (S original by Kenneth Hess and R port by R. Gentleman 2019) to generate a smooth hazard function and then output the maximum hazard in a defined time window, as well as the time at which this maximum is reached. To assess the appropriate amount of smoothing, we tested and visualised results for four values (1, 2, 3 and 5) of the smoothing parameter, *b*, which was specified using *bw.grid* (Moore 2016). We present the results from $b = 2$, but all of the other values gave qualitatively similar results (see Figure S1). We used *bw.method="global"* to allow a constant smoothing parameter across all times. The defined time window was zero to 20 days post injection. We removed one replicate (92 CFU for *E. cloacae* infection) because there was no mortality in the first 20 days and therefore the maximum hazard could not be estimated. This gave final sizes of n = 14 for *E. cloacae* and n = 15 for each of the other three species.

$$\text{Model 1: } \log(\text{maximum hazard}) \sim \text{bacterial species}$$

2.7.2. Are virulence differences due to variation in parasite exploitation or PPP?

To test whether the bacterial species vary in PPP, we performed a linear model with the natural log of the maximum hazard as the dependent variable, bacterial species as a factor, and the

natural log of infection intensity as a covariate. We also included the interaction between bacterial species and infection intensity: a significant interaction would indicate variation in the reaction norms, *i.e*., variation in PPP. The package "emmeans" (Lenth 2020) was used to test which of the reaction norms differed significantly from each other. We extracted maximum hazard values from time of death data for each bacterial species/dose/experimental replicate as described in 2.7.1. We also calculated the maximum hazard for the Ringer's control groups, which gives the maximum hazard in the absence of infection (the y-intercept). We present the results from $b = 2$, but all of the other values gave qualitatively similar results (see results). We wanted to infer the causal effect of bacterial load upon host survival (and not the reverse), therefore we reasoned that the bacterial load measures should derive from flies homogenised before the maximum hazard had been reached. For *E. cloacae*, *L. lactis*, and *Pr. burhodogranariea*, for all smoothing parameter values, the maximum hazard was reached after two days post injection, although for smoothing parameter value 1, there were four incidences where it was reached between 1.8- and 2-days post injection. Per species/dose/experimental replicate we therefore calculated the geometric mean of infection intensity combined for days 1 and 2 post injection. In order to include flies with zero load, we added one to all load values before calculating the geometric mean. This was done using the R packages "dplyr" (Wickham, François et al. 2020), "plyr" (Wickham 2011) and "psych" (Revelle 2020). Each mean was calculated from the bacterial load of eight flies, except for four mean values for *E. cloacae*, which derived from four flies each.

For *Ps. entomophila* the maximum hazard was consistently reached at around day one post injection, meaning that bacterial sampling happened at around the time of the maximum hazard, and we therefore excluded this bacterial species from the analysis. We removed two replicates (Ringer's and 92 CFU for *E. cloacae* infection) because there was no mortality in the first 20 days and therefore the maximum hazard could not be estimated. One replicate was removed because the maximum hazard occurred before day 1 for all *b* values (92,000 CFU for *E. cloacae*) and six replicates were removed because there were no bacterial load data available for day one (experimental replicate three of *L. lactis*). This gave final sample sizes of n = 15 for *E. cloacae* and n = 12 for *L. lactis*, and n = 18 for *Pr. burhodogranariea.*

$$\text{Model 2: } \log(\text{maximum hazard}) \sim \log(\text{geometric mean bacterial load}) \times \text{bacterial species}$$

To test whether there is variation in parasite exploitation (infection intensity measured as bacterial load), we performed a linear model with the natural log of infection intensity as the dependent variable and bacterial species as a factor. Similar to the previous model, we used the geometric mean of infection intensity combined for days 1 and 2 post injection, for each bacterial species/dose/experimental replicate. The uninfected Ringer's replicates were not included in this model. Post-hoc multiple comparisons were performed using "emmeans". *Ps. entomophila* was excluded for the reason given above. The sample sizes per bacterial species were: n = 13 for *E. cloacae*, n = 10 for *L. lactis* and n = 15 for *Pr. burhodogranariea.*

$$\text{Model 3: } \log(\text{geometric mean bacterial load}) \sim \text{bacterial species}$$

2.7.3. Are persistent infection loads dose-dependent?

We tested whether initial injection dose is a predictor of bacterial load at seven days post injection (Duneau, Ferdy et al. 2017, Chambers, Jacobson et al. 2019). We removed all flies that had 0 CFU as they are not informative for this analysis. The response variable was natural log transformed bacterial load at seven days post-injection and the covariate was natural log transformed injection dose. Separate models were carried out for each bacterial species. Experimental replicate and person were fitted as fixed factors. By day seven none of the flies injected with 92,000 CFU of *L. lactis* were alive. The analysis was not possible for *Ps. entomophila* infected flies because all flies were dead by seven days post injection.

$$\text{Model 4: } \log(\text{day 7 bacterial load}) \sim \log(\text{injection dose}) + \text{replicate} + \text{person}$$

2.7.4. Calculation of clearance indices

To facilitate the analyses of clearance we calculated clearance indices, which aggregate information about clearance into a single value for each bacterial species/dose/experimental replicate. All indices were based on the estimated proportion of cleared infections (defined as samples with measured zero bacterial load) of the whole initial population. For this purpose, we first used data on bacterial load in living flies to calculate the daily proportion of cleared infections in live flies for the days that we sampled. Then we used the data on fly survival to

calculate the daily proportion of flies that were still alive. By multiplying the daily proportion of cleared flies in living flies with the proportion of flies that were still alive, we obtained the proportion of cleared infections of the whole initial population – for each day on which bacterial load was measured. We then used these data to calculate three different clearance indices, which we used for different analyses. For each index we calculated the mean clearance across several days. Specifically, the first index was calculated across days one and two post injection (clearance $index_{1,2}$); the second index was calculated across days three and four (clearance $index_{3,4}$); and the third index was calculated from days seven, 14 and 21 (clearance $index_{7,14,21}$).

2.7.5. Does injection dose affect bacterial clearance?

For this purpose, we aimed to assess clearance that occurs shortly after injection. Accordingly, we used the clearance index that was calculated for days one and two post injection. We suspected that the effect of dose on clearance might differ between bacterial species. Therefore, we ran separate tests for each species. The distribution of clearance values did not conform to the assumptions of linear models, therefore we used Spearman rank correlations to test whether clearance changes with injection dose.

Model 5: clearance $index_{1,2} \sim$ injection dose

2.7.6. Do the bacterial species differ in clearance?

To test whether the bacterial species differed in clearance, we used clearance $index_{3,4}$, which is the latest timeframe for which we could calculate this index for all four species: due to the high virulence of *Ps. entomophila* we were not able to assess bacterial load and thus clearance for later days. The distribution of clearance values did not conform to the assumptions of a linear model. We therefore used a Kruskal-Wallis test with pairwise Mann-Whitney-U post hoc tests. To control for multiple testing we corrected the p-values of the post hoc tests using the method proposed by Benjamini and Hochberg (Benjamini and Hochberg 1995) that is implemented in the R function *pairwise.wilcox.test*.

Model 6: clearance $index_{3,4} \sim$ bacterial species

2.7.7. Do exploitation or PPP predict variation in clearance?

To assess whether exploitation or PPP predict variation in clearance we performed separate analyses for clearance index$_{3,4}$ and clearance index$_{7,14,21}$. As discussed above, this precluded analysing *Ps. entomophila*. Exploitation and PPP were calculated based on bacterial load from days 1 and 2 (see section 2.7.2), therefore we did not perform an analysis for clearance index$_{1,2}$. For each of the two indices we fitted a linear mixed effects model with the clearance index as the response variable. As fixed effects predictors we used the replicate-specific geometric mean log bacterial load (see section 2.7.2) and the species-specific PPP (see section 3.2). In addition, we included species as a random effect.

In our analysis we faced the challenge that many measured clearance values were at, or very close to zero. In addition, clearance values below zero do not make conceptual sense. To appropriately account for this issue, we used a logit link function (with Gaussian errors) in our model, which restricts the predicted clearance values to an interval between zero and one. Initial inspections of residuals indicated violations of the model assumption of homogenously distributed errors. To account for this problem, we included the log bacterial load and PPP as predictors of the error variance, which means that we used a model in which we relaxed the standard assumption of homogenous errors and account for heterogenous errors by fitting a function of how errors vary. For this purpose, we used the option *dispformula* when fitting the models with the function *glmmTMB*.

$$\text{Model 7: clearance index}_{3,4} \text{ or clearance index}_{7,14,21} \sim \log(\text{geometric mean bacterial load}) + \text{PPP} + \text{bacterial species}_{\text{random}}$$

2.7.8. Is the infection cleared before death, and is clearance dependent upon the injection dose?

Using binomial logistic regressions, we tested whether initial injection dose affected the propensity for flies to clear an infection with *E. cloacae* or *Pr. burhodogranariea* before they died. The response variable was binary whereby 0 denoted that no CFUs grew from the homogenate and 1 denoted that CFUs did grow from the homogenate. Natural log transformed injection dose was included as a covariate as well as its interaction with day post injection, and

person was fitted as a fixed factor. Replicate was included in the *Pr. burhodogranariea* analysis only, because of unequal sampling across replicates for *E. cloacae. L. lactis* injected flies were not analysed because only 4 out of 39 (10.3 %) cleared the infection. *Ps. entomophila* infected flies were not statistically analysed because of a low sample size (n = 12). The two bacterial species were analysed separately.

Model 8: $\text{CFU presence/absence}_{\text{dead}} \sim \log(\text{injection dose}) \times \text{day post injection} + \text{replicate} + \text{person}$

2.7.9. Do the proportions of dead and live uninfected flies correlate with each other?

To test whether the proportion of live uninfected flies was a predictor of the proportion of dead uninfected flies, we separately summed up the numbers of uninfected and infected flies for each bacterial species and dose, giving us a total sample size of n = 20 (four species × five doses). For live and for dead homogenised flies we had a two-vector (proportion infected and proportion uninfected) response variable, which was bound into a single object using cbind. The predictor was live flies, and the response variable was dead flies, and it was analysed using a generalized linear model with family=quasibinomial.

Model 9: $\text{cbind}(\text{dead uninfected}, \text{dead infected}) \sim \text{cbind}(\text{live uninfected}, \text{live infected})$

3. Results

3.1. Bacterial species vary in virulence

Fly survival after infection with five doses of the four different bacterial species is shown in Figure 1A-D. As predicted, the bacterial species differed significantly in virulence, given as the maximum hazard ($F_{3,55} = 193.05$, $p < 0.0001$; Figure 1E). All species differed significantly from each other ($p < 0.0017$ in all cases; Table S1): *E. cloacae* was the least virulent and *Ps. entomophila* the most virulent bacterium. *Pr. burhodogranariea* and *L. lactis* were intermediate, with the former being less virulent than the latter. In all figures, the bacterial species are thus presented in order of virulence.

3.2. Differences in virulence are due to variation in parasite exploitation and PPP

We assessed infection intensity over time post injection (Figure 2) and used the geometric mean of the values for the first two days post injection, as a proxy for parasite exploitation (see methods for rationale). Bacterial species varied significantly in exploitation of their hosts ($F_{2,35}$ = 35.90; $p < 0.0001$; Figure 3A). The least virulent bacterium, *E. cloacae*, had a significantly lower infection intensity, and thereby lower parasite exploitation, compared to either of the other species (Tukey contrasts: *E. cloacae vs. Pr. burhodogranariea*: t = -5.24, $p < 0.0001$; *E. cloacae vs. L. lactis*: t = -8.36, $p < 0.0001$). The more virulent bacterium, *L. lactis*, had the highest infection intensity, and differed significantly compared to the less virulent *Pr. burhodogranariea* (*L. lactis vs. Pr. burhodogranariea*: t = 3.50, p = 0.0018).

The slopes of the relationship between infection intensity and maximum hazard differed significantly across bacterial species, suggesting that the bacterial species differed in their PPP (infection intensity × bacterial species: $F_{2,39}$ = 7.35, p = 0.0020; Figure 3B). *E. cloacae* had a relatively flat reaction norm, indicating a minimal increase in hazard with an increase in bacterial load, and thus a significantly lower PPP compared to both *Pr. burhodogranariea* (Tukey contrast: t = -3.74; p = 0.0017) and *L. lactis* (t = -3.34; p = 0.0052). In contrast, the latter two species had similar PPP to each other (t = -0.68; p = 0.78); both species had negative reaction norms, indicating an increase in hazard with an increase in bacterial load. There was no significant effect of bacterial load ($F_{1,39}$ = 0.19, p = 0.67) or bacterial species $F_{2,39}$ = 0.50, p = 0.61) on the maximum hazard. Qualitatively similar results were obtained using the three alternative smoothing parameters (Figure S1).

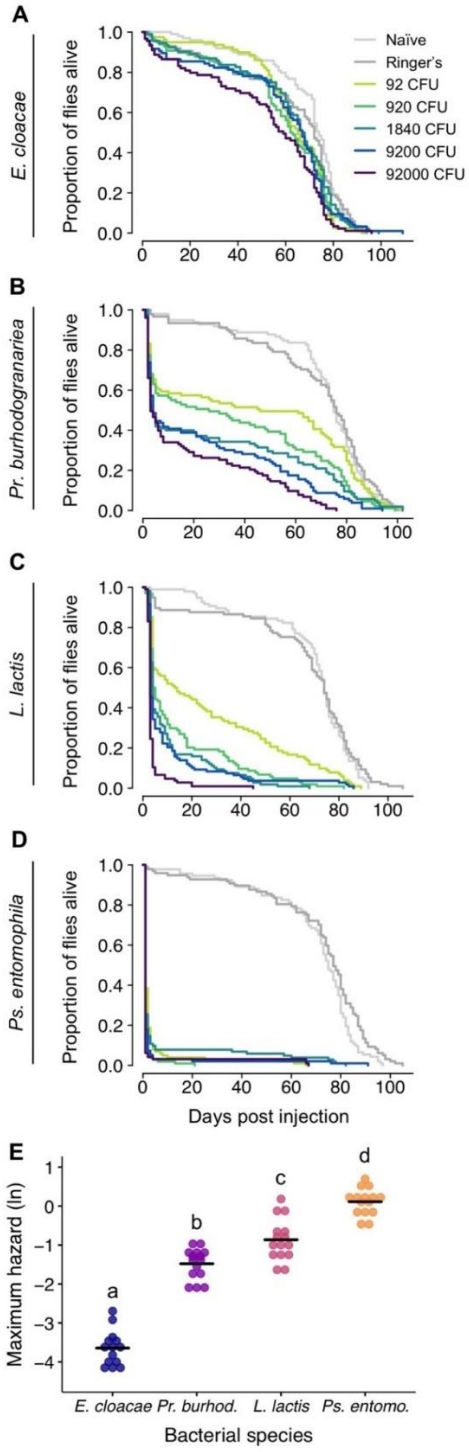

Figure 1. Fly survival after injection with one of four bacterial species**. A** – **D**. Survival curves after injection with four bacterial species, each at one of five doses. Controls were either injected with Ringer's solution or received no injection (naïve). Each survival curve is from n = 79 to 108 flies. The legend in panel **A**, shows the treatments for all survival curves. **E**. The natural log of maximum hazard for all bacterial species, where each data point is the maximum hazard calculated from one replicate per dose. Ringer's injected and naïve flies are not included. Black lines show means. Different letters denote means that are significantly different from one another (Tukey multiple comparison test).

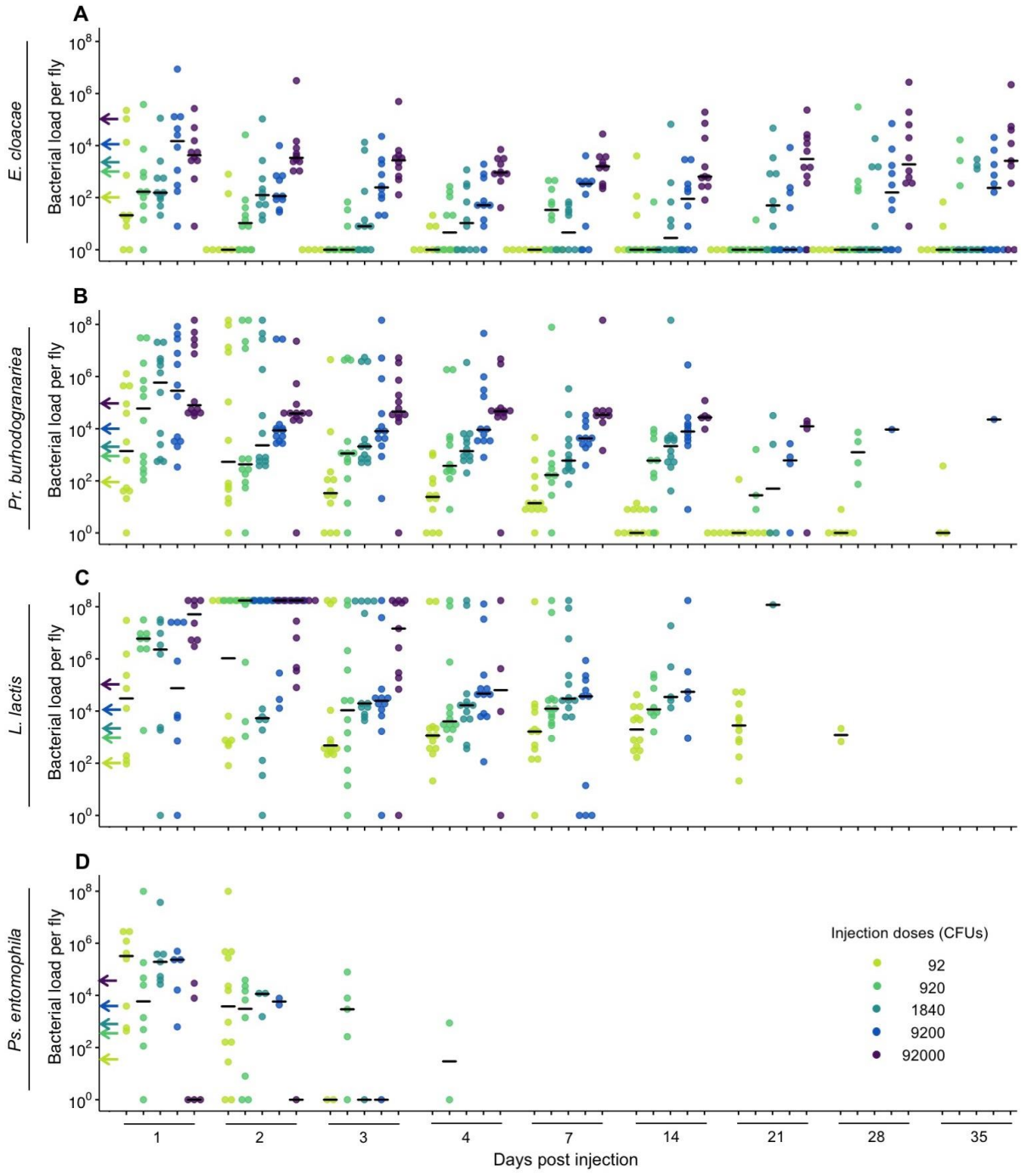

Figure 2. Bacterial load per living fly after injection with one of four bacterial species (**A** – **D**). Flies were homogenised at between 1- and 35-days post-injection. The injection dose legend for all panels is shown in **D**. The arrows on the y-axis indicate the approximate injection doses. Missing data are due to increasing fly death over time. Black lines show medians.

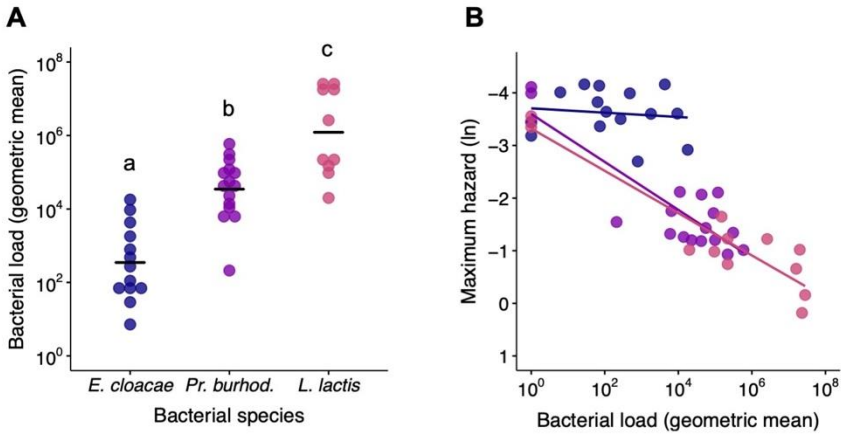

Figure 3. Virulence decomposition. **A**. Parasite exploitation given as infection intensity/bacteria load across bacterial species. Each data point is from one injection dose per bacteria, per experimental replicate, and gives the geometric mean of bacterial load for days one and two post injection. The circles are jittered along the x-axis to aid visualisation of overlapping data points. Black lines show means. Different letters denote means that are significantly different from one another (Tukey multiple comparison test). **B**. PPP given as the relationship between bacterial load and maximum hazard. The bacterial load data is the same as that given in **A** but with the addition of the Ringer's control group. To allow inclusion of the uninfected Ringer's control group to the figure we added one CFU to all mean bacterial load values. The natural log of maximum hazard data is estimated from survival data for the corresponding injection doses and experimental replicates. Maximum hazard is plotted as the inverse, such that the hazard (virulence) increases with proximity to the x-axis. Lines show linear regressions.

3.3. All bacterial species established persistent infections

By homogenising living flies, we found that the two bacterial species with lower virulence, *E. cloacae* and *Pr. burhodogranariea* were able to persist inside the fly until at least 35 days post injection (Figures 2A and 2B respectively). The persistence estimates for *L. lactis* (28 days; Figure 2C) and *Ps. entomophila* (four days; Figure 2D) were both shorter, because the high mortality caused by these bacterial species meant that we could not test later time points. However, by testing for the presence or absence of bacteria in homogenised dead flies, we found that infections could persist for considerably longer, *i.e.*, around two and a half months: *E.* cloacae = 77 days, *Pr. burhodogranariea* = 78 days, *L. lactis* = 76 days and *Ps. entomophila* = 75 days (Figure 4A-D).

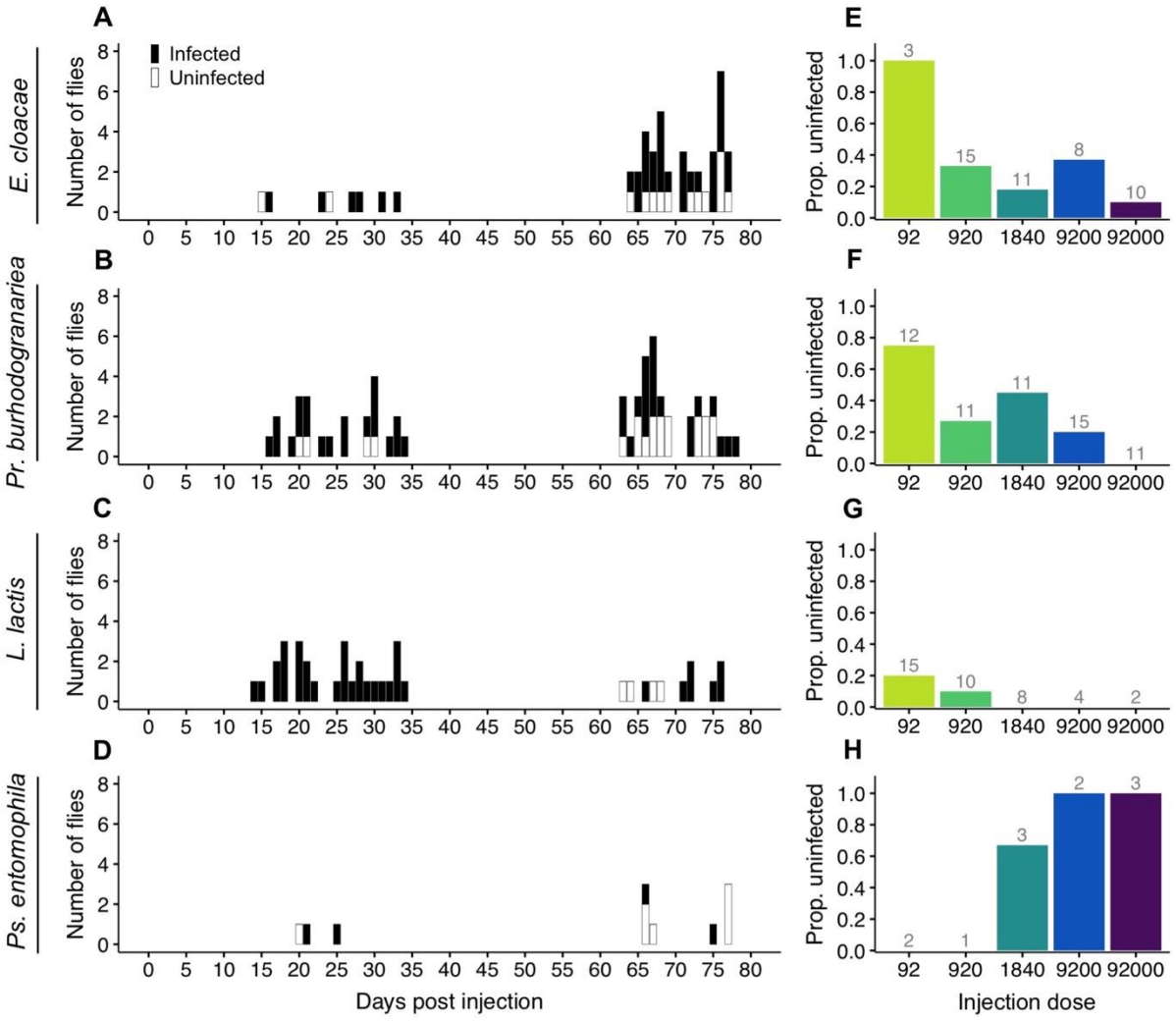

Figure 4. Bacterial clearance in dead flies. Each row shows flies that had been injected with one of four bacterial species. **A-D** The proportion of dead flies that were infected and uninfected according to the day post injection at which they died and were homogenised. Dead flies were homogenised at between 14 and 35, and 56 and 78, days post injection. **E-H** The same data as shown in the left-hand panels but graphed by injection dose. Numbers above the bars indicate the total numbers of flies from which the proportions were calculated, *i.e.*, the total numbers of flies homogenised. Note that we cannot distinguish between flies that had cleared the infection and those where the bacterial load was below our detection limit.

3.4. Lower doses of *E. cloacae* are cleared more quickly than higher doses

Summing up across all doses and days, 39.4 % (177 of 449) of *E. cloacae*-injected flies, 11.8 % (45 of 381) of *Pr. burhodogranariea*-injected flies, 3.7 % (11 of 301) of *L. lactis*-injected flies, and 21.4 % (15 of 70) of *Ps. entomophila*-injected flies cleared the infections (Figure 6).

To account for mortality in our estimates of bacterial clearance we calculated clearance indices. Using the clearance index for the early infection phase, i.e., days one and two post injection (clearance $index_{1,2}$), we found that flies injected with lower doses of *E. cloacae* were more likely to clear the infection compared to flies injected with higher doses (Spearman rank correlation: $\rho = -0.86$, $p < 0.001$, Figure 7A). However, the other three bacterial species did not show dose-dependent clearance (Figure 7B-D)

Table 1. The effect of initial injection dose on bacterial load at seven days post injection (Model 4). Experimental replicate and the person performing the injection were also included as factors in the models. *Ps. entomophila* was not analysed because it caused high fly mortality. Statistically significant factors are in bold.

Injected bacterium	*Tested effect*	*df*	*F*	*P*
E. cloacae	Log(Injection dose)	1,25	26.41	**<0.0001**
	Person	1,25	0.16	0.69
	Replicate	2,25	1.78	0.19
Pr. burhodogranariea	Log(Injection dose)	1,47	37.33	**<0.0001**
	Person	1,47	0.23	0.63
	Replicate	2,47	2.11	0.13
L. lactis	Log(Injection dose)	1,37	3.81	0.058
	Person	1,37	0.71	0.40
	Replicate	2,37	1.98	0.15

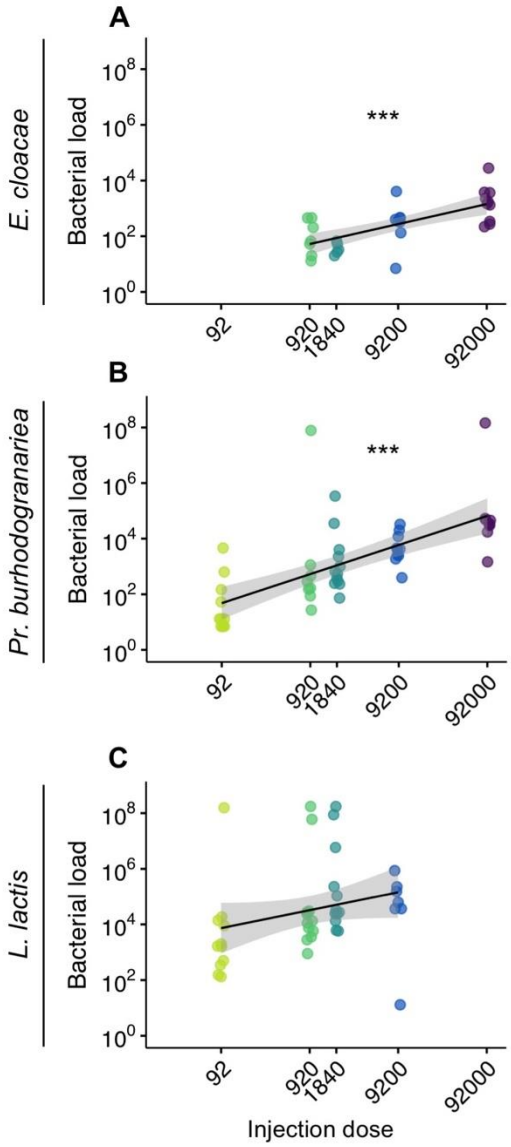

Figure 5. The relationship between bacterial load at seven days post injection, and the initial injection doses. Each row shows data from one bacterial species. Panel **A** contains no flies injected with 92 CFUs because all flies had a bacterial load of zero at day seven; **C** contains no flies injected with 92,000 CFUs because all flies had died by this time point. Each circle is the bacterial load of one fly, they are jittered along the x-axis to aid visualisation of overlapping data points, and they are coloured according to the injection dose. Flies with zero bacterial load are not shown (see methods). Linear regression lines are shown in black with 95 % confidence intervals. Asterisks denote significant correlations, where $p < 0.0001$.

3.5. Bacterial species vary in clearance

The four bacterial species used in this study covered a broad spectrum of clearance on days three and four post injection (Figure 7E). There was a statistically significant difference among species ($p = 0.002$, $Chisq = 15.309$, $df = 3$), and after p-value correction, the post hoc tests indicated statistically significant differences among the following species pairs: *E. cloacae* and *Pr. burhodogranariea* ($p = 0.024$), *E. cloacae* and *L. lactis* ($p = 0.011$), *Ps. entomophila* and *Pr. burhodogranariea* ($p = 0.048$), *Ps. entomophila* and *L. lactis* ($p = 0.011$). Rather than matching the virulence gradient across species (Figure 1E), clearance formed a U-shaped pattern with the species with the highest virulence (*Ps. entomophila*) and lowest virulence (*E. cloacae*) showing higher levels of clearance compared to the two species of intermediate virulence (*Pr. burhodogranariea* and *L. lactis*).

3.6. Exploitation but not PPP predict clearance

Our analyses of clearance $index_{3,4}$ and clearance $index_{7,14,21}$ showed similar results. In both cases we found no statistically significant effect of PPP, but a significant negative effect of exploitation, such that as bacterial load increased, clearance decreased (Figure 7F, G Table 2). Similar results were obtained using the three alternative smoothing parameters for calculating PPP (Figure S2).

Table 2. The effect of log bacterial load (exploitation) and PPP on two different clearance indices. Statistically significant factors are in bold.

Response variable	*Tested effect*	*df*	*Chisq*	*P*
Clearance index$_{3,4}$	Log (geometric mean bacterial load)	1	14.16	< **0.001**
	PPP	1	0.38	0.535
Clearance index$_{7,14,21}$	Log (geometric mean bacterial load)	1	36.34	< **0.001**
	PPP	1	0.35	0.557

3.7. Bacterial clearance before death is dose dependent

We homogenised flies that died during the chronic phase of the infection (between 14 and 35 days and between 56- and 78-days post injection) to test whether they died whilst being infected, or whether they were able to clear the infection before death. Flies were indeed able to clear the infection before death, but the degree to which this occurred varied across bacterial species (Figure 4). Furthermore, for all bacterial species in both homogenisation phases there were flies where the infection persisted until death, and flies that were uninfected at death (Figure 4A-D). Lower injection doses of *E. cloacae* were more likely to be cleared before death than higher injection doses (Figure 4E; Table 3), but there was no significant effect for *Pr. burhodogranariea* ($p = 0.051$; Figure 4F; Table 3). Summing up across all doses and days, 29.8 % (14 out of 47) of *E. cloacae*-injected flies, 33.3 % (20 out of 60) of *Pr. burhodogranariea*-injected flies, 10.3 % (4 out of 39) of *L. lactis*-injected flies, and 66.7 % (8 out of 12) of *Ps. entomophila*-injected flies cleared the infection before death.

Table 3. The effect of injection dose on presence/absence of infection in dead flies (Model 8). Person performing the injection was also included as a factor in the models, and replicate was included for the analysis for *Pr. burhodogranariea* infections. Statistically significant factors are in bold.

Injected bacterium	*Tested effect*	*df*	*LR Chisq*	*P*
E. cloacae	Log(Injection dose)	1	3.82	0.051
	Day post injection	1	0.0037	0.95
	Person	1	1.22	0.27
	Log(Injection dose) × Day post injection	1	0.013	0.91
Pr. burhodogranariea	Log(Injection dose)	1	13.26	**0.00027**
	Day post injection	1	2.99	0.084
	Person	1	4.33	**0.037**
	Replicate	1	0.038	0.54
	Log(Injection dose) × Day post injection	1	1.75	0.19

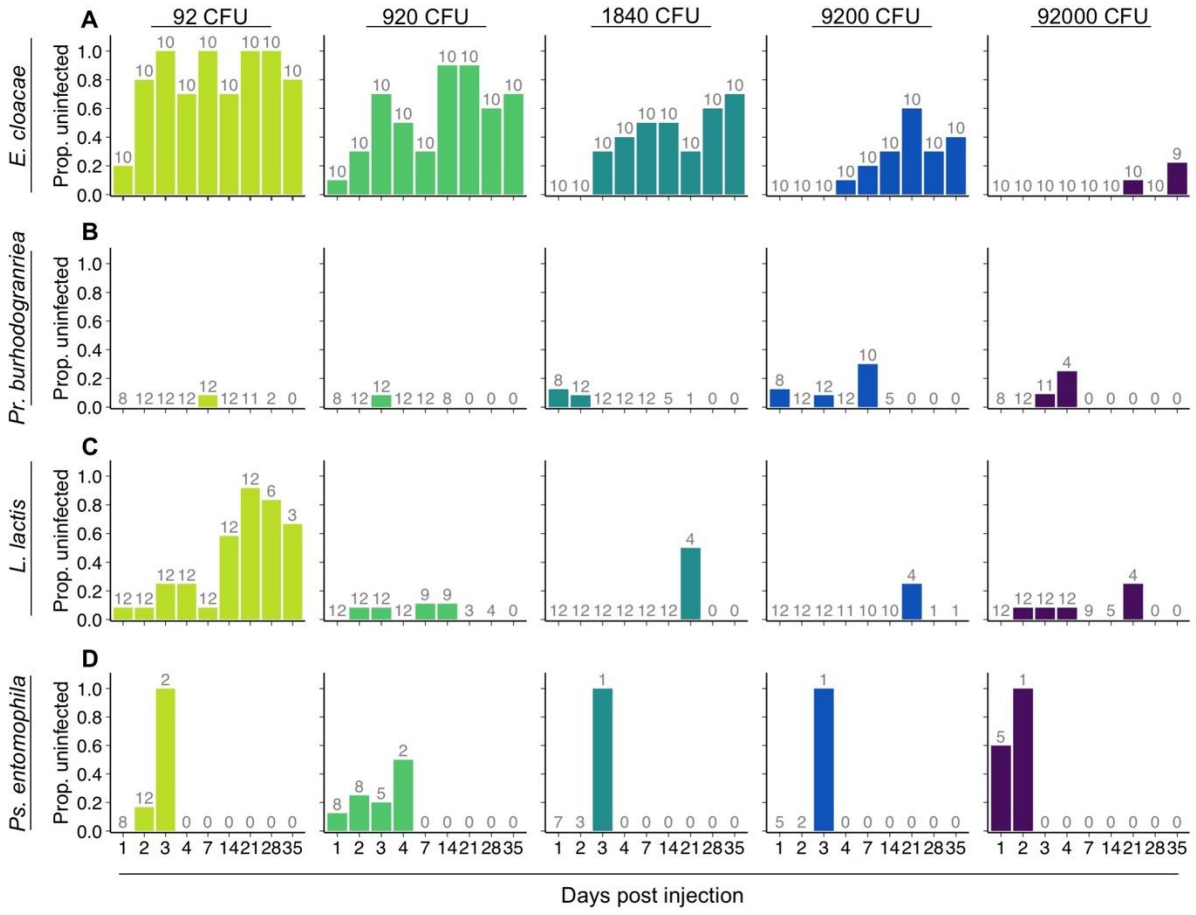

Figure 6. Bacterial clearance by living flies. Each row shows flies that had been injected with one of four bacterial species. **A-D** The proportion of live flies that were uninfected. Each column shows a different injection dose. Numbers above the bars indicate the total numbers of flies from which the proportions were calculated, *i.e.*, the total numbers of flies homogenised. Note that we cannot distinguish between flies that had cleared the infection and those where the bacterial load was below our detection limit.

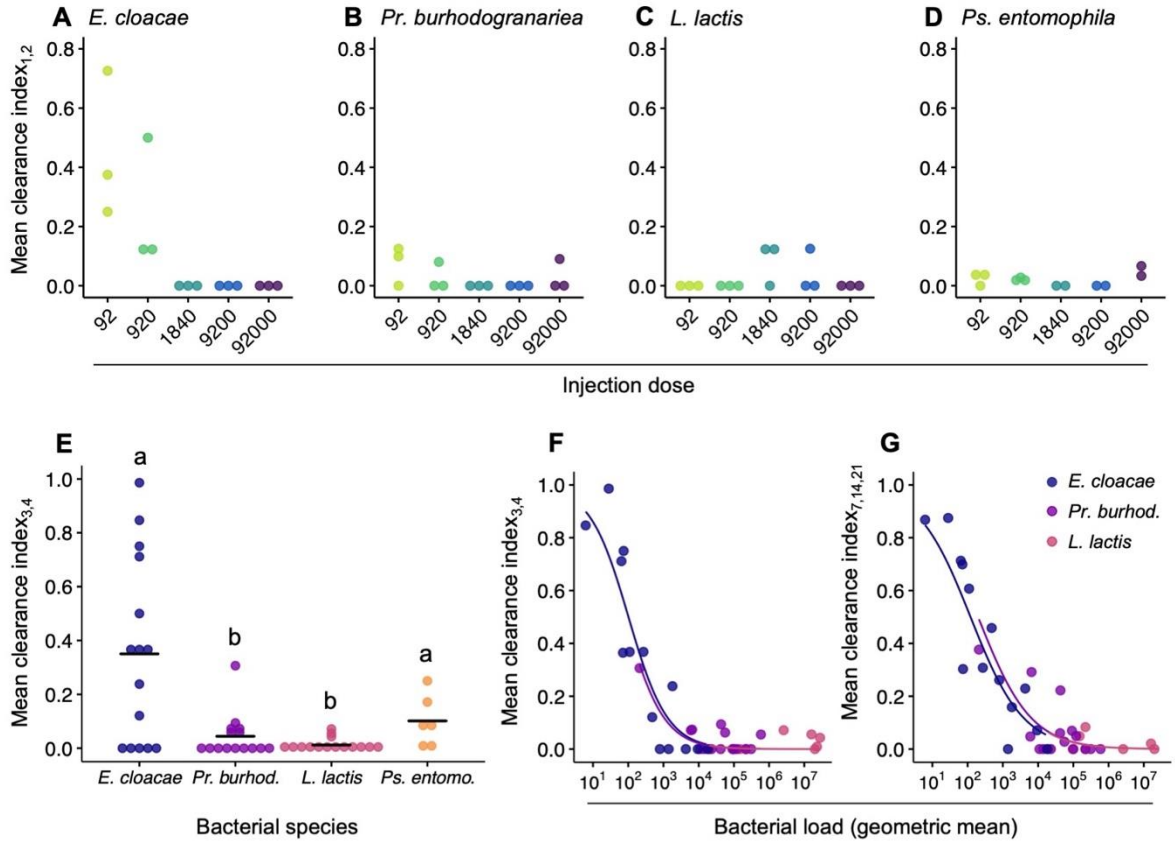

Figure 7. Effects of injection dose, species and exploitation on bacterial clearance. For all figures, each data point is from one injection dose per bacteria, per experimental replicate, and gives the mean proportion of cleared infections (out of the initial infected population) on days one and two (clearance index$_{1,2}$), days three and four (clearance index$_{3,4}$) or days seven, 14 and 21 (clearance index$_{7,14,21}$). **A**-**D** Effect of injection dose on clearance index$_{1,2}$ for each bacterial species. There was a statistically significant negative correlation for **A**. *E. cloacae* ($\rho = -0.86$, $p < 0.001$), but not for **B**. *Pr. burhodogranariea* ($\rho = -0.36$, $p = 0.182$), **C**. *L. lactis* ($\rho = 0.13$, $p = 0.657$) or **D**. *Ps. entomophila* ($p = 0.982$, $\rho = -0.01$). **E**. Mean species differences in clearance index$_{3,4}$. The circles are jittered along the x-axis to aid visualisation of overlapping data points. Black lines show means. Different letters denote means that are significantly different from one another (Mann-Whitney-U post hoc tests). The effect of parasite exploitation, given as bacterial load, upon **F**. mean clearance index$_{3,4}$ and **G**. mean clearance index$_{7,14,21}$. The geometric mean of bacterial load was calculated from days 1 and 2 post injection, *i.e.*, the same values as in figure 3. There was a negative relationship between the two variables. Statistics are given in the main text and the species legend for both panels is shown in panel G.

3.8. A similar proportion of live and dead flies are uninfected

Despite variation in the time post infection at which live and dead flies were sampled, across bacterial species and doses, the proportion of living flies that cleared an infection was a predictor for the proportion of dead flies that cleared an infection (Figure S3; LR = 7.11, df = 2,17, p = 0.0285).

4. Discussion

In this study we demonstrate that bacterial virulence differences can be explained by a combination of variation in exploitation and PPP. Sustained persistent infection and clearance are both possible outcomes for bacteria showing a range of virulence when they infect female *D. melanogaster*. We show that lower doses of the bacterium with the lowest virulence, *E. cloacae*, are cleared more quickly than higher doses, and that clearance rates are species specific. Furthermore, higher exploitation of the host leads to lower bacterial clearance. Finally, we show that bacteria of all species can persist inside the host for at least 75 days.

4.1. Differences in virulence are due to variation in exploitation and PPP

The infecting bacterial species showed pronounced differences in virulence. To understand why this was the case, we decomposed virulence into its two components: exploitation and PPP (Råberg and Stjernman 2012, Råberg 2014). Exploitation, given as infection intensity or bacterial load, is the more frequently tested explanation for variation in virulence (Råberg and Stjernman 2012). There is ample evidence that exploitation varies across parasite genotypes (e.g., monarch butterflies and their protozoan parasites: de Roode, Pedersen et al. 2008, de Roode and Altizer 2010, *Daphnia magna* infected with the bacterium *Pasteuria ramosa*: Clerc, Ebert et al. 2015), and also, unsurprisingly, that it varies across parasite species infecting the same host genotype (Kutzer and Armitage 2016, Duneau, Ferdy et al. 2017, Chambers, Jacobson et al. 2019). Indeed, in the current study, all bacterial species tested showed significant differences in exploitation, where bacterial load increased as virulence increased. Chambers et al. (Chambers, Jacobson et al. 2019) observed that the two bacterial species in their study that

caused lower mortality showed little initial proliferation inside the host, but that the species causing more mortality showed an initial increase in the bacterial load: these results support our findings.

However, variation in virulence is not only determined by the load that a pathogen attains: Råberg & Stjernman (Råberg and Stjernman 2012) proposed that pathogen genotypes may also vary in PPP *i.e.*, the harm or damage caused per parasite (Råberg and Stjernman 2012, Råberg 2014). Some pathogens may cause more damage to the host independently of their density, through specific mechanisms that directly affect the host homeostasis. For example, *Bacillus anthracis* produces two anthrax toxins which are responsible for impairing the host immune system and disrupting basic cellular functions, ultimately killing the host (Liu et al. 2014). By comparison, the genetically similar *Bacillus cereus* usually only produces a mildly virulent gastro-intestinal infection (Helgason et al. 2000; Koritanta et al. 2000; Radnedge et al. 2003). Variation in PPP can be observed when different parasite genotypes show different reaction norms for the relationship between host health and infection intensity, when infecting the same host genotype (Råberg 2014). Variation in PPP has been demonstrated for rats infected with different clones of *Plasmodium chabaudii*, the agent of rodent malaria (Råberg and Stjernman 2012), for different strains of protozoan parasites infecting monarch butterflies (de Roode and Altizer 2010), and for humans infected with different HIV-1 genotypes (Bertels, Marzel et al. 2018). Here we found a significant overall effect of PPP across bacterial species, whereby *Pr. burhodogranariea* and *L. lactis* had significantly more negative slopes compared to *E. cloacae*. This finding, combined with the exploitation results, implies that *E. cloacae* is less virulent towards its host compared to the other two species, because of a combination of lower PPP and less exploitation. On the other hand, given that *Pr. burhodogranariea* and *L. lactis* both showed similar levels of PPP, it suggests that the variation in virulence between these two species is due to higher exploitation by *L. lactis*, rather than differences in PPP. Some of our *L. lactis* counts were underestimates because there were too many CFUs to count, so it is possible that the slope of the reaction norm might have differed slightly had we not encountered this issue. Nonetheless, had we only examined exploitation as a source of variation, we might have concluded that load alone explains the differences that we found in virulence. These two sources of variation, exploitation and PPP, have not frequently been explored in the same study, so it is generally difficult to ascertain the relative importance of the two sources of variation. However,

variation in PPP was demonstrated to explain more of the variance in virulence across HIV-1 genotypes than did set point viral load (Bertels, Marzel et al. 2018).

4.2. All bacterial species established persistent infections

All four bacterial species were able to establish persistent infections in *D. melanogaster*. *E. cloacae* and *Pr. burhodogranariea* could be retrieved from live homogenised flies up to 35 days, *L. lactis* up to 28 days, and *Ps. entomophila* up to four days post injection. The reduced estimates for the latter two species are due to higher mortality, meaning that no flies were alive to sample at later time points. However, by homogenising flies that had died, we show that all bacterial species can persist inside the host for at least 75 days. To the best of our knowledge these estimates are far beyond the currently known length of persistent infections after injection in insects (28 days: Haine, Moret et al. 2008, Kutzer, Kurtz et al. 2019). The duration of infection can be of key ecological and potentially also evolutionary importance, because persistence determines the prevalence of infection in a population, and therefore could affect transmission.

It is unclear how these bacteria are able to persist for so long inside the host, although there are a number of theoretical possibilities, for example, through surviving inside host tissue, forming biofilms or existing as persister or tolerant cells (Ellner, Buchon et al. 2021). *Salmonella typhimurium* (Shinzawa, Nelson et al. 2009) and *S. aureaus* (McGonigle, Purves et al. 2016) can survive inside insect haemocytes. The bacterial species that we used are not known to be intracellular, *e.g.*, some *Providencia* strains were able to survive, but not replicate, at low numbers 24 hours after infecting a *D. melanogaster* S2 cell line (Galac and Lazzaro 2011). Both *E. cloacae* and *L. lactis* are able to produce biofilms *in vitro* (Nyenje, Green et al. 2013, Chodorski, Hauth et al. 2020, respectively), but it is unknown whether this is the case inside an insect host. *Pr. burhodogranariea* is not able to form biofilms *in vitro* (Galac and Lazzaro 2011) although it is unknown if it might still be possible *in vivo*. Biofilms can cause chronic infections such as *Pseudomonas aeruginosa* in cystic fibrosis patients (Høiby, Bjarnsholt et al. 2010), and oral infection of *D. melanogaster* with *Ps. aeruginosa* resulted in biofilm production in the crop (Mulcahy, Sibley et al. 2011), but it is unknown whether *Ps. entomophila* forms biofilms *in vivo*. Lastly, bacteria could potentially survive inside the host in persistent or tolerant cell states,

as is discussed in relation to the failure of antibiotic treatments (Brauner, Fridman et al. 2016). However, persistent cells typically make up a small proportion (< 1%) of the bacterial population (Brauner, Fridman et al. 2016), so this might not explain the high numbers of CFUs that are retrieved from the flies. Future research will test the likelihood of these and other mechanisms.

D. melanogaster that are able to control a bacterial infection during the acute infection phase have been shown to have a relatively constant bacterial load in the chronic infection phase, which Duneau *et al.* (Duneau, Ferdy et al. 2017) found remains stable until at least ten days post injection for *Pr. rettgeri*. Our bacterial load data (Figure 2) suggests that *E. cloacae*, *Pr. burhodogranariea* and *L. lactis*, show relatively stable loads from around day three to four post infection, lending support to the SPBL concept. In addition, Duneau *et al*. (Duneau, Ferdy et al. 2017) observed that, per host, bacteria with low virulence had a SPBL of a few hundred bacteria, whereas bacteria of intermediate virulence had a SPBL of a few thousand bacteria. Our data also lend support to the idea that virulence relates to SPBL, given that low virulence *E. cloacae* had a persistent load of tens to hundreds of bacteria, and high virulence *L. lactis* had a load of tens of thousands of bacteria. This finding is supported by the virulence decomposition analysis (see section 4.1), which shows that as virulence increases, so does exploitation of the host over the first couple of days post infection. Therefore, more virulent bacteria have higher initial proliferation rates as shown by exploitation. Given that the infection load stays relatively constant in the longer term, the initial proliferation differences likely explain the relationship between SPBL and virulence.

4.3. Injection dose correlates with persistent infection loads

The bacterial load at day seven post injection, positively correlated with the initial injection dose for *E. cloacae*, *Pr. burhodogranariea* and *L. lactis* (but see results section for the latter). Our results expand the known bacterial species for which this relationship exists, and they lend weight to the idea that this may be a more general phenomenon in *D. melanogaster* bacterial infections. Previous studies found that this relationship held for bacterial load at seven- and fourteen-days post injection (*Pr. rettgeri*: Duneau, Ferdy et al. 2017; , *E. faecalis*, *Pr. rettgeri* and *S. marcescens*: Chambers, Jacobson et al. 2019). It has been suggested that the SPBL will

remain at around the bacterial load at which the infection was controlled (Duneau, Ferdy et al. 2017, Chambers, Jacobson et al. 2019). Given that insects can show dose dependent inducible immune activation (Jent, Perry et al. 2019), and given that the antimicrobial peptide Drosocin has been shown to control *E. cloacae* infections and that a combination of Drosocin, Attacins and Diptericins control *Pr. burhodogranariea* infections (Hanson, Dostálová et al. 2019), one could hypothesise that these AMPs are to some degree involved. However, the mechanisms that allow a dose-dependent persistent infection remain to be uncovered. Unfortunately, it was not possible to test *Ps. entomophila* given its high mortality during the acute infection phase.

4.4. Lower doses of *E. cloacae* are cleared more quickly than higher doses

The likelihood of clearing *E. cloacae* was dose dependent, although we did not find a dose threshold below which there was complete clearance in all flies. The finding of dose-dependent clearance, whilst maybe not surprising, could explain some discrepancies across studies in terms of whether evidence of persistent infections is found. Just as stochastic variation explains variation in the outcome of the early infection phase (Duneau, Ferdy et al. 2017), perhaps stochasticity plays a role in the clearance of bacteria (Coates, Park et al. 2018), particularly where infection loads are low such as in *E. cloacae,* for example through variation in expression of Drosocin. In comparison to *E. cloacae,* most replicates of the other species showed no clearance in the early infection phase, i.e., one- and two-days post infection, thus dose did not influence clearance for these species. Although we note that our lower detection limit is ~7 CFUs per fly, therefore we cannot discriminate between clearance of the bacteria and a load that is below our detection limit.

4.5. Bacterial species vary in clearance

Across species, we uncovered a U-shaped pattern in bacterial clearance, where the species with the lowest and highest virulence had higher levels of clearance compared to the two species of intermediate virulence. Our finding that the low virulent species could be cleared, is supported by evidence from infections with low virulence *E. coli* and *Erwinia carotovora Ecc15*, where an injection dose of 30,000 bacteria was cleared in 22 % and 8 % of flies, respectively (Duneau,

Ferdy et al. 2017). The clearance of intermediate and high virulence pathogens in *D. melanogaster* has been described as being rare, because no bacteria were cleared from any of the previously infected hosts over the seven-days post injection (Duneau, Ferdy et al. 2017). *Pr. rettgeri* and *Enterococcus faecalis* were described as having intermediate virulence in that study, with a survival of around 50-60% seven days post infection (Duneau, Ferdy et al. 2017), which is in the region of the survival that we found for our intermediate virulence species, *Pr. burhodogranariea* and *L. lactis*. Although clearance in our intermediately virulent bacteria was lower than for the low and high virulent bacteria, our results challenge the finding that clearance is rare, given that our three more virulent bacteria all appear to be clearable to differing degrees, including within the first seven days of infection. Our results thereby show that persistent infections are not inevitable. This finding is supported by the observation that *Pr. burhodogranariea* was cleared in flies seven- to ten-days post injection (Galac and Lazzaro 2011; although low sample sizes). Similarly to the current study, Kutzer & Armitage (Kutzer and Armitage 2016) also found that a few female flies, inoculated with a dose of *L. lactis* in common with this study (1,840 CFUs), cleared the infection (3 out of 141; 2.1 %). Lastly, we expected that there may be selection for a fast and efficient early clearance of infection by *Ps. entomophila*, because of its high virulence. Clearance of *Ps. entomophila* was indeed higher than for the intermediate bacteria, although mortality was too high to assess clearance in living flies for longer than four days post injection. Nonetheless, there is evidence from other studies that *Ps. entomophila* has high virulence and can be cleared from other *D. melanogaster* populations/genotypes (Martins, Faria et al. 2013, Kutzer, Kurtz et al. 2018, Kutzer, Kurtz et al. 2019). The ability to resist *Ps. entomophila* infection varied across host genotypes, similarly, five out of the ten tested genotypes contained some individuals who could clear this pathogen (Kutzer, Kurtz et al. 2018). Host genotypic variation in clearance may thereby more generally explain why some studies find clearance and others do not.

4.6. Exploitation but not PPP predicts clearance

We next sought to understand how the two components that determine variation in virulence affect clearance of the infection, whilst framing our argumentation in the context of the benefits to costs ratio to the host of clearing an infection. Because a higher PPP should directly result in a higher host death rate, we predicted that as PPP increases it would become more beneficial

for the host to clear an infection. We found no support for this prediction. However, the statistical power of detecting the predicted effect was low, because in contrast to exploitation, PPP by definition, cannot not vary within replicates of a species. In addition, only three species could be included in this analysis due to high mortality after injection with *Ps. entomophila*. *Ps. entomophila* produces a pore-forming toxin called Monalysin (Opota, Vallet-Gély et al. 2011) in association with activation of stress-induced pathways and an increase in oxidative stress (Chakrabarti, Liehl et al. 2012). Ultimately this leads to a lack of tissue repair in the gut, and in most cases fly death (reviewed in Buchon, Broderick et al. 2013). Monalysin toxic activity is dependent on the cleavage by bacterial and fly proteases (Nonaka, Salim et al. 2020). One of the bacterial proteases is AprA, also described as a virulence factor due to its role in AMP degradation (Liehl, Blight et al. 2006). If similar pathologies are induced in the haemocoel after infection, contrarily to other bacterial species, sustaining a persistent bacterial load in the face of high levels of tissue damage might only rarely be a viable option. Instead, the fly host might activate a stronger immune response to attempt to clear the infection (Lazzaro and Rolff 2011, Moreno-García, Condé et al. 2014). However, a few flies in ours and other studies, did survive into the chronic phase whilst testing positive for a *Ps. entomophila* infection. In *D. melanogaster* Drosocrystallin expression in the gut has been shown to confer protection to Monalysin and lead to higher individual survival (Kuraishi, Binggeli et al. 2011, Shibata, Maki et al. 2015); perhaps individuals surviving into the chronic infection phase exhibit higher expression of such mechanisms, allowing them to tolerate the damage caused by infection. Nonetheless, we suggest that the higher virulence of *Ps. entomophila* could have been driven by an increased PPP, because of the above-mentioned toxins, but additional experiments would be required to test this idea.

Host exploitation affected clearance at days three and four post injection, and also at days seven to 21: as exploitation across species increased, so clearance decreased. This data supports the second of our two predictions for exploitation, whereby increasing loads are costlier to clear and thereby the benefits of clearance are outweighed by the costs. This finding is in contrast to data from vertebrate viral infections (reviewed in Althouse, Durbin et al. 2014; , e.g., Ben-Shachar and Koelle 2018), where larger viral loads led to a faster decline in viral load or in shorter durations of viremia. Our data would support the notion that increasing exploitation could be an advantageous strategy for some pathogens, namely when increased pathogen load results in increased transmission. Nevertheless, a pathogen relying on an exploitation strategy

faces a trade-off between virulence and transmission (Anderson and May 1982, Alizon, Hurford et al. 2009): high pathogen loads will result in increased mortality, thereby reducing the time window where it can reproduce and transmit. A strategy relying on exploitation for transmission might then be more successful when combined with intermediate levels of virulence. For example, *Pasteuria ramosa*, a spore-forming bacterium that is transmitted upon death of its host after being released in the environment (Ebert 2005), has the highest bacterial loads at intermediate virulence (Jensen, Little et al. 2006, Ben-Ami 2017). Our different bacterial species fall along the continuum of the negative relationship between exploitation and clearance. *L. lactis* and *P. burhodogranariea*, have high levels of exploitation, are less often cleared, and coincidentally show intermediate levels of mortality, compared to the other two bacterial species. It would be informative to extend this kind of analysis to other host-pathogen interactions, particularly where the mode of transmission is well-described.

4.7. Clearance in live and dead flies

Even though dead flies were sampled for a longer period post-injection (up to 78 days) compared to live flies (up to 35 days), the patterns of bacterial clearance in dead flies largely reflected the results for live flies, in that dead flies that had been infected with *E. cloacae* showed dose dependency in clearance. Once again, comparatively few dead individuals had cleared *L. lactis* infections, whereas proportionally more had cleared *Ps. entomophila*. As expected, the proportion of live flies that cleared a particular species and dose of bacteria was a predictor of the proportion of dead flies that did the same; most of the data points lie above, rather than on, the diagonal (Figure S3) possibly because the dead flies were on average homogenised later on in the infection, therefore allowing for more clearance to take place before being sampled. Because we processed dead flies up to 24 hours post-death we did not analyse the number of CFUs, however it would be interesting to test whether the bacterial load upon death (BLUD) (Duneau, Ferdy et al. 2017) remains constant even after many weeks of infection.

To conclude, ours, and the results of others, suggest that PPP is an important component driving variation in virulence, and that disentangling its contribution towards virulence, in combination with the contribution of exploitation, will undoubtedly help our mechanistic and evolutionary

understanding of host-pathogen interactions. We also suggest that such a decomposition of virulence can be used to better understand how virulence relates to other infection processes such as clearance during persistent infections. Future research will be needed to test the generality of the relationships we have uncovered between virulence decomposed as exploitation and PPP, and the persistence and clearance of infections.

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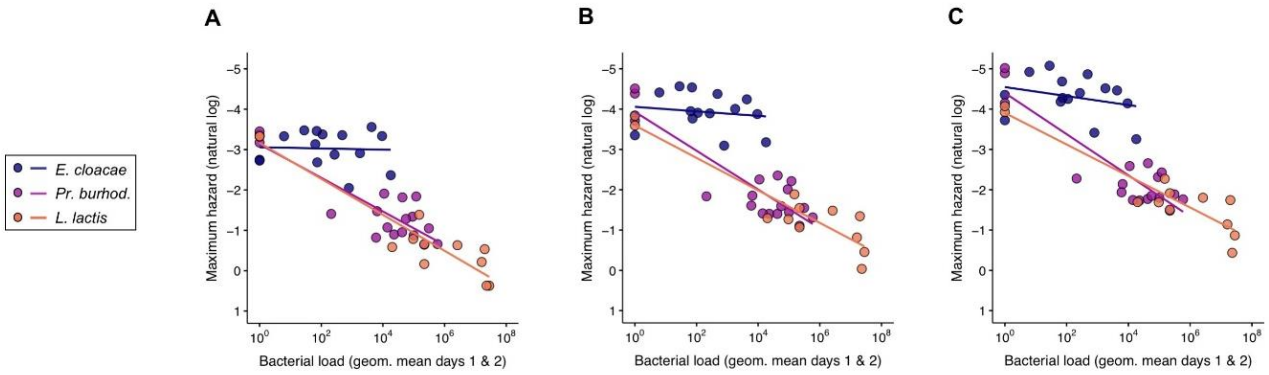
Supporting Information

Table S1. Tukey multiple comparisons between bacterial species for differences in virulence, measured as maximum hazard (model 1). Statistically significant comparisons are in bold.

Contrast	*df*	*t*	*P*
E. cloacae – L. lactis	55	-17.23	< **0.0001**
E. cloacae – Pr. burhodogranariea	55	-13.42	< **0.0001**
E. cloacae – Ps. entomophila	55	-23.29	< **0.0001**
L. lactis – Pr. burhodogranariea	55	3.88	**0.0016**
L. lactis – Ps. entomophila	55	-6.17	< **0.0001**
Pr. burhodogranariea – Ps. entomophila	55	-10.04	< **0.0001**



	A			B			C		
	df	F	P	df	F	P	df	F	P
Bacterial species	2,39	0.047	0.95	2,39	0.71	0.50	2,39	1.34	0.27
Bacterial load	1,39	0.027	0.87	1,39	0.34	0.56	1,39	1.30	0.26
Bacterial species × bacterial load	2,39	8.92	**0.00065**	2,39	7.29	**0.0020**	2,39	6.02	**0.0053**

Figure S1. Per parasite pathogenicity using different smoothing parameter values to estimate the maximum hazard. Per parasite pathogenicity is given as the relationship between bacterial load and maximum hazard. The bacterial load data is the same as that given in Figure 6A, with the addition of the Ringer's treatment control. The maximum hazard data is estimated from survival data for the corresponding injection doses and experimental replicates. Maximum hazard is plotted as the inverse, such that the hazard (virulence) increases with proximity to the x-axis. The maximum hazard was estimated from time to death data using four different values (1, 2, 3 and 5) for the smoothing parameter, b, as specified using "bw.grid". Shown above are **A**. b= 1, **B**. b= 3, **C**. b= 5. Grey and black lines show the linear regressions. The corresponding statistical results are shown below each panel, where maximum hazard was the dependent variable. Statistically significant p-values are in bold.

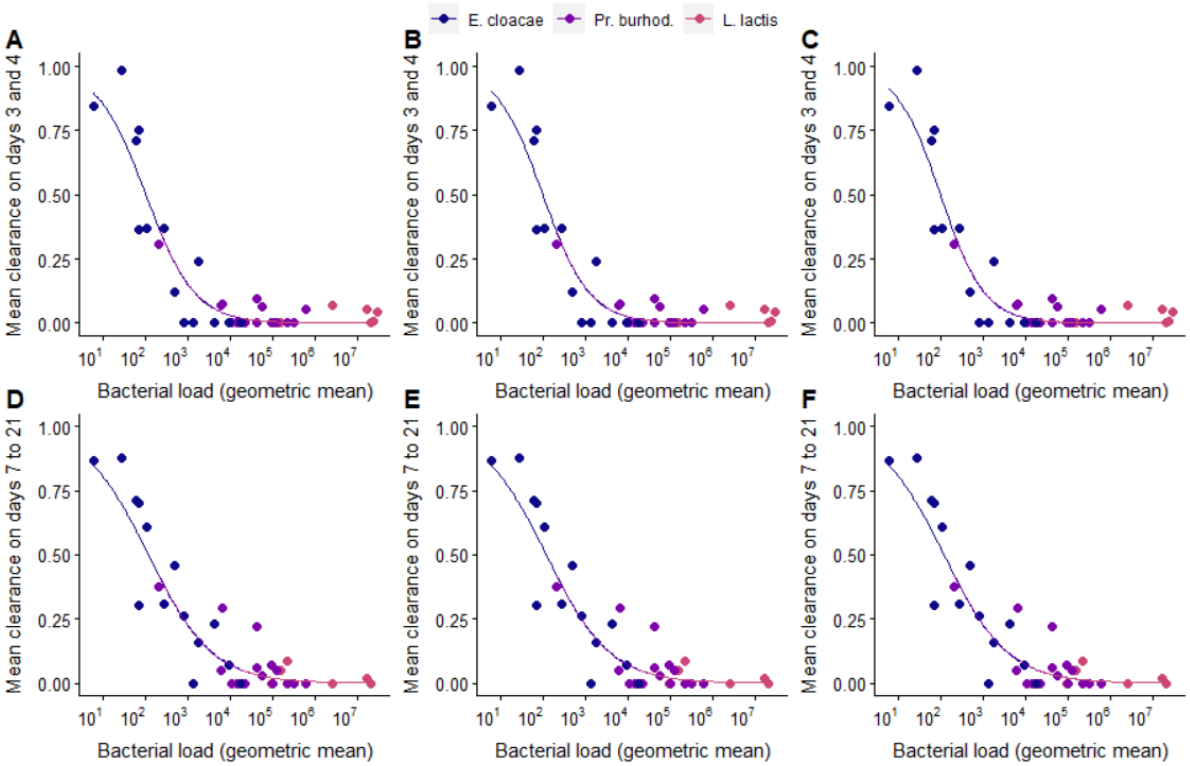


Figure S2. Effect of exploitation (bacterial load) on clearance for different values of the smoothing parameter b in the estimation of maximum hazard that is used for the calculation of PPP: b = 1 (A, D), b = 3 (B, E), b = 5 (C, F). The geometric mean of bacterial load was calculated from days 1 and 2 post injection. Each data point is from one injection dose per bacteria, per experimental replicate, and gives the mean proportion of cleared infections on days three and four (A-C) and days 7 to 21 (D-F).

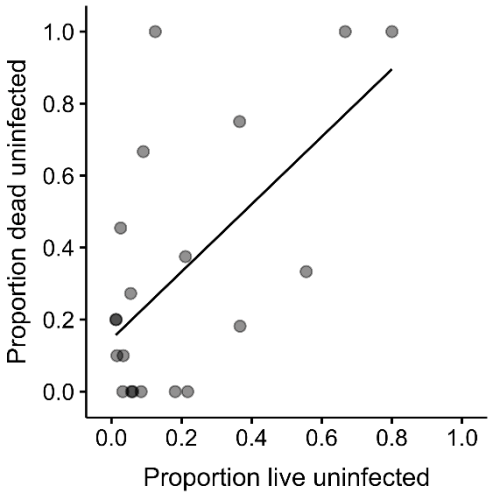


Figure S3. Proportion of live and dead flies that were uninfected across bacterial species and doses. Each data point is the proportion for one bacterial species and dose. Darker circles are due to overlapping data points. The black line shows the linear regression.

CHAPTER 2:

HOST RESISTANCE TO BACTERIAL INFECTION VARIES OVER TIME, BUT IS NOT AFFECTED BY A PREVIOUS EXPOSURE TO THE SAME PATHOGEN

Host resistance to bacterial infection varies over time, but is not affected by a previous exposure to the same pathogen

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Author Contributions

SA conceived the idea and designed the experiments together with BAH. BAH collected the data, conducted the statistical analyses with advice from SA, and wrote the first draft of the manuscript. Both authors contributed critically to the drafts.

Unpublished manuscript.

Abstract

Immune priming describes the phenomenon whereby after a primary pathogen exposure, a host more effectively fights a lethal secondary exposure (challenge) to the same pathogen. Conflicting evidence exists for immune priming, potentially due to heterogeneity across studies in the pathogen species tested, the antigen preparation for the primary exposure, and the phenotypic trait used to test for priming. To explore these factors, we injected *Drosophila melanogaster* with one of two bacterial species, *Lactococcus lactis* or *Providencia burhodogranariea*, which had either been heat-killed or inactivated with formaldehyde, or we injected a 1:1 mixture of the two inactivation methods. Survival and resistance (i.e., the inverse of bacterial load) were assessed after a live bacterial challenge. In contrast to our predictions, none of the primary exposure treatments provided a survival benefit after challenge compared to the controls. Resistance in the acute phase (one day post challenge) separated into a lower and higher group, however, neither group varied according to the primary exposure. In the chronic phase (seven days post challenge), infection intensity was also unaffected by the primary exposure. Our multi-angled study supports a view of immune priming that requires specific circumstances to occur, rather than it being a ubiquitous aspect of insect immunity.

1. Introduction

Research on immune defences in the past few decades has changed our understanding of immune memory. The definition of immune memory has been extended beyond a phenomenon restricted to vertebrate adaptive immunity to include invertebrates, plants and bacteria (Pradeu and Du Pasquier 2018). In the case of invertebrates, evidence for a memory-like phenomenon has been found across a broad range of taxa (Milutinović and Kurtz 2016; Contreras-Garduño et al. 2016; Pradeu and Du Pasquier 2018). This phenomenon, termed "immune priming" (Little and Kraaijeveld 2004), has been defined as the ability of an immune system to store or use the information on a previously encountered antigen or parasite, upon a secondary exposure (Milutinović and Kurtz 2016).

There is considerable evidence supporting immune priming in invertebrates (Milutinović and Kurtz 2016; Contreras-Garduño et al. 2016), with some of the mechanistic bases including antimicrobial peptides (Dhinaut, Chogne, and Moret 2018; Patrnogic et al. 2018; Chambers et al. 2019) and haemocyte-mediated defences (Pham et al. 2007; Rodrigues et al. 2010). However, a number of studies testing immune priming have not found evidence to support its existence (Pham et al. 2007; Reber and Chapuisat 2012; Longdon et al. 2013; Wu et al. 2015; Duneau, Ebert, and Du Pasquier 2016; Patrnogic et al. 2018; Kutzer, Kurtz, and Armitage 2019) (reviewed in Milutinović and Kurtz 2016; Contreras-Garduño et al. 2016). It has been suggested that the inconsistent findings are due to the heterogeneity in the way this phenomenon is tested across studies (Pradeu and Du Pasquier 2018; Milutinović and Kurtz 2016). Although this list is by no means exhaustive, heterogeneity has come in the form of variation in the pathogen species tested, the methods used to prepare the pathogen for the previous exposure, and the phenotypic read-out used to assess whether there is evidence for priming or not. Our experimental design encompasses testing variation in all three of these factors.

First, evidence that priming could be pathogen species dependent, comes from studies where within one experiment, priming has been found against one species of pathogen but not against another (Pham et al. 2007; Roth et al. 2009). The evolutionary history and ecology of the host-pathogen interaction studied might also play a role. For example, previous exposure to a Gram-positive bacterium conferred *Tenebrio molitor* a more effective protection against infection compared to a Gram-negative bacterium (Dhinaut, Chogne, and Moret 2018). The authors

suggested that since many pathogenic bacteria naturally present in the environment of *T. molitor* are Gram-positive, immune priming might have only evolved against these bacteria as they represent a significant threat to the host (Dhinaut, Chogne, and Moret 2018; Dubuffet et al. 2015). Here we test two species of bacteria, both isolated from the host species.

Second, Milutinović and Kurtz (2016) proposed that using different antigen preparation methods for the primary exposure might result in the antigens being recognised in contrasting ways by the host immune system, leading to inconsistent results between studies (Milutinović and Kurtz 2016). Antigen preparations have ranged from cell components and toxins (Miyashita et al. 2014; Milutinović, Fritzlar, and Kurtz 2014; Miyashita et al. 2015; Miyashita 2017; Karp and Rheins 1980; Rheins, Karp, and Butz 1980) to varying doses of live (Castro-Vargas et al. 2017; Chambers et al. 2019) or inactivated pathogens (Lin et al. 2013; Pham et al. 2007). The use of live compared to dead pathogens for the primary exposure might lead to different priming responses (Milutinović and Kurtz 2016). A live primary infection can lead to an initial phase of host mortality, after which survivors are challenged with a secondary infection. This first exposure may act as a filter, selecting for fitter hosts. Compared to non-primed individuals, these hosts are predicted to survive the challenge better due to their higher fitness, rather than an ability to store and recall information on a previous encounter with the pathogen (Kurtz 2005; Milutinović and Kurtz 2016). Moreover, a live pre-exposure could lead to a persistent infection and result in differential bacterial loads across hosts (e.g. Acuña Hidalgo et al. 2021), and thereby introduce heterogeneity in the immunological history of the pre-exposed flies. Therefore, we here focus on the use of inactivated pathogens.

Pathogens can be inactivated using a number of methods including heat-killing (Wu et al. 2014; Pham et al. 2007; González-Tokman et al. 2010; Longdon et al. 2013; Riessberger-Gallé et al. 2015; Kutzer, Kurtz, and Armitage 2019) and chemical compounds like formaldehyde (Dhinaut, Chogne, and Moret 2018; Wang, Zhang, and Wang 2009; Zhuang et al. 2011) and glutaraldehyde (Faulhaber and Karp 1992; Rosengaus et al. 1999). There are a limited number of studies directly comparing whether the antigenic preparation method affects the likelihood of uncovering a priming effect (Lin et al. 2013; Miyashita et al. 2014). Lin *et al*. (2013) found that the immune system of the white shrimp *Litopenaus vannamei* is activated more quickly by heat-killed *Vibrio alginotylicus*, but that the response induced after challenge is stronger in shrimp primed with formalin-inactivated *V. alginotylicus* (Lin et al. 2013). The authors argued

that this might be due to how the inactivation methods affect the antigenicity of the bacterial cells (Lin et al. 2013). Heating bacterial cells can lead to membrane disruption (Russell 2003), releasing lipopolysaccharides (Katsui et al. 1982; Tsuchido et al. 1985), which are highly stimulant to the host immune system. This would lead to a fast response, but cause the bacterial cells to retain less antigenicity (Lin et al. 2013). On the other hand, formaldehyde cross-links the molecules present on the surface of the cell (Fraenkel-Conrat and Olcott 1948; Feldman 1973) leading to formalin-inactivated bacteria to retain a high level of antigenicity (Spitznagel and Trainer 1949; Arshadi et al. 2020).

Third, the phenotypic trait that has been measured to test whether there is increased protection upon the secondary encounter, varies across studies. This protection has most frequently been tested by monitoring survival after the secondary exposure (Contreras-Garduño et al. 2016), showing an increased longevity in some (Dhinaut, Chogne, and Moret 2018; Pham et al. 2007; Wu et al. 2014; Lin et al. 2013; Christofi and Apidianakis 2013; Faulhaber and Karp 1992; Boman, Nilsson, and Rasmuson 1972; Roth et al. 2009; Lafont et al. 2017; Miyashita et al. 2014; Castro-Vargas et al. 2017; Futo, Armitage, and Kurtz 2016), but not all previously exposed hosts (Pham et al. 2007; Boman, Nilsson, and Rasmuson 1972; Kutzer, Kurtz, and Armitage 2019). In the traditional sense of immune memory, it would be expected that this increased survival results from the host immune system inducing a stronger and more efficient immune response upon secondary exposure (Pradeu and Du Pasquier 2018), which can be quantified at the level of the host immune effectors (Lin et al. 2013; Zhao et al. 2013; Wu et al. 2014; Vargas, Cime-Castillo, and Lanz-Mendoza 2020). These changes in the immune response are expected to increased host resistance to the infection, which is defined as the host ability to reduce the pathogen load (Råberg, Graham, and Read 2009; Graham et al. 2011). Increased resistance upon secondary exposure has been demonstrated, (Pham et al. 2007; Boman, Nilsson, and Rasmuson 1972; Sadd and Schmid-Hempel 2006; Miyashita et al. 2014), but a primary exposure can also lead to a reduction in host resistance (Kutzer, Kurtz, and Armitage 2019), potentially because a host can tolerate an infection instead of eliminating it (Kutzer and Armitage 2016a). Despite its relevance as a phenotypic read-out for immune priming, host resistance has not frequently been assayed. Furthermore, while chronic infections can persist in insects for weeks (Haine et al. 2008; Hotson and Schneider 2015; Acuña Hidalgo et al. 2021), the effects of a primary exposure on resistance post-secondary exposure are not well understood in the chronic infection phase (but see Vargas, Cime-Castillo, and Lanz-Mendoza 2020;

Rodrigues et al. 2010; Kutzer, Kurtz, and Armitage 2019; Contreras-Garduño et al. 2015), with some studies showing that pathogens are not always eliminated in primed hosts(Rodrigues et al. 2010; Contreras-Garduño et al. 2015; Kutzer, Kurtz, and Armitage 2019).

Here, using *D. melanogaster* as our host, we explored the effect of pre-exposure to two bacterial species isolated from wild flies, Gram-positive *L. lactis*(Lazzaro 2002) and Gram-negative *P. burhodogranariea*(Juneja and Lazzaro 2009), which are considered opportunistic pathogens and and are able to establish an infection in the fly with intermediate levels of mortality(Lazzaro 2002; Lazzaro, Sackton, and Clark 2006; Galac and Lazzaro 2011; Kutzer and Armitage 2016b; Acuña Hidalgo et al. 2021). We asked whether pre-exposure affords protection against both species of bacteria, and whether the inactivation method affects the level of protection. We hypothesised that flies simultaneously pre-exposed to formaldehyde-inactivated and heat-killed bacteria would benefit from both types of antigenicity and show a higher level of protection compared to a pre-exposure with only one method of inactivation. We also asked whether pre-exposure affects survival and resistance after a homologous challenge with live bacteria. By quantifying resistance as the pathogen load in the acute and chronic phases of infection (one- and seven-days post-infection), we aimed to determine the strength and duration of the immune priming response, as well as its effect on bacterial persistence (Pradeu and Du Pasquier 2018).

2. Materials and Methods

2.1. Experimental animals

We used an outbred population of *D. melanogaster*, naturally infected with the intracellular bacterium *Wolbachia* (gift from Élio Sucena). This population was established from 160 fertilised females collected in Azeitão, Portugal in 2007 (Martins et al. 2013). The flies were reared and maintained at a density of at least 5,000 flies inside a population cage with non-overlapping generations of 14 days on a 12:12 hour light-dark cycle, at 60-80 % relative humidity and a temperature of 24.8 ± 0.5 °C. They were maintained on a sugar yeast agar medium (SYA medium: 970 mL water, 100 g brewer's yeast, 50 g sugar, 15 g agar-agar, 30 mL 10 % Nipagin solution and 3 mL propionic acid; Bass et al. 2007).

Experimental flies were produced after two generations of density control. The first density-controlled generation was obtained by placing four purple grape juice agar plates (25 g agar-agar, 300 mL red grape juice, 21 mL 10% Nipagin solution, 550 mL water; Wensing, Koppik, and Fricke 2017) coated with a thin layer of baker's yeast paste, inside the population cage and letting the flies lay eggs for 24 hours. Larvae were collected 24 hours after the end of the oviposition period and placed in groups of 100 larvae in plastic vials (95 x 25 mm) containing 7 mL of SYA medium. They were left to develop for eight days under standard conditions. The second density-controlled generation was produced by placing four-day old adults in two embryo cages, allowing 600-800 adults per cage to oviposit on a purple grape juice agar plate for 24 hours. Larvae were again collected 24 hours later at a density of 100 larvae per vial and allowed to develop. One day after the start of eclosion, adults were collected, placed in vials in mixed sex groups of five males and five females.

2.2. Preparation of the bacterial solutions

In this study, we used two bacterial species isolated from wild-caught *D. melanogaster* (gifts from Brian Lazzaro), *L. lactis* (Lazzaro 2002) and *P. burhodogranaria* strain B (Juneja and Lazzaro 2009) (DSMZ; type strain: DSM-19968). Culturing of these bacteria was performed as in Kutzer and Armitage (Kutzer and Armitage 2016b). In brief, bacteria were streaked on lysogeny broth (LB) agar directly from aliquots stored in 34.4 % glycerol at -80 ºC. After an incubation period of 24 hours at 30 ºC, four colony-forming units (CFUs) were added to 100 mL of sterile LB medium and incubated at 30 ºC and 200 rpm. Two individual bacterial cultures were incubated per bacteria. The next morning, approximately 15 hours later, the liquid cultures were prepared for the primary exposure or challenge injections.

2.2.1. Preparation of inactivated bacteria for primary injections

After the incubation period of 15 hours, the concentration of the overnight cultures was determined as follows. A volume of 500 µL was sampled from each of the two bacterial cultures per species and pooled together in three replicates, centrifuged at 21ºC and 2,880 rcf for five minutes and washed two times in *Drosophila* Ringer's solution (182 mmol·L^{-1} KCl; 46 mol·L^{-}

[1] NaCl; 3 mmol·L^{-1} $CaCl_2$; 10 mmol·L^{-1} Tris·HCl; Werner et al. 2000). The optical density (OD) of each sample was measured using an Ultrospec10 classic spectrophotometer (Amersham, 600 nm), and the OD values were averaged to calculate the total concentration of the overnight cultures for each species. For each bacterial species we had pre-determined the relationship between OD and the number of live bacteria by plating serial dilutions of bacterial solutions with known ODs. The overnight cultures were centrifuged at 2880 rcf and 21 ºC for 10 minutes. The supernatant was discarded, and the remaining pellet was resuspended in sterile distilled water. No washing steps with distilled water were performed to limit the exposure of bacteria to osmotic lysis.

For formaldehyde inactivation, a solution containing 5 % formaldehyde in sterile distilled water was added to the bacterial solution to achieve a final concentration of 0.5% formaldehyde (Pereira et al. 2009; Dhinaut, Chogne, and Moret 2018). The solution was then placed on a shaker (Biosan ES20) at 1,000 rpm at room temperature. We previously determined the inactivation time needed for each of the two bacterial species by exposing an overnight culture to 0.5% formaldehyde for 10, 30, 120 minutes or 24 hours at room temperature, then plating the bacterial solutions on LB agar plates in triplicate for each time tested and verifying the absence of colonies after 24 and 48 hours. Formaldehyde inactivates the bacterial cells by cross-linking proteins of the cell wall (Fraenkel-Conrat and Olcott 1948; Feldman 1973). Our aim was to kill the cells while preserving the conformation of the membrane as much as possible, and thus the antigenicity of the bacterial solution, therefore we aimed for the shortest amount of time possible. No colonies grew on the agar plates after two hours of exposure to formaldehyde for *L. lactis*, and after 10 minutes of exposure for *P. burhodogranariea*. The inactivated bacterial solution was centrifuged at 21 ºC at 2,880 rpm for 10 minutes, the supernatant was removed, and the pellet was resuspended in 7 ml Ringer's solution. This step was repeated two more times to remove the formaldehyde from the solution. To verify that the bacteria had been inactivated, we plated 100 µL of the solution onto LB agar plates and checked for the absence of bacterial colony growth after an incubation period of 24 and 48 hours at 30 ºC. The solution was then aliquoted into 1.5 mL microcentrifuge tubes, snap frozen in liquid nitrogen and stored at -80 ºC. Since the bacterial solution was washed in Ringer's solution three times, we expected that a portion of the inactivated bacterial cells might have been lost during that process. Hence, we once again measured again the concentration of the solutions. One tube

per bacteria was defrosted at room temperature and serially diluted in *Drosophila* Ringer's, and the cells were counted using a haemocytometer (Thoma, 0.02 mm deep, 0.0025 mm^2).

For heat-killing, the bacterial solution was serially diluted to achieve double of the aimed concentration, i.e., 2×10^8 CFUs/mL. The solution was pipetted into several 1.5 mL microcentrifuge tubes and placed on a heat-block (Eppendorf ThermoMixer® C) at 90 ºC and 1,000 rpm. Prolonged exposure to heat can lead to protein denaturation, and thus reduce recognition of the antigens present in the solution by the host immune system. Therefore, we tested for the shortest amount of heat-killing time that would lead to the inactivation of the bacterial cells. The time needed to kill each of the two bacteria was previously tested by exposing them to this treatment for 5, 10 and 20 mins. The bacterial solutions were then plated in triplicates on LB agar plates to verify the absence of colonies after 24 hours, and then again after 48 hours. No *L. lactis* colonies grew on the plates after 10 minutes of heating, and no *P. burhodogranariea* colonies grew after five minutes of heating. The bacterial solutions were then aliquoted into 1.5 mL microcentrifuge tubes. The final solutions for the injections were made by adding double concentrated Ringer's solution to the tubes in a 1:1 volume ratio, diluting the concentration to 1×10^8 CFUs/mL. Subsequently, 100 µL per tube were plated onto LB agar and checked for the absence of bacterial colony growth after 24 hours of incubation at 30 ºC. The tubes containing the inactivated bacteria were frozen in aliquots in liquid nitrogen and stored at -80 ºC until use.

Before injection of the primary exposure, the inactivated bacterial aliquots were allowed to defrost at room temperature. For the formaldehyde-inactivated bacteria, three serial dilutions per bacteria were performed and pooled together to adjust the solution to a concentration of 1×10^8 CFUs/mL. For the combination treatment, an equal volume of the formaldehyde-inactivated and heat-killed bacteria were pooled together. A volume of 50 µL of each solution was plated onto LB agar to confirm the absence of bacterial colony growth.

2.3. Bacterial preparation for challenge injections

For each experimental replicate, the overnight bacterial cultures were produced following the same protocol as described above. After an incubation period of 15 hours, the liquid cultures

were centrifuged at 2880 rcf and 4 ºC for 10 minutes. The supernatant was removed, and the cultures were washed twice with *Drosophila* Ringer's solution. The concentration of the bacterial solution was estimated measuring the optical density of 500 µL of the bacterial solution after serial dilution. The concentration was adjusted to 5 $\times 10^6$ CFUs/mL for *L. lactis* (equivalent to an injection of 92 CFUs per fly) and to 5 $\times$ 10^7 CFUs/mL for *P. burhodogranariea* (equivalent to 920 CFUs per fly). We chose these concentrations because we aimed to infect the flies with a dose that caused an intermediate mortality, i.e. 50-60% of dead flies by day seven (Acuña Hidalgo et al. 2021). To verify these concentrations, we performed three serial dilutions of the bacterial solution from 1:1 to 1:10^4, plated 5 µL of the solution onto agar eight times and counted the number of CFUs that grew after incubation for 20 hours at 30 °C.

2.4. Previous exposure and challenge injections

The experiment was performed in five independent experimental replicates. Three replicates assessed the effect of pre-exposure on survival and bacterial load, and two replicates assessed only the effect on survival. Four days after having been placed in vials with five males and five females, females were exposed to a pre-exposure injection, and then to a challenge injection after seven days (Figure 1). The previous exposure injections were performed in a randomised block design. Flies were anesthetized with CO_2 for a maximum of five minutes in groups of 10 flies. A total volume of 18.4 nL of the primary exposure solution containing 1 $\times$ 10^8 CFUs/mL (equivalent to 1,840 CFUs per fly (Kutzer, Kurtz, and Armitage 2019)) was injected on the right side of the thorax using a fine glass capillary (Ø 0.5 mm, Drummond), pulled to a fine tip with a Narishige PC-10, and connected to a Nanoject II™ injector (Drummond). Flies were injected with one of the three previous exposure treatments per bacteria, i.e. F: formaldehyde-inactivated bacteria, HK: heat-killed bacteria or F+HK: a solution containing equal volumes of the two types of inactivated bacterial solutions. Control flies were injected with 18.4 nL of *Drosophila* Ringer's solution (treatment R). In total, per each pre-exposure treatment with dead bacteria, 260 flies were injected (40-60 flies per experimental repeat), and 460 flies were given a control injection with Ringer's, (80-100 flies per repeat). Flies were then transferred to vials containing 7 mL of fresh SYA medium, kept in groups of 10 at 25 ºC and 70 % relative humidity and flipped into new food vials every two to four days. For each group of 10 flies, one aliquot containing the injection solution was used. At the end of the injections, the remaining volume

of each aliquot was plated onto LB agar and incubated at 30 °C for 15 hours to confirm that there was no contamination. No CFUs grew on any the incubated plates.

The secondary exposure to live bacteria (challenge injections) was carried out seven days after the previous exposure(Pham et al. 2007; Kutzer, Kurtz, and Armitage 2019). Before the injections, the survival of pre-exposed flies was assessed. For the injections, flies were anesthetised and injected on the left side of the thorax with a volume of 18.4 nL of live bacterial solution or *Drosophila* Ringer's solution. Therefore, flies injected with *L. lactis* were given a dose of approximately 92 CFUs and those injected with *P. burhodogranariea* were given a dose of approximately 920 CFUs (Acuña Hidalgo et al. 2021). Across experimental repeats, 138 flies per primary exposure treatment were injected with either live *L. lactis* or *P. burhodogranariea* (24-30 flies per repeat), and 78 were injected with Ringer's solution (12-18 flies per repeat). After injection, flies were placed in vials containing fresh SYA medium in groups of six flies(Kutzer, Kurtz, and Armitage 2018) A single aliquot of bacterial solution or Ringer's solution was used for each group of six flies and each of them was plated at the end of the injections to check for potential contamination, which we did not find. Additionally, to verify the dose of bacteria that had been injected we prepared three serial dilutions from 1:1 to $1:10^4$ for *L. lactis* and 1:1 to 10^5 for *P. burhodogranariea*. Eight droplets of 5 µL per dilution were plated for the three highest dilutions before and after the challenge injections, and counted after 20 hours of incubation at 30 °C. The injected doses were thereby on average 136 ± 5.22 CFUs for *L. lactis* and 1,168 ± 37.60 CFUs for *P. burhodogranariea*.

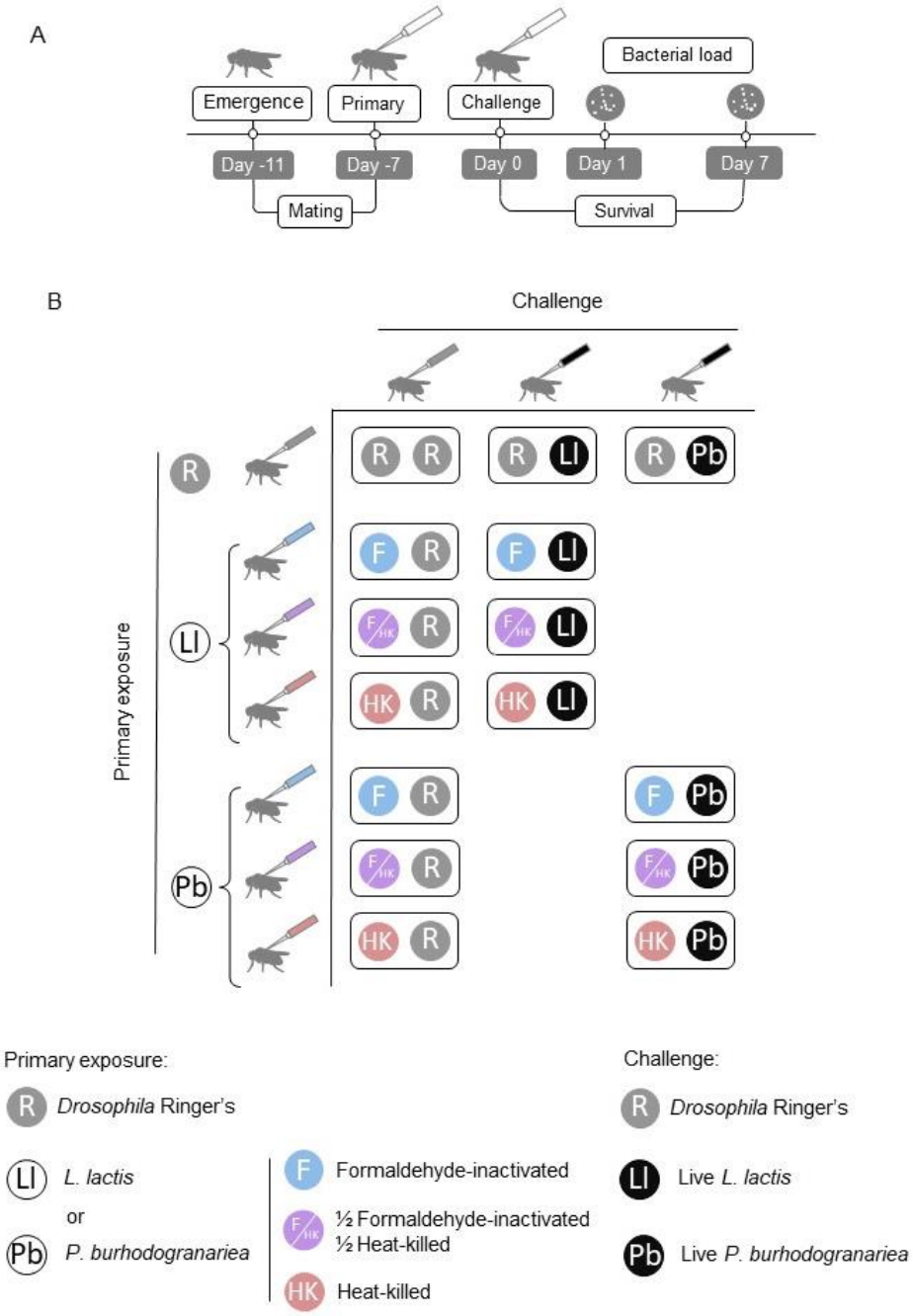

Figure 1. Experimental design. **A**. Timeline of the experiment with essential steps and assaying timepoints (white text in black boxes). **B**. Previous exposure and challenge treatment combinations used for this experiment. For each primary exposure-challenge combination treatment where flies were challenged with 92 colony forming units (CFUs) of *L. lactis* or 920 CFUs of *P. burhodogranariea*, a total of 96 flies were monitored for survival, and 21 flies were homogenised to measure their bacterial load.

2.5. Survival and bacterial load assays

After the challenge injections, flies were placed in fresh food vials and flipped into new food vials every three to four days. A portion of the vials from each replicate were randomly allocated to survival, which was monitored daily for seven days for a total of 96 bacteria-infected flies per primary exposure and challenge treatment (18-30 flies per experimental repeat), and for a total of 60 Ringer's injected flies (12-18 flies per experimental repeat).

The remaining vials from each replicate were randomly allocated to bacterial load measures. At one- and seven-days post challenge, flies from randomly allocated vials were homogenised. A total of 21 flies per previous exposure and challenge treatment (seven flies for each of the three experimental repeats) were allocated to each timepoint. For homogenisation, flies were anesthetized with CO_2, removed from their vial, and transferred into a 1.5 mL microcentrifuge tube containing 100 µL of LB media and one stainless steel bead (Ø 3 mm, Retsch) and immediately placed on ice. The tubes were placed in a Retsch Mill (MM300) inside holders that had been previously chilled for 30 minutes at 4 ºC. The flies were homogenised at a frequency of 20 Hz for 45 seconds. The tubes were subsequently centrifuged at 420 rcf for one minute at 4 ºC. The homogenate was re-suspended and 80 µL were placed in a 96-well plate, and one serial dilution from 1:10 to $1:10^5$ was performed for each sample. For each of the 6 dilutions, three droplets of 5 µL per fly were placed onto LB agar and incubated at 30 ºC for approximately 20 hours. The number of CFUs per droplet were counted for the dilutions with droplets containing between approximately 10-60 CFUs. and the bacterial load per fly was estimated by averaging the counts for the three droplets and back-calculating the number of CFUs in each fly based on the number of dilutions. *D. melanogaster* microbiota does not easily grow under the above culturing conditions (e.g., Hanson et al. 2019; Kutzer, Kurtz, and Armitage 2019). Nonetheless we homogenised flies that had been challenged with Ringer's as a control. Of the 147 Ringer's-injected flies, four flies had more than 2 CFUs in the 1:1 dilution. Of the remaining 437 bacteria-challenged flies, 11 flies (six challenged with *L. lactis* and five challenged with *P. burhodogranariea*) had more than 2 CFUs and were excluded from the analyses. One of the *L. lactis*-injected flies had too many CFUs to count in the highest dilution factor ($1:10^5$); therefore, its bacterial load was replaced by the highest bacterial load from the same bacteria, experimental replicate and day post-challenge, i.e., 3,133,333 CFUs.

2.6. Statistical analyses

All statistical analyses were performed using R studio (R version 3.6.2). Figures were created using plyr (Wickham 2011), dplyr (Wickham et al. 2020) and ggplot2 (Wickham 2009). For each model, the effects of the explanatory variables and interactions on the response variable were tested using a Wald test (Bolker et al. 2009). As explanatory variables, all the models included the previous exposure treatment and the experimental repeat, as well as the interaction between these two variables unless stated otherwise. For all the analyses, each model was tested independently for each bacterial species, and the same group of control flies i.e., injected with Ringer's was used as a comparison.

2.6.1. Fly survival after a previous exposure with bacteria

We tested the effect of the previous exposure treatment on survival seven days after the pre-exposure, by comparing the survival of dead bacteria-injected flies to Ringer's injected flies. We used a generalised linear model using the *glm* function of the *nlme* package with a binomial distribution. Using the function cbind, the number of flies that died and the number of flies that survived per vial was combined into a vector, which we used as a response variable. Previous exposure treatment (F, F + HK and HK), experimental repeat, and their interaction were used as factors. Model 1a tested the survival after pre-exposure for *L. lactis*, and model 1b for *P. burhodogranariea*:

$$\text{Model 1a, b: Survival post-priming}_{\textit{L. lactis, P. burhodogranariea}} \sim \text{Previous exposure} \times \text{Repeat}$$

2.6.2. Survival post-challenge of flies previously exposed with bacteria

For each bacterial species, we compared the survival of flies after a challenge with live bacteria between the previous exposure treatments. Our data met the proportionality assumptions of the Cox model and we included the identification number of the vial the flies had been kept in for the survival assay, as a random effect factor. Therefore, this was tested with a Cox proportional hazards model with random effects using the function *coxme* in the *survival* package (Therneau

and Grambsch 2000; Therneau 2020). The variable tested was a survival object constructed for each individual fly with the function *Surv* in the survival package. This vector contained two variables: a binary censor variable that indicates whether the fly is dead (1) or alive (0), and the day in which the fly died, or in the case of censored flies (i.e., that were still alive at the end of the assay) the last survival check day (seven days post-infection). Models 2a and 2b tested survival after a challenge with *L. lactis* and *P. burhodogranariea*, correspondingly:

$$\text{Model 2a,b: Survival post-challenge}_{\textit{L. lactis, P. burhodogranariea}} \sim \text{Previous exposure} \times \text{Repeat} + (1|\text{Vial ID})$$

2.6.3. Resistance of previously exposed flies at one day post-infection

For both bacterial species, visual inspection of the log transformed bacterial load suggested that the data distribution on day one post challenge was not unimodal. This was statistically tested using a Hartigan's Dip test for unimodality (Hartigan and Hartigan 1985) with the *dip.test* function from the *diptest* package (Maechler 2016) by simulating 5,000 p-values. Then, using the k-means clustering method (Forgy 1965; MacQueen 1967; J. A. Hartigan and Wong 1979; Lloyd 1982), we found the data to be bimodal, a phenomenon previously described by in the literature. Duneau et al. (2017) showed that in the acute phase of infection, hosts can be classified into two groups with low vs. high pathogen burdens (Duneau et al. 2017). These two groups are expected to have different fates, with highly infected hosts predicted to die in this acute phase, while hosts with a low burden survive with a persistent infection because they were able to control the pathogen growth (Duneau et al. 2017). Following this rationale, we sub-set the bacterial load data for day one post challenge into two groups. We determined the cut-off point between these groups as the local minima in the interval between the highest values for both modes. We divided the data into two subsets comprised of flies with a “low” (i.e. below the cut-off point) or “high” (above the cut-off point) bacterial load. Both subsets were analysed separately for each bacterial species. The effect of the previous exposure and the experimental repeat on bacterial load was tested with a linear model on a natural log transformation of the bacterial load using the *lm* function. Models 3a and b for the low and high subsets of flies infected with *L. lactis* correspondingly, and model 3d for the high subset infected with *P. burhodogranariea*. The low subset for *P. burhodogranariea* (Model 3c) was overdispersed,

therefore we used a generalised linear model (function *glm*) with a quasipoisson distribution, where the bacterial load response variable was not transformed. Additionally, we detected three data points in model 3c, which may have been influential (i.e., they were above 0.5 Cook's distance) and gave Bonferroni p-values below 0.05 (tested with the outlierTest function in the *car* package). We therefore analysed the low subset of data with (model 3c) and without these three data points (model 3e). Both models 3c and 3e gave qualitatively similar results (see Table S1 for the results from model 3e). We did not include the identity of the vial in which flies had been kept as a random variable, because the flies were sampled at random from the vials. We did not include the interaction between previous exposure and repeat because some combinations of previous exposure and experimental repeat contained only had one individual.

Models 3a,b,d: Log (Bacterial load day 1 *L. lactis* or *P. burhodogranariea* + 1) ~ Previous exposure + Repeat

Model 3c,e: Bacterial load day 1 *P. burhodogranariea* ~ Previous exposure + Repeat

2.6.4. Resistance of previously exposed flies at seven days post-infection

Bacterial load data seven-days post challenge was found to be unimodal using the Hartigan's Dip test for unimodality as described above (Hartigan and Hartigan 1985). The effect of previous exposure treatment on the bacterial load seven days post-challenge was tested using a linear model (function *lm*) with log transformed bacterial load (Model 4a for a challenge with *L. lactis*, and 4b for *P. burhodogranariea*).

Model 4: Log (Bacterial load day 7 *L. lactis* or *P. burhodogranariea* + 1) ~ Previous exposure × Repeat

3. Results

3.1. Survival after a previous exposure to inactivated bacteria

Fly survival directly before the live bacterial challenge was higher than 96 % across all treatments and experimental repeats (Figure S1). There was no significant effect of the previous exposure treatment or experimental repeat for either bacterial species, and there was no interaction between these two factors (Table S2).

3.2. Survival after a live bacterial challenge

As expected, fly survival was high seven days after challenge with *Drosophila* Ringer's, and it was unaffected by the previous exposure injection: across all seven control groups there was 98.95 % survival (three out of 287 flies died). Contrary to our expectations, we did not find any significant differences in survival between flies injected with the different pre-exposure treatments, whether they were challenged with *L. lactis* or *P. burhodogranariea* (Table 1; Figure 2), meaning that there were no survival benefits to any of the primary bacterial exposure treatments compared to the Ringer's primary control exposure.

Table 1. The effects of previous exposure and experimental repeat on fly survival for the seven days post challenge. Previous exposure treatments include *Drosophila* Ringer's solution, or bacteria that had been inactivated in different ways, i.e., formaldehyde-inactivated bacteria, heat-killed bacteria or a mixture of the two. Flies were then injected with a homologous live challenge of either *L. lactis* (Model 2a) or *P. burhodogranariea* (Model 2b).

Challenge injection	*Tested effect*	X^2	*df*	*p*
L. lactis	Previous exposure	3.22	3	0.36
	Repeat	0.98	2	0.61
	Previous exposure × repeat	7.18	6	0.30
P. burhodogranariea	Previous exposure	2.22	3	0.53
	Repeat	2.71	2	0.26
	Previous exposure × repeat	1.81	6	0.07

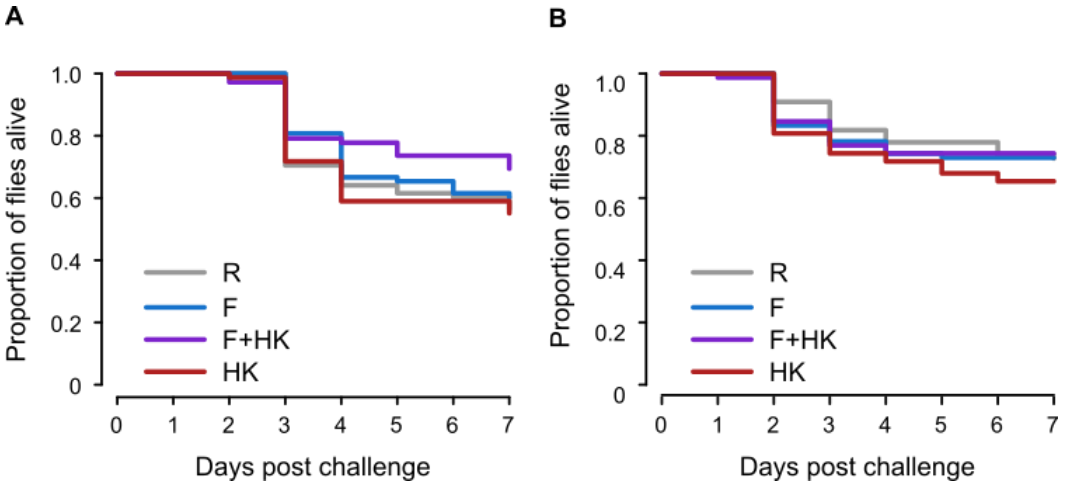

Figure 2. Experimental design. A. Timeline of the experiment with essential steps and assaying timepoints (white text in black boxes). B. Previous exposure and challenge treatment combinations used for this experiment. For each primary exposure-challenge combination treatment where flies were challenged with 92 colony forming units (CFUs) of *L. lactis* or 920 CFUs of *P. burhodogranariea*, a total of 96 flies were monitored for survival, and 21 flies were homogenised to measure their bacterial load.

3.3. Resistance after a live bacterial challenge

Host resistance, i.e., the inverse of bacterial load was assessed on days one and seven after challenge (Figure 3). We found that eleven flies across both days cleared the infection: five out of 168 flies had no *L. lactis* CFUs, and six out of 168 flies had no *P. burhodogranariea* CFUs. On day one post challenge, regardless of treatment and bacterial species, bacteria-infected flies showed large variation in their bacterial load (Figure 3A and B). The data did not follow a unimodal distribution (*L. lactis: D* = 0.071, $p < 0.01$; *P. burhodogranariea: D* = 0.072, $p < 0.01$), with some flies showing a high bacterial load while most flies had a lower bacterial load. Therefore, by calculating the local minima between the highest values for each group of flies, a cut-off point was determined to split the data into two subsets. The data was analysed separately for flies belonging to the low (below the cut-off point) or high (above the cut-off point) subsets (Figure 3A and B) for both bacterial species. We found for both subsets and bacterial species that the pre-exposure treatment did not have a significant effect on the mean bacterial load on day one post challenge (Table 2). For *P. burhodogranariea*, experimental repeat had a significant effect on the bacterial load of the low subset (Table 2). This effect was mainly driven by the presence of a replicate with two flies pre-exposed with heat-killed bacteria

that cleared the infection, as clearance was not found in any other treatment for this bacterial species and day post-challenge.

On day seven post challenge, the bacterial load for the two bacterial species did not differ significantly from a unimodal distribution (*L. lactis*: $D = 0.040$, $p = 0.48$; *P. burhodogranariea*: $D = 0.026$, $p = 0.99$) (Figure 3C and D). We did not find any significant effect of the priming treatment on the bacterial load seven days after challenge (Table 3).

Table 2. The effects of previous exposure and experimental repeat on bacterial load on day one post-challenge. Bacterial load data was split into "low" and "high" subsets by cutting off the data at the local minima between the highest bacterial load values for each subset. These subsets were analysed separately. Previous exposure treatments include *Drosophila* Ringer's solution, formaldehyde-inactivated bacteria, heat-killed bacteria, or a mixture of the two. Flies were then injected with a homologous live challenge of 92 colony forming units (CFUs) of *L. lactis* (Models 3a,b), or 920 of *P. burhodogranariea* (Models 3c,d). Statistically significant factors are shown in bold.

		Low subset			High subset		
Challenge injection	*Tested effect*	*Sum squares*	*df*	*p*	*Sum squares*	*df*	*p*
L. lactis	Previous exposure	4.72	3	0.45	3.72	3	0.46
	Repeat	5.69	2	0.21	2.8	2	0.75
P. burodogranariea	Previous exposure	7.03	3	0.070	6.6	2	0.29
	Repeat	10.62	2	**0.005**	7.68	3	0.40

Table 3. The effects of previous exposure and experimental repeat on bacterial load on day one post-challenge. Bacterial load data was split into "low" and "high" subsets by cutting off the data at the local minima between the highest bacterial load values for each subset. These subsets were analysed separately. Previous exposure treatments include *Drosophila* Ringer's solution, formaldehyde-inactivated bacteria, heat-killed bacteria, or a mixture of the two. Flies were then injected with a homologous live challenge of 92 colony forming units (CFUs) of *L. lactis* (Models 3a,b), or b: 920 of *P. burhodogranariea* (Models 3c,d). Statistically significant factors are shown in bold.

Challenge injection	*Tested effect*	*Sum squares*	*df*	*p*
L. lactis	Previous exposure	1.53	3	0.99
	Repeat	54.26	2	0.11
	Previous exposure × repeat	17.76	6	0.96
P. burodogranariea	Previous exposure	38.24	3	0.25
	Repeat	21.07	2	0.32
	Previous exposure × repeat	21.37	6	0.88

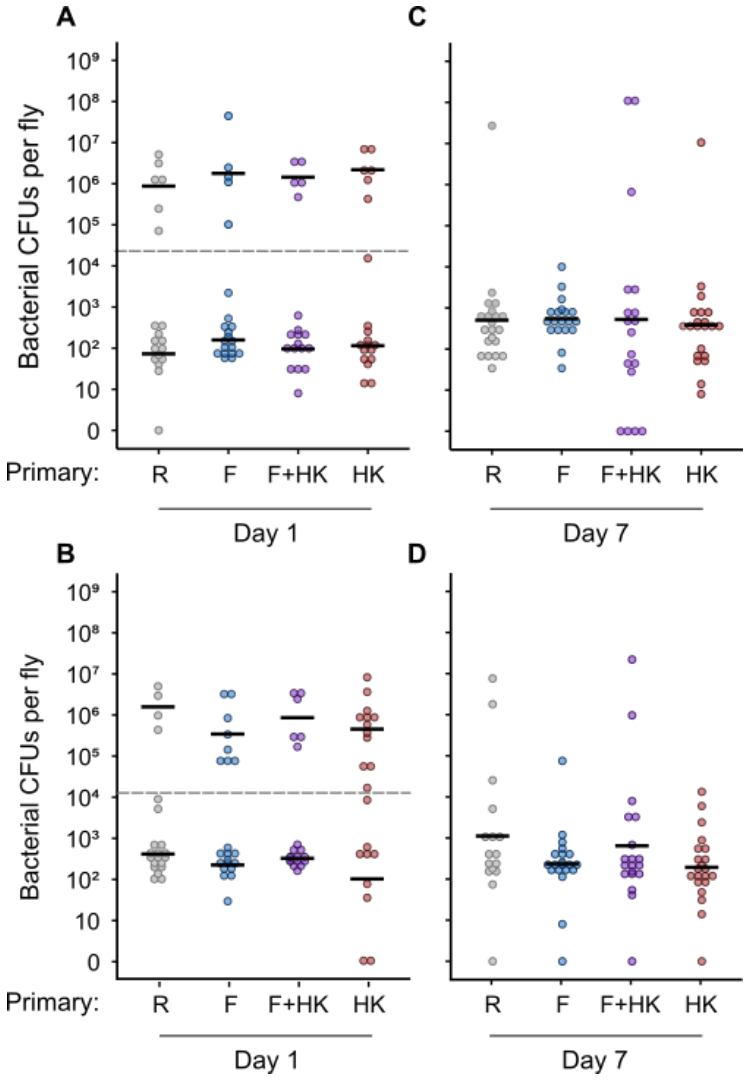

Figure 3. Bacterial load of individual flies after a homologous challenge with 92 colony forming units (CFUs) of *L. lactis*, at A. one and C. seven days post-infection; or with 920 CFUs of *P. burhodogranariea* at B. one and D. seven days post-infection. Bacterial load on the y-axis was quantified as the number of colony-forming units per fly. Here, we present a log transformation of the CFU (+1) for ease of interpretation. On the x-axis, previous exposure treatments are presented as R: Drosophila Ringer's solution, F: formaldehyde-inactivated bacteria, F + HK: a mixture of formaldehyde-inactivated and heat-killed bacteria, HK: heat-killed bacteria. Black lines show the geometric mean of the bacterial load per treatment, and per subset for bacterial load one-day post-challenge. The grey dotted line represents the cut-off point dividing the low and high bacterial load subsets, which were analysed separately. We did not find any effect of the previous exposure on bacterial load for either of the two days assayed. For statistics, see Table 2.

4. Discussion

Our study addresses whether pre-exposure to two bacterial species inactivated with different methods, affects subsequent host survival and resistance against a secondary challenge. We found no enhanced host survival or resistance after a primary exposure to dead bacteria, which was consistent across inactivation treatments and bacterial species. Our results highlight the dynamic nature of host resistance over the infection course, and they raise questions as to whether immune priming is a universal trait of invertebrate immunity.

4.1. Pre-exposure injection does not select for fitter flies

As predicted, we found that a primary injection with inactivated bacteria resulted in high survival (>96%) and similar mortality compared to a primary injection with Ringer's solution. We used dead bacteria for the primary exposure, which for priming experiments has potential advantages over live bacteria: first there is usually minimal mortality after injection with dead bacteria meaning that unlike after the injection of live bacteria, there is no self-selection for a sub-group of fitter flies that survive until challenge; in the case of live bacterial injection, these latter flies may themselves then be predicted to have increased survival after a second infection. Second, a primary exposure with live bacteria will likely reach varying densities across flies by the time of the secondary challenge or even be cleared (Duneau et al. 2017; Acuña Hidalgo et al. 2021); this will result in heterogeneity in the immunological history of the population of flies that are to be challenged. Bacterial infections in insects have been shown to be highly persistent and to lead to sustained antimicrobial responses in the host (Haine et al. 2008; Chambers et al. 2019; Acuña Hidalgo et al. 2021). Using live bacteria for the pre-exposure can lead to persistent infections inside the host, as well as the maintenance of a high level of immune activity, in turn advantaging the host when fighting a secondary bacterial infection(Chambers et al. 2019). However, it is important to note that immune priming responses to inactivated bacteria can persist over time, e.g., antimicrobial responses to heat-killed *S. aureus* can be sustained in *T. molitor* for at least 21 days (Makarova et al. 2016).

4.2. Pre-exposed flies have neither increased survival nor resistance, but resistance varies over the course of infection

An advantage of a pre-exposure to fighting a secondary bacterial challenge has most frequently been measured in terms of increased survival to the secondary infection (Dhinaut, Chogne, and Moret 2018; Pham et al. 2007; Wu et al. 2014; Lin et al. 2013; Christofi and Apidianakis 2013; Faulhaber and Karp 1992; Boman, Nilsson, and Rasmuson 1972; Roth et al. 2009; Lafont et al. 2017; Miyashita et al. 2014; Castro-Vargas et al. 2017; Futo, Armitage, and Kurtz 2016). Contrary to our expectations, we did not find that pre-exposed flies survived the bacterial challenge better than non-exposed flies. Although less commonly tested in the context of immune priming, host resistance, as measured by pathogen load, has been shown to be increased in hosts previously exposed to pathogens (Pham et al. 2007; Boman, Nilsson, and Rasmuson 1972; Sadd and Schmid-Hempel 2006; Miyashita et al. 2014). However, we did not find pre-exposed hosts to be more resistant to a live bacterial challenge in the acute (one day post-challenge) or chronic (day seven post-challenge) phases of infection. While our results contrast with some pathogen infections in *D. melanogaster* (Boman, Nilson & Rasmuson 1972, Pham *et al.* 2007), they are consistent with those of a recent study by Kutzer, Kurtz and Armitage (2019) which showed that four inbred fly genotypes pre-exposed to heat-killed *L. lactis* did not have a higher survival in the 28 days post-homologous challenge, and they did not have increased resistance one and 28 days post challenge (Kutzer, Kurtz, and Armitage 2019). Despite using a lower challenge dose in our current study, an outbred fly population, and different antigen production methods, the results of the two studies are consistent in that pre-exposure does not offer any significant advantages.

While resistance did not differ between pre-exposure treatments, bacterial load varied over the course of the challenge infection. One day post-challenge, bacterial load appeared to follow a bimodal distribution (Figure 3a,b), consistent with previous data on the dynamics of bacterial infections (Duneau et al. 2017). Duneau *et al.* showed that the early dynamics of bacterial load follow a bimodal distribution for intermediately virulent bacterial species, with different predicted outcomes of infection for each of the modes (Duneau et al. 2017). Hosts with high pathogen burden are not able to control the infection and will die during the acute phase of infection. Meanwhile, other hosts will manage to control the pathogen growth and will survive, entering a phase of chronic infection with a constant pathogen load, the set point bacterial load

(Duneau et al. 2017). We expected that, if the primary exposure affected acute phase resistance, it would be apparent as increased resistance of the flies in the lower subgroup. Seven days after infection, we observed that clearance of the bacteria was rare, and bacterial load was unimodally distributed. Our results highlight the importance of measuring bacterial load as a measure of resistance at several points in the infection.

4.3. Resistance is not influenced by the inactivation method

Heat-killing (Wu et al. 2014; Pham et al. 2007; González-Tokman et al. 2010; Longdon et al. 2013; Riessberger-Gallé et al. 2015; Kutzer, Kurtz, and Armitage 2019) and formaldehyde-inactivation (Dhinaut, Chogne, and Moret 2018; Wang, Zhang, and Wang 2009; Zhuang et al. 2011) are two of the most frequently used methods to inactivate pathogens in priming studies. Based on Lin et al (2013) we had reason to hypothesise that host responses would vary according to the inactivation protocol and to our knowledge, a combination of these two methods has not been tested before. Based on the properties of both types of antigenic preparations, we predicted that combining bacterial cells inactivated with both treatments would result in a synergistic effect in which hosts would benefit from the high antigenicity of formaldehyde-inactivated bacteria, and a fast trigger of the immune response caused by the lipopolysaccharides freed upon cell membrane disruption during heat-killing(Lin et al. 2013). However, our results showed that the method used to inactivate the bacteria for the pre-exposure did not influence host resistance. It could be that these treatments still induce differential immune responses in terms of strength, speed and duration (Pradeu and Du Pasquier 2018) but lead to similar outcomes in terms of bacterial load, however we did not test this. Interestingly host survival in the Lin *et al.* (2013), study was not different between hosts pre-exposed to different antigen preparations despite the differences measured in the immune response to both types of inactivated bacteria (Lin et al. 2013).

4.4. Can we consider priming as a ubiquitous aspect of innate insect immunity?

Our study offers a multi-angled evaluation of the effects of pre-exposure on a secondary challenge. Despite this, we did not find any advantage of previous exposure against a bacterial

infection across any pre-exposure treatments. Other studies have identified a priming response in *D. melanogaster* (Boman, Nilsson, and Rasmuson 1972; Pham et al. 2007) but similar to our study, priming is not always found (Kutzer, Kurtz, and Armitage 2019; Pham et al. 2007; Reber and Chapuisat 2012). In addition, many experimental parameters can be explored to achieve priming, including the pre-exposure and challenge doses and bacterial species. While *L. lactis* and *P. burhodogranariea* were isolated from *D. melanogaster* (Lazzaro 2002; Juneja and Lazzaro 2009), and can cause intermediate virulence and persistent infections (Acuña Hidalgo et al. 2021), it could be that pre-exposure against other pathogens with different infection dynamics might result in other outcomes. For example, Kutzer, Kurtz and Armitage (2019) found that pre-exposure with heat-killed *Pseudomonas entomophila*, a more virulent bacterium than the two bacteria tested in this study resulted in a lower resistance across genotypes (Kutzer, Kurtz, and Armitage 2019). A theoretical consideration of immune priming suggested that virulence plays a role in how a pre-exposed host will respond to the infection (Best et al. 2013). Tolerance is another host defence strategy that quantifies the ability of the host to maintain its fitness in the face of an infection (Råberg, Graham, and Read 2009), and which has been rarely explored in priming studies (but see Kutzer, Kurtz, and Armitage 2019). In the case of fecundity as a measure for fitness, Kutzer, Kurtz and Armitage (2019) found no effect of previous exposure on fecundity-tolerance (Kutzer, Kurtz, and Armitage 2019), and although we did not explicitly test it here, the fact that survival and bacterial load did not differ across treatments suggests no effect of survival tolerance under these experimental conditions.

Finally, as mentioned above, while our study and several others did not find priming, it might be that this phenomenon only occurs only under certain circumstances, such as specific host-pathogen combinations (Roth et al. 2009; Pope et al. 2011). For instance, Pope *et al*. (2011) found that white shrimp can be primed using the bacteria *Vibrio harveyi* but not *Bacillus subtilis* (Pope et al. 2011). They argued that shrimp pre-exposed to *V. harveyi* might have an advantage against a live challenge since this bacterium is a known pathogen present in the host natural environment, to which the host may have evolved priming defences, while *B. subtilis* is not naturally present in this environment (Pope et al. 2011). Because it allows the host to reduce or avoid the negative effects of an infection on host fitness, immune priming might be expected to be subjected to a strong selection pressure (Best et al. 2013). However, if it is the case that priming is only elicited in specific experimental circumstances, one could argue about the adaptive value of this phenomenon. Immune priming might then not be a general trait of the

innate immune system, but rather a defence trait specific to populations where it gives a significant evolutionary advantage against pathogens.

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Supporting information

Table S1. The effects of previous exposure treatment and experimental repeat on survival seven days after the previous exposure, i.e. immediately before the challenge. Flies were previously exposed to either Drosophila Ringer's solution, bacteria from the species *L. lactis* (Model 1a) or *P. burhodogranariea* (Model 1b) that were formaldehyde-inactivated or heat-killed bacteria, or a mixture of bacteria inactivated with these methods.

Challenge injection	*Tested effect*	X^2	*df*	*p*
L. lactis	Previous exposure	3.49	4	0.76
	Repeat	8.35	4	0.71
	Previous exposure × repeat	7.62	9	1.00
P. burodogranariea	Previous exposure	0.11	3	0.95
	Repeat	16.79	4	0.25
	Previous exposure × repeat	11.97	12	0.93

Table S2. The effects of previous exposure and experimental repeat on bacterial load of the low subset on day one post-challenge with live *P. burhodogranariea*, when influential data points were removed from the analyses. Bacterial load data was split into "low" and "high" subsets by cutting off the data at the local minima between the highest bacterial load values for each subset. Previous exposure treatments include *Drosophila* Ringer's solution, formaldehyde-inactivated bacteria, heat-killed bacteria or a mixture of the two. Statistically significant factors are shown in bold.

Tested effect	*Sum squares*	*df*	*p*
Previous exposure	6.17	3	0.10
Repeat	35.77	2	< **0.001**

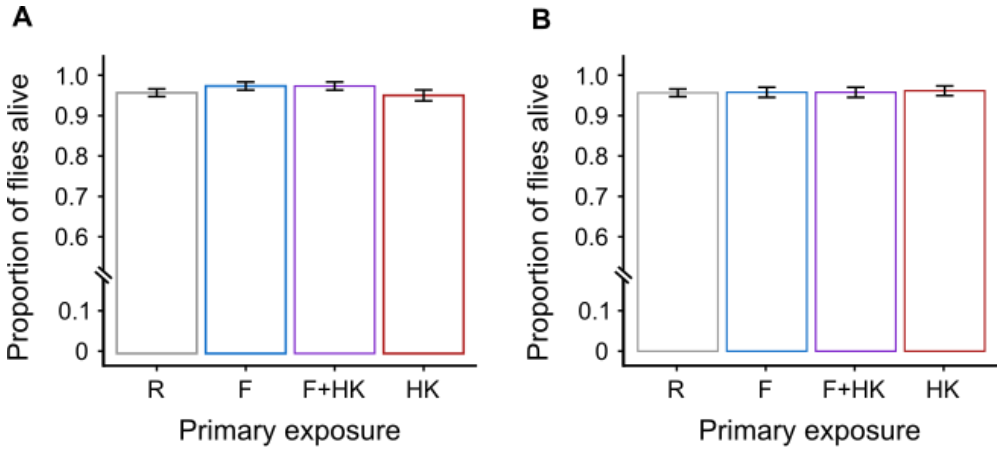

Figure S1. Proportion of flies that were alive before challenge, i.e. seven days after the previous exposure for flies previously exposed to **A**. *L. lactis*, or **B**. *P. burhodogranariea*. Flies were previously exposed to one of the following treatments: R: *Drosophila* Ringer's solution, F: formaldehyde-inactivated bacteria, F + HK: a mixture of formaldehyde-inactivated and heat-killed bacteria, HK: heat-killed bacteria. Mean survival and standard error are shown for all each pre-exposure treatment. For statistics, see Table S1.

CHAPTER 3:

HOSTS THAT CONTROL EARLY PATHOGEN GROWTH ARE MORE TOLERANT TO FECUNDITY COSTS DURING THE CHRONIC PHASE

Hosts that control early pathogen growth are more tolerant to fecundity costs during the chronic phase

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Author Contributions

SA conceived the overall idea. BAH, LS and SA designed the experiments and collected the data. BAH, LS, MF and SA wrote the manuscript. MF & RRR conceived the virulence decomposition and clearance analyses and MF, RRR & SA analysed the data. All authors contributed critically to the drafts.

Unpublished manuscript.

Abstract

Resistance and tolerance contribute towards improving host health through parasite reduction and damage control, respectively. In order to survive an infection, hosts might switch between these two strategies. Although this switch in the defence strategy employed has been shown, how this switch affects the likelihood of the host surviving is poorly understood. Here, adapting a predictive model of infection outcome, we assessed how resistance and fecundity-tolerance differ in *Drosophila melanogaster* throughout infection phases and bacterial burden levels when infected with *Providencia burhodogranariea* or *Lactococcus lactis*. Our results indicate that resistance differs between the acute and chronic phase of infection with *P. burhodogranariea* but not with *L. lactis*. In contrast, in *L. lactis* there is evidence for changes in tolerance across time and/or bacterial burden level. Moreover, for this bacterial species we have indication for a positive correlation between resistance and tolerance during the first days of infection. Hence, at a populational level we demonstrate that differences in immune strategies, and particularly in tolerance, might explain why some individuals succumb to infection, while others manage to persist or clear it. These observations emphasize the need for a multi-level analysis approach to infection dynamics and the danger of universality when inferring from distinct and variable host populational responses.

1. Introduction

When faced with an infection, a host can defend itself by either limiting parasite growth, a strategy know as resistance, or by reducing the detrimental effects of the infection on its fitness, a strategy named tolerance (Kutzer and Armitage 2016a; Råberg, Sim, and Read 2007). Resistance mechanisms come with usage costs, as they are often based on inducible components of the immune system (Moret and Schmid-Hempel 2000; Schmid-Hempel 2005; Alves et al. 2019; Miller and Metcalf 2019). Mounting an immune response requires energetic resources, which may be redirected from other life-history traits towards the immune system, resulting in a trade-off between resistance and other traits (Lawniczak et al. 2007), such as reproductive fitness (Gwynn et al. 2005; Fedorka et al. 2007; Short and Lazzaro 2010; Naim et al. 2020). In contrast to resistance, tolerance is expected to be less energetically costly because it instead carries functional costs, as the evolution of tolerance mechanisms is often dependent on pre-existing elements that can be co-opted for a given infection (Miller, White, and Boots 2006; Huen et al. 2020). In some cases, disease severity can be buffered through tolerance mechanisms, and thereby increasing the survival of the host (Schofield et al. 2002; Seixas et al. 2009; Silva et al. 2020). Therefore the most resistant host is not necessarily the fittest (Ayres and Schneider 2012; Boots and Begon 1993). Moreover, the evolutionary constraints of tolerance on the pathogen growth might be more relaxed than those imposed by host resistance (Boots and Begon 1993; Roy and Kirchner 2000; Miller, White, and Boots 2006). Research on host tolerance as an immune strategy has implications for biomedicine, for example, in the context of the antibiotic-resistance crisis and the increase in persistent infections (Medzhitov, Schneider, and Soares 2012; Vale et al. 2016; Soares, Teixeira, and Moita 2017; Mok et al. 2020).

Nevertheless, what we observe in natural host populations is a mosaic of both resistance and tolerance strategies within a population (Hayward et al. 2014). Furthermore, these immune strategies have been found to be positively (Howick and Lazzaro 2014; Zeller and Koella 2016) or negatively correlated (Råberg, Sim, and Read 2007; Vincent and Sharp 2014; Balard et al. 2020), while others have found no evidence for such a correlation (Lefèvre, Williams, and de Roode 2010; Sternberg et al. 2012; Mazé-Guilmo et al. 2014; Decker, de Roode, and Hunter 2018).This may indicate that the relationship between these strategies is specific to each host-pathogen relationship.

Infection is an inherently dynamic process, characterised by distinct phases of infection (Lough et al. 2015; Louie et al. 2016; Torres et al. 2016). Thus we would expect that the host immune strategy will vary accordingly to what is more advantageous in a given infection(Howick and Lazzaro 2014; Hayward et al. 2014; Lough et al. 2015; Kutzer and Armitage 2016b). For instance, Howick and Lazzaro (2014) unveiled that in *D. melanogster* infected with *Providencia rettgeri*, different forms of tolerance (i.e., mortality-tolerance and fecundity-tolerance) are only present in the acute phase of infection, while resistance prevails throughout the beginning of the chronic phase. Howick & Lazzaro (2014) described the acute phase as being during days one to three post infection with *P. rettgeri* where they observed high mortality, high bacterial burden and low fecundity, whereas during the chronic phase (days four and five post-infection) there were constant bacterial levels, and uninfected-like levels of mortality and fecundity. However, the two studies mentioned above assessed point-tolerance, as opposed to range-tolerance on which we focus in this study (Howick and Lazzaro 2014; Lough et al. 2015). While the former is based on the pathogen load of a single individual, the latter is estimated by assessing the burden of a group of individuals (Kutzer and Armitage 2016b). Therefore, our conclusions on tolerance will greatly differ, depending on whether we trace a tolerance reaction norm based on one individual or a population, as it has been discussed by Little *et al.* (Little et al. 2010). This was experimentally shown in a study on house finches infected with a pathogenic bacteria (Adelman et al. 2013). Kutzer & Armitage (2016b) analysed how immune strategies vary at a population level across time. Their results showed that host tolerance already differs within the acute phase and that, in agreement with the study from Howick and Lazzaro, diet strongly affects tolerance but not resistance (Kutzer and Armitage 2016b). However, none of the studies focused on how subpopulations differ in resistance or tolerance through infection kinetics.

Pathogen loads can vary considerably across a host population, particularly in the early infection stages. This variation can be partly explained by bifurcation within the host population, resulting in a bimodal infection outcome within the first 24 hours post infection (Duneau et al. 2017). Based on their pathogen load in the acute phase, individuals can be classified in two populations characterized by the outcome of infection: (i) hosts that die in the acute phase due to an uncontrolled bacterial proliferation, carrying a host-bacteria specific burden, coined bacterial load upon death (BLUD), and (ii) hosts that survive with a persisting infection at a constant set-point bacterial load (SPBL) (Duneau et al. 2017). Duneau *et al.*

(2017) showed that the likelihood of falling into one of these populations can be estimated by the individual bacterial burden after a given time interval, designated the time to control (t_c). According to these authors, each bacterial species has a time interval during which an infection can be controlled by the host, and small variations in this time can predict if the individuals are fated to survive or die. While in general the bacterial growth within-host during the first hours post-infection is a strong indicator of bacterial virulence and host survival to the infection (Faucher et al. 2020), in intermediately virulent infections the host t_c may be the solo main predictor (Duneau et al. 2017). Given the distinct outcomes of each of these two populations, it is expectable that individuals from each population might differ in their response to the infection. For instance, individuals with high uncontrolled bacterial burdens might not be as resistant or tolerant to the infection, and consequently succumb to the infection.

In the present study, we examined how resistance and fecundity-tolerance are expressed during infection. Through a novel approach, we aimed to understand how categorization of individual hosts into groups, based on their pathogen burden, explain the population response. We classified individuals into a high load group, i.e., individuals assumably fated to die, and a low load group, with individuals fated to survive. We predicted that individuals from the low load category, i.e., which are expected to survive the infection might exhibit distinct resistance and tolerance signatures comparatively to the ones fated to die. In order to assess if these signatures are pathogen species-specific, we infected mated female *Drosophila melanogaster* with one of two opportunistic bacterial species extracted from wild-caught flies, *Providencia burhodogranariea* and *Lactococcus lactis*, both of which can persist within the fly for a number of weeks (Acuña Hidalgo et al. 2021). Relative to other bacterial pathogens, both species are of intermediate virulence (Acuña Hidalgo et al. 2021). However, *L. lactis* was shown to be more virulent than *P. burhodogranariea* due to higher exploitation, i.e. because it reaches higher pathogen burdens (Acuña Hidalgo et al. 2021). At intermediate levels of virulence, the host t_c is expected to dictate infection outcome (Duneau et al. 2017). Because *L. lactis* can reach higher pathogen loads than *P. burhodogranariea*, the host may allocate more energetic resources to reduce its bacterial burden, therefore diverging energetic resources towards mounting an immune response. Therefore, we would expect a higher investment in tolerance opposed to resistance strategies, as the costs of the latter would tend to infinite (Restif and Koella 2003; 2004). We measured the dynamics of resistance and fecundity-tolerance during the acute and early chronic phases of infection. Resistance was measure as the inverse of the bacterial load at

each time-point, while fecundity-tolerance was measured as the slope of the relationship between bacterial load and fecundity. Our measures estimated variation in resistance and tolerance within an outbred population of fruit flies (Martins et al. 2013), through the measurement of individual bacterial load and reproductive fitness for each of the treatment groups (Råberg, Sim, and Read 2007; Råberg, Graham, and Read 2009; Graham et al. 2011; Sternberg et al. 2012; Kutzer and Armitage 2016b; Kutzer, Kurtz, and Armitage 2019).

2. Materials and Methods

2.1. Fly maintenance and production of experimental animals

We used an outbred population of *D. melanogaster* established from 160 fertilised females collected in Azeitão, Portugal (Martins et al. 2013). The population is naturally infected with *Wolbachia* and was gifted to us by Élio Sucena. Flies were maintained at a minimum population density of ~5,000 flies on standard sugar yeast agar medium (SYA medium: 970 mL water, 100 g brewer's yeast, 50 g sugar, 15 g agar, 30 mL 10 % Nipagin solution and 3 mL propionic acid; Bass et al. 2007)) with non-overlapping generations of 15 days. The population and experimental flies were stored at 24.3 ± 0.2°C, on a 12:12 hours light-dark cycle, at 60-80 % relative humidity.

To obtain the experimental animals, grape juice agar plates (50 g agar, 600 mL red grape juice, 42 mL Nipagin (10 % w/v solution) and 1.1 L water) were smeared with a thin layer of active yeast paste, placed inside the population cage for egg laying and removed 24 hours later. After an overnight incubation, first instar larvae were collected and placed into plastic vials (95 x 25 mm) containing 7 mL of SYA medium. Each vial contained 100 larvae to control for density during development. One day after the start of adult eclosion, the flies were placed in fresh food vials in groups of five males and five females and allowed to mate for four days, before allocating the females to treatment groups.

2.2. Bacterial culturing and preparation

We used two bacterial species: Gram-positive bacterium *L. lactis*, and Gram-negative bacterium *P. burhodogranariea* strain B (gifts from Brian Lazzaro, DSMZ; type strain: DSM-19968). Bacterial preparation was performed as in Kutzer and Armitage (2016) (Kutzer and Armitage 2016b). In brief, bacteria were plated on lysogeny broth (LB) agar from bacterial stock aliquots stored in 34.4 % glycerol at -80 ºC, and incubated for 24 hours at 30 ºC. Four colony forming units (CFUs) were added to 100 mL of sterile LB medium in a 500 mL Erlenmeyer flask. Two liquid cultures per bacterial species were incubated overnight (approximately 15 hours) at 30 ºC and 200 rpm. The two liquid cultures per species were centrifuged at 2880 rcf and 4 ºC for 10 minutes and the supernatant was removed. The bacteria were washed twice in 45 mL sterile *Drosophila* Ringer's solution (182 mmol·L^{-1} KCl; 46 mol·L^{-1} NaCl; 3 mmol·L^{-1} $CaCl_2$; 10 mmol·L^{-1} Tris·HCl; Werner et al. 2000) by centrifugation at 2880 rcf at 4°C for 10 minutes. Then, the liquid cultures from the two flasks were combined into a single bacterial solution and the optical density (OD) of 500 µL of the solution was measured in a Ultraspec 10 classic (Amersham) at 600 nm in order to calculate the concentration of the bacterial solution. Based on previous assays, this concentration was adjusted to $5x10^7$ CFU/mL. To confirm the concentration estimated by the OD *post hoc*, we serially diluted the solution to from 1:10 to $1:10^7$, plated eight droplets of 5 µl of the bacterial solution on three LB agar plates, and counted the number of CFUs.

2.3. Infection assays

The injections were performed on four-to-five-day-old female flies randomly allocated to one of four treatments: (i) injection with *L. lactis*; (ii) injection with *P. burhodogranariea*; (iii) injection control inoculated with Ringer's solution; (iv) naïve, i.e., non-injected treatment. The infection assays were carried out in two replicates, i.e., on two different days. In each replicate, the injections were split into two blocks with equal representation of treatments. Injections were performed by two different experimenters. In total, 321 female flies were processed for *L. lactis*, 324 for *P. burhodogranariea*, 55 for Ringer's and 57 for Naïve. A fraction of the flies was sacrificed for bacterial load estimation at day two (*L. lactis*: 69, *P. burhodogranariea*: 69, Ringer's: 6, Naïve: 8) and day four (*L. lactis*: 69, *P. burhodogranariea*: 71, Ringer's: 8, Naïve: 8). remaining individuals had their fecundity assessed for the following days (See section 2.4

below). Females were anesthetized with CO_2 for a maximum of five minutes in groups of 8 or 9 flies. They were injected in the lateral side of the thorax using a fine glass capillary (Ø 0.5 mm, Drummond), pulled to a fine tip with a Narishige PC-10, and then connected to a Nanoject II™ injector (Drummond). A volume of 18.4 nL of bacterial solution, or Ringer's solution as a control, was injected into each fly. For the bacterial solutions, this inoculates each fly with approximately 920 CFUs. For each group of 8 or 9 flies, we used an individual aliquot containing Ringer's or the bacterial solution. At the end of the injections, 50 µL of these aliquots were plated on LB agar to check for potential contamination. No bacteria grew from the Ringer's solution and there was no obvious evidence of contamination in any of the bacterial replicates. In addition, serial dilutions up to $1:10^5$ were prepared and plated before and after the injections for each experimental replicate to ensure that there the concentration of the inoculum remained constant from beginning to end of the experimental day. Full controls, i.e., naïve flies, underwent the same procedure but without any injection. After being treated, flies were maintained individually in plastic vials containing 7 ml of SYA medium and transferred into a new vial for the duration of their experimental treatment.

2.4. Fecundity assay

All the flies were placed into new food vials every 24 ± 0.5 hours for four days in the same order as they were processed on injection day. Because we observed notable variation in the number of adult offspring produced from one day to the other, we decided to assess fecundity based on a 48-hour time window. Fecundity was assayed as the number of adult offspring produced on two timepoints, days one and two, and days three and four post-infection. The vials were frozen upside down at these timepoints and the number of adults were counted.

2.5. Bacterial load assay

Resistance, measured as the inverse of bacterial load, was assayed in separate cohorts of flies at two, four- and ten-days post injection. For days two and four 69-71 flies were assayed per treatment group and for day ten 17-49 flies. Live flies from each treatment were randomly selected to be sacrificed at each infection time-point. Flies were first lightly anesthetized with CO_2, removed from their vial, and placed in a 1.5 mL microcentrifuge tube containing 100 µL

of pre-chilled LB media and one stainless steel bead (Ø 3 mm, Retsch) on ice. The flies were homogenised in a Retsch Mill (MM300) at a frequency of 20 Hz for 45 seconds, following which the tubes were centrifuged for one minute at 420 rcf and 4 ºC. After resuspending the solution, 80 mL of the homogenate from each fly was pipetted into a 96-well plate and then serially diluted from 1:1 to $1:10^5$. Per fly, three droplets of 5 µL of every dilution were plated onto LB agar. Additional tests on the detection of bacteria in homogenised flies indicated that our lower detection limit was of 5 colony-forming units per fly. The plates were incubated at 30 ºC and the number of CFUs was counted after ~20 hours. Individual bacterial loads per fly were back calculated using the average of the three droplets from the lowest countable dilution in the plate. *D. melanogaster* microbiota does not grow easily under the above culturing conditions (BAH et al), however we still homogenised flies that had been injected with Ringer's solution (n = 47) and naïve flies (n = 48). We found foreign CFUs grew from only one naïve fly. Furthermore, three out of 338 bacteria-injected flies had what appeared to be one foreign CFU per droplet in the 1:1 dilution.

2.6. Statistical analyses

All statistical analyses were performed in RStudio version 1.3.1073 (R Core Team 2019). Figures were produced using RStudio and Prism 7.0a. We used the following packages in our statistical analyses “lme4” (Bates et al. 2014) , “glmmTMB” (Brooks et al. 2017), “car” (Fox and Weisberg 2018) and the following for plotting our data: “ggplot2” (Wickham 2016). To include a factor as a random effect in a model it has been suggested that there should be more than five to six random-effect levels per random effect (Bolker et al. 2009), so that there are sufficient levels to base an estimate of the variance of the population of effects (Crawley 2007). In our experimental designs, the low numbers of levels within the factors ‘experimental replicate’ (two levels) and ‘person’ (two levels), meant that we fitted them as fixed effect, rather than random effect, factors (Crawley 2007).

2.6.1. Resistance

As is commonly seen with insect pathogenic bacterial load data (e.g., Duneau et al. 2017; Kutzer, Kurtz, and Armitage 2019; Acuña Hidalgo et al. 2021), the distribution of the loads for flies injected with both bacterial species appeared not to be unimodal. Following the findings of Duneau et al (2017) we hypothesised that we had two sub-groups of flies, i.e., those with a high constant load that did not control the infection in the first hours post-exposure. Therefore, we predicted that resistance would not vary in this group. As for the lower load population, this group has supposedly controlled bacterial growth and thus is more likely to survive the infection. We expected that this group may vary in resistance over time.

To test these hypotheses, for each bacterial species we fitted a mixture model with a log-normal error distribution with bacterial load as a response variable. We included the day post-infection (DPI), person and experimental replicate as explanatory variables. We estimated the parameters using the 'optim' function. To assess the statistical significance of day post-infection (DPI) in the lower load population we used a likelihood ratio test that compares the full model to the reduced model without DPI. Finally, based on the estimated model, for each data point, we calculated the probability that the bacterial load belonged to the lower load population. This information was then used to categorise each data point as belonging either to the lower or upper population (based on a 50% cut-off). This categorical variable was used as a predictor in the tolerance analysis.

$$\text{Bacterial load} \sim \begin{cases} \text{DPI} + \text{Replicate} + \text{Person} & \text{, with probability p} \\ \text{Replicate} + \text{Person} & \text{, with probability } 1 - \text{p} \end{cases}$$

2.6.2. Fecundity

The fecundity models were fitted with a generalised linear model using the package "lme4" with a quasipoisson error structure. First, we tested whether fecundity, measured as the number of adult offspring produced by each female in a 48-hour period, was affected by treatment (i.e., Naïve, Ringer's, *L. lactis* and *P. burhodogranariea*), replicate or person. We applied this model separately for fecundity combined for days one and two, and combined for days three and four post-infection:

$$\text{Fecundity}_{\text{DPI } 1+2 \text{ or } 3+4} \sim \text{Treatment} + \text{Replicate} + \text{Person}$$

We tested if fly fecundity was different between the high and low bacterial load groups, as determined with the methods described above (section 2.6.1.), at days one and two, and days three and four post-infection. Only *L. lactis* and *P. burhodogranariea* injected flies were included in the model. Burden population allocation (low or high burden), replicate and person were included in the model. Additionally, we included the interaction between the treatment and burden allocation:

$$\text{Fecundity}_{\text{DPI } 1+2 \text{ or } 3+4} \sim \text{Treatment}_{\text{Infected}} * \text{Burden} + \text{Replicate} + \text{Person}$$

2.6.3. Tolerance

Here we asked whether fecundity-tolerance differed (i) between populations of flies having either a high or a low load (see previous section) and (ii) between different days post infection (i.e., days two and four). We fitted a generalized linear model with negative binomial error structure using the "glmmTMB" package. As response variable we used the number of adult offspring produced by each individual female over an egg-laying period of 48 hours before bacterial load estimation (e.g., day one and two fecundity for tolerance at day two). We used a longer egg-laying period to reduce inter-day variation. As predictors we included individual $\log_{10}$-transformed bacterial load, day post-infection (DPI, i.e., two or four), burden population, person, and experimental replicate. In addition, we included all pairwise interactions among bacterial load, DPI and burden and their three-way interaction. A separate model was run for each of the two bacterial species:

$$\text{Fecundity}_{\text{Infected}} \sim \log_{10}(\text{Bacterial load}) * \text{DPI} * \text{Burden} + \text{Replicate} + \text{Person}$$

To test for statistical significance we employed a Wald χ^2 test (Bolker et al. 2009) using the Anova function in "car" package. More specifically, for the main effects we used a type II and in the presence of at least one interaction we used a type III Anova. In the data set of *L. lactis*, an influential data point belonging to DPI two and the lower load population was detected based on Cook's distance and was removed from the analysis.

3. Results

3.1. Resistance to *L. lactis* decreases over time

The bacterial load after infection with both bacterial species resolved into a lower and upper population, i.e., flies that were more and less resistant, respectively. Based on the rationale that flies with a higher load will shortly succumb to infection, we fixed the bacterial load of this population so that it was not allowed to vary over time and tested for changes in the low load group only. In this lower group, flies infected with *L. lactis* showed a significant increase in bacterial load over time (D =6.67, df = 1, p = 0.0098; Figure 1A), i.e., a decrease in resistance from two to four days post-infection. Under similar conditions, *P. burhodogranariea* infected flies did not exhibit any changes in bacterial load with time (D =1.66, df = 1, p = 0.20; Figure 1B). We note that when we allowed the upper groups to vary over time, it did not change our interpretation of the results (Figure S1). There was a similar result with an increase in bacterial load with time for *L. lactis* (D = 6.49, df = 1, p = 0.011; Figure S1A), indicating a reduction in resistance within this time interval. Similarly, there was no change for *P. burhodogranariea* between these two time-points (D = 1.66, df = 1, p = 0.20; Figure S1B).

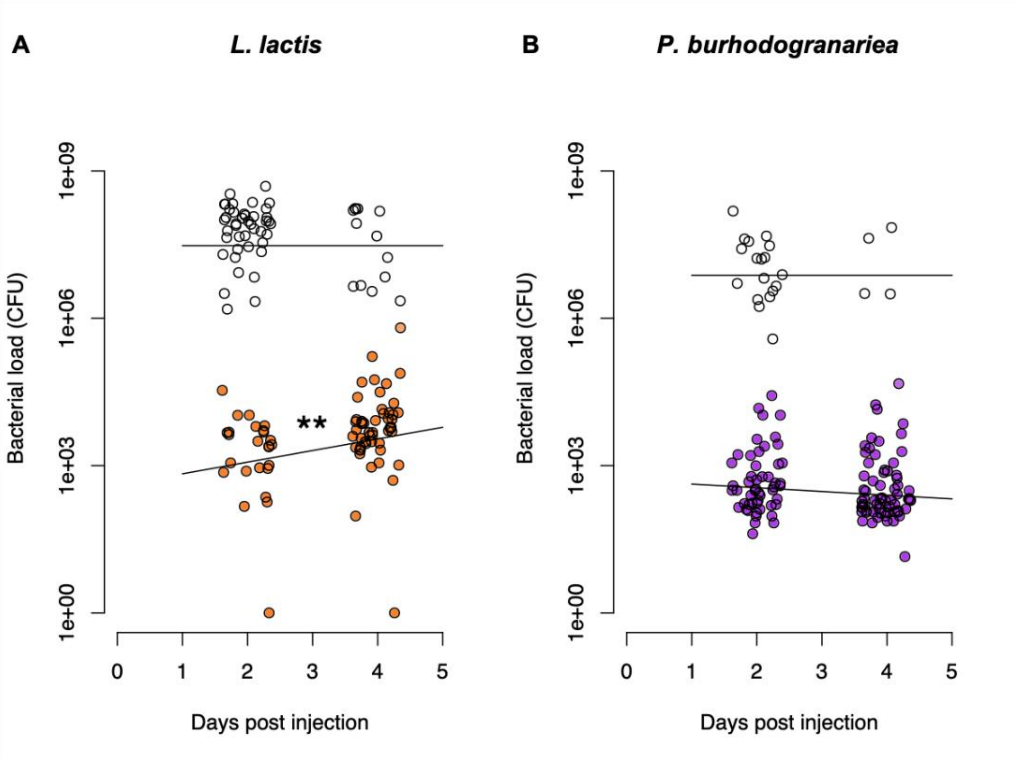

Figure 1. Bacterial load per fly after injection with **A**: *L. lactis* or **B:** *P. burhodogranariea*. Flies were assigned to high or low burden population. Only the lower burden population was allowed to vary over time (see methods for details and Figure S1 where the upper population was allowed to vary). Each data point is from one individual. The darker the colour of the data point, the higher the likelihood is that the fly belongs to the low burden population. The asterisk indicates a significant increase in bacterial load across time ($p < 0.01$). For statistics, see Results.

3.2. Fecundity costs of infection are apparent in the chronic infection phase

We tested whether infection was costly in terms of reduced offspring production. There was no effect of treatment on fecundity measured on days one and two post-infection (Figure 2A, Table S1). However, there was a significant effect by days three and four (Table S1): Both *L. lactis* and *P burhodogranariea* infected flies had lower fecundity compared to Ringer's injected flies, although they did not differ significiantly compared to naïve flies (Figure 2B, Table S1). We then tested whether high infection loads are more costly in terms of reduced fecundity, compared to low inf because ction loads. Once again there were no effects on fecundity at days one and two (Figure 2C, Table S2), but by days three and four, fecundity was higher in the low load population for the two bacterial species (Figure 2D, Table S2).

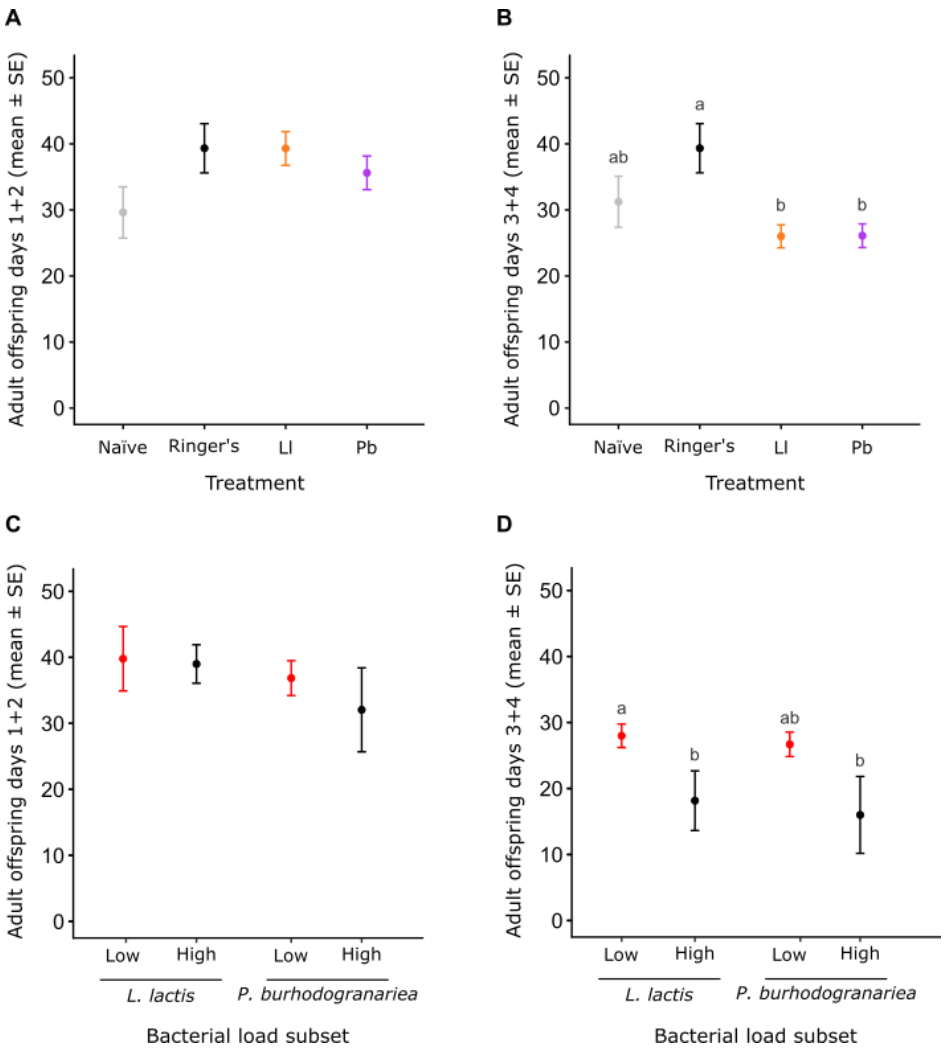

Figure 2. Fecundity estimated as the number adult offspring produced. The mean fecundity per treatment for (A) days one and two, and (B) days three and four post injection. The flies were injected with *L. lactis* (Ll) or *P. burhodogranariea* (Pb), or received a control injection (Ringer's), or received no injection (Naïve). The mean fecundity per bacterial species and bacterial load subset (i.e., low or high) for (C) days one and two and (D) days three and four. Error bars show the standard error. Means with the same letter above them do not differ significantly from one another. For statistics see Tables S1 and S2.

3.3. Reduction in tolerance by day and burden for L. lactis

We measured fecundity-tolerance of flies to the two bacterial species at two time points (days two and four post infection) in the low and high bacterial load populations. There was no

significant three-way interaction between bacterial load, DPI and bacterial burden population. *L. lactis* infections showed evidence for variation in fecundity tolerance (Table 1, Figure 3D-F): there was a significant reduction in fecundity tolerance with time post infection, i.e., day two *versus* day four (significant interaction between bacterial load and day post infection; Table 1, Figure 3E). Furthermore, fecundity-tolerance varied significantly by bacterial load population, whereby the hosts categorised with higher loads were less tolerant to the infection, than the hosts with lower loads (significant interaction between bacterial load and population; Table 1, Figure 3F). Contrary to *L. lactis*, *P. burhodogranariea* infected flies did not show variation for fecundity-tolerance. Instead, fecundity varied significantly by the load population, whereby flies from the lower population were less fecund than the high load counterparts (Table 1). There was also a significant effect of the experimenter (Table 1).

Table 1. The effects of bacterial load, day post infection (DPI), burden, experimental replicate, and person on the response variable fecundity, measured as the number of adult offspring. Each bacterial species was analysed separately. Statistically significant values are shown in bold.

	P. burhodogranariea			*L. lactis*		
Tested effect	***df***	χ^2	***p***	***df***	χ^2	***p***
Bacterial load	1	1.10	0.293	1	0.29	0.593
DPI	1	6.38	0.080	1	5.48	**0.019**
Burden	1	3.06	**0.012**	1	3.43	0.064
Replicate	1	2.18	0.140	1	1.30	0.253
Person	1	5.84	**0.016**	1	0.18	0.675
Bacterial load × DPI	1	0.41	0.523	1	7.39	**0.007**
Bacterial load × Burden	1	0.0022	0.962	1	5.53	**0.019**
Burden × DPI	1	0.35	0.553	1	3.32	0.069
Bacterial load × DPI × Burden	1	0.30	0.583	1	1.62	0.203

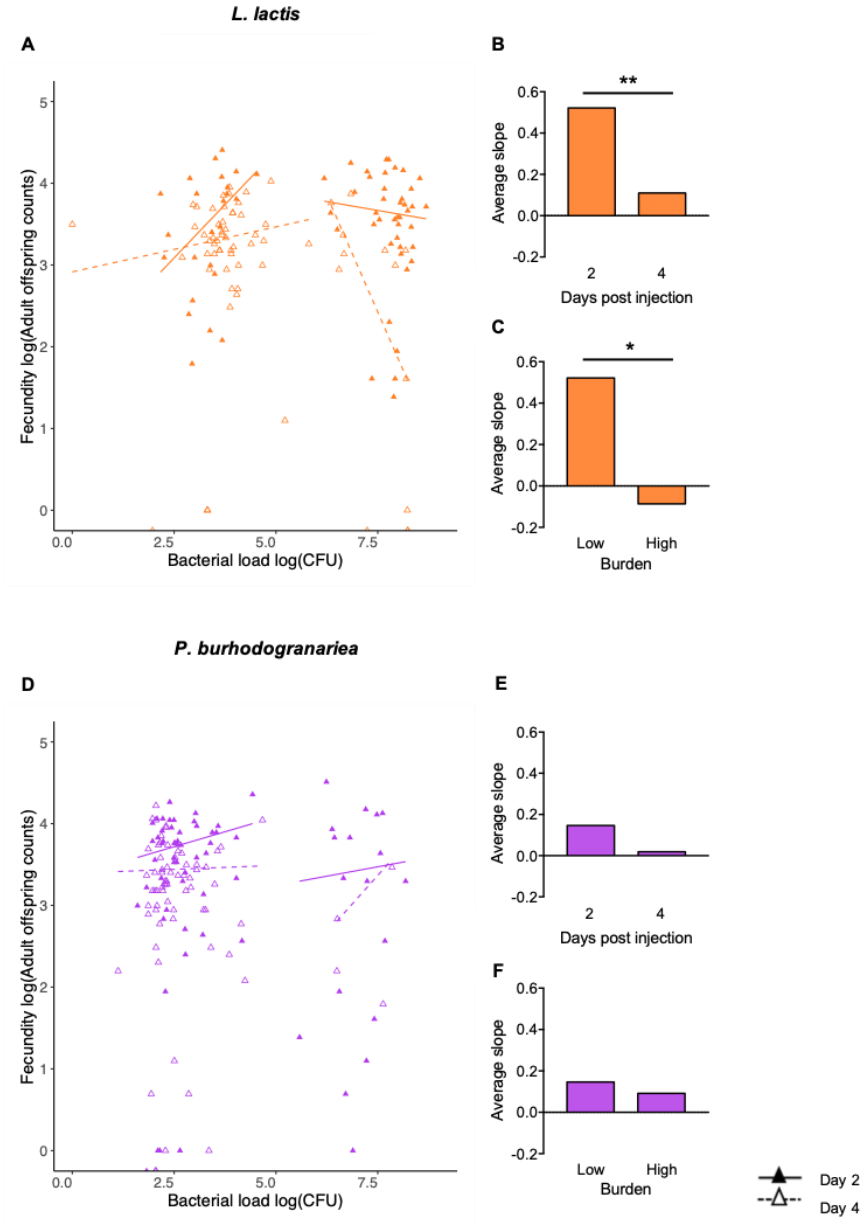

Figure 3. Fecundity tolerance reaction norms are plotted for each bacterial species across different days post injection and bacterial load populations. (A-C) Flies that had been injected with *L. lactis*: (A) Tolerance slopes estimated based on the statistical model described in the Materials and Methods. To aid interpretation of the statistical results, the average slopes for day post injection (B) and bacterial load population (C) are also plotted. (D-F) Flies that had been injected with *P. burhodogranariea*: (D) Tolerance slopes estimated based on the statistical model. The average slopes for day post injection (E) and bacterial load population (F). The asterisks indicate a significant difference in the slopes where * indicates $p < 0.05$ and ** $p < 0.01$. For statistics, see Table 2.

4. Discussion

In the present study, we offer a novel approach to infection dynamic studies through the inclusion of a binary outcome mixture model (Duneau et al. 2017). Our results show that infection comes with costs that are expressed through a reduction in fecundity. Furthermore, we found that tolerance and resistance can vary on a temporal scale and between subgroups within a population based on their likelihood of survival to the acute phase of the infection. We emphasise the importance of considering the population structure and infection stage when addressing the dynamics of infection.

4.1. Infection induces fecundity costs later in the infection and more strongly in high burden flies

Infections can be costly for a host, for instance due to host tissue damage caused by the pathogen, e.g., via bacterial toxins (Opota et al. 2011), or by the host immune system, i.e. immunopathology (Sadd and Siva-Jothy 2006; Khan, Prakash, and Agashe 2017). Moreover, infections have also been shown to be costly in terms of trade-offs with life history traits such as reduced fecundity (Gwynn et al. 2005; Brandt and Schneider 2007). Infections with *L. lactis* and *P. burhodogranariea* have negative consequences on the fitness of hosts as it results in reduced survival over a four-day window (Acuña Hidalgo et al. 2021), therefore, we predicted that there would also be a cost in terms of reduced fecundity. Days one and two saw no effects on infection on fecundity, but by days three and four, bacterial infection reduced fecundity compared to Ringer's injected flies. That costs might only become apparent later in the infection, has been shown for flies infected with *Salmonella typhyrium*, in which fecundity decreases over the course of the infection (Brandt and Schneider 2007). Furthermore, bacteria that elicit a stronger antimicrobial response, also cause a stronger decrease in fecundity, pointing to the induced immune response as a possible cause of these fecundity costs (Brandt and Schneider 2007). Induced immune responses are energetically costly (Moret and Schmid-Hempel 2000; Chambers et al. 2019). Thus, over the course of the infection, hosts might progressively deplete their energetic resources by investing them in fighting the infection, making the costs for fecundity only apparent later on.

Our finding that flies with high burdens of *L. lactis* had a lower fecundity at three- and four-days post-infection compared to the low burden group, indicates that the costs are load-dependent. With higher bacterial burdens, they might have less resources available for reproduction compared to flies with lower loads due to the redirection of resources from other life-history traits like reproduction to mounting an immune response, as explained above. Nevertheless, flies from both bacterial load populations sustain these costs, and higher load flies are predicted to die as a consequence of not being able to control the infection (Duneau et al. 2017). This suggests that their immune response is not sufficient to control the growth of the pathogen, indicating that they might sustain additional costs unrelated to the induced immune response. This could be due to the pathogen consuming the host resources (Hurd 2001; Cassat and Skaar 2013). Alternatively, these resources may be allocated towards repairing damage caused during the infection, rather than towards reproduction.

4.2. Resistance and fecundity-tolerance to infection

Immune strategies can fluctuate throughout infection (Kutzer and Armitage 2016b; Howick and Lazzaro 2014). Based on the models built by Duneau *et al.* (Duneau et al. 2017), we can predict whether individual flies will succumb or survive an infection by assessing their bacterial load after a given time, defined by the authors as t_c i.e. the time to control the infection. Here we focused on post-branching variation in resistance and tolerance. We propose that flies in the upper population have reached a plateau burden that eventually will lead to host death, defined by Duneau et al. (2017) as the bacterial load upon death (BLUD). For this reason, we predicted that the bacterial load of flies with high burdens would remain constant. However, because in our model we could not estimate whether the bacterial load of these individual flies remains constant after 48 hours, we also ran analyses where we considered the possibility of bacterial load variation within this high load population. The reasoning behind the latter is that flies can survive with high loads for extended periods of time. For example, a previous study found that high *P. burhodogranariea* loads could be retrieved from flies up to 14 days post-infection (Acuña Hidalgo et al. 2021) and that the bacterial load of this bacterial species can decrease over time (Acuña Hidalgo, Silva & Armitage, unpublished data). Therefore, flies from the high load population might see their loads decrease over time.

L. lactis infected flies showed a reduction in both resistance and fecundity tolerance from day two to four post infection. This fecundity shift between day two and four might support the terminal investment hypothesis (Williams 1966; Clutton-Brock 1984). When infected with *L. lactis*, female flies increase their investment towards early reproduction in the first 48 hours to potentially maximize their reproductive success. This resource allocation towards fecundity is particularly relevant for the high load population of flies, as they are unlikely to mitigate this cost of infection during their short lifetime. On the other hand, flies in the low load population bear a strong early immune response that successfully allows them to control infection (Duneau et al. 2017), as well as a higher reproductive effort compared to the flies fated to die from the uncontrolled pathogen growth. These accumulated costs are likely to drive the reduction in resistance and fecundity-tolerance between days two and four post infection in flies fated to survive. To date, there are a few examples in literature of positive correlation between these immune strategies (Howick and Lazzaro 2014; Zeller and Koella 2016). In our system there is indication for a positive correlation between resistance and fecundity-tolerance, at least, during the acute phase of infection with *L. lactis*. This result suggests these immune mechanisms might be interlinked or even dependent on each other, and the absence of a strong resistance response might lead to an uncontrollable infection as seen in the high load population. In contradiction to the BLUD hypothesis, we observe a reduction in the bacterial load with time when we allow the high load population to vary over time in our model (Figure S1). This suggests that either the flies in the high load population are able to control the infection later on, or flies in the low load population are unable to do so, potentially due to accumulated costs of infection. In either of the scenarios, there is an indication that hosts vary in their pathogen load over time, interchanging between high burden and low burden populations according to their individual infection dynamics, specially later on in the infection.

For *P. burhodogranariea* we noted a different overall pattern (Figure 2B and 3D-F). We did not observe any differences between day two and four in terms of resistance or fecundity-tolerance. More interestingly, flies in the low load population which are likely to survive the infection, do not seem to pay a price in reproductive fitness for their long-term increase in resistance, comparatively to flies in the high load population. Taking in account the early branching between survivors and hosts that succumb (Duneau et al. 2017), it is possible that for this species we are in the presence of a different infection dynamic compared to *L. lactis*, and that what we observe is already the chronic phase of infection. If that would be the case, we

might not be able to observe changes in immune strategies like we did in *L. lactis* infection flies, seemingly more frequent in the early stages of infection. According to Howick and Lazzaro (2014), during the chronic phases of infection there is not a clear mark of fecundity-tolerance (Howick and Lazzaro 2014). Nevertheless, we must be careful with our assumptions due to the limited sample size in the high load population at day four post infection. Interestingly, in the latter we now see a significant increase in resistance in the high load population as well, however due to the low sample size, this cannot be confirmed.

Moreover, our data proposes that the hypothetical low survival ability of the high load population individuals might be due to a reduced average tolerance comparatively to their low load counterparts, as it has been shown in mice infected with *Listeria monocytogenes* (Lough et al. 2015). Mice fated to die from the infection exhibit less tolerance or resistance comparatively to surviving mice. Based on this result, we can hypothesise that surviving flies might handle better this infection through an early and stronger investment in tolerance. The underlying cause for this disparity is unknown to us. Although Duneau *et al.* (2017) has hypothesized that t_c, the time to control the infection, is the most decisive host parameter to explain the binary outcome and disease severity, given the outbred nature of the population tested we cannot discard genotype differences. This natural variation might confer different opportunity for host mechanism co-option or higher resilience. Unpublished data suggests *P. burhodogranariea* has an earlier t_c than *L. lactis* (Acuña Hidalgo, Silva and Armitage, unpublished data). Therefore, it is possible our time window does now allow for detection of strong changes in *P. burhodogranariea*, as they might have happened earlier than for *L. lactis*. The latter highlights the need to study infection host-pathogen model dynamics over time and the danger of universality in temporal dynamic studies.

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Supporting Information

Table S1. The effects of treatment (*L. lactis*, *P. burhodogranariea*, Ringer's and Naïve), experimental replicate and person on the response variable fecundity, estimated as the number of adult offspring produced in hours. Statistically significant factors are shown in bold.

Tested effect	***df***	χ^2	***p***
Days 1+2			
Treatment	3	73.27	0.13
Person	1	13.63	0.31
Replicate	2	38.33	0.23
Days 3+4			
Treatment	3	167.01	**0.0013**
Person	1	10.02	0.33
Replicate	2	23.45	0.33

Table S2. The effects of bacterial species (*L. lactis* or *P. burhodogranariea*) the bacterial load population to which flies are allocated (i.e., low or high load), experimental replicate, person as well the interaction between the bacterial species and population allocation on the response variable fecundity, estimated as the number of adult offspring produced over two days. Statistically significant factors are shown in bold.

Tested effect	***df***	χ^2	***p***
Days 1+2			
Treatment	1	12.37	0.30
Subset	1	5.38	0.50
Person	1	68.80	**0.02**
Replicate	1	5.60	0.49
Treatment × subset	1	2.50	0.64
Days 3+4			
Treatment	1	0.01	0.97
Subset	1	60.70	**0.01**
Person	1	0.16	0.89
Replicate	1	0.54	0.80
Treatment × subset	1	0.31	0.84

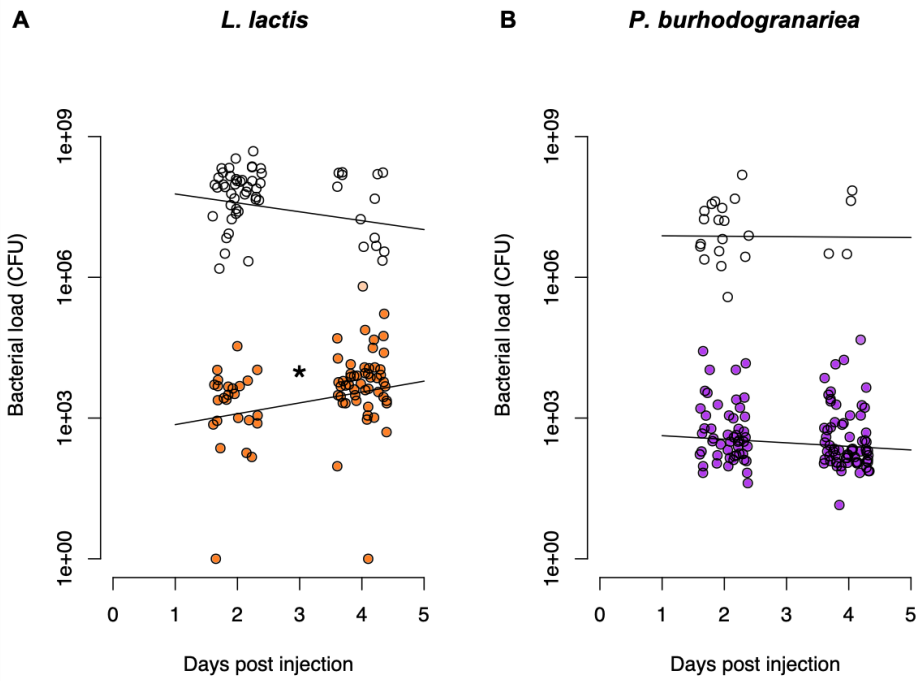

Figure S1. Bacterial load per living fly after injection (A) *L. lactis* or (B) *P. burhodogranariea*. Flies were assigned to higher or lower load population and the linear relationship of both populations was allowed to vary over time. Each data point is from one individual. The darker the colour of the data point, the higher the likelihood is that the fly belongs to the low burden population. The * asterisk indicates a significant increase in bacterial load across time (p-value < 0.05). See Results section for statistics.

CHAPTER 4:

SUSTAINED HOST ANTIMICROBIAL RESPONSE TO A PERSISTENT BACTERIAL INFECTION IN *DROSOPHILA MELANOGASTER*

Sustained host antimicrobial response to a persistent bacterial infection in *Drosophila melanogaster*

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Author Contributions

ARR, BAH and SA conceived the idea and designed the experiments. BAH conducted the infection experiment. ARR prepared the *in vitro* bacterial samples, as well as the samples for proteomics. BK ran the liquid chromatography-mass spectrometry analyses. BAH conducted the preliminary analyses and wrote the first draft of the manuscript with advice from SA and ARR.

Unpublished manuscript.

Abstract

Persistence is a recurrent outcome of infection. Varying species of bacterial pathogens can be recovered from a host for up to weeks post-exposure. Studies on insect infections have shown that chronically infected hosts sustain antimicrobial responses for days after the initial growth of the pathogen has been controlled, yet the perspective of the pathogen has rarely been explored. With the aim to offer a full view of how the host and pathogen interact after the onset of a chronic infection, we infected *Drosophila melanogaster* with an intermediately virulent and persistent bacterium, *Providencia burhodogranariea*, and measured the protein expression of both host and pathogen seven days post-exposure. By measuring the protein expression of the fly, we confirm that hosts express a strong antimicrobial peptide response during the chronic infection. Moreover, we uncovered that flies may use iron depletion as a strategy to fight the infection. Due to experimental constraints, we were unable to detect enough bacterial proteins inside the fly, indicating that other experimental methods focused on specific proteins of interest might be more appropriated to provide the perspective of the pathogen. Nonetheless, our study provides a sound base for future studies focusing on the bacterial perspective of persistent infections.

1. Introduction

Upon encountering a bacterial infection, insect hosts typically go through an acute phase of infection, characterised by high mortality and high pathogen burden (Howick and Lazzaro 2014). Some hosts will succumb to the infection during this phase, as they fail to control the pathogen growth, and other hosts will manage to control the infection and survive this initial acute phase with a persistent infection (Duneau et al. 2017). While clearance of the infection is a possible outcome in hosts that survive the acute phase, persistence seems to be a recurrent scenario across many studies on bacterial infections (Gorman and Paskewitz 2000; Brandt and Schneider 2007; Haine et al. 2008; Kutzer and Armitage 2016; Chambers et al. 2019; Kutzer, Kurtz, and Armitage 2019; Acuña Hidalgo et al. 2021). Different types of bacteria, ranging from opportunistic pathogens to well-studied entomopathogenic bacteria, can cause chronic infections. Acuña Hidalgo et al. (2021) tested the ability of four bacterial species across a wide range of virulence to chronically infect *Drosophila melanogaster*. They showed that all bacterial species were able to persist in at least some flies, regardless of their level of virulence (Acuña Hidalgo et al. 2021). Moreover, they found that these bacteria can persist inside their hosts for extended periods of time, namely up to eleven weeks (Acuña Hidalgo et al. 2021), surpassing previous estimates in fruit flies, i.e. 28 days post-infection (Kutzer, Kurtz, and Armitage 2019), as well as in other insect models, e.g. 28 days in mealworm beetles (Haine et al. 2008).

There are several studies focusing on the factors predicting whether hosts will survive or succumb to a systemic infection (Duneau et al. 2017; Jent et al. 2019; Ellner et al. 2021), yet little is known about how the host and pathogen interact when a chronic infection has been established. Current evidence focusing on the host side indicates that chronically infected hosts express a persistent antimicrobial response against the bacterial pathogen (Uttenweiler-Joseph et al. 1998; Lowenberger et al. 1999; Chambers et al. 2019). The antibacterial immune response of insects is mediated in part by the secretion of proteins aimed to kill the pathogen (Wu, Patočka, and Kuča 2018). In *D. melanogaster*, various antimicrobial peptides (AMPs) are upregulated in chronically infected hosts at seven days post-exposure with bacteria (Chambers et al. 2019). While this sustained response is beneficial against a secondary infection (Chambers et al. 2019), how it affects bacteria in the context of persistent infections remains unclear. Evidence from *Tenebrio molitor* shows that most of the bacteria are cleared from the host

haemolymph before the onset of the antimicrobial peptide response, suggesting that it might serve the purpose of eliminating the leftover cells that survived the first wave of the constitutively active immune response (Haine et al. 2008). However, the dynamics of infection in *D. melanogaster* are considerably different. Within the first hours of infection, there is a substantial increase in the bacterial load, paired with an upregulation of AMP expression (Clemmons, Lindsay, and Wasserman 2015; Khalil et al. 2015; Duneau et al. 2017). The bacterial growth stabilizes after a few hours in hosts that survive the acute phase, suggesting that the antimicrobial response plays a key role in the early stages of infection by preventing an uncontrolled bacterial growth, which would lead to host death (Duneau et al. 2017).

This raises the following question: if AMPs act by controlling early pathogen growth, why is it then that they remain active for several days after the resolution of the acute infection phase? Antimicrobial peptides are part of the inducible immune response, and one would expect that energetic costs may arise from this long-lasting persistent response, as resources are diverted from other functions towards the immune system (Schmid-Hempel 2003; Brandt and Schneider 2007; Bajgar and Dolezal 2018). This is the case for chronically infected flies sustaining an AMP response: they are less resistant to starvation, suggesting that this response is energetically straining for the host (Chambers et al. 2019). The costs of long-lasting antimicrobial responses would only be justified if they are out weighted by the benefits of expressing these responses (Moreno-García et al. 2014). For instance, if the bacteria are still replicating, hosts that sustain an antimicrobial response may be able to keep the bacterial growth at bay. Hosts that enter a chronic phase of infection can in some instances carry a constant bacterial load during the rest of the infection, the set point bacterial load (Duneau et al. 2017; based on the set point viral load Fraser et al. 2014). If bacteria are actively replicating during the chronic phase, this indicates that the host might be killing the bacteria at the same rate at which the bacteria are reproducing.

Nevertheless, experimental evidence from antibiotic resistant chronic infections shows that bacteria can undergo physiological changes that shield them from the immune response of the host, often at the cost of their growth (Fisher, Gollan, and Helaine 2017). In the case of chronic infections in insect hosts, it is possible that the pathogen may employ the same strategy. In their mathematical model of a bacterial infection, Ellner et al. (2021) showed that allowing pathogens to exist in a protected state inside the host results in theoretical outcomes that match those

observed in experimental studies (Ellner et al. 2021). To our knowledge, empirical evidence of persistent bacteria trading off their growth for a shielded state against the insect immune response has not been tested for. Therefore, in the present study we infected *D. melanogaster* with the bacterial species *P. burhodogranariea* with the goal of simultaneously examining the proteomes of both organisms during a persistent infection. We aimed to offer a complete view on the interaction between host and pathogen in the context of a persistent infection. *P. burhodogranariea* causes dose-dependent intermediately virulent infections in *D. melanogaster*, and it is persistent at high inoculation doses (Acuña Hidalgo et al. 2021). Thus, we expected that infected flies would vary considerably in their physiological state compared to non-infected flies, and based on previous transcriptomic evidence in this host species, we predicted that infected *D. melanogaster* would sustain an antimicrobial response at the assayed timepoint (Chambers et al. 2019).

Regarding the perspective of the pathogen, we could make several hypotheses based on the evidence from experimental studies. Persistent bacteria have been shown to evade the immune system via two furtive strategies. Firstly, they can invade host tissues where they will be protected from the immune effectors (Fisher, Gollan, and Helaine 2017). For example, *Mycobacterium tuberculosis*, the causative agent of tuberculosis, manages to persist by surviving inside the macrophages after being phagocytosed (McDonough, Kress, and Bloom 1993; Schnappinger et al. 2003). Interestingly *S. aureus*, another bacterium that uses this strategy to chronically infect humans (Kubica et al. 2008), is capable of invading the phagocytes of *T. molitor*, allowing it to persist for several weeks inside the host (McGonigle, Purves, and Rolff 2016). Secondly, some bacterial species can form biofilms, a consortium of bacterial cells adhering to each other and embedded in an extracellular matrix (Hall-Stoodley, Costerton, and Stoodley 2004). This structure protects the bacterial cells from external aggressions by the immune system and antibiotic treatments, allowing them to persist without a dramatic decrease in numbers (Joo, Fu, and Otto 2016). Galac and Lazzaro (2011) showed that *P. burhodogranariea* (and all their *Providencia* isolates) does not form biofilms in vitro, but this was not tested in vivo. Moreover, there is no current evidence that *P. burhodogranariea* is not capable of invading host cells, and other bacteria of the same genus, *Providencia alcalifaciens* was capable of infecting S2 *Drosophila* cells in an antibiotic protection assay (Galac & Lazzaro 2011). Therefore, biofilms and phagocyte invasion by *P. burhodogranariea* are scenarios that cannot be discarded. However, we here decided to focus on a third possibility.

Instead of physically evading the host immune response, bacterial cells can switch their physiological state into a "persister" state, characterised by a slowed down or arrested growth (Balaban et al. 2004; Conlon et al. 2016; Westblade, Errington, and Dörr 2020; Shan et al. 2017), or a decrease in metabolic activity (Amato et al. 2014). While a small proportion of persister cells seem to already be present in bacterial populations before any growth occurs, a switch from a growing state to a persister state can happen during the exponential phase, as it was shown for *Escherichia coli* (Balaban et al. 2004; Harms, Maisonneuve, and Gerdes 2016). Moreover, persister cells have a decreased susceptibility to killing by antibiotics (Keren et al. 2004; Harms, Maisonneuve, and Gerdes 2016). Thus, it might be that *P. burhodogranariea* switchs to a dormancy state upon infection of the fly, protecting it from the immune response and allowing it to chronically infect the host. By comparing the proteome of *P. burhodogranariea* inside the fly to that of *in vitro* bacteria sampled during the replicating or dormant stages, we aimed to understand whether this bacterium is still replicating inside the host, or if it exists inside the host in a protected state, at the expense of its growth.

2. Material and Methods

2.1. Infection model

We produced our experimental flies from an outbred population of *D. melanogaster* (gift from Élio Sucena from Instituto Gulbenkian de Ciência, Portugal), which was established from 160 fertilised females collected in Azeitão, Portugal in 2007 (Martins et al. 2013). This population is naturally infected with the intracellular bacterium *Wolbachia*. The population was reared at a density of 5,000 flies, with non-overlapping generations of 14 days. They were maintained in a population cage on a 12:12 hour light-dark cycle, at 60-80% relative humidity and a temperature of 25 °C. They were fed with a sugar yeast agar (SYA) medium (970 mL water, 100 g brewer's yeast, 50 g sugar, 15 g agar-agar, 30 mL 10 % Nipagin solution and 3 mL propionic acid; Bass et al. 2007).

Experimental flies were produced after two generations of density control. To do this, we placed four purple grape juice agar plates (25 g agar-agar, 300 mL red grape juice, 21 mL 10% nipagin solution, 550 mL water; Wensing, Koppik, and Fricke 2017) coated with a thin layer of baker's

yeast paste inside the population cage. We let the flies lay eggs for 24 hours and removed the plates. After another 24 hours, larvae were collected in groups of 100 individuals in plastic vials (95 x 25 mm) containing 7 mL of SYA medium. These individuals were left to develop for eight days under the conditions described above. Four days after they had emerged as adults, they were placed in two embryo cages in groups of 600-800 adults and allowed to mate and lay eggs on a purple grape juice agar plate for 24 hours. Another 24 hours later, larvae were collected as above and allowed to develop. Newly emerged adults were collected one day after emergence and placed in fresh food vials in groups of five males and five females.

2.2. Preparation of the bacterial solutions

In this study, we infected the flies with *P. burhodogranariea* strain B (DSMZ; type strain: DSM-19968; gift from Brian Lazzaro from Cornell University, USA), a bacterial species isolated from wild-caught *D. melanogaster* (Juneja and Lazzaro 2009). It is able to establish an infection in *D. melanogaster*, causing intermediate levels of mortality (Lazzaro 2002; Lazzaro, Sackton, and Clark 2006; Galac and Lazzaro 2011; Acuña Hidalgo et al. 2021).

2.2.1. Infecting bacterial solution

We grew an overnight culture of the bacterium as previously described in Kutzer and Armitage (Kutzer and Armitage 2016). We streaked bacteria on a lysogeny broth (LB) agar plate from a 34.4 % glycerol stock kept at -80 °C. We let the colony-forming units (CFUs) grow for 24 hours at 30 °C, then picked four colonies and inoculated 100 mL of sterile LB medium. Two overnight bacterial cultures were grown for 15 hours, at 30 °C and 200 rpm. After an incubation period of 15 hours, the cultures were centrifuged at 2880 g and 4 °C for 10 minutes. After removing the supernatant, the cultures were twice washed in *Drosophila* Ringer's solution (182 $mmol \cdot L^{-1}$ KCl; 46 $mol \cdot L^{-1}$ NaCl; 3 $mmol \cdot L^{-1}$ $CaCl_2$; 10 $mmol \cdot L^{-1}$ Tris·HCl; Werner et al. 2000). We measured the optical density of 500 µL of this bacterial solution, and estimated the concentration based on xxxx and adjusted it to 5 $\times 10^9$ CFUs/mL. We performed three serial dilutions to verify this concentration, from 1:1 to 1:10^6, and plated eight droplets of 5 µL of

$1:10^4$ to $1:10^6$. After an incubation period of 20 hours at 30 °C, we counted the number of CFUs and back calculated the concentration of the solution.

2.2.2. *In vitro* generation of dormant and replicating bacteria

In this study, we aimed to determine whether the bacteria inside the fly were replicating or in a dormant stage. Therefore, we used two *in vitro* treatments as controls for the protein expression of bacteria inside the fly: bacteria in a replicating stage (PB-R), and bacteria in a dormant stage (PB-D).

All steps for growing the overnight cultures were performed as described above, but the bacterial colonies were used to inoculate 10 mL of LB medium, instead of 100 mL. We maintained the same air to medium ratio as in the previous section to achieve almost identical growing conditions. We grew six independent overnight cultures for each of the two treatments. After an incubation period of 15 hours, we performed one centrifugation step at 2880 g and 4 °C for 10 minutes. The supernatant was discarded and replaced by the same volume of LB medium for the PB-R treatment and of 0.9 % sodium chloride solution (ref?) for the PB-D treatment. The sodium chloride solution maintained the dormant bacterial cells in a viable state, while not providing enough nutrients for them to grow. We measured the OD of a 1:10 dilution of each solution to verify that the bacterial concentration was around 10^9 CFUs/mL.

The bacterial solutions from the PB-R treatment were serially diluted $1:10^3$ times, to achieve a concentration of $\sim 10^6$ CFUs/mL. Then, they were allowed to grow for two hours at 30 °C and 180 rpm, until they had reached the mid-exponential growth phase and a concentration of $\sim 10^7$ CFUs/mL. The solutions from the PB-D treatment were serially diluted to a concentration of $\sim 10^7$ CFUs/mL and kept at 30 °C with shaking at 180 rpm for 24 hours to trigger dormancy (stationary phase) of the bacterial cells. One millilitre of each of the twelve bacterial cultures was transferred to microcentrifuge tubes and placed at -80 °C for later proteomic analyses. To estimate the concentration of the final bacterial cultures, we extracted two times 100 µL per solution and serially diluted twice the solution from 1:1 to $1:10^5$. Three droplets of 5 µL were plated per serial dilution and the CFUs counted after 20 hours incubation at 30 °C.

To confirm morphologically that the bacteria were indeed replicating/dormant, during the above-described methods, we examined the bacterial cells under a Nikon Ti-2 inverted microscope (Nikon, Japan). Five hundred microlitres of the final bacterial solution were centrifuged at 10,000 rpm for three minutes and 450 µL of the supernatant discarded. The pellet was resuspended in the remaining volume. The bacterial cells were stained with SYODE® 9 by adding 0.05 µL of the Live/Dead BacLight Bacterial Viability (Thermo Fisher Scientific) kit solution to each sample. Five microlitres of bacterial solution was placed on a small square piece of LB agar and observed under the green fluorescence channel with the 100x objective, using the Nis Element AR Software.

2.3. Bacterial injections

Four to five days after eclosion, female flies were injected with bacteria in groups of ten flies. For this, they were anesthetised with CO_2 and injected on the side of the thorax with a volume of 18.4 nL of bacterial solution (N = 150 flies) or *Drosophila* Ringer's solution (N = 30 flies). The group of ten flies was then placed in a vial containing 7 mL of SYA medium and flipped to new food medium every four days. The injection dose we aimed for was ~ 92000 CFUs per individual fly. Based on Acuña Hidalgo, Silva et al. (2021) we estimated that flies would carry a similar bacterial load at seven days post-infection (Acuña Hidalgo et al. 2021). We used a single aliquot of bacterial solution or Ringer's solution for each group of six flies injected. To verify the bacterial dose, we performed three serial dilution from 1:1 to $1:10^6$ and, before and after injections, plated eight droplets of 5 µL per dilution for the three highest dilutions.

2.4. Proteomics assay

2.4.1. Whole body sample preparation from the flies

Seven days after infection, flies were prepared for liquid chromatography-mass spectrometry (LC-MS) analyses as follows, based on Rodríguez-Rojas and Rolff (Rodríguez-Rojas and Rolff 2020). The flies were split into groups of 10 flies, with six replicates for the treatment infection with *P. burhodogranariea*, and three replicates each split in two, for a total of six replicates for the non-infected controls (injected with Ringer's solution). Each sample with infected flies was therefore estimated to contain around 1,000,000 bacterial cells. The samples were placed on ice

until use. For each replicate, 10 flies were placed on a previously chilled mortar, which was filled with liquid nitrogen. The flies were ground to a powder with a cold pestle. Then, 250 µl of urea denaturing buffer (6 M urea, 2 M thiourea,10 mM HEPES; pH 8.0) and 250 µL of 0.9 % sodium chloride were added into the mortar and mixed with the powder. The insect solution was transferred to microcentrifuge tubes and kept at -80 °C until further use. Before further sample processing, the samples were thawed on a heat block (Eppendorf ThermoMixer® C) at 37 °C, and centrifuged at room temperature? at 20,000 g for five minutes. Fifty microlitres of the supernatant was recovered and placed into new microcentrifuge tubes, whilst taking care in not disturb the pellet.

2.4.2. Bacterial sample preparation

The bacterial solution controls were thawed on a heat block at 37 °C. They were centrifuged at 5,000 g for 20 minutes, the supernatant was removed, and 50 µL of urea denaturing buffer were added. In order to break down the bacterial cells and facilitate the digestion, the samples were exposed to five freeze-thaw cycles alternating freezing at -80 °C and thawing at 37° C.

2.4.3. Bacteria and fly sample treatment and digestion

An ammonium bicarbonate buffer (NH_4HCO_3 50 mM, [ABC]) was freshly prepared to be used over the course of the experiment (40 mg ABC, 10 mL distilled water). The treatment and digestion of the samples was done as follows. After the addition of 2 µL of dithiothreitol 10 mM (DTT: 1.54 g dithiothreitol, 10 mL distilled water) to each sample, the samples were incubated for 30 minutes at room temperature. Thereafter, 2 µL of iodoacetamide 55 mM (10.2 mg iodoacetamide, 1 mL ABC buffer) were added and the samples were incubated in the dark at room temperature for 30 minutes. The samples were then diluted with 200 µL of ABC buffer. Finally, we added 2 µL of a freshly prepared trypsin 0.5 µg/µl solution (1 mg trypsin protease sequencing grade, 2 mL ABC buffer), and incubated the samples overnight at 37 °C. The following day, the digestion was stopped by acidifying the samples by adding 7.5 µL of a freshly prepared buffer A* (2.5 mL acetonitrile, 1.5 mL trifluoracetic acid, 46 mL distilled water).

2.4.4. Peptide purification and elution

All purification and elution steps were done at 25 °C. The StageTip purification tips were prepared according to (Rappsilber, Mann, and Ishihama 2007; Rodríguez-Rojas and Rolff 2020). A C18 reserve phase matrix disk (0.4 mm to 0.6 mm 3M™ Empore™ C18 Extraction Disks) was folded twice. Using a biopsy punch, the disk was punched once, introduced into a 200 µL filter-less tip, and tightly packed inside the tip close to its narrower end. The tip was placed through the previously perforated cap of a 2 mL microcentrifuge tube, which played the role of a collecting reservoir for the tip in all further steps. The tips were activated by adding 100 µL of liquid chromatography–mass spectrometry grade methanol and centrifuging for five minutes at 1,200 rpm and 25 °C. Then, they were equilibrated by adding 200 µL of freshly prepared buffer A (2.5 mL acetonitrile, 50 µL formic acid 99.8%, 47.5 mL distilled water) and centrifuged for 5 minutes at 1200 rpm, 25 °C.

The acidified samples were added in their entirety to the tips and centrifuged for 10 minutes at 5,000 g. The tips were then washed by adding 200 µL of buffer A and centrifuging at 5,000 g for 10 minutes. Prior to the LC-MS analyses, 100 µL of elution buffer B (300 µL TFA, 8 mL acetonitrile, 2 mL distilled water) were added to the samples, which were centrifuged for 10 minutes at 5,000 g.

2.4.5. Liquid chromatography and mass spectrometry data analysis

After vacuum centrifugation, the dried peptides were reconstituted in 0.1% trifluoroacetic acid, 4% acetonitrile in water, and approximately 0.5-2 µg of peptides were analysed by a reversed-phase nano liquid chromatography system (Ultimate 3000, Thermo Scientific) connected to a Q Exactive HF mass spectrometer (Thermo Scientific). The peptides were concentrated on a trap column (PepMap100 C18, 3 µm, 100 Å, 75 µm i.d. x 2 cm, Thermo Scientific). After switching the trap column inline, LC separations were performed on a capillary column (Acclaim PepMap100 C18, 2 µm, 100 Å, 75 µm i.d. x 25 cm, Thermo Scientific) at an eluent flow rate of 300 nl/min at 40°C. Mobile phase A contained 0.1 % formic acid in water, and mobile phase B contained 0.1% formic acid in 80 % acetonitrile, 20% water. Peptides were separated using a gradient of 5–44% B within 70 min and further increase to 95% B within 4

min, followed by a 7 min plateau before re-equilibration. Mass spectra were acquired in a data-dependent mode utilising a single MS survey scan (m/z 350–1650) with a resolution of 60,000 at m/z 200, and MS/MS scans of the 15 most intense precursor ions with a resolution of 15,000 at m/z 200 using an isolation window of 1.4 m/z. Higher-energy collisional dissociation MS/MS scans were performed with a normalized collision energy of 27. Only 2+ to 5+ charged precursors were selected for fragmentation. The dynamic exclusion time was set to 20 s. Automatic gain control (AGC) was set to $3x10^6$ for MS scans using a maximum injection time of 20 ms. For MS2 scans the AGC target was set to $1x10^5$ with a maximum injection time of 25 ms.

MS and MS/MS raw data were analysed with the MaxQuant software package (version 1.6.14) with the implemented Andromeda peptide search engine and label-free quantification (LFQ algorithm) (Tyanova, Temu, and Cox 2016). Data were searched against the reference proteome of *D. melanogaster* (22,045 proteins, taxonomy 7227, last modified June 2020) and the proteome of *P. burhodogranariea* DSM 19968 (3,888 proteins, taxonomy 1141662, last modified June 2020), both downloaded from the UniProt website. Default parameters were used for MaxQuant except the following: Label-free quantification was used with the match between runs option enabled. Filtering and statistical analysis was carried out using the software Perseus (version 1.6.14) (Tyanova et al. 2016). Protein hits from decoy database, potential contaminants and proteins that were identified exclusively by one site modification were excluded from the analysis. For further processing, proteins identified from *D. melanogaster* and *P. burhodogranariea* were analysed separately. Only protein hits with measured intensity values from at least three out of six replicates were used for downstream analysis. Missing values were replaced from a normal distribution (imputation) using the default settings (width 0.3, down shift 1.8). Student's *t*-tests were performed using permutation-based false discovery rate (FDR) of 0.05 for the creation of volcano plots.

3. Results

3.1. Low abundance of *P. burhodogranariea* proteins in vivo

We aimed to quantify the protein expression of *P. burhodogranariea* inside the fly at seven days post-infection and compare it to in vitro replicating (PB-R) and dormant (PB-D) bacteria.

However, the bacterial proteins were not abundant enough for reliable detection against the background of highly abundant fly proteins. Forty-four of the bacterial proteins that we found were reproducibly identified as belonging to *P. burhodogranariea*, but we could not differentiate most of the proteins from background signal of other bacterial species. Therefore, we were unable to compare the expression of bacteria in the fly to that of the replicating and dormant *in vitro* bacterial controls.

3.2. Confirmation of dormant and replicating bacterial cells via microscopy

After growing for two hours in LB medium (PB-R), replicating bacterial cells were observed to be larger in size (approximately 5 µm) and most cells were in the process of dividing, as indicated by the presence of septa(?) (Figure 1a). After 24 hours in sodium chloride 0.9 % (PB-D), dormant bacterial cells appeared to be smaller in size (ranging from 1 to 2 µm), with almost all cells in non-dividing state (Figure 1b).

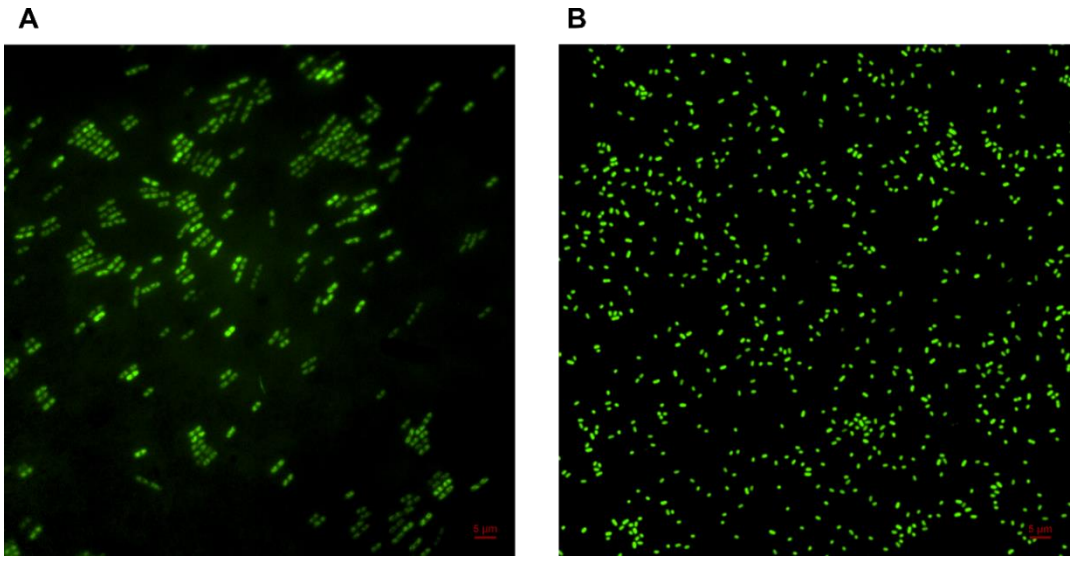

Figure 1. Viable bacterial cells stained with SYTO® 9 under green-fluorescent channel after A. two hours in a nutrient-rich medium (lysogeny broth), i.e., replicating cells (PB-R) or B. 24 hours in a nutrient-depleted environment (NaCl 0.9 %), i.e., dormant cells (PB-D).

3.3. Differential protein abundance in dormant vs. replicating bacterial cells *in vitro*

The protein expression changes of *P. burhodogranariea* were measured in its *in vitro* replicating and dormant phases, i.e., after two hours in nutrient-rich medium and 24 hours in a nutrient-depleted medium, respectively. We identified 2112 proteins at a 0.05 % FDR, and quantified 1818 proteins in at least three out of six replicates. Compared to dormant bacteria, replicating *P. burhodogranariea* showed a notably different expression in its proteins: overall, 1117 proteins were found to be significantly differentially expressed, i.e., 61.4 % of all quantified proteins, with 30.9 % (563 proteins) significantly upregulated, and 30.5 % (554 proteins) significantly downregulated (Figure 2).

3.2. Changes in protein expression in infected flies

3.2.1. A wide host protein expression perturbation during infection

We quantified the protein expression changes of *D. melanogaster* at seven days post-infection with the bacterium *P. burhodogranariea* and compared it to non-infected flies injected with Ringer's solution. Overall, 2463 proteins were identified at a 0.05 % false discovery rate, out of which 1773 proteins were quantified in at least three out of six replicates. Seven days post-infection with *P. burhodogranariea,* flies showed a wide perturbation of their protein expression compared to non-infected flies. In total, 453 proteins were differentially expressed compared to controls, accounting for 25.5 % of all quantified proteins, with 11.6 % being significantly upregulated (205 proteins) and 13.9 % significantly downregulated (248 proteins) (Figure 3, Table S2).

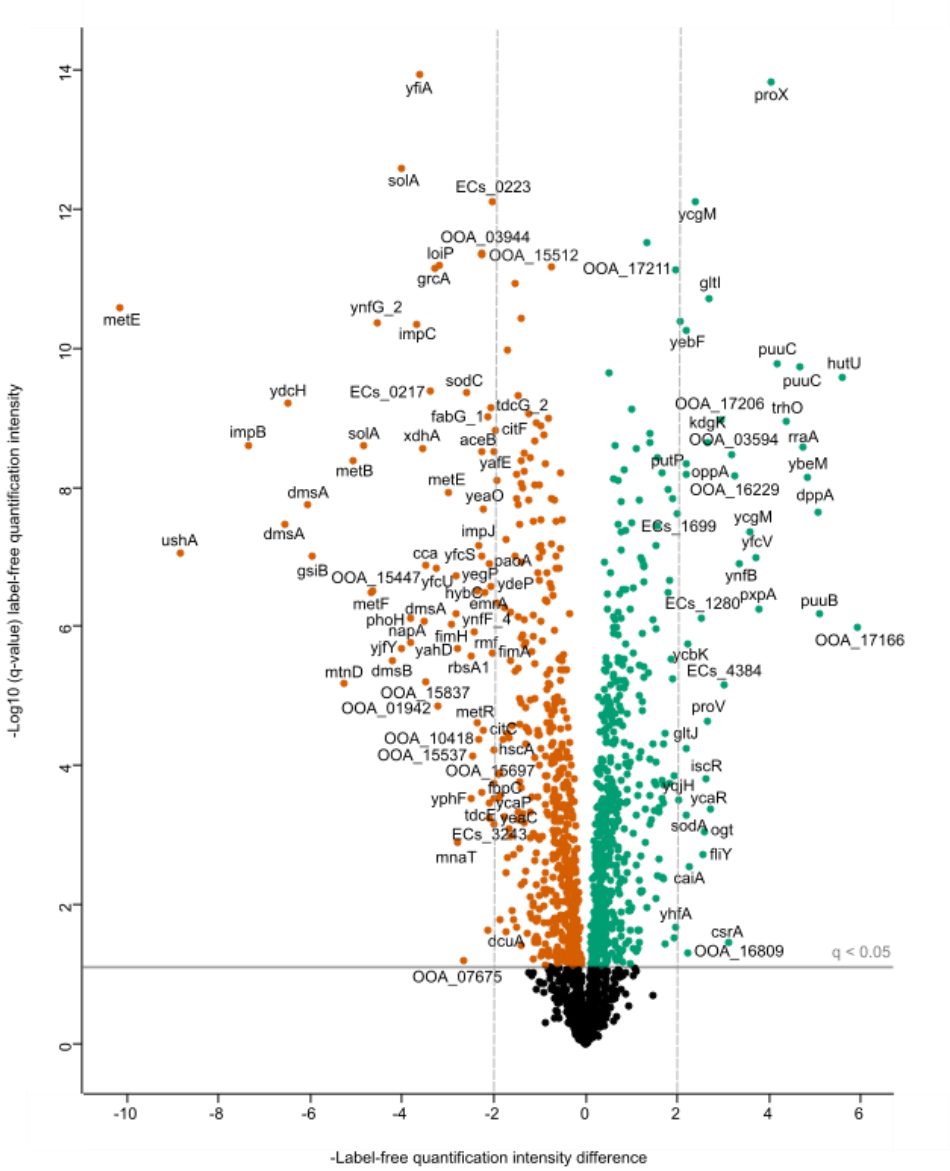

Figure 2. Volcano plot of –log q values against log2 fold change of protein intensity measured by LC-MS of *P. burhodogranariea* replicating in vitro control (PB-R), compared to dormant in vitro bacteria (PB-D). Black dots represent not significantly expressed proteins, green and red dots show significantly upregulated and downregulated proteins, respectively (Student t-test q-value < 0.05). For improved visualization of this plot, only proteins with a four-fold change, or above, in protein expression are labelled with the gene names. Gene names shown are those of *E. coli* K12 genes which we found to be homolog in *P. burhodogranariea*; when a homolog was not found, we kept the original gene name (see Table S1 for details).

3.2.2. Proteins differentially expressed in response to infection

For the proteomics of the host, we focused on the differentially expressed proteins of infected flies that were involved in the host response to infection. We categorised them into three functional classes based in their role in the immune response: (i) Receptors, i.e., molecules involved in recognition, (ii) Signalling, i.e., molecules that take part in the signalling transduction cascades, and (iii) Effectors, i.e., directly acting to fight the infection response (Sackton et al. 2007; Seto and Tamura 2013; Wertheim 2015) (Figure 4). Proteins for which the function in this response is unclear were categorised as "Undetermined".

In total, among the proteins significantly differentially expressed, we found 14 differentially expressed proteins involved in the fly response to infection, from which 12 were upregulated and two were downregulated (Table 1, Figure 5). Infected flies had one significantly upregulated receptor protein involved in the detection of bacterial cells, namely the Gram-negative bacteria-binding protein-like protein 3 (GNBP-like3). We found only one protein involved in the signal transduction cascade: the phagocyte signalling-impaired protein (psidin), a signalling molecule that triggers the humoral and phagocytosis responses. This protein was downregulated in infected hosts when compared to uninfected. Six of the most upregulated proteins were effector molecules, AMPs. They consisted of Attacin A and Attacin C, Diptericin A and Diptericin B, Cecropin C and Metchkinowin. We found that Transferrin 1 (Tsf1) was upregulated upon infection (Figure 5). Transferrin is a major transporter for iron and its concentration is highly modified under stress conditions or infection (Yoshiga et al. 1997; Yoshiga et al. 1999; De Gregorio et al. 2001; Levy, Bulet, and Ehret-Sabatier 2004; Troha et al. 2018; Iatsenko et al. 2020). Even though its activity is not involved in directly killing the pathogen, we classified it as an effector of the host response as it plays an essential role in iron sequestration during infection. Another upregulated effector protein we found was the peptidoglycan recognition protein SB1 (PGRP-SB1), which plays the role of degrading peptidoglycans during infection (Mellroth, Karlsson, and Steiner 2003; Zaidman-Rémy et al. 2011). Additionally, one effector protein was downregulated, Phenoloxidase 2 (PPO2), this is an enzyme involved in melanin synthethesis (Table 1, Figure5). Finally, we observed the upregulation of three proteins that are infection responsive, but for which the precise function in the immune response is undetermined: immune-induced molecule 18 (IM18), Elevated during infection (Edin), and Turandot M (TotM). Edin and IM18 are two peptides thought to

be involved in the humoral response to bacteria. Although Edin has been shown to have a signalling role in the encapsulation process in *D. melanogaster* larvae, its role during bacterial infections in fly adults remains unclear (Vanha-aho et al. 2012; 2015). The third upregulated protein for which the function is undetermined is Turandot M (TotM). This protein is involved in stress responses to various triggers, including bacterial infections (Ekengren and Hultmark 2001).

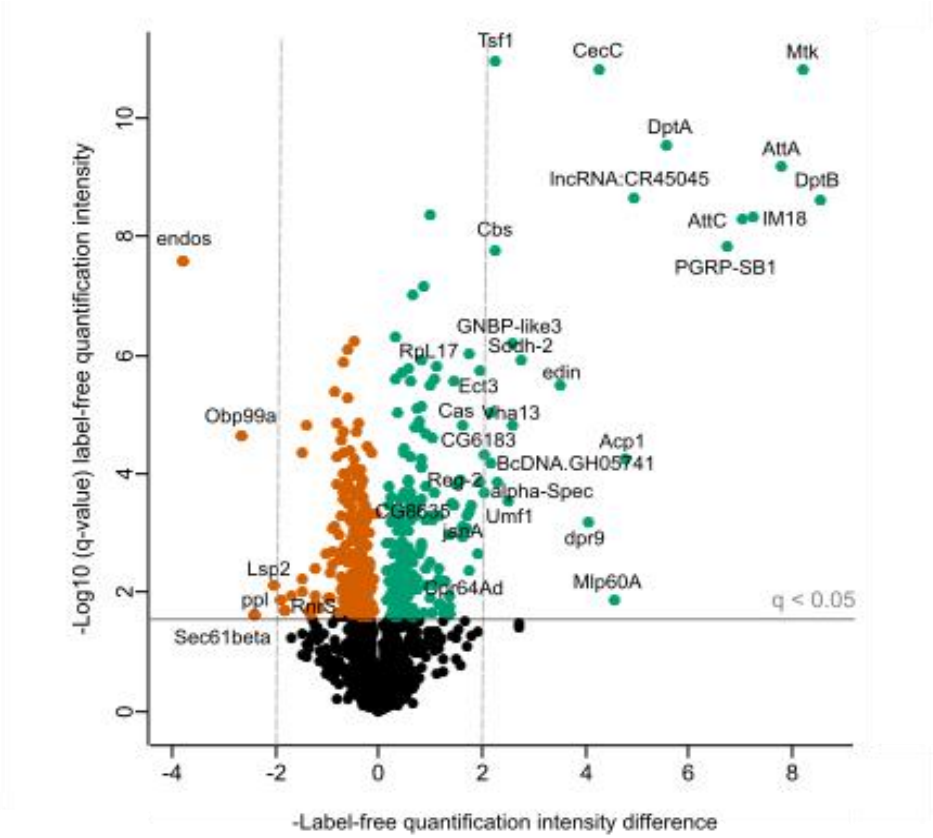

Figure 3. Volcano plot of –log q values against the log2 fold-change of protein intensity measured by LC-MS of bacteria-infected *D. melanogaster*, compared to control flies injected with Ringer's solution. Black dots represent non-significantly expressed proteins, green and red dots show significantly upregulated and downregulated proteins, respectively (Student's t-test q-value < 0.05). For improved visualisation of this plot, only proteins with a four-fold change, or above, are labelled with the gene names (see Table S2 for details).

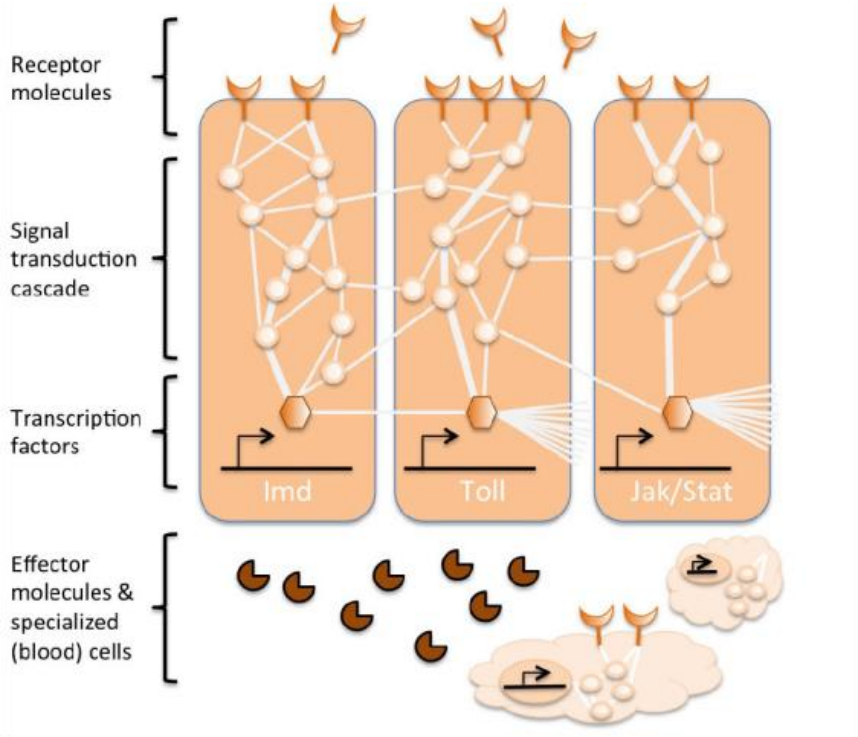

Figure 4. Schematic representation of the networks that coordinate the *D. melanogaster* immune response, modified from Wertheim (2015). Cell-surface and circulating proteins act as receptor molecules that will detect the presence of pathogens by binding to pathogen-associated molecular patterns. Their interaction with other proteins involved in the signal transduction cascade will regulate the expression of transcription factors (hexagonal symbols). The activation of three core signal transduction pathways, i.e., Imd, Toll and Jak/Stat triggers the production of effector proteins (e.g., antimicrobial peptides, pie-shaped symbols), as well as the differentiation and proliferation of specialized blood cells (cloud-shaped symbols).

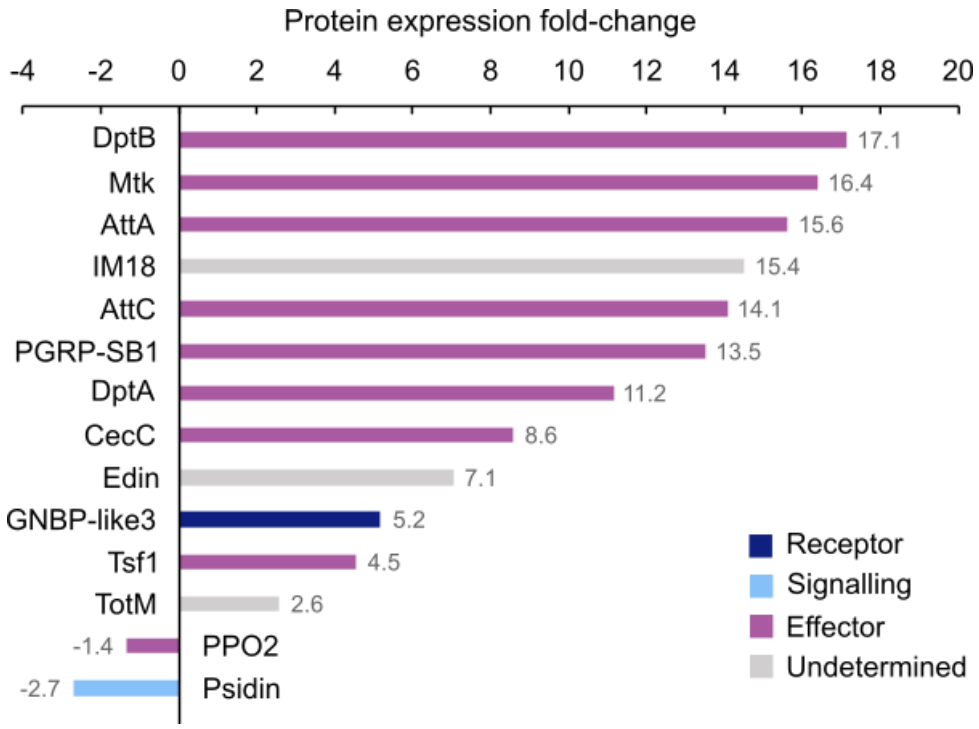

Figure 5. Fold-change of proteins significantly upregulated involved in the response to infection for infected flies compared to uninfected flies (injected with Ringer's solution). Protein names and function are detailed in Table 1. Fold-change values for each protein are indicated in grey. Colours represent the function of the protein in the host response to infection as specified in the legend (based on Wertheim (2015), see Figure 4).

Table 1. *D. melanogaster* immunity-related proteins that were differentially expressed in infected flies compared to controls, and their role in the host response to infection. The column "Function" represents the molecular function of the protein in the immune response (see Figure 2).

UniProt ID	Abbreviated name	Full name	Function	Bacterial process	References
P45884	AttA	Attacin-A	Effector	Antimicrobial response to Gram-negative bacteria	1-5
Q95NH6	AttC	Attacin-C	Effector	Antimicrobial response to Gram-negative bacteria	2-3, 6-7
O16829	CecC	Cecropin C	Effector	Antimicrobial response to Gram-negative bacteria	2-3, 8

P24492	DptA	Diptericin A	Effector	Antimicrobial response to Gram-negative bacteria	1, 7, 9-11
A1ZBF6	DptB	Diptericin B	Effector	Antimicrobial response to Gram-negative bacteria	2, 3, 5
Q8IQR7	edin	Elevated during infection	Undetermined	Signalling role in encapsulation in larvae, undetermined role in adult defences against bacteria	3, 12-13
A1ZBU5	GNBP-like3	GNBP-like3	Receptor	Bacterial cell-membrane lipopolysaccharide binding	7, 14
P82701	IM18	Immune-induced peptide 18	Undetermined	Undetermined role in humoral response to bacteria and nematodes	6-7, 14
Q24395	Mtk	Metchnikowin	Effector	Antimicrobial response to fungi, Gram-negative and Gram-positive bacteria	1, 5-7, 15-16
Q70PY2	PGRP-SB1	Peptidoglycan-recognition protein SB1	Effector	Bacterial cell-wall peptidoglycan catabolic process	17-19
Q9VDQ7	psidin	Phagocyte signalling-impaired protein	Signalling	Activation of humoral response and phagocytosis	20-21
Q9V521	PPO2	Phenoloxidase 2	Effector	Melanin biosynthesis in melanisation response	22-24
Q9VMR8	TotM	Turandot M	Undetermined	Undetermined role in tolerance to heat stress and humoral response to bacteria	6, 25
Q9VWV6	Tsf1	Transferrin-1	Effector	Iron binding and sequestration during infection	7, 26

[1] Lemaitre, Reichhart, and Hoffmann (1997)
[2] Verleyen et al. (2006)
[3] Gordon et al. (2008)
[4] Wang et al. (2010)
[5] Tattikota et al. (2020)
[6] Uttenweiler-Joseph et al. (1998)
[7] Levy, Bulet, and Ehret-Sabatier (2004)
[8] Tryselius et al. (1992)
[9] Wicker et al. (1990)
[10] Berkey, Blow, and Watnick (2009)
[11] Cronin et al. (2009)
[12] Vanha-aho et al. (2012)
[13] Vanha-aho et al. (2015)
[14] Arefin et al. (2014)
[15] Levashina et al. (1995)
[16] Reed et al. (2008)
[17] Werner et al. (2000)
[18] Mellroth, Karlsson, and Steiner (2003)
[19] Zaidman-Rémy et al. (2011)
[20] Brennan et al. (2007)
[21] Stephan et al.(2012)
[22] Binggeli et al. (2014)
[23] Dudzic et al. (2015)
[24] Schmid et al. (2019)
[25] Ekengren and Hultmark (2001)
[26] Iatsenko et al. (2020)

4. Discussion

In the present study, we aimed to evaluate the perspectives of both host and pathogen in the context of a persistent *P. burhodogranariea* infection in *D. melanogaster*. While we were unable to measure the protein expression changes of the bacteria inside the fly, we are able to offer an insight into the proteomic response of flies during a chronic infection. Infected flies show an active and complex response against the pathogen, whereby they combine various mechanisms to fight the infection.

4.1. Low abundance of bacterial proteins inside infected flies

We detected a small number of *P. burhodogranariea* proteins in the infected fly samples, however we were unable to distinguish them from background noise. Due to the high ratio of fly to bacterial protein in the infected *D. melanogaster* samples, it was unfortunately not possible to obtain a clear overview of the protein expression of this bacterium inside the host, and consequently to determine whether the bacteria were in a protected dormant state inside the flies. To overcome this issue in the future, it would be necessary to increase the concentration of bacteria in the samples. This could be achieved by increasing the inoculation dose: the bacterial load of *P. burhodogranariea* at seven days post-infection has been shown to be

correlated to the infectious exposure dose, although higher initial doses are also correlated to increased host mortality (Acuña Hidalgo et al. 2021). Alternatively, the number of flies per proteomics sample could be increased. To increase the concentration of bacterial cells in each sample by at least one order of magnitude, both options would require significantly increasing the sample size of injected flies, which would be highly challenging to achieve from a logistical point of view.

4.2. Differential protein expression between dormant and replicating in vitro baceria

We were able to measure the protein expression of *P. burhodogranariea* in the *in vitro* controls (PB-D and PB-R) (see Table S1, Figure 2), where a high proportion of the proteins had differential expression between the two states (61.4 % of all identified proteins). Further analysis of this data will be used to identify key proteins differentially expressed between replicating and dormant bacteria. By measuring the expression of these selected proteins in infecting bacteria via RT-qPCR for example, it would be possible in future studies to determine whether the persistent *P. burhodogranariea* bacterial cells are in a dormant or replicating state.

4.3. An active response against infection in chronically infected flies

In this study, we aimed to determine the response of *D. melanogaster* to a persistent *P. burhodogranariea* infection by measuring the host protein expression changes at seven days post-infection. We detected a systemic response, with many proteins (25.5 %) differentially expressed in infected flies compared to control flies injected with Ringer's solution. Further gene ontology analyses will be necessary to obtain a more general view of the fly response. In our results, we focused on 14 proteins, 12 upregulated and two downregulated, involved in the host response to infection, which we categorized by their function in this response, i.e., receptors, signalling molecules or effectors (Figure 2) (Sackton et al. 2007; Seto and Tamura 2013; Wertheim 2015). Most of the upregulated proteins were antimicrobial peptides, but we observed other peptides and proteins expressed by the host, consistent with other transcriptome and proteome-level studies (De Gregorio et al. 2001; Irving et al. 2001; Boutros, Agaisse, and

Perrimon 2002; Vierstraete et al. 2004; Levy, Bulet, and Ehret-Sabatier 2004; Ramond, Dudzic, and Lemaitre 2020).

4.3.1. The pathogen may still be actively recognised by the immune system

We identified one receptor protein that was upregulated in infected flies: GNBP-like3 (Table 1), among the most strongly upregulated proteins (Figure 5). GNBP-like3, detects cell-membrane lipopolysaccharides of Gram-negative bacteria (Lee et al. 1996), and has been shown to play a role in the immune response against nematobacterial infections (Arefin et al. 2014). Another protein we found to be upregulated was the effector protein PGRP-SB1, which binds to and degrades bacterial peptidoglycans (Mellroth, Karlsson, and Steiner 2003; Zaidman-Rémy et al. 2011). It has been found to be the most strongly induced PGRP upon infection with various bacterial species, as well as persisting in the host for at least two days (Zaidman-Rémy et al. 2011). The upregulation of these two upon infection has also been demonstrated in *D. melanogaster* infections including bacteria (Levy, Bulet, and Ehret-Sabatier 2004; Seto and Tamura 2013; Arefin et al. 2014; Ramond, Dudzic, and Lemaitre 2020), and indicates that the host immune system may still be detecting the presence of *P. burhodogranariea* at our sampling timepoint. If this is the case, then it is possible that at least a proportion of *P. burhodogranariea* might not be evading recognition by the immune system.

4.3.2. Strong upregulation of antimicrobial peptides in infected flies

We detected a strong antimicrobial response in infected flies at seven days post-infection, with six known AMPs amongst the most highly upregulated proteins (Figure 5). Four of these AMPs are associated with antimicrobial responses against Gram-negative bacteria: Attacin-A and -C and Diptericin A and B, while Cecropin C has been reported to act against bacteria and fungi (Tryselius et al. 1992; Lemaitre, Reichhart, and Hoffmann 1997; Levy, Bulet, and Ehret-Sabatier 2004; Verleyen et al. 2006; Cronin et al. 2009; Wang et al. 2010). Metchkinowin is usually attributed to anti-fungal responses (Brennan and Anderson 2004; Seto and Tamura 2013), but it is also upregulated during bacterial infections (Levashina et al. 1995; Levy, Bulet, and Ehret-Sabatier 2004; Reed et al. 2008; Wagner, Isermann, and Roeder 2009; Kutzer, Kurtz,

and Armitage 2019). The simultaneous presence of a cocktail of AMPs upon infection is characteristic of the immune response of *D. melanogaster* against pathogens (Seto and Tamura 2013; Ramond, Dudzic, and Lemaitre 2020). AMPs act in a specific way against different pathogen species. Through knockdown of full AMPs families via CRIPSR technology, Hanson et al. determined that *D. melanogaster* expresses a specific antimicrobial response against *P. burhodogranariea*, whereby Drocosin, the Attacins and the Diptericins act in synergy to resist an infection by this bacterium in the first hours post-infection (Hanson et al. 2019). Consistent with this study, we found two dipericins and two attacins to be upregulated during infection by this bacterial species; however, we did not detect Drosocin. This could be due to the genetic background of our fly population being different, as it was established from wild-caught flies (Martins et al. 2013). Host genotype affects the regulation of immune response effectors: for example, polymorphism of genes involved in the immune system signalling cascade can lead to differential expression of immune effectors (Sackton, Lazzaro, a nd Clark 2010). Alternatively, Drosocin might only be upregulated early in the infection, instead of in the chronic phase.

In the first hours after infection, AMP responses allow hosts to control the growth of the pathogen and avoid succumbing to an infection (Duneau et al. 2017). Hosts that control the infection survive the acute phase with a persistent infection, that can last up to weeks and is often never cleared (Acuña Hidalgo et al. 2021). A persistent upregulation of various AMPs has been detected in *D. melanogaster* during bacterial infections (Chambers et al. 2019). Consistent with this, our results suggest the persistence of an antimicrobial peptide response in flies infected with *P. burhodogranariea* at seven days post-infection. The role of this upregulated response during chronic infections remains unknown, and because we could not obtain the proteome of the bacteria inside the fly (see Results section), we were unable to provide any insight on this. Alternatively, to a persistent production of AMPs during the chronic infection, it might be possible that these peptides are produced in the beginning of the infection without being cleared out of the host system. The intensity of the immune responses is dependent on the inoculation dose (Leulier et al. 2003; Jent et al. 2019). The inoculation dose we used here is considerably high (approximately 92000 CFUs) and might induce a very strongly upregulated antimicrobial response. While AMPs are very stable molecules due to the presence of intramolecular di-sulfide bridges, it is unclear for how long they can remain in the

fly system. Our experimental setup allowed us to detect the presence of these proteins inside the host, but it does not assure that AMPs are being expressed at that timepoint.

4.3.3. Upregulation of transferrin as an iron sequestration strategy

We found Transferrin 1 to be upregulated in infected flies (Figure 5). Transferrin is a molecule capable of binding to iron, causing its sequestration and restricting microbial growth during infection (Hood and Skaar 2012; Cassat and Skaar 2013). The upregulation of this protein is triggered in *D. melanogaster* upon infection by various types of pathogens (Toyoshi Yoshiga et al. 1999; De Gregorio et al. 2001; Levy, Bulet, and Ehret-Sabatier 2004; Troha et al. 2018), as well as during chronic infections with bacteria (e.g., 132 hours, Troha et al. 2018).

Iron sequestration is an evolutionarily advantageous strategy against pathogens (Barber and Elde 2014). It seems that the success of this defence strategy may be linked to the dependency of pathogen virulence on iron availability (Iatsenko et al. 2020). Iron sequestration through Transferrin 1 determines host mortality for pathogens which greatly rely on iron for their growth, (Iatsenko et al. 2020). Along with other species from the *Providencia* genus, *P. burhodogranariea* possesses genes encoding a direct heme uptake system (hmuRSTUV), which allows *Proteus* sp. bacterial cells to use hemin and hemoproteins as an iron source (Yuan et al. 2020). This system has been shown to regulate virulence in a bacterial species responsible for urinary tract infections, *Proteus mirabilis* (Lima et al. 2007; Schwiesow et al. 2018). It is possible then that the host might be combining an active resistance response through antimicrobial peptides and iron depletion to fight the infection. In our experiment, the Tsf1 protein is four times more abundant in infected flies, compared to non-infected controls, meaning that iron is depleted eight times more during infection at the sampled timepoint because each transferrin protein binds to two iron molecules. This iron depletion could partially explain the observed absence of further increase in the bacterial load during the chronic phase of infection, as the bacteria lacks this essential mineral.

4.3.4. Downregulation of some branches of the immune response

Interestingly, we found the downregulation of two immunity-related proteins (Figure 5): PPO2 and psidin. Phenoloxidase 2 (PPO2) is a molecule responsible for melanisation of pathogens as part of the humoral immune response (Binggeli et al. 2014; Dudzic et al. 2015; 2019; Schmid et al. 2019). Psidin is a signalling molecule that activates the phagocytosis response, although it has been shown to play a role in the humoral response (Brennan et al. 2007). In the context of an infection, it might seem counterintuitive at first to see the downregulation of two essential branches of the immune response, but these responses might actually be costly for the host to maintain during a persistent infection. Firstly, immune responses come with energetic costs (Schmid-Hempel 2003; Bajgar and Dolezal 2018). For instance, the production and maintenance of the phenoloxidase cascade is energetically costly and dependent on resource intake (Siva-Jothy and Thompson 2002; Rantala et al. 2003; González-Tokman et al. 2011; González-Santoyo and Córdoba-Aguilar 2012). Additionally, an immune response can cause damage to the host (i.e. immunopathology) (Graham, Allen, and Read 2005; Medzhitov, Schneider, and Soares 2012). The phenoloxidase cascade produces reactive oxygen species, which are toxic to the host tissues (Khan, Agashe, and Rolff 2017). The downregulation we observe in *D. melanogaster* during the chronic infection might be in fact a tolerance response, whereby the host reduces the costs of the infection without affecting the pathogen burden (Råberg, Sim, and Read 2007; Råberg, Graham, and Read 2009; Frank and Schmid-Hempel 2019). Chronically infected hosts might then employ a joint resistance and tolerance strategy, by investing in a strong antimicrobial response against the pathogen while downregulating other branches of the immune system to avoid infection-related costs.

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Supporting information

Table S1. *P. burhodogranariea* proteins that were at least four-fold differentially expressed between the replicating and the dormant *in vitro* controls. Because the proteome of *P. burhodogranariea* has not been thoroughly characterised, the names that have been given to the identified genes of *P. burhodogranariea* are quite cryptic and make it hard to associate many proteins with specific functions. Moreover, for this same reason, this species is not available in the most commonly used gene ontology tools. To avoid the tedious work of searching the function of each protein one by one, we ran analyses to find their homologs in a closely related and well-characterised species, *Escherichia. coli*. The complete proteomes of *E. coli* K12 (4437 proteins, taxonomy 83333, last modified October 2020) and *P. burhodogranariea* (see Materials and Methods) were downloaded from the UniProt database. For each *P. burhodogranariea* protein, the homolog in the proteome of *E. coli* was identified as the best BLAST hit using the program blastp (Altschul et al. 1990). We found 91 out of 106 homolog proteins in *E. coli* with an expectation value below 0.05.

***P. burhodogranariea* protein name**	**Gene name**	**UniProt ID**	**Protein expression fold-change**	**Homolog in *E. coli***	**Gene name**	**UniProt ID**	**Label in Fig. S1**	**Alignment score**	**e-value**
4-hydroxyphenylacetate 3-monooxygenase, oxygenase component	OOA_17166	K8W1R9	11.82	Fimbrial protein	yadL	Q8X919	yadL	27.7	1.9
Urocanate hydratase	hutU	K8WGA4	11.21	N-acetyl-alpha-D-glucosaminyl-diphospho-ditrans,octacis-undecaprenol 4-epimerase	gnu	Q8X7P7	hutU	28.5	1.6
Urocanate hydratase	hutU	K8WGA4	11.21	Peptide chain release factor 3	prfC	P0A7I6	hutU	30.4	0.47
Oxidoreductase	OOA_16224	K8W4D1	10.18	Gamma-glutamylputrescine oxidoreductase	puuB	Q8X7G7	puuB	252	2.00E-80
Dipeptide ABC transporter periplasmic substrate-binding protein DppA	OOA_14665	K8WI07	10.13	Dipeptide/heme ABC transporter periplasmic binding protein	dppA	A0A0H3JJ33	dppA	934	0
CN hydrolase domain-containing protein	OOA_09768	K8WWZ3	9.66	Deaminated glutathione amidase	ybeM	P58054	ybeM	162	5.00E-50
Dimethylmenaquinone methyltransferase	OOA_09773	K8WMR7	9.50	Regulator of ribonuclease activity A	rraA	P0A8R2	rraA	49.7	1.00E-08
Phenylacetaldehyde dehydrogenase	OOA_16234	K8W681	9.36	Aldehyde dehydrogenase	puuC	Q8X7G6	puuC	351	4.00E-117
tRNA uridine(34) hydroxylase	trhO	K8WY29	8.72	tRNA uridine(34) hydroxylase	trhO	Q8X8P2	trhO	543	0
5-carboxymethyl-2-hydroxymuconate semialdehyde dehydrogenase	OOA_17216	K8W1S7	8.32	Aldehyde dehydrogenase	puuC	Q8X7G6	puuC	347	7.00E-116
Glycine betaine transporter periplasmic subunit	proX	K8WZ44	8.07	Glycine betaine/proline betaine-binding periplasmic protein	proX	P0AFM3	proX	489	1.00E-176
5-oxoprolinase subunit A	pxpA	K8WVH0	7.54	5-oxoprolinase subunit A	pxpA	Q8X9C8	pxpA	203	3.00E-66

Fimbrial protein	OOA_00980	K8X9G1	7.42	Uncharacterized fimbrial-like protein YfcV	yfcV	Q8X563	yfcV	52.4	2.00E-09
HpaG2 protein	OOA_17221	K8WDX5	7.13	Isomerase/hydrolase	ycgM	Q8XDM0	ycgM	133	2.00E-39
UPF0482 protein OOA_05326	OOA_05326	K8X481	6.71	UPF0482 protein YnfB	ynfB	Q8X7A0	ynfB	103	5.00E-31
Cupin_3 domain-containing protein	OOA_16229	K8WGL0	6.50	Ethanolamine utilization protein EutQ	eutQ	Q8XBG1	eutQ	26.2	0.82
Putative hydro-lyase OOA_03594	OOA_03594	K8X2N3	6.34	L-arabinose-inducible transporte	araJ	Q8X5A5	araJ	28.1	0.76
Translational regulator CsrA	csrA	K8WA20	6.22	Translational regulator CsrA	csrA	P69915	csrA	112	4.00E-36
Uncharacterized protein	OOA_18619	K8VYY1	6.02	Heme utilization carrier protein	ECs_4384	Q8X5N5	ECs_4384	272	3.00E-96
5-carboxymethyl-2-hydroxymuconate delta-isomerase	OOA_17206	K8WAC0	5.93	Ribonucleoside-diphosphate reductase 1 subunit beta	nrdB	P69925	nrdB	28.1	0.16
UPF0434 protein OOA_09336	OOA_09336	K8WNE2	5.46	UPF0434 protein YcaR	ycaR	P0AAZ9	ycaR	93.2	2.00E-28
Glutamate and aspartate transporter subunit	OOA_10591	K8WLF9	5.38	Glutamate/aspartate periplasmic binding protein	gltI	Q8XBL6	gltI	486	2.00E-176
2-dehydro-3-deoxygluconokinase	OOA_08112	K8WXR7	5.33	2-dehydro-3-deoxygluconokinase	kdgK	Q8X5M4	kdgK	338	1.00E-117
Glycine betaine/L-proline ABC transporter ATP-binding protein	OOA_10716	K8WLI0	5.32	Glycine betaine/proline ABC transporter periplasmic binding protein	proV	Q8X914	proV	612	0
Iron-sulfur cluster transcriptional regulator	OOA_02652	K8WXK4	5.26	Gene HTH-type transcriptional regulator IscR	iscR	P0AGL0	iscR	209	2.00E-71
Methylated-DNA--protein-cysteine methyltransferase	OOA_10691	K8WLH6	5.15	Methylated-DNA--protein-cysteine methyltransferase	ogt	Q8X8N5	ogt	97.8	2.00E-27
Family 3 extracellular solute-binding protein	OOA_01035	K8WYU3	5.08	Cystine transporter subunit	fliY	Q8XBC5	fliY	123	1.00E-34
Fimbrial protein	OOA_00200	K8WY20	5.04	Major pilin protein	ECs_1280	Q8XAP4	ECs_1280	60.5	1.00E-12
HpaG1 protein	OOA_17226	K8W3L0	4.80	Isomerase/hydrolase	ycgM	Q8XDM0	ycgM	72.4	2.00E-16
Butyryl-CoA dehydrogenase	OOA_12962	K8WI04	4.51	Crotonobetainyl-CoA reductase	caiA	P60586	caiA	127	3.00E-34
Uncharacterized protein	OOA_16809	K8W6J6	4.44	Siroheme synthase	cysG	P0AEA9	cysG	28.1	0.58
Uncharacterized protein	OOA_09291	K8X0T1	4.43	Uncharacterized protein YcbK	ycbK	P0AB08	ycbK	268	7.00E-94
Uncharacterized protein	OOA_04692	K8WST4	4.41	Protein YebF	yebF	Q8XCK0	yebF	86.7	4.00E-24
Sodium/proline symporter	OOA_08482	K8WQM9	4.40	Sodium/proline symporter	putP	Q8XAT3	putP	749	0
Oligopeptide ABC transporter periplasmic binding protein	OOA_04967	K8X1Z4	4.39	Oligopeptide ABC transporter periplasmic binding protein	oppA	A0A0H3JE39	oppA	787	0
Glutamate/aspartate ABC transporter permease GltJ	OOA_10596	K8WLM1	4.39	Glutamate/aspartate ABC transporter permease	gltJ	Q8XBL7	gltJ	436	3.00E-158
Superoxide dismutase	OOA_16082	K8WF89	4.36	Superoxide dismutase	sodA	P66828	sodA	357	4.00E-128
3,4-dihydroxyphenylacetate 2,3-dioxygenase	OOA_17211	K8W1I7	4.11	Integrase	ECs_4534	Q7DB95	ECs_4534	28.1	0.79
Uncharacterized protein	OOA_06898	K8X211	4.08	Siderophore interacting protein	yqjH	Q8XAN3	yqjH	67	6.00E-14

Iron compound ABC transporter substrate-binding protein	OOA_18914	K8VXU1	4.00	ABC transporter ATP-binding protein	ECs_1699	Q8XDH9	ECs_1699	283	7.00E-95
Uncharacterized protein	OOA_14425	K8WM91	3.93	Protein YhfA	yhfA	P0ADX3	yhfA	233	1.00E-81
Citrate lyase alpha chain	OOA_08012	K8WNN2	-3.92	Citrate lyase alpha chain	citF	A0A0H3JGB8	citF	804	0
Chaperone protein HscA	hscA	K8WXL0	-3.97	Chaperone protein HscA	hscA	P0A6Z2	hscA	926	0
dTDP-4-dehydrorhamnose 3,5-epimerase	OOA_15697	K8WIL4	-3.98	Hca operon transcriptional regulator	hcaR	Q8XA74	hcaR	27.3	0.72
Sucrose-6-phosphate hydrolase	OOA_00360	K8WWI3	-4.00	Sucrose-6-phosphate hydrolase	ECs_3243	A0A0H3JGP6	ECs_3243	553	0
Uncharacterized protein	OOA_17256	K8WAC7	-4.00	Uncharacterized protein	ycaP	Q8XEA8	ycaP	43.5	4.00E-06
Type 11 methyltransferase	OOA_15677	K8W879	-4.02	Uncharacterized protein	yafE	Q8X7Z4	yafE	45.1	3.00E-06
Type VI secretion ATPase, ClpV1 family protein	OOA_15330	K8WM62	-4.04	ATP-dependent Clp proteinase	ECs_0223	Q8X7V7	ECs_0223	635	0
Major mannose-resistant fimbrial protein	OOA_12033	K8WJI5	-4.07	Major fimbrium subunit FimA type-1	fimA	A0A0H3JJS9	fimA	69.7	4.00E-16
Putative oxidoreductase	OOA_07490	K8WYG6	-4.13	Protein YdeP	ydeP	Q8XAX1	ydeP	1107	0
L-serine dehydratase	OOA_09698	K8WMQ4	-4.14	L-serine dehydratase	tdcG_2	Q8X6S0	tdcG_2	767	0
Uncharacterized protein	OOA_04832	K8WUP6	-4.19	Uncharacterized protein	yeaC	Q8XDU7	yeaC	117	4.00E-37
2Fe-2S iron-sulfur cluster binding domain-containing protein	OOA_15517	K8W664	-4.19	Aldehyde oxidoreductase iron-sulfur-binding subunit PaoA	paoA	Q8X6I9	paoA	90.5	2.00E-23
Formate C-acetyltransferase	OOA_09468	K8WWQ7	-4.21	Formate C-acetyltransferase	tdcE	Q8XEB4	tdcE	1434	0
Anaerobic C4-dicarboxylate transporter	OOA_12650	K8WVN5	-4.25	Anaerobic C4-dicarboxylate transporter DcuA	dcuA	P0ABN7	dcuA	696	0
Short-chain dehydrogenase/reductase SDR	OOA_11903	K8WTG6	-4.28	3-oxoacyl-[acyl-carrier-protein] reductase	fabG_1	Q8X8I5	fabG_1	134	2.00E-39
Multidrug efflux system protein EmrA	OOA_10741	K8WLI5	-4.38	Multidrug efflux system protein	emrA	Q8X905	emrA	497	1.00E-177
Uncharacterized protein	OOA_15070	K8W945	-4.45	Uncharacterized protein	yeaO	Q8XDS9	yeaO	117	3.00E-36
[Citrate [pro-3S]-lyase] ligase	OOA_07997	K8WQD6	-4.49	[Citrate [pro-3S]-lyase] ligase	citC	Q8XBS1	citC	495	5.00E-178
Malate synthase	OOA_18799	K8W014	-4.50	Malate synthase	aceB	Q8X609	aceB	818	0
Pili chaperone protein	OOA_18354	K8W856	-4.51	Periplasmic pilin chaperone	yfcS	Q8XCP6	yfcS	225	5.00E-75
Gluconate 2-dehydrogenase	OOA_15512	K8W5Z9	-4.52	Uncharacterized protein	ECs_1094	A0A0H3JH42	ECs_1094	26.2	0.6
Uncharacterized protein	OOA_03944	K8X2V4	-4.54	Adhesin	iha	Q9LAP1	iha	27.7	2
ABC-2 type transporter ATP-binding protein	OOA_15682	K8WF21	-4.55	Fe(3+) ions import ATP-binding protein FbpC	fbpC	Q7AH43	fbpC	87.8	5.00E-20
Uncharacterized protein	OOA_15310	K8WBF4	-4.68	Type VI secretion system protein ImpJ	impJ	Q8X7V2	impJ	154	7.00E-43
Uncharacterized protein	OOA_10418	K8WMC3	-4.68	Toxin B	toxB	A0A0H3JC22	toxB	26.2	1.9
Hydrogenase 2 large subunit	OOA_12168	K8WX56	-4.71	Hydrogenase-2 large chain	hybC	P0ACE1	hybC	947	0
HTH-type transcriptional regulator MetR	OOA_19399	K8VXQ3	-4.76	HTH-type transcriptional regulator MetR	metR	P0A9G0	metR	466	1.00E-167

Ribosome modulation factor	rmf	K8WNB4	-4.85	Ribosome modulation factor	rmf	P0AFW3	rmf	93.2	2.00E-28
2-oxo-3-deoxygalactonate kinase	OOA_15537	K8W603	-4.95	Type II secretion system protein J	etpJ	Q7BSV6	etpJ	26.9	1.5
ABC transporter-like protein	OOA_06983	K8WYR8	-4.98	Ribose import ATP-binding protein RbsA 1	rbsA1	Q8XAW7	rbsA1	416	4.00E-142
Sugar ABC transporter periplasmic sugar-binding protein	OOA_05676	K8WSN0	-5.02	Sugar ABC transporter periplasmic binding protein	yphF	A0A0H3JHJ1	yphF	45.8	3.00E-06
Superoxide dismutase [Cu-Zn]	OOA_05132	K8X5I5	-5.18	Superoxide dismutase [Cu-Zn]	sodC	P0AGD2	sodC	182	2.00E-60
Ribose-5-phosphate isomerase B	OOA_07675	K8WPF2	-5.33	NAD(P)-binding succinyl-CoA synthase	yahF	Q8X6B0	yahF	28.9	0.14
Putative acyltransferase domain protein	OOA_03679	K8WTH3	-5.57	CDK-activating kinase assembly factor MAT1	mnaT	Q8X9W8	mnaT	30.8	0.037
Putative ankyrin repeat protein YahD	OOA_15612	K8W618	-5.57	Ankyrin domain-containing protein	yahD	Q8X6B2	yahD	291	2.00E-102
Dimethyl sulfoxide reductase chain A	OOA_05956	K8X043	-5.64	S-and N-oxide reductase subunit A	ynfF_4	A0A143EF68	ynfF_4	1225	0
DUF1508 domain-containing protein	OOA_07550	K8WPD2	-5.64	UPF0339 protein YegP	yegP	Q8X7I0	yegP	54.7	1.00E-12
Minor fimbrial subunit (Mannose-resistance fimbriae), MrfF protein	OOA_18369	K8WBN7	-5.88	Type 1 fimbrin D-mannose specific adhesin (Protein FimH)	fimH	Q8XBA6	fimH	59.7	5.00E-11
5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	OOA_15842	K8W6B3	-6.01	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	metE	Q8X8L5	metE	115	3.00E-29
Metalloprotease yggG	OOA_18009	K8W0P2	-6.40	Metalloprotease	loiP	Q8XCY1	loiP	333	1.00E-117
Uncharacterized protein	OOA_01942	K8WXU5	-6.47	Arabinose efflux transporter	ynfM	A0A0H3JEF8	ynfM	27.7	2.8
Fimbrial biogenesis outer membrane usher protein	OOA_18349	K8W1G0	-6.54	Outer membrane usher protein	yfcU	A0A0H3JFW3	yfcU	833	0
Autonomous glycyl radical cofactor	grcA	K8X4H1	-6.57	Autonomous glycyl radical cofactor	grcA	P68067	grcA	215	8.00E-75
Type VI secretion-associated protein	OOA_15432	K8WEX8	-6.78	Uncharacterized protein	ECs_0217	A0A0H3JBT0	ECs_0217	50.4	2.00E-07
Uncharacterized protein	OOA_15837	K8W658	-6.95	Membrane protein	yqhH	Q8XBS8	yqhH	25	3.5
HD_domain domain-containing protein	OOA_08037	K8WNN8	-6.98	Multifunctional CCA protein	cca	Q8XBL4	cca	34.3	0.014
Anaerobic dimethyl sulfoxide reductase chain a	OOA_05966	K8WQW1	-7.04	Anaerobic dimethyl sulfoxide reductase subunit A	dmsA	A0A0H3JCX2	dmsA	794	0
Aldehyde oxidase and xanthine dehydrogenase	OOA_15522	K8WII4	-7.13	Xanthine dehydrogenase molybdenum-binding subunit	xdhA	Q8X6C7	xdhA	88.2	1.00E-18
YhbH sigma 54 modulator	OOA_17734	K8W165	-7.21	Ribosome-associated factor Y	yfiA	P0AD51	yfiA	167	4.00E-56
Uncharacterized protein	OOA_15305	K8WM57	-7.40	Type VI secretion system protein ImpC	impC	Q8X7T9	impC	414	5.00E-142
Periplasmic nitrate reductase	napA	K8WWV2	-7.61	Periplasmic nitrate reductase	napA	Q8XE47	napA	1552	0
PhoH domain-containing protein	OOA_06908	K8WYQ6	-7.61	Protein PhoH	phoH	P0A9K2	phoH	386	9.00E-137

Exported amino acid deaminase	OOA_06406	K8WQ44	-8.00	N-methyl-L-tryptophan oxidase	solA	P58523	solA	56.2	2.00E-09
DUF1471 domain-containing protein	OOA_04127	K8X1K4	-8.05	Uncharacterized protein YjfY	yjfY	P0AF88	yjfY	34.3	2.00E-04
Anaerobic dimethyl sulfoxide reductase	OOA_03629	K8WTG7	-8.40	Anaerobic dimethyl sulfoxide reductase subunit B	dmsB	Q8X4K5	dmsB	282	9.00E-99
Dimethyl sulfoxide reductase subunit B	OOA_11878	K8WTG1	-9.11	Oxidoreductase	ynfG_2	Q8X4Q0	ynfG_2	362	4.00E-130
Methylenetetrahydrofolate reductase	metF	K8WCW5	-9.31	Methylenetetrahydrofolate reductase	metF	Q8X766	metF	521	0
Uncharacterized protein	OOA_15447	K8WIH4	-9.35	Uncharacterized protein	yihF	Q8X8H3	yihF	26.2	1.8
Amino acid deaminase	OOA_11788	K8WL55	-9.71	N-methyl-L-tryptophan oxidase	solA	P58523	solA	49.3	4.00E-07
Cystathionine gamma-synthase	OOA_17864	K8W2P9	-10.17	Cystathionine gamma-synthase	metB	Q8X768	metB	594	0
Acireductone dioxygenase	mtnD	K8WSM5	-10.53	Gentisate 1,2-dioxygenase	gtdA	Q8X655	gtdA	29.6	0.14
Putative solute-binding protein	OOA_12208	K8WJL8	-11.91	Glutathione-binding protein GsiB	gsiB	Q8X6V9	gsiB	105	5.00E-25
Anaerobic dimethyl sulfoxide reductase subunit A	OOA_11873	K8WL68	-12.13	Anaerobic dimethyl sulfoxide reductase subunit A	dmsA	A0A0H3JCX2	dmsA	1369	0
Uncharacterized protein	OOA_01807	K8WVY5	-13.01	Uncharacterized protein	ydcH	Q8X9V2	ydcH	69.7	1.00E-18
Anaerobic dimethyl sulfoxide reductase chain a	OOA_03634	K8X654	-13.13	Anaerobic dimethyl sulfoxide reductase subunit A	dmsA	A0A0H3JCX2	dmsA	790	0
Type VI secretion protein	OOA_15300	K8W9K7	-14.72	Type VI secretion system protein ImpB	impB	Q8X7T5	impB	116	2.00E-34
5-nucleotidase	OOA_03949	K8WTT0	-17.70	UDP-sugar hydrolase	ushA	Q8XD35	ushA	124	4.00E-31
5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	metE	K8W9Q5	-20.29	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	metE	Q8X8L5	metE	1211	0

Table S2. *D. melanogaster* proteins that were at least four-fold significantly differentially expressed in infected flies compared to Ringer's injected flies.

UniProt ID	Protein names	Gene names	Fold-change
A1ZBF6	Diptericin B	DptB	17.12
Q24395	Metchnikowin	Mtk	16.40
P45884	Attacin A	AttA	15.59
P82701	Immune-induced peptide 18	IM18	14.51
Q95NH6	Attacin C	AttC	14.08
Q70PY2	Peptidoglycan-recognition protein SB1	PGRP-SB1	13.50
P24492	Diptericin A	DptA	11.18
A0A6H2EDS4	Uncharacterised protein	lncRNA:CR45045	9.89
Q26416	Adult cuticle protein 1	Acp1	9.53
B7YZP9	Muscle LIM protein at 60A	Mlp60A	9.13
O16829	Cecropin C	CecC	8.57
Q9VFD9	Defective proboscis extension response 9	dpr9	8.09
Q8IQR7	Elevated during infection	edin	7.06
O96299	Sorbitol dehydrogenase-2	Sodh-2	5.51
Q9XZH6	V-type proton ATPase subunit G	Vha13	5.20
A1ZBU5	Gram-negative bacteria-binding protein-like 3	GNBP-like3	5.15
A0A0B4LFP4	Ubiquitin-fold modifier 1	Ufm1	4.97
P13395	Spectrin alpha chain	alpha-Spec	4.58
Q9VWV6	Transferrin 1	Tsf1	4.54
Q9VRD9	Cystathionine beta-synthase	Cbs	4.53
Q9XZU1	Exportin-2	Cas	4.32

Q9Y141	Carboxylic ester hydrolase	BcDNA.GH05741	4.30
Q7JWE2	Uncharacterised protein	CG6183	4.12
Q7JWR9	Zinc finger CCCH domain-containing protein 15 homolog	CG8635	4.05
Q24388	Larval serum protein 2	Lsp2	-4.03
P60468	Protein-transport protein Sec61	Sec61beta	-4.81
Q9VAJ4	General odorant-binding protein 99a	Obp99a	-5.33
Q9VUB8	Endosulfine	endos	-7.60

GENERAL DISCUSSION

The work presented in this thesis offers a multi-angled investigation on the dynamics of host defences against persistent infections in *Drosophila melanogaster*. By combining various parameters of the infection, including measures of two proxies of host fitness (i.e., survival and fecundity) and bacterial load at various timepoints over the course of the infection, we explored the contribution of various aspects of host defences to the outcome of persistent infections. **Chapter 1** provides a long-term perspective of the dynamics of infection and the role of pathogen-specific factors in virulence. In the two following chapters, we focused on two precise aspects of host defences, immune priming, and pathogen control in the early phase of infection, and how they shape the variation of host defences during the infection. While in **Chapter 2** we did not observe any effects of a previous encounter with a pathogen on resistance against persistent infections, in **Chapter 3** we found that whether a host has controlled the pathogen growth in the early infection can determine how well it can sustain reproduction costs caused by the disease. In **Chapter 4**, we took a different approach by studying the proteome of chronically infected hosts and showed that the host response is complex and involves several mechanisms related to both host metabolism and immunity.

1. The consequences of bacterial persistence

1.1. Persistence and host fitness

While sustaining a chronic infection is a better outcome of infection than early death due to uncontrolled pathogen growth, persistence may not be without costs for host fitness. In Chapter 3, we found that hosts infected with *Lactococcus lactis* and *Providencia burhodogranariea* had lower fecundity on days three and four post-infection compared to Ringer's injected controls. A decrease in fecundity linked to bacterial persistence has also been observed in other studies (Brandt and Schneider 2007; Bashir-Tanoli and Tinsley 2014). This fecundity decrease illustrates a cost of the infection, which could potentially arise from damage caused by the presence of the pathogen. For example, female flies infected with *Salmonela typhimurium* sustain a decrease in fecundity due to the degeneration of the ovaries by the colonisation of

bacteria, which is most dramatic on days two and three post-infection (Brandt and Schneider 2007).

Alternatively, a reduction in fecundity could arise from energetic costs, because mounting an immune response against the pathogen is costly in terms of resources (Boots and Begon 1993; Chambers, Song, and Schneider 2012; Bajgar et al. 2015; Bajgar and Dolezal 2018). Animal hosts face a trade-off between immunity and other life-history traits such as fecundity (Nystrand and Dowling 2020). Bashir-Tanoli and Tinsley (2014) showed that a decrease in fecundity was observed in the beginning of the infection (days one to three post-infection) in flies injected with live or dead pathogens, suggesting that this reduction was due to the activation of the immune response (Bashir-Tanoli and Tinsley 2014). In Chapter 4, we found an upregulated antimicrobial peptide (AMP) response against the persistent infection, consistently with other studies (Haine et al. 2008; Chambers et al. 2019). Chambers *et al*. (2019) showed that this sustained AMP response is energetically costly for *D. melanogaster* (Chambers et al. 2019). Thus, sustaining a persistent antimicrobial response could divert resources from reproduction, into mounting this response. However, whether these fecundity costs are due to the persistent AMP response is not clear. Firstly, one should note that the sustained AMP response reported in Chapter 4 and in Chambers *et al*. (2019), and the energetic costs observed by these authors, were both measured at seven-days post-infection, while the decrease in fecundity we showed in Chapter 3 happened earlier, i.e., three to four days after the infection. In our study, we did not initially observe a lowered fecundity in infected flies when we assayed this trait at one- and two-days post-infection, indicating that these costs arose after these timepoints. This suggests that fecundity costs may vary over the course of the infection, thus might not be the same at seven days post-infection. Secondly, these costs may be due to other branches of the immune response than AMPs. In their study, Bashir-Tanoli and Tinsley (2014) found similar costs in terms of reduced fecundity (but also metabolic rate and food intake) between bacteria injected with fungal and bacterial pathogens. They argued that the immune response inducing these costs was likely independent of the Imd and Toll pathways, which mediate specific responses to bacteria and fungi (e.g., AMPs), respectively (Bashir-Tanoli and Tinsley 2014). The observed costs could be due, for instance, to the melanisation response, which has been to be linked to energetic depletion in flies infected with *Listeria monocytogenes* (Chambers, Song, and Schneider 2012).

1.2. Long-term persistence and infection transmission

Persistent bacterial infections have been shown to occur in various insect species, including *D. melanogaster, Tenebrio molitor* and *Anopheles gambiae* (Gorman and Paskewitz 2000; Haine et al. 2008; McGonigle, Purves, and Rolff 2016; Boman, Nilsson, and Rasmuson 1972; Hotson and Schneider 2015; Kutzer, Kurtz, and Armitage 2019). Until now, the estimations for the maximum duration of persistence were limited to 28 days post-infection in both *D. melanogaster* and *T. molitor* (Haine et al. 2008; Kutzer, Kurtz, and Armitage 2019). Chapter 1 offers a new estimate far beyond this duration: we were able to retrieve bacteria up to 78 days post-infection. By assaying the presence of bacteria in dead flies, we observed that many hosts died while carrying a persistent infection. These observations could have significant implications for the spread of pathogenic bacteria in natural insect populations. If hosts can sustain a persistent infection for extended periods of time and even during their entire lifetime, they may become reservoirs for the pathogen, as it is observed for some agents of human infectious diseases such as *Salmonella* Typhi (Gal-Mor 2018), *Mycobacterium tuberculosis* (Gomez and McKinney 2004) and *Treponema pallidum* (Garnett et al. 1997; LaFond and Lukehart 2006). The duration of persistence can be important because it will increase the number of susceptible individuals the infected host will come into contact with.

Nevertheless, the way systemic bacterial infections spread horizontally from one insect host to the other remains unclear. While it has been suggested that ingestion of micro-organisms may be the main route of bacterial infections in insects (Vallet-Gely, Lemaitre, and Boccard 2008), individuals may also get infected via penetration of bacteria through injuries. These injuries may occur at various points over the course of the host lifetime. Both male and female *D. melanogaster* engage in same-sex fighting (Nilsen et al. 2004), which can result in wing injuries as it was shown on males during territorial aggression (Hoffmann 1987; Davis et al. 2018). Whether females sustain injuries has not been investigated; however, when kept in groups for several days in the same vial, females sometimes show missing limbs and wing damage (personal observation). Infection via septic injuries may occur under the assumption that hosts shed the pathogen into the environment, which could be possible through faeces if the bacteria reach the gut, for example. Another scenario in which a systemic infection could be horizontally spread is during mating, as it has been shown to occur in other pathogenic organisms (reviewed

in Knell and Webberley 2004), although sexually transmitted bacteria have not been studied enough to support this hypothesis (Otti 2015).

2. Host defences are complex and dynamic

Duneau *et al*. (2017) showed that host resistance plays a key role in the beginning of the infection by determining whether the host controls the pathogen growth. Early in the infection bacterial load increases in all individuals up to six to eight hours post-infection, around which two groups of hosts can be distinguished. These two groups vary in their resistance: some hosts carried high loads and other hosts sustained lower constant loads (Duneau et al. 2017). In our measures of bacterial load in the first days after the inoculation, i.e., days one to four, we also observed two distinct groups of hosts (Chapters 2-3). In Chapter 3, we assessed the fecundity-tolerance of these two groups at days two and four post-infection. Fecundity-tolerance varied between the two groups at four-days post-infection for one of the two bacterial species we tested, *L. lactis*. Hosts carrying high bacterial loads showed a lower tolerance to infection compared to those carrying low loads, indicating that they were less able to counterbalance the fecundity costs of the infection. These two groups of hosts are predicted to go through different outcomes of infection: hosts with high loads are predicted to succumb to uncontrolled pathogen growth, while hosts carrying low loads are expected to sustain chronic infections (Duneau et al. 2017). This was consistent with a previous study where mice infected with *Listeria monocytogenes* varied in their health-tolerance dynamics (as measured by body weight) depending on whether they ultimately controlled the infection and survived or died (Lough et al. 2015). This study also demonstrated that infections resulting in different outcomes will also follow different paths of expression of tolerance over time (Lough et al. 2015; Torres et al. 2016). Our results support this idea and underline the importance of considering the path hosts may be following, i.e., whether they are able to control pathogen growth, when assessing the host defences.

In Chapter 3, we aimed to determine whether tolerance can also vary over the course of the infection. We found that fecundity-tolerance to a *L. lactis* infection decreased between days two and four post-infection. This observation was consistent with previous studies on fecundity-tolerance (Kutzer and Armitage 2016b) and health-tolerance (Lough et al. 2015). These results

support the idea that host-pathogen interactions are no static, and that therefore we can learn more about infections by assaying the parameters of interest at various timepoints, rather than taking a single screenshot of the infection (Boughton, Joop, and Armitage 2011; Schneider 2011; Ayres and Schneider 2012; Lough et al. 2015). The costs sustained by the host will vary over the course of the infection (e.g., decreased fecundity in Chapter 3), thus hosts may differently invest in their defence strategies at different moments in the infection, depending on those costs (Lough et al. 2015). For instance, it has been suggested that hosts may invest in resistance early in the infection to control the pathogen growth but shift to managing a persistent infection at a later timepoint (Lazzaro and Rolff 2011). Tolerance could play a role in maintaining host fitness during persistent infections. The fecundity-tolerance measures observed in Chapter 3 would be consistent with this hypothesis for *Pr. burhodogranariea*, although tolerance to *L. lactis* decreases between the two timepoints measured in hosts carrying low loads. However, to confirm this hypothesis it would be necessary to measure tolerance at an earlier timepoint, i.e., during the resolution phase before the onset of persistence, and compare it with a later timepoint. Alternatively, hosts could invest in other types of tolerance to infection independent from fecundity. In Chapter 4, at seven-days post-infection with *Pr. burhodogranariea*, we observed the downregulation of two proteins, phenoloxidase 2 (PPO2) and psidin in the host which are involved in melanisation and phagocytosis, respectively (Brennan et al. 2007; Binggeli et al. 2014; Dudzic et al. 2015; 2019; Schmid et al. 2019). Melanisation can be energetically costly (Siva-Jothy and Thompson 2002; Rantala et al. 2003; González-Tokman et al. 2011; González-Santoyo and Córdoba-Aguilar 2012), in addition to causing host tissue damage through the reactive oxygen species, a side product of the synthesis of melanin (González-Santoyo and Córdoba-Aguilar 2012; Khan, Prakash, and Agashe 2017). This downregulation may be part of a tolerance strategy to reduce the costs of the infection (Schneider 2007; Frank and Schmid-Hempel 2019). Because we also found AMPs to be amongst the most upregulated proteins in flies infected with *Pr. burhodogranariea* one-week post-infection, we suspected that resistance may also be employed at this point in the infection, although we were unable to determine if bacteria were replicating (cf. Chapter 4, Section 3.1). Thus, host carrying a persistent infection may combine resistance and tolerance, highlighting the importance of measuring both aspects of host defences in order to understand how they manage the infection.

Furthermore, while many studies on persistent infections have mainly focused on the host AMP response (but see De Gregorio et al. 2001; Irving et al. 2001; Levy, Bulet, and Ehret-Sabatier 2004; Troha et al. 2018), there are other aspects of immunity that may play a role in fighting the infection and that should be considered. For example, nutritional immunity plays an important role in host defences (Hood and Skaar 2012; Núñez, Sakamoto, and Soares 2018). Concordantly, we found that flies chronically infected with *Pr. burhodogranariea* overexpressed Transferrin 1 compared to non-infected flies (Chapter 4). This has also been observed in *D. melanogaster* upon invasion by various pathogens (Yoshiga et al. 1999; De Gregorio et al. 2001; Levy, Bulet, and Ehret-Sabatier 2004) but also during persistent infections with bacteria (Troha et al. 2018). Transferrin is a glycoprotein that binds to iron and causes the sequestration of this nutrient from the haemolymph towards the fat body during infection, increasing host survival to infection in fruit flies (Iatsenko et al. 2020). Iron sequestration depletes the nutrient resources in the haemolymph, making them unavailable for the pathogen, thus limiting its growth (Hood and Skaar 2012; Cassat and Skaar 2013). Because of its high evolutionary advantages when it comes to facing infections (e.g., in apes, Barber and Elde 2014), this defence strategy is widespread across different vertebrate and invertebrate host taxa (Hood and Skaar 2012).

3. The contribution of pathogens to the outcome of infection

In Chapter 1, we characterised the long-term dynamics of host survival and bacterial load for four bacterial species. Based on host survival, we could place the different bacterial species on a gradient of virulence ranging from the least to most virulent: *Enterobacter cloacae*, *Pr. burhodogranariea*, *L. lactis* and *Pseudomonas entomophila*. We observed that both persistence and clearance could occur for all bacterial species, although at varying degrees: *En. cloacae* and *Ps. entomophila* were cleared more often than *L. lactis* and *Pr. burhodogranariea*. These differences were explained by a difference in exploitation between these bacterial species, i.e., a differential reduction in fitness as a side effect of an increase in pathogen load (Råberg and Stjernman 2012). In our study, we defined exploitation as the initial bacterial load, i.e., measured in the first two days post-infection. Exploitation was higher in *L. lactis*, followed by *Pr. burhodogranariea* and *En. cloacae* (*Ps. entomophila* was excluded from these analyses, cf. Chapter 1, Section 2.7.2). Exploitation contributes to shaping the host defence responses to

infection by influencing how well it can control the infection, but also how much damage will be caused by the pathogen growth. Bacteria that vary in exploitation will likely vary in the costs to host fitness; therefore, these costs may be managed differently according to this parameter. For instance, *L. lactis* and *Pr. burhodogranariea* both cause a reduction in fitness, as measured by fecundity, in infected hosts between the two timepoints we assayed in Chapter 3 (days one and two *vs.* days three and four post-infection). When it comes to fecundity-tolerance, we found the same pattern for *L. lactis*, indicating that the ability of hosts to maintain their fecundity for a given pathogen load decreased over the course of the infection. However, we did not find the same pattern for *Pr. burhodogranariea* as fecundity-tolerance stayed constant between the two timepoints assayed. This could potentially be due to the higher virulence of *L. lactis:* while these two bacteria did not vary in per-parasite pathogenicity, we found that *L. lactis* presented a higher exploitation on days one and two post-infection, as assayed in Chapter 1. Flies infected with *L. lactis* sustain higher loads in the beginning of an infection compared to those infected with *Pr. burhodogranariea*. Thus, by day four post-infection, they may have sustained more damage than flies infected with *Pr. burhodogranariea*, making them less capable of managing the associated fecundity costs. It may be that they have invested more in controlling the infection in the first hours (Duneau et al. 2017), and they have no energetic resources left to invest in maintaining their fecundity.

In Chapter 1, we explored how we can conceptually decompose virulence into various pathogen factors to explain the outcome of an infection (Råberg and Stjernman 2012; Regoes et al. 2014). However, to understand what host immune responses are pushing against, it is necessary to look at the pathogen behaviour with a higher resolution (Schneider 2011). In Chapter 4, our aim was to determine whether *Pr. burhodogranariea* is reproducing inside the flies or if it is in a dormant stage during the chronic infection. Because we did not detect enough bacterial protein expression signal in the host samples, we were unable to answer this question. Nevertheless, we were able to obtain the protein expression of the bacterial in vitro controls for which replication or dormancy were induced. The most differentially expressed proteins between these two treatments might be useful as the focus of future studies testing the expression of specific proteins (e.g., through RT-PCR). By uncovering whether the bacteria are replicating or not, we may be able to understand, firstly, how the pathogen is able to persist inside the host. Bacteria can achieve persistence through several mechanisms, some of which involve a slowed down or arrested growth state (Grant and Hung 2013; Fisher, Gollan, and Helaine 2017).

Secondly, we may be able to determine the role of the sustained AMP response observed in chronically infected hosts in Chapter 4. Virulence is the contribution of both host and pathogen processes, therefore investigating the side of the pathogen is essential to fully understand the outcome of an infection (Casadevall and Pirofski 1999; 2003; Råberg and Stjernman 2012).

Conclusion

Virulence results from the interplay of a complex set of host defence mechanisms and strategies, and the processes through which pathogens induce damage and fitness costs to the host. These mechanisms and processes will vary over time depending on the path taken by hosts towards the outcome of infection. Thus, host defences should be understood in the context of the specific host-pathogen interaction at play and phase of the infection at which they are measured.

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APPENDIX 1: PROTEIN ABUNDANCE OF *WOLBACHIA*

In all the studies conducted in the present work, we used an outbred population of *Drosophila melanogaster*, naturally infected with the intracellular bacterium *Wolbachia* (gift from Élio Sucena). This population was established from 160 fertilised females collected in Azeitão, Portugal in 2007 (Martins et al. 2013).

We tested for the presence of *Wolbachia* in our experimental flies, using the liquid chromatography-mass spectrometry results obtained in Chapter 4. We searched all the fly samples against the reference proteome of *Wolbachia pipientis* wMel (1,159 proteins, taxonomy 163164, last modified October 2020) downloaded from the UniProt website. This strain of *Wolbachia* was isolated from *D. melanogaster* naturally carrying this pathogen (Wu et al. 2004). We identified 2,491 proteins from the fly database, 17 proteins from the *Providencia burhodogranariea* base and 16 proteins (cf. Chapter 4, Section 3) from the *W. pipiensis* wMel database. Only three of these 16 *Wolbachia* proteins were found in all replicates: the surface antigen Wsp, 60kDa chaperonin and 10 kDa chaperonin.

The surface antigen Wsp is a highly polymorphic immunogenic surface protein which is used for constructing phylogenies of different strains of *Wolbachia* (Roehrdanz and Wichmann 2013). Wsp is one of the most dominant proteins in another proteomics study on another strain of Wolbachia in a nematode host (Darby et al. 2012). Therefore, the presence of this protein in the samples indicated that *Wolbachia* was likely present inside the fly.We hypothesised that *Wolbachia* should be present at the same abundance in both uninfected and infected flies. Thus, we tested whether there was a difference in Wsp protein abundance (as a proxy for *Wolbachia* abundance) between the *P. burhodogranariea* infected and Ringer's injected fly samples. We found that there was no difference in protein abundance between the two treatments ($t = -0.92254$, $df = 10$, p-value = 0.378). We concluded that the flies used in this experiment were infected with *Wolbachia.*

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APPENDIX 2: TIME COURSE OF AN INFECTION

As part of Chapter 4, we conducted a preliminary experiment to obtain information on the growth of persistent bacteria in the first hours of infection. The aim was to determine when the bacteria are in the exponential phase of infection; i.e., the best timepoint to assay replicating bacteria, e.g., to measure the expression of relevant genes/proteins. Four days after mating, flies were injected with approximately 92,000 colony-forming units of *Providencia burhodogranariea*. The bacterial load was assayed immediately after the infection, then every two hours up to 16 hours post-infection, and at a final 24-hour timepoint (Figure 1). Bacterial load did not seem to increase early in the infection as we had expected. At around 12 hours, we started to see two groups of flies with high and low loads, as observed in Chapters 2-3. This is contrast with the study conducted by Duneau *et al.* (2017) where bacterial load initially increases in all the flies. We hypothesised that because of the high inoculation dose we chose, bacterial growth might be limited by a lack of resources, or a strong immune response triggered by the intensity of the infection. Bacterial load likely increased only for a portion of the flies, i.e., those that did not manage to control the infection past the 12-hour timepoint.

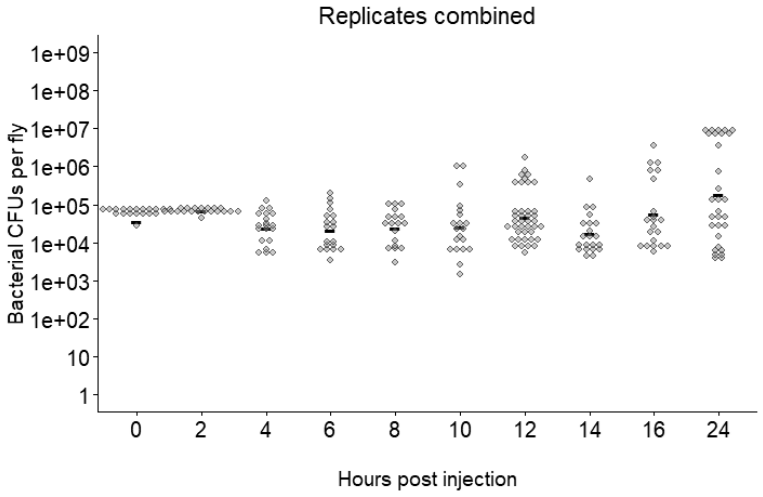

Figure 1. Time course of the bacterial load of flies infected with *P. burhodogranariea.*

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SELBSTSTÄNDIGKEITSERKLÄRUNG

Freie Universität Berlin

DECLARATION FOR SUBMISSION OF A MONOGRAPH

according to § 7 (2a) and (4) of the Doctorate Regulations of the Department of Biology, Chemistry, Pharmacy
based on Official Announcements at Official Gazette of Freie Universität Berlin
No. 21/2018, 31st of May 2018

Acuña Hidalgo	Beatriz
Last name of doctoral student	First name of doctoral student

Hereby I confirm that I have prepared my doctoral thesis entitled

Living with the enemy: Understanding the dynamics of host defences against persistent infections

independently and without impermissible help.

☒ Hereby I confirm that my doctoral thesis is not based on my Diploma / Master thesis.

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☒ Hereby I confirm that I did not publish my thesis completely or partly.

☐ Hereby I confirm that I have published my thesis completely or partly in compliance with my supervisor _____
Name of the supervisor

____ Number of publications

☐ A printout of my publications is attached to each copy of my monographic thesis separately.

Hereby I confirm that the present doctoral thesis has not been filed anywhere before.

16.05.2021	
Date	Signature of doctoral student