Pathogen infection dynamics and the evolution of host resistance and tolerance

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I hereby declare this dissertation is my own work and it does not contain no material previously published or written by another person or institution. Any contribution is explicitly acknowledged in the thesis.

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Chapter 1

Summary

1.1 Summary

Since the dawn of time, multicellular life has been exposed to a wide range of microbes. A share of them will not interfere with the host development or daily homeostasis, some might even contribute to it and potentially become essential, such as gut microbiota. However, a smaller, but significant, fraction of them will threaten the correct livelihood of their host, often defined as parasites or pathogens, depending on their properties or the scientific school that is addressing them. Hosts face this kind of organisms on a daily basis. Most parasites we face every day present low virulence and frequently their menace happens by chance, commonly referred as opportunistic pathogens, as is the case of microbiota that following a given stress might grow uncontrollably. On the other side of the spectrum, we have parasites that under normal conditions hosts might be exposed much less often but exhibit a higher level of virulence and pathogenicity, the so-called pathogens. Therefore, just by focusing on a parasite virulence we observe a wide range of threats to the host. Nevertheless, there are an immense range of properties intrinsic to the parasite that might influence the outcome of a host-parasite, or better a host-pathogen, interaction. These properties can include infection site, transmission rate and which resources they hitchhike. In face of these threats, hosts were selected to develop an equivalent wide set of immune responses with different costs and benefits to themselves. Immunity can be further divided into innate and adaptive. The latter is a complex branch only found in vertebrates, and therefore, I will focus on the most universal branch of the immune system throughout this thesis, the innate immune responses. Hosts evolved resistance and tolerance strategies, two disparate sets of mechanisms that in a *stricto sensu* aim to clear or cope with the infection. Naturally, these strategies will have very distinct consequences for the ecology and evolution of a host-parasite interaction and each of the populations' evolutionary history. Moreover, in the current medicine state and health crisis, the study of these strategies offers new insights and solutions for immunology and epidemiology. Up to now, when we address an infection, fighting and hopefully clearing the parasite burden is the first solution that comes to mind. We define the latter as resistance, particularly quantitative resistance as it reduces the parasite number. Nevertheless, resistance comes with a number of cons. Resistance mechanisms are usually very costly, either by the effectors induction or the collateral damage caused by these (e.g. oxidative stress and consequent inflammation). Furthermore, resistance acts on the parasite number and therefore selects for increased virulence alleles on the parasite side that will in turn select for further host increased resistance and so forth. This antagonistic coevolution between the host and parasite is often referred as Red Queen dynamics and it is

one of the major selective forces in nature. On the other hand, tolerance acts by reducing or controlling the damage employed by either the parasite itself or by the immune response. Hence, this strategy does not affect the parasite burden and consequently, it is not expected to select for higher virulence. Experimentally, we quantify tolerance as the reduction of a given infection cost in fitness measures, such as fecundity-tolerance for reproductive fitness or mortality tolerance for survival. Unfortunately, tolerance studies are scarce and often reflect theoretical predictions from modelling or studies from plant biology, where most of our understanding comes from.

In this thesis, we started by investigating how different pathogens shape infection dynamics outcome in an outbred population of *Drosophila melanogaster* and second, how does a host population respond to the exposure to different pathogens in the short and long-term. In Chapter 3, we exposed a *D. melanogaster* population to a variety of parasites from low to high virulence and low to high inoculum size. This allowed us to characterize bacterial infections during the host lifetime. From the host side, we focused on common points such as clearance ability, while on the parasite side we turned to its aptitude to persist within the host. Furthermore, we decompose parasitic virulence into smaller factors, such as host exploitation or per-parasite pathogenicity, i.e. how well the bacterial species survive and replicate within the host, and the amount of damage they inflict. In Chapter 4, we turn our focus to the host and addressed how the variation in parasite burden at given time-points during infection reflects variation in the immune strategies. Here we showed that throughout a bacterial infection with *Lactococcus lactis*, individuals become less tolerant and less resistant across the two time-points measured, potentially indicating immunity costs. Curiously, we also observed that individuals unable to control parasite burden in the acute phase are particularly less tolerant than their counterparts. Lastly, in Chapter 5 we examined what are the evolutionary implications of selection for host resistance and fecundity-tolerance in a specific host-parasite interaction, *D. melanogaster* and *L. lactis*. Interestingly, our results show that after eight generations of selection, tolerance seems to be the favoured immune strategy to evolve in both resistance and tolerance selection regimes. This result suggests tolerance mechanisms might offer less costs or be more promptly available under the settings of this study. Altogether, our results help to clarify some of the implications and properties of these strategies on the dynamics of host-parasite interactions, as well as bring to light a new set of questions regarding tolerance and all its underpinnings.

1.2 Zusammenfassung

Tiere sind täglich einer Vielzahl von Mikroorganismen ausgesetzt. Die Wechselwirkung zwischen ihnen kann von positiv bis negativ reichen. Letztere Art von Mikroorganismen werden je nach ihren Eigenschaften als Parasiten oder Krankheitserreger bezeichnet. Wirte reagieren auf eine Infektion mit verschiedenen Strategien, nämlich mit Resistenz oder Toleranz. Resistenz zielt darauf ab, die Anzahl der Parasiten zu reduzieren oder eine Infektion vollständig zu beseitigen. Auf der anderen Seite erlaubt Toleranz dem Wirt, mit den Auswirkungen einer Infektion und der Immunantwort zurechtzukommen. Natürlich führen diese beiden Strategien zu sehr unterschiedlichen Ergebnissen sowohl in der Evolution, als auch in der Ökologie der Wirt-Parasit-Interaktion, aber auch zu unterschiedlichen Implikationen für Medizin, Immunologie und Epidemiologie. Resistenz wirkt sich auf die Parasitenzahl aus und selektiert daher auf erhöhte Virulenz. Auf der anderen Seite beeinflusst Toleranz nicht die Parasitenlast und sollte daher laut Studien einen neutralen bis positiven Effekt auf die Parasitenprävalenz und die Evolution haben.

In dieser Arbeit untersuchten wir: i) wie verschiedene Parasiten die Infektionsdynamik und deren Ergebnis in einer Population von *Drosophila melanogaster* beeinflussen; ii) wie ein Wirt kurzfristig auf eine Infektion mit einem bestimmten Parasiten reagiert; iii) ob der Wirt langfristig eine Resistenz oder Toleranz entwickeln kann. In Kapitel 3 wurde unsere Population von *D. melanogaster* mit verschiedenen Krankheitserregern in unterschiedlichen Inokulationsdosen infiziert. Die Infektionsdynamik wurde über die gesamte Lebensdauer des Wirts verfolgt. Auf der Seite des Wirts konzentrierten wir uns auf die Clearance-Fähigkeit. Auf der Seite der Parasiten analysierten wir deren Persistenz im Wirt. Wir zerlegten die Virulenz der Parasiten weiter in kleinere Faktoren. In Kapitel 4 konzentrierten wir uns auf die Dynamik der Wirtsresistenz und -toleranz während verschiedener Phasen der Infektion. Unsere Ergebnisse zeigen, dass bei einer Infektion mit *Lactococcus lactis* der Wirt mit der Zeit weniger tolerant und weniger resistent wird. Darüber hinaus sind Individuen, die in der akuten Phase nicht in der Lage sind, die Parasitenzahl zu kontrollieren, weniger tolerant als ihre Gegenspieler. In Kapitel 5 haben wir bei *D. melanogaster*, die mit *L. lactis* infiziert wurde, auf Wirtsresistenz und Fruchtbarkeitstoleranz selektiert. Unsere Ergebnisse zeigen, dass sich nach acht Generationen der Selektion die Toleranz sowohl bei der Resistenz-, als auch bei der Toleranzselektion entwickelt. Dieses Ergebnis deutet darauf hin, dass Toleranz unter den Bedingungen dieser Studie weniger Energie verbraucht und für den Wirt schneller verfügbar

ist. Insgesamt zeigen unsere Ergebnisse die Auswirkungen und Eigenschaften dieser Immunstrategien auf die Dynamik von Wirt-Parasit-Interaktionen und bringen eine Reihe neuer Fragen zur Toleranz und ihren Grundlagen ans Licht.

Chapter 2

General introduction

2.1 - Immunity and host-microbe interactions

Classically, the perceived immune system sole function was to detect and destroy any invading microbes (Burnet 1961). This view underwent a radical set of shifts throughout the decades to come with the surfacing of layers of complexity the original model did not take in consideration. The first big turn encompassed microbe recognition. The immune system was formerly expected to only differentiate between self and non-self, however, today we are aware the immune system carefully regulates the microbes by ranking them from beneficial to dangerous (Matzinger 2002). Contrarily to previously thought, most microbes have a neutral or beneficial interaction to the host (Lee and Mazmanian 2010). The mechanisms through which the host is able to differentiate between different types of interactions remains unclear however the most currently accepted hypothesis states a parasite would not only exhibit microbe-associated molecular patterns (MAMPs) but also induce the release of danger-associated molecular patterns (DAMPs) from the infected tissue (Lazzaro and Rolff 2011). On the other extreme, a symbiont would release MAMPs but not induce the release of DAMPs.

With the growing literature on beneficial microbes came the establishment of the microbiota field and the successful characterization of the microbiome of a series of organisms, namely in humans (Turnbaugh et al. 2007). Microbiota comprises all microorganisms that live inside or on a host, including protozoa, bacteria, viruses or fungi. Although most of the microbiota is environmentally acquired either through diet or vertical transmission (Chandler et al. 2011; Engel and Moran 2013), its composition does not reflect the microbial intake. Instead, we observe a strong host regulation and at least a fraction of the microorganisms are potentially population/species-specific and in some developmental stage-specific. For instance, studies in Hydra showed that the host shapes its microbial composition through differential antimicrobial peptides (AMPs) expression across different species (Fraune and Bosch 2007; Franzenburg et al. 2013). This specific fraction of the microbiota has been referred to core microbiota and has also been found in several organisms across a range of phyla (Thongaram et al. 2005; Ochman et al. 2010; Brucker and Bordenstein 2012; Dishaw et al. 2014; Pais et al. 2018). A reason for this strong link between specific host and microbe populations is potentially their coevolutionary history. Growing evidence has shown that microbiota is essential for the correct tissue development (Sommer and Bäckhed 2013; Mazmanian et al. 2005; Koropatnick et al. 2004), immune maturation/response (Futo, Armitage, and Kurtz 2015; Chung et al. 2012; Leger et al. 2017) and often a protective effect against parasites (Herren et al. 2020), as is the

case of the highly popularized *Wolbachia* (Teixeira, Ferreira, and Ashburner 2008; Martinez et al. 2014). Host-microbiota-parasite interactions have received special attention as potential routes for controlling vector-borne diseases such as dengue or malaria (Herren et al. 2020; Hoffmann et al. 2011). The strong interaction between host and microbiota led to the development of the holobiont concept, where microbiota is treated as an extension of the host which is potentially under the same selection (Rosenberg and Zilber-Rosenberg 2014; Catania et al. 2017). Nevertheless, in most cases it might be difficult to draw the line between a neutral and a positive/negative interaction and this can evolve to either a mutualistic or pathogenic scenario (Hooper and Gordon 2001).

On the other side of the microbial spectrum, we have the parasites that can either be obligatory or opportunistic. Either per chance or evolutionary history, the agonistic pressure is ubiquitous and one of the strongest and most decisive evolutionary driving-force (Woolhouse et al. 2002; Obbard et al. 2009). Hosts which are able to adapt to the parasite can exert similar levels of selection on it, leading to a coevolutionary arms race know as Red queen dynamics (Van Valen 1973; Lively and Apanius 1995). This coadaptation has been shown to occur rapidly (Routtu and Ebert 2015; Papkou et al. 2019) and select for a broad range of responses and the evolution of the immune system (Dybdahl and Lively 1998; Kaufman 2010; Obbard et al. 2009).

2.1.1 Insect immunity

Insects have evolved an array of recognition and sophisticated defence mechanisms against different types of parasites. As invertebrates, they lack the adaptive (acquired) immunity, therefore these mechanisms are part of the innate (inborn) immune response which are in common with vertebrates (Schmid-Hempel 2005). To successfully establish an infection, a parasite faces the following lines of host defence: i) behavioural mechanisms, such as pathogen avoidance; ii) physical barriers, namely the chitin exoskeleton; iii) a large set of immune responses (Siva-Jothy, Moret, and Rolff 2005).

Once the outer barriers are surpassed, the immune system will be activated. The innate immune system mainly relies on the cellular and humoral mechanisms that are strictly coordinated and often act in synergy. Moreover, cellular immunity is based on haemocytes, the blood cells of invertebrates. In *Drosophila*, this branch has been fairly studied and based on their function, haemocytes can be divided into three different types: plasmatocytes, crystal cells and

lamellocytes (Lemaitre and Hoffmann 2007). Plasmatocytes are responsible for the phagocytosis of microorganisms, apoptotic cells and their constituents whereas crystal cells are larval gut-specific nonphagocytic cells involved in oxidoreductases reservoirs which is essential for phenol oxidase activity during melanization (Rizki, Rizki, and Grell 1980; Lebestky et al. 2000; Meister 2004). In the presence of big intruders, such as parasitoid wasp eggs, lamellocytes differentiation is induced by circulating plasmatocytes. Therefore, the cell layers around the invader forming a capsule. The capsule goes then through melanogenesis, where reactive oxygen species (ROS) and other cytotoxic products are released, killing the internal parasite (Nappi et al. 1995; Lavine and Strand 2002; Marmaras and Lampropoulou 2009).

The encapsulation process is a neat example of the interplay between cellular and humoral responses. ROS are a hallmark of the humoral response. In *Drosophila*, ROS can be expressed within haemocytes or in specific regions of the gut. As early as one hour into the infection, nonphagocytic cells will generate a strong ROS response, while after ninety minutes we will have a response by phagocytic cells that engulfed the parasite (Myers et al. 2018). In the gut epithelium, specifically foregut and hindgut, there is a basal level of ROS produced by the membrane-associated dual oxidase (DUOX), a NADPH oxidase enzyme (Buchon, Broderick, and Lemaitre 2013; Ha, Oh, Bae, et al. 2005). Upon infection, there is an increase in its production and induction of proinflammatory compounds. Inflammation is a local response that aims to limit the damage self-inflicted by ROS. Due to the high volatile properties of ROS, the host not only strictly limits the circulating ROS precursors but also expresses redox enzymes such as an extracellular immune-regulated catalase to regulate the excessive respiratory burst levels (Ha, Oh, Ryu, et al. 2005). In a similar manner, parasites evolved ways to dodge ROS (Imlay 2008) and manipulate the host detoxification system (Bahia et al. 2013). The other fundamental element of the humoral response is the antimicrobial peptides (AMPs) production (Zasloff 2002). These small cationic peptides can either be inducible or constitutively expressed (Tzou et al. 2000). In Drosophila, different bacterial species activate different pathways through pattern recognition receptors (PRRs) binding (Lemaitre and Hoffmann 2007; Hanson et al. 2019). Toll and Immune deficiency (Imd) pathways are known to differentially regulate AMP synthesis in response to Gram-positive bacteria and fungi in the former case , and Gram-negative bacteria for the latter (Hillyer 2016; Lemaitre and Hoffmann 2007). In *Drosophila*, there is evidence for seven AMP families that totalize in a 21 AMPs and AMP-like genes (Hanson and Lemaitre 2020). These act by destabilizing parasite's anionic cell

membranes, for instance, through pore formation (Brown and Hancock 2006; Joo, Fu, and Otto 2016). Depending on the host species, antimicrobial expression might be active for a long period of time after infection (Makarova et al. 2016), although there is evidence for a short half-life for some AMPs given their unstable nature (Knappe et al. 2014). In the last years, a number of AMPs has been proposed as an alternative for reviving the lost killing ability of the conventional antibiotics. The diversity of eukaryotic AMPs which are naturally synthesised in synergistic cocktails limits the possibility of evolutionary bacterial resistance leading to successful translation of them into medical applications of drug combinations (Lazzaro, Zasloff, and Rolff 2020).

2.1.2 Infection dynamics

Infection is an intrinsically dynamic process sectioned in different phases, each affected by host and parasite properties. Although, different authors set the border between acute and chronic phase in distinct time-points, reflecting the variable nature of the respective experimetnal model, in this study we follow Howick and Lazzaro definition (Howick and Lazzaro 2014). According to the authors, the acute phase is characterized by high host mortality and peak pathogen intensity (e.g. 1-3 days post infection in their case), while chronic phase of infection still presents a negative impact of infection but less strong and often a number of persisting parasite population. In the last decades, acute phase has been shown to be decisive to infection outcome. During this phase, and within only the first hour of infection, inducible immunity is activated and heavily expressed (Haine et al. 2008; Myers et al. 2018). Some individuals will be able to control the infection within a certain time-frame, while other will succumb to infection. A study from Duneau and colleagues has proposed there is a set of variables found across infections and timely specific to different pathogens (Duneau et al. 2017) and that often infections should follow one of two paths: i) pathogen growth and eventual host death; ii) pathogen control and host survival. For instance, for a given pathogen, a host will have a time to control its burden, if not the parasite will grow until it reaches a plateau load, designated bacterial load upon death, named after this individual's destiny. Opposed to this, individuals that are able to control the infection, will still carry a low pathogen number that might persist throughout their lifetime (Duneau et al. 2017), named as set-point bacterial load. Both set-point bacterial load and bacterial load upon death are supposedly universal within a host-parasite species infection. However, other authors have observed that pathogen load can actually continue to decrease (Kutzer and Armitage 2016b; Haine et al. 2008; Zanchi, Johnston, and Rolff 2017), potentially even reaching clearance (Duneau et al. 2017) or even

increase later into infection (C. V. L. Miller and Cotter 2017). Altogether, it is fair to say there is a lot of contradictory evidence regarding infection dynamics and what exactly goes on within each phase. Both hosts are parasites are highly diverse and we are aware that both the host and the parasite can switch their strategies during the course of infection, either due to environmental cues, such as diet, or to their genetic programme (Duneau et al. 2017; Howick and Lazzaro 2014; Lough et al. 2015; Ganeshan et al. 2019).

2.1.3 Immune strategies: resistance and tolerance

Although parasite clearance mechanisms are undoubtedly crucial and a great fraction of the immune response, they are not always the optimal investment in terms of host fitness. Mechanisms that aim to clear or limit parasite growth are classified as resistance strategies. These strategies can be further classified in qualitative or quantitative resistance. The first englobes avoidance and/or clearance of the parasite, while the second regards reduction/control of the parasite burden (Råberg, Graham, and Read 2009; Restif and Koella 2004). A classic example of a resistance mechanism is ROS release. Both ROS and the consequent inflammation process are rather costly both energetically and in tissue damage. In extreme scenarios the cost might be great enough that the individual might succumbs to death by the self-inflicted damage of the immune response, named immunopathology, instead of the one inflicted by the parasite. At a population level, we might observe trade-offs with other life-history-traits (Boots and Begon 1993) and ultimately population divergence (Maan et al. 2008; MacColl and Chapman 2010). Therefore, in some occasions might be more beneficial to reduce the detrimental effects of an infection, what is defined as tolerance (Kutzer and Armitage 2016a). A rather well-studied example of tolerant hosts advantage in the wild is in regard to heme oxygenase, reviewed in (Silva et al. 2020). Heme is a fundamental precursor of oxygen transporters, namely haemoglobin, because of its high affinity to oxygen. Nevertheless, the latter also confers it highly prooxidant and proinflammatory properties, reason why it is mainly buffered intracellularly within haemoproteins (e.g. haemoglobin, myoglobin). Upon infection with *Plasmodium*, replication within red blood cells leads to a massive release of heme. In many cases this heme unleash explains on its own disease severity and *Plasmodium* pathogenicity (Ferreira et al., 2008). Heme oxygenase is a naturally occurring enzyme that catalases the degradation of heme. Curiously, some individuals exhibit an overexpression of a form of heme oxygenase, HO-1, and are found to have higher survival comparatively to control individuals carrying the same parasite burden (Seixas et al., 2009). Hence, these individuals

maximize their fitness by reducing the circulating heme and therefore infection damage by infection in opposition to parasite load. This example shows that evolution might favour tolerance phenotypes in particular disease conditions and this strategy potential applications in biomedicine (Schofield et al., 2002).

Although tolerance has only been studied more recently in animals (Råberg, Sim, and Read 2007), its role in plant defences against herbivores has been exhaustively studied and a significant number of the theoretical assumptions come from these systems (Simms and Triplett 1994; Herms and Mattson 1992). Tolerance has been defined as the ability to limit the negative consequences of a parasite load on fitness/health (Kutzer and Armitage 2016a). It can be measured as range-tolerance or point-tolerance. While in the first we perform a reaction norm between individual fitness and parasite load, in the second we average the fitness of a population/genotype and plot it against one parasite load value. Due to the different nature of the measures, different inferences can be made from the same dataset as it has been shown in a house finches study (Adelman et al. 2013). Distinct studies have assessed range-tolerance from a linear to a four-parameter regression (Louie et al. 2016; Simms 2000). A key factor in a tolerance measure is its dependent variable, the fitness measure. This will depend on the model and question constrains (Rohr, Raffel, and Hall 2010). For instance, in long-lived model such as a mouse infected with *Plasmodium*, both weight loss and red blood cell counts might represent a better assessment of the infection cost on health, as it is less laborious and more relevant for the disease pathogenesis in question. On the other hand, when handling short-lived models such as insects, might be more appropriate to use reproductive or survival fitness, as repeated measures are often not possible because of destructive nature of the bacterial load assessment in these models. Thereupon, I will mainly focus on the latter in this thesis, to which we refer as fecundity-tolerance and mortality-tolerance, respectively. These are expected to exhibit very different outcomes in both pathogen prevalence and host population adaptation.

As a result of their properties, one immune strategy might be more advantageous than the other under certain conditions. When facing a parasite with high transmission rate and low virulence, tolerance is likely to be the more favourable. Under opposite conditions, resistance would be selected and in an intermediate scenario both could be evolve depending on their associated costs or if they are linked to each other (Restif and Koella 2003). Moreover, host damage is likely to play a part in the decision between resistance and tolerance, as suggested by Moreno-Garcia and colleagues on what was proposed as damage threshold hypothesis (Moreno-García

et al. 2014). To date, there are several examples in literature of positive (Zeller and Koella 2016; Howick and Lazzaro 2017), negative (Råberg, Sim, and Read 2007; Vincent and Sharp 2014) and even no correlation (Sternberg et al. 2012; Mazé-Guilmo et al. 2014; Lefèvre, Williams, and de Roode 2010; Decker, de Roode, and Hunter 2018) between both immune strategies suggesting this relationship might be infection model specific and dependent on the underlining immune mechanisms.

Resistance mechanisms usually present a regulatory cost as they are often based on inducible components of immune response (Schmid-Hempel 2005; Moret and Schmid-Hempel 2000; Alves et al. 2019; I. F. Miller and Metcalf 2019) (Table 1). These costs are evident in frequent trade-offs with other life-history traits, such as fecundity (Simms and Triplett 1994; Brandt and Schneider 2007; Partridge, Gems, and Withers 2005; Lawniczak et al. 2007). A classic example of the latter is a study from Kraaijeveld and colleagues where they conducted experimental evolution for host resistance in *Drosophila melanogaster* infected with the parasitoid wasp *Asobara tabida* (Kraaijeveld and Godfray 1997). This species is an endoparasitoid in which parasite larvae feed within the host larvae leading to its death before pupation. Some fruit flies are able to mount an early immune response to the parasitoid wasp, including encapsulation and melanization, and successfully develop to pupae and later to adult. Therefore, this particular parasite imposes a very strong selective pressure as for host survival a strong and time-strict immune response is fundamental. Within this, the authors in this study artificially selected for higher encapsulation rate for nine generations in four different fly food volumes. Probably due to the high selective pressure, resistance evolved quite quickly with a plateau around 5 generations after the start of selection. More interestingly, individuals grown in different food volumes differed in their larval competitive ability. Selected larvae performed worse in low food volumes, exhibiting a trade-off between resistance and larval competition ability, but not in high food volumes. This study clearly showed that resistance evolution is often coupled with life-history costs that might not be always evident in rich laboratory conditions (McKean et al. 2008).

Table 1 - Studies that performed experimental evolution for host resistance. Studies that might have evolved a form of tolerance are marked with **(T)**. For further information on experimental evolution, see section 1.2 below.

Host	Parasite	Selection	Selected trait	Number of generations	Selection outcome	Trade-offs	Reference
Aedes aegypti	*Plasmodium gallinaceum*	-	Susceptibility	4	Plasmodium refractoriness	-	(Thathy, Severson, and Christensen 1994)
Biomphalaria glabrata	*Schistosoma mansoni* (flatworm)	Artificial	Resistance / susceptibility	5	Increased qualitative resistance	Reduced fecundity and survival compared to control lines	(Webster and Woolhouse 1998)
Drosophila melanogaster	*Asobara tabida* (Parasitoid wasp)	Natural	Encapsulation ability	19	Higher encapsulation rate	-	(Hughes and Sokolowski 1996)
		Artificial	Encapsulation ability	8	Higher encapsulation rate	Reduced larval competitive ability	(Kraaijeveld and Godfray 1997)
		Artificial	Encapsulation ability	5	Higher encapsulation rate; genomic changes	-	(Jalvingh et al. 2014)
	Leptolina boulardi (Parasitoid wasp)	Artificial	Encapsulation ability	9	Higher encapsulation rate	Reduced larval competitive ability	(Fellowes, Kraaijeveld, and Godfray 1998)
		Artificial	Encapsulation ability	33	Higher encapsulation rate; Immune-induced genes were constitutively expressed dependent on lamellocyte differentiation	-	(Leitao et al. 2020)
	Tubulinosema kingi (microsporidian)	Natural	Survival	73 weeks	Early life fecundity and increased longevity when infected **(T)**	Reduced fecundity and larval competitive ability in the absence of infection	(Vijendravarma, Kraaijeveld, and Godfray 2009)
	Beauvaria bassiana (fungi)	Artificial	Survival	15	No increased resistance; Higher late fecundity **(T)**	Reduced early and lifetime fecundity	(Kraaijeveld and Godfray 2008)

Drosophila melanogaster	*Bacillus cereus* (bacteria)	Artificial	Survival	18-24	Increased egg production **(T)**	Delayed developmental time	(Ma et al. 2012)
	Pseudomonas aeruginosa (bacteria)	Natural	Survival	10	Higher survival; Rapid development; Toll-Imd synergy	Reduced longevity and larval viability; Rapid trait loss	(Ye, Chenoweth, and McGraw 2009)
	Pseudomonas entomophila (bacteria)	Natural	Survival	24/34	Distinct infection route genetic basis	Susceptibility to viral infections	(Martins et al. 2013)
		Natural	Survival	45	High survival	-	(Gupta et al. 2016)
	Drosophila C Virus (Virus)	Natural	Survival	20	Higher survival; Cross-resistance to other viruses; 3 genes involved	-	(Martins et al. 2014)
Galleria mellonella	*Beauvaria bassiana* (bacteria)	Artificial	Survival	25	Increased resistance and cuticle phenol oxidase activity	Reduced haemolymph PO activity upon infection with the fungi *Metarhizium anisopliae*	(Dubovskiy et al. 2013)
Plodia interpunctella	*Granulosis virus* (virus)	Natural	Survival	24 months	Higher survival and pupal weight	Delayed developmental time; Reduction egg viability; Reproductive cost in the absence of infection	(Boots and Begon 1993)
		Natural	Survival	14	Different genetic architecture of resistance mechanisms between different nutrition environments	Reduced growth rate in low resistance-selected lines grown in low-nutrition	(Roberts et al. 2020)

On the other hand, there are very few examples of tolerance mechanisms (Seixas et al. 2009; Troha et al. 2018; Huen et al. 2020; Soares, Gozzelino, and Weis 2014; Mace, Pearson, and McGinnis 2005; Ganeshan et al. 2019; Clark et al. 2013; Ayres and Schneider 2009; Shinzawa et al. 2009) and these mechanisms strongly differ between host-parasite species and types of infection. As infection scenarios can greatly vary, encompassing different tissue sites and physiological processes, mechanisms that aim to reduce infection damages are expected to be equally diverse (Medzhitov, Schneider, and Soares 2012). Nevertheless, the general consensus is that tolerance strategy should be energetically less costly than resistance (M. R. Miller, White, and Boots 2006) and its cost be mostly adaptive/functional, as in dependent on the presence of host elements that can be co-opted when faced with a given infection (Huen et al. 2020). Regarding costs on intrinsic growth rate, there is contrasting theories. Some authors argue tolerance might be the predominant defence strategy during energy-restricted developmental stages, such as infancy where resources are fulcrum for the correct tissue development/growth (Harbeson et al. 2018), whereas others argue that during early and late stages of development, tolerance might actually be compromised because of development constrains and impaired tissue repair capacity, respectively (M. R. Miller, White, and Boots 2006; Medzhitov, Schneider, and Soares 2012; Sheffield et al. 2020).

A considerable appeal for tolerance studies in biomedicine and pest control for the past years has been due to its predicted effect on pathogen evolution (Rausher 2001; Vale, Fenton, and Brown 2014). Currently, we are quite aware of parasite resistance evolution in face of resistance mechanisms, either natural or artificial (e.g. antibiotics). Since resistance acts on the pathogen number, increased virulence is selected. The alarmingly increasing abuse of antibiotics and the growing concern regarding parasites' resistance throughout the world prompted us to explore new defence pathways (May 2014). Within this framework, tolerance has been proposed as a silver bullet as it does not have a negative impact on parasite burden and therefore arguably should not select for higher virulence (Schneider and Ayres 2008; Vale, Fenton, and Brown 2014). An example of an artificial tolerance mechanism is the anti-Glycosylphosphatidylinositol (GPI) vaccine for malaria developed by Schofield and collaborators (Schofield et al. 2002). In the latter, immunized mice infected with *Plasmodium berghei* exhibited higher survival and delayed disease severity under the same parasite burden as sham-immunized controls. Unfortunately, they did not assess the vaccine effect on the parasite population, this marks a potential shift in the current medical strategies. Nevertheless, some authors fairly argue that virulence should still slowly evolve in a tolerance scenario, as

in the absence of parasite control there is still intra-competition and likely high replication/transmission evolution (Little et al. 2010).

2.2 Experimental evolution

Experimental evolution has been an increasingly used tool to test evolutionary questions often in a controlled laboratory environment. An exception to the typical experimental evolution studies is the work from Reznick performed in the wild (Reznick, Bryga, and Endler 1990). These tools have been further reviewed by Kawecki and colleagues (Kawecki et al. 2012), but the workflow is the following: i) a population is exposed to a novel environment or stressor with most of the environmental settings under control by the experimenter; ii) changes in the different treatment populations, often referred to as selection lines, are tracked during and after selection; iii) depending on the question and traits under selection, experiments are conducted on the evolved populations, ranging from life history traits, physiological, morphological and, nowadays often performed, resequencing of the populations (Jha et al. 2015; Kelly and Hughes 2018; Turner et al. 2011; Barghi et al. 2017; Baldwin-Brown, Long, and Thornton 2014; Long et al. 2015; Kofler and Schlötterer 2013). Each experimental evolution assay is specific to the question and working model (e.g. selective pressure, number of generations and populations' sample size) (Fuller et al. 2005). Selection can be employed by the setting itself, defined as natural selection experiment (e.g. selection for survival to infection), or employed by the experimenter depending on the quantification of a specific trait, so-called artificial selection (e.g. dot size in butterflies' wings) (Hill, Caballero, and Systematics 1992; Jeffrey 2003). The key take-home message from this experimental tool is that by selecting a trait under laboratory conditions, we are able to see and quantify gradual changes and populational evolution over a few generations that can range from weeks to years, from bacteria to insects, respectively. Experimental evolution has exhaustedly been used in regard to immunity to quantify changes in host defences in face of parasites (Ebert 1998).

2.3 The aims of this thesis

This thesis is comprised of three different studies covering bacterial infection dynamics and host immune strategies role and evolution in a fruit fly-bacteria model.

In **Chapter 3,** we studied how is infection outcome and its parameters affected by bacterial species and inoculum size. For this, we infected female *D. melanogaster* with a range of

bacterial species at different injection doses and measure survival and bacterial load across lifetime infection. Some of the parameters we measured include bacterial clearance during life and upon death, set-point bacterial load and persistence, and per-parasite pathogenicity.

In **Chapter 4,** we characterized the temporal dynamics of both resistance and fecundity-tolerance to two bacterial species: *Lactococcus lactis* and *Providencia burhodogranariea*. Given the debate around immune strategies variation across infection, we assessed daily fecundity and bacterial load across different time-points during acute and chronic phase for each of the infections. Based on individual bacterial load, we categorized flies in "likely to die" and "likely to survive" subgroups and subsequently assessed each subgroup changes in quantitative resistance and fecundity-tolerance after two and four days post-infection.

In **Chapter 5,** we focused on the evolutionary implications of resistance and tolerance. For this, we evolved an outbred population of *D. melanogaster* for high quantitative resistance or high fecundity-tolerance to a *L. lactis* infection for eight generations. We assessed their changes in immune and reproductive parameters during and after selection. *L. lactis* was incubated inside evolved individuals and later retrieved for re-infection experiments to measure virulence/persistence evolution for each of the immune strategy's selection regime. In sum, we evaluated how resistance and tolerance affect both sides of a host-parasite interaction.

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Chapter 3

Decomposing virulence in persistent infections

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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 then sought to understand the basis of variation in virulence, by asking if it is due to differences in bacterial load sustained within the host or due to differences in damage, i.e., per-parasite pathogenicity. 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 in flies that had died up to ~10 weeks post infection. We show that sustained chronic infection and clearance are both possible outcomes for bacterial species showing a range of virulence. Lower injection doses of *E. cloacae* could be cleared more quickly than higher doses, but bacteria of all species could persist inside the host for at least 75 days. Furthermore, we show that bacterial virulence differences can be explained by a combination of variation in host exploitation, i.e., how well the bacteria can survive and replicate inside the host, and per-parasite pathogenicity, i.e., the amount of damage per-parasite that is inflicted on the host, and that these factors relate to the proportion of flies with persistent infections of each bacterial species. Separating the contributions of both actors in the dynamics of infection will help us to gain insight into the intricacies of host-pathogen interactions.

1. Introduction

Once a host has become infected, the immune system will potentially limit pathogen growth, a response termed host resistance (Best, White, and Boots 2008; Råberg, Graham, and Read 2009; Schmid-Hempel 2011). Resistance can therefore be quantified as the inverse of pathogen load (Råberg, Graham, and Read 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 and usage costs for the host (Boots and Begon 1993; Kraaijeveld and Godfray 1997; Armitage et al. 2003; 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 et al. 2015), into mounting an immune response. Furthermore, immune responses can lead to self-inflicted damage to the host, namely immunopathology (Graham, Allen, and Read 2005; Sadd and Siva-Jothy 2006; Khan, Agashe, and Rolff 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 chronic 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 chronic systemic infections, for example in the mosquito *Anopheles gambiae* (Gorman and Paskewitz 2000), the fruit fly *D. melanogaster* (Boman, Ingrid, and Bertil 1972; Hotson and Schneider 2015) and the yellow mealworm beetle *Tenebrio molitor* (Haine et al. 2008). These experimentally-induced infections can persist for at least 28 days in both *T. molitor* (Haine et al. 2008) and *D. melanogaster* (Kutzer, Kurtz, and Armitage 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, and Bertil 1972; Chambers et al. 2019; Kutzer and Armitage 2016; Kutzer, Kurtz, and Armitage 2019; Duneau et al. 2017; Hotson and Schneider 2015; Dionne et al. 2006; Brandt et al. 2004). Persistent infections could be influential because the inability to clear an infection will result in more infected individuals in a population, and may thereby potentially increase pathogen transmission rate. In the chronic infection phase in *D. melanogaster*, the bacterial

load has been shown to stabilise around a relatively constant pathogen load over time (Duneau et al. 2017; Hotson and Schneider 2015), which has been termed the set point bacterial load (SPBL; Duneau et al. 2017), after the set point viral load (e.g., Regoes 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 et al. 2008; Zanchi, Johnston, and Rolff 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 is relatively uncommon in insects and may be related to the costs and benefits of immune system activation, and the costs of 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. On the one hand, if the damage caused by an infection is low, and the costs of mounting an immune response exceed the benefit of clearing the infection, one might predict a host to manage a persistent infection (Lazzaro and Rolff 2011). On the other hand, if the damage or virulence is low and the clearance costs are low, then the pathogen might be cleared in some instances. For example *E. coli* and *Erwinia carotovora*, species that did not lead to death in *D. melanogaster*, were cleared in 22 % and 8 % of flies, respectively (Duneau et al. 2017). Furthermore, *E. coli* infections of the mosquito, *Aedes aegypti*, were cleared in 2 % of cases (Hillyer et al. 2005). *Micrococcus luteus* infections of *Anopheles gambiae* were all cleared, although *A. gambiae* was not able to clear *E. coli* (Gorman and Paskewitz 2000). In contrast, Duneau et al (2017) found that infections with bacteria of intermediate virulence, *Providencia rettgeri* and *Enterococcus faecalis*, were never cleared. For bacteria of high virulence, one might predict selection for a fast and efficient early clearance of the infection: Individuals who do not clear the infection will die in the acute phase because even a low dose of the pathogen can be deadly, and longer-term survivors will have cleared the infection early on and thus will be uninfected. Tentative support for this hypothesis comes from *Pseudomonas entomophila* infections of *D. melanogaster*, where the few individuals surviving until 28 days post injection were all uninfected (Kutzer, Kurtz, and Armitage 2019).

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 et al. 2019). Not only is dose-dependent survival frequently reported in response to bacterial infections (Louie et al. 2016; Miller and Cotter 2017; Chambers et al. 2019), but bacterial load later in the infection has been demonstrated to correlate with the initial inoculation dose (Duneau et al. 2017; Chambers et al. 2019). One could predict that lower inoculation doses are more likely to be cleared, because a smaller bacterial population is likely to be more susceptible to the host immune defences, but the extent to which this kind of pattern is generalizable is unknown.

Virulence will be influenced by both host and parasite traits, i.e., it depends on defence 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 host exploitation, that is an increase in virulence is a side effect of an increase in pathogen load (Råberg 2014; Råberg and Stjernman 2012). However, variation in virulence could also be due to differences in per-parasite pathogenicity, whereby the slopes of the reaction norms linking infection intensity (pathogen load) and host fitness differ across parasite strains (Råberg 2014; Råberg and Stjernman 2012). A parasite genotype causing a steeper negative slope across a range of infection intensities, suggests higher per-parasite pathogenicity compared to a parasite genotype infection resulting in a shallower slope. Here we use the concepts of host exploitation and per-parasite pathogenicity to disentangle the causes of variation in virulence caused by infection with different bacterial species.

Here we first injected flies with four candidate bacterial species at a range of infection doses to test whether they varied in virulence, which was measured as survival after bacterial injection. We then asked whether all four bacterial species establish a persistent infection by assessing chronic infection status up to 35 days post injection, d. Third, given evidence from different studies that both low and more virulent bacteria can be cleared but to a limited degree, we set out to assess whether clearance in living flies is affected by bacterial virulence, whether there is a dose threshold below which the host can clear the infection, and if time post infection affects the infection outcome. Fourth, by assessing the infection status of flies that had died up to two and a half months post-infection, 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. Lastly, we asked whether differences in bacterial virulence are due to variation in parasite exploitation (infection intensity) or due to variation in per-parasite pathogenicity.

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 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 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 burhodogranaria* 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 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; Kutzer, Kurtz, and Armitage 2018, 2019; Duneau et al. 2017; Hanson 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, and Armitage 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 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 colony forming units (CFUs).

The experimental flies were reared at constant larval density for 1 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 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 microliters 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. Preliminary tests showed that three droplets gave a similar mean value to counting eight droplets per fly (25,333 ± 390 CFUs per fly for eight droplets and 27,000 ± 1053 CFUs per fly for three droplets; n = 12). Additional tests on the detection of bacteria in homogenised flies, we found that our lower detection limit was five colony-forming units per fly. The plates were incubated at 30 ºC and the numbers of CFUs were 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, which was usually between 10 and 60 CFUs per droplet.

We did not use bacteria with antibiotic resistance, and consequently selective medium for bacterial growth. Therefore, in theory, bacteria other than the infecting species could grow on the plates that we use to incubate the fly homogenate. However, by homogenizing control flies (Ringer's injected and naïve) we showed that foreign CFUs grew infrequently. 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, and Clark 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. burhodogranariea* (7 out of 381 flies) and *Ps. entomophila* (1 out of 71 flies) there were too many CFUs to count at the highest dilution. In these cases, we denoted these flies as having the highest countable number of CFUs found in any fly for that bacterium and at the highest dilution. 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. Similarly, 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: "grid", "gridExtra" (Baptiste 2017), "ggplot2" (Wickham 2016), "scales" (Wickham and Seidel 2020) and "survival" (Therneau 2020; Therneau and Grambsch 2000). 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 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).

2.7.1. How does infection dose affect survival, and do the bacteria vary in virulence?

To analyse survival after injection, fly survival until death was analysed using day of death as the response variable. All flies were followed until death, therefore we have no censored cases. In Model 1A, we tested whether bacterial injection had a negative effect on survival compared to injection with the Ringer's injection control alone, and whether the Ringer's differed significantly from the survival of the naïve group; this analysis was done using all treatment groups (naïve, Ringer's and all bacterial doses). In Model 1B, we tested whether bacterial injection dose affected survival in a dataset where we excluded the two control groups. We then compared the survival of all bacterial doses against each other to test whether survival responds to dose. To account for the increased likelihood of type I errors due to multiple testing, we corrected the p-values with the Benjamini and Hochberg (1995) method in R using p.adjust and the false discovery rate ("fdr"). We also noted the scale parameters from Model 1B, which indicate whether the risk of death (hazard) decreases or increases with age (Crawley 2007). Treatment, person and experimental replicate were included as factors in both models. We first used Cox proportional hazard models and the "survival" package (Therneau 2020; Therneau and Grambsch 2000), but for all bacteria, neither Model 1A nor Model 1B fulfilled the assumptions of proportional hazards over time when tested using cox.zph. We therefore stratified the factor that least well fulfilled the assumptions of proportional hazards, which allows for a different baseline hazard function for each level of that factor. However, after stratification, the assumptions of proportional hazards over time were still not met for one or both of the models. Therefore, we used accelerated failure-time models using survreg and with

an extreme error distribution for *E. cloacae*, a lognormal error distribution for *Pr. burhodogranariea* and a loglogistic error distribution for *L. lactis* and *Ps. entomophila*.

Model 1A, B: day of death ~ treatment + person + replicate

2.7.2. Is there a set point bacterial load for each bacterium?

To be able to test whether there is a set point bacterial load across different bacterial species, we generated datasets for each infection species that contained the same doses and days post injection, and where there was a minimum of four flies for each dose/day post injection combination (fly mortality led to lower sample sizes for later time points). This allowed us to examine days 1 to 14 and injection doses 1840 and 9200 CFU for *E. cloacae*, *Pr. burhodogranariea* and *L. lactis*. We removed all flies that had 0 CFU as they are not informative for this analysis. To allow us to compare our findings to Duneau *et al* (2017), similarly to their analyses, we carried out linear regressions. The response variable was natural log transformed bacterial load and separate models were carried out for each bacterial species. Days post injection at which the fly was homogenised was included as a covariate, and injection dose, replicate and person were fitted as fixed factors. We also fitted the interaction between injection dose and days post injection.

Model 2: log(bacterial load) ~ days post injection + injection dose + days post injection × injection dose + replicate + person

2.7.3. Does injection dose correlate with the bacterial load at four- or seven-days post injection?

We tested whether initial injection dose is a predictor of the bacterial load for the bacterial species that we tested, at both four- and seven-days post injection. Similarly, to the set point bacterial load analysis, 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 either four- or 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 only two flies survived to day four and all flies were dead by seven days post injection.

Model 3: log(day 4 or 7 bacterial load) ~ log(injection dose) + replicate + person

2.7.4. Is the ability of living flies to clear an infection affected by injection dose or time post injection?

Using a binomial logistic regression, we tested whether initial injection dose or time post injection affected the propensity for flies to clear an infection with *E. cloacae* (note that this is indistinguishable from an infection that is below our detection limit). 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, and days post injection at which the fly was homogenised, replicate and person were fitted as fixed factors. We also fitted the interaction between injection dose and days post injection. *L. lactis* injected flies were not analysed because only 11 out of 301 (3.7 %) cleared the infection. Although more *Pr. burhodogranariea* injected flies cleared the infection (45 out of 381 [11.8 %]) compared to *L. lactis*, a number of doses/days had zero clearance so these flies were not statistically analysed. *Ps. entomophila* infected flies were not statistically analysed because of 100 % mortality by day seven post-injection.

Model 4: CFU presence/absence$_{live}$ ~ days post injection + log(injection dose) + days post injection × log(injection dose) + replicate + person

2.7.5. 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, 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.* We did not fit dpi as a factor because of the unequal samplings across days. *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).

Model 5: CFU presence/absence$_{dead}$ ~ log(injection dose) + replicate + person

2.7.6. 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 6: cbind(dead uninfected, dead infected) ~ cbind(live uninfected, live infected)

2.7.7. Are virulence differences due to variation in parasite exploitation or per-parasite pathogenicity?

To test whether the bacterial species vary in per-parasite pathogenicity, 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 per-parasite pathogenicity. The package "emmeans" (Lenth 2020) was used to test which of the reaction norms differed significantly from each other. The hazard function in survival analyses gives the instantaneous failure rate, and the maximum hazard gives the point at which this rate is highest. We extracted maximum hazard values from time of death data for each bacterial species/dose/replicate. 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). Each maximum hazard per species/dose/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 uses 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 four values (1, 2, 3 and 5) for the smoothing parameter, *b*, which was specified using "bw.grid" (Moore 2016). We present the results from bw.grid=2, but all of the other values gave qualitatively similar results (see results). 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 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/replicate we therefore calculated the geometric mean of infection intensity combined for days 1 and 2 post injection. This was done using the R packages "dplyr" (Wickham 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 bw.grid 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.*

Model 7: log(maximum hazard) ~ log(geometric mean bacterial load) × 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. Similarly, 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/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.*

Model 8: log(geometric mean bacterial load) ~ bacterial species

Results

3.1. Bacterial species vary in virulence and dose can affect survival

As predicted, the bacterial species chosen for infection showed a range of virulence (Figure 1), with *E. cloacae* being the least virulent and *Ps. entomophila* being 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.

There was a significant effect of treatment on *E. cloacae* injected and control flies (Table 1; Figure 1A). Ringer's injected flies had higher survival than the 920 and 92000 CFU doses (Table S1), and there was no significant survival difference between the Ringer's injected flies and the naïve group or the remaining three bacterial doses (Table S1). In the analysis where the two controls were excluded, there was no significant effect of treatment (Table 1), indicating that survival was not dependent upon *E. cloacae* injection dose. The scale parameter of the analysis containing only the bacterially-injected flies was 16.3, indicating that the risk of death increased with age.

There was a significant effect of treatment on *Pr. burhodogranariea* injected and control flies (Table 1; Figure 1B). Ringer's injected flies had a higher survival than all bacterially-injected flies (Table S1). There was no significant survival difference between the Ringer's injected and naïve flies (Table S1). In the analysis where the two control groups were excluded, survival was dependent upon injection dose (Table 1). After correcting for multiple testing, comparisons of all injection doses with each other, showed that 92 CFU had higher survival than all doses except 920 CFU, and that 920 CFU had higher survival than 9200 and 92000 CFU (Table S2). The scale parameter of the analysis containing only the bacterially-injected flies was 1.49, which being more than one, indicates that the risk of death increased with age.

There was a significant effect of treatment on *L. lactis* injected and control flies (Table 1; Figure 1C). Ringer's injected flies had a higher survival than all bacterially-injected flies (Table S1). There was no significant survival difference between the Ringer's injected and naïve flies (Table S1). In the analysis where the two control groups were excluded, survival was dependent upon injection dose (Table 1). After correcting for multiple testing, comparisons of all injection

doses with each other, showed that apart from comparisons between 1840 and one dose either side of it, all survival curves differed significantly from each other (Table S3). The scale parameter of the analysis containing only the bacterially-injected flies was 0.543, which being less than one, indicates that the risk of death decreased with age.

There was a significant effect of treatment on *Ps. entomophila* injected and control flies (Table 1; Figure 1D). Ringer's injected flies had a higher survival than all bacterially-injected flies (Table S1). There was no significant survival difference between the Ringer's injected and naïve flies (Table S1). In the analysis where the two controls were excluded, survival was partly dependent upon injection dose (Table 1). After correcting for multiple testing, comparisons of all injection doses with each other showed that 92 CFU had significantly higher survival than all other doses except for 1840 CFU (Table S4). None of the other doses differed significantly from each other (Table S4). The scale parameter of the analysis containing only the bacterially-injected flies was 0.285, indicating that the risk of death decreased with age.

3.2. All bacterial species established persistent infections

By homogenising living flies we found that the two bacterial species with lower virulence, *E. cloacae* (Figure 2A) and *Pr. burhodogranariea* (Figure 2B) were able to persist inside the fly until at least 35 days post injection. 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 (data not shown).

3.3. Not all bacteria establish a set point bacterial load

There was a relatively stable bacterial load across for time for infections with *E. cloacae* and *L. lactis* (Table 2, non-significant effect of time post injection; Figure 3), suggesting that these species show evidence of a set point bacterial load when injected at a dose of 1840 or 9200 CFUs. In contrast, *Pr. burhodogranariea* bacterial load tended to decrease over time (Table 2; Figure 3), suggesting that there was no strict set point bacterial load under these conditions. *E. cloacae* was the only species to show a significant effect of injection dose, where an initial dose of 9200 resulted in a higher load than an initial dose of 1840 (Table 2; Figure 3).

3.4. Injection dose correlates with the bacterial load at four- and seven-days post injection

At four- and seven-days post injection, *E. cloacae* (Figure 4A & B) and *Pr. burhodogranariea* (Figure 4C & D) loads were significantly positively correlated with the initial injection dose (Table 3). *L. lactis* loads showed no significant correlation with the initial injection dose (Figure 4E & F), but there was a significant effect of replicate on *L. lactis* load at day four (Table 3). We hypothesised that the lack of significant relationship between *L. lactis* load and injection dose might be due to our underestimation of the load of some flies injected with this bacterial species: this is because some flies had too many CFUs to count even at the lowest dilution, and they were therefore assigned a maximum bacterial load value (see methods), which was necessarily lower than their actual load. When we excluded the four flies at day four and the two flies at day seven that had been assigned the maximum value, the relationship became significant for both days (Day 4: $F_{1,42} = 5.61$; $p = 0.023$; Day 7: $F_{1,35} = 4.59$; $p = 0.039$).

3.5. Infections in living flies can be cleared, and lower doses of E. cloacae *are cleared more quickly than higher doses*

The rate at which *E. cloacae* infections were cleared depended upon the initial injection dose (day post injection × injection dose: Table 4), whereby lower injection doses were cleared more quickly than higher doses (Figure 5A). Although we note that we cannot discriminate between flies that had cleared the infection and those where the infection was below our detection limit. In addition, there was dose-dependent clearance whereby lower doses were more likely to be cleared (Table 4). The other three bacterial species could not be statistically analysed (see methods), but for all species and all doses, there was at least one fly that cleared the infection (Figure 5B-D). 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.

3.6. 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 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 5E-H). 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 (data not shown). For the two injected bacterial species that we were able to statistically analyse, *E. cloacae* and *Pr. burhodogranariea*, lower initial injection doses were more likely to be cleared before death than higher injection doses (Figure 5E & F; Table 5). 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.

3.7. 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 S1; LR = 7.11, df = 2,17, p = 0.0285).

3.8. Differences in virulence are due to variation in parasite exploitation and per-parasite pathogenicity

Bacterial species varied significantly in exploitation of their hosts ($F_{2,35}$ = 35.90; p < 0.0001; Figure 6A). The least virulent bacterium, *E. cloacae*, had a significantly lower infection intensity, and thereby lower host 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 differ in their per-parasite pathogenicity (infection intensity × bacterial species: $F_{2,39}$ = 7.35, p = 0.0020; Figure 6B). *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 per-parasite pathogenicity 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 per-parasite pathogenicity 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 S2).

Discussion

In this study we demonstrate that sustained chronic infection and clearance are both possible outcomes for bacteria showing a range of virulence when they infect female *D. melanogaster*. We show that lower injection doses could be cleared more quickly than higher doses, but that bacteria of all species could persist inside the host for at least 75 days. Furthermore, we show that bacterial virulence differences can be explained by a combination of variation in host exploitation and per-parasite pathogenicity.

4.1. Bacterial species vary in virulence and dose can affect survival

In line with our predictions, the four bacterial species used in this study covered a broad spectrum of virulence, where virulence is defined as a reduction in host survival. The virulence gradient – from low to high – E. *cloacae*, *Pr. burhodogranariea*, *L. lactis* and *Ps. entomophila*, is complemented by the scale parameter of the survival analyses, which was highest in *E. cloacae*, indicating that death increased with age, and lowest in *Ps. entomophila*, indicating death decreased with age. Survival was not dose-dependent for the least virulent bacterium, *E. cloacae.* Chambers *et al.* (2019) showed similar results for *D. melanogaster* injected with *Enterococcus faecalis*, where there was little mortality over a similar injection dose range to the one used in this study. Survival was only partly dose-dependent for the most virulent pathogen, *Ps. entomophila*, likely due to swift killing of the host. However, the bacteria of intermediate virulence, *Pr. burhodogranariea* and *L. lactis*, had stronger dose-dependency over the injected range. Dose-dependent survival is in accordance with numerous studies across host-pathogen systems (e.g., Louie et al. 2016; Miller and Cotter 2017; Chambers et al. 2019).

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 test 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 in insects (28 days: Haine et al. 2008; Kutzer, Kurtz, and Armitage 2019).

It is unclear how the bacteria are able to persist for so long inside the host. One hypothesis is that the bacteria may be able to survive inside the insect haemocytes, as has been demonstrated for *Salmonella typhimurium* (Shinzawa et al. 2009) and *S. aureaus* (McGonigle, Purves, and Rolff 2016). The bacteria may also be able to persist in biofilms, i.e., multicellular aggregations, where the bacteria are embedded in a self-produced matrix of extracellular polymeric substances, such as polysaccharides, proteins and nucleic acids (Flemming and Wingender 2010). Biofilms can cause chronic infections such as *Pseudomonas aeruginosa* in cystic fibrosis patients (Høiby et al. 2010), and oral infection of *D. melanogaster* with *Ps. aeruginosa* resulted in biofilm production in the crop (Mulcahy et al. 2011). Both *E. cloacae* and *L. lactis* are able to produce biofilms *in vitro* (Nyenje, Green, and Ndip 2013; Chodorski et al. 2020, respectively). It is unknown whether *Pr. burhodogranariea* produces biofilms, but there is evidence that *Providencia stuartii* can form biofilms *in vitro* and in humans (El Khatib et al. 2017), and it is unknown whether *Ps. entomophila* forms biofilms.

4.3 Not all bacteria establish a set point bacterial load

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.* (2017) found remains stable until at least ten days post injection for *Pr. rettgeri*. Two of the species that we tested, *E. cloacae* and *L. lactis*, showed relatively stable loads across the first 14 days post infection for inoculation doses of 1840 and 9200 CFUs, which lends support to the SPBL concept. In addition, Duneau *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 SPBL of tens to hundreds of bacteria, and high virulence *L. lactis* had a SPBL of tens of thousands of bacteria. This finding is supported by the virulence decomposition analysis (see section 4.6), which shows that as virulence increases, so does host exploitation of the host over the first couple of days post infection. Therefore, more virulent bacteria have higher initial proliferation rates as shown by host exploitation, and given that the infection load stays relatively constant

in the longer term (SPBL), the initial proliferation differences explain the relationship between SPBL and virulence.

In contrast to the aforementioned two bacterial species, *Pr. burhodogranariea* load showed a shallow but significant decline over time. A significant reduction in bacterial load over time has been documented for *S. aureaus* infections of *T. molitor* (Haine et al. 2008; Zanchi, Johnston, and Rolff 2017). Duneau *et al.* (2017) hypothesised that different dynamics after infection in *D. melanogaster* and *T. molitor* could be due to the two host species utilising the different arms of the immune response, i.e. humoral and cellular defences, to differing degrees. However, *L. lactis* infections in a different *D. melanogaster* host population to the one used in the current study also declined over the course of a week post-injection (Kutzer and Armitage 2016), and we note the caveat that *L. lactis* loads in the current study were in some cases too numerous to count for days two to four post-injection, therefore these days will be underestimated and may hide a negative relationship. Further work is necessary to uncover the factors influencing the stability of bacterial loads.

4.4. Injection dose correlates with the bacterial load at four and seven days post injection

The bacterial load at days four and 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 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 et al. 2017*; E. faecalis, Pr. rettgeri* and *S. marcescens*: Chambers et al. 2019). It has been suggested that the SPBL will remain at around the bacterial load at which the infection was controlled (Chambers et al. 2019; Duneau et al. 2017). Given that insects can show dose dependent inducible immune activation (Jent 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 et al. 2019), one could hypothesise that these AMPs are to some degree involved. However, the mechanisms that allow a dose-dependent persistent infection have yet to be uncovered. Unfortunately, it was not possible to test *Ps. entomophila* given its high mortality during the acute infection phase.

4.5 Bacterial infections with different levels of virulence can be cleared

At least two predictions can be made concerning the clearance of low virulence bacteria: if the damage that they cause is low and the clearance costs are low then the pathogen may be cleared, but if the damage is low and the costs of clearing the infection are greater than the benefits, then a persistent infection might ensue. Similarly, to other studies on low virulence infections (Hillyer et al. 2005; Gorman and Paskewitz 2000; Duneau et al. 2017), some individuals (~39 %) were able to clear an *E. cloacae* infection whilst others remained infected. 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. As we had predicted, the likelihood of clearing *E. cloacae* was dose dependent, but interestingly it also depended upon the time after the initial injection: lower doses were cleared more quickly than higher doses. This finding of dose-dependent clearance, whilst it may not be 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 et al. 2017), perhaps stochasticity plays a role in the clearance of bacteria, particularly where infection loads are low such as in *E. cloacae*, for example through variation in expression of Drosocin.

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 et al. 2017). Our data challenge this finding, given that the three more virulent bacteria all appear to be clearable to differing degrees, including within the first seven days of infection. The combined effects of dose and time after infection could not be statistically analysed for *Pr. burhodogranariea*, *L. lactis* and *Ps. entomophila*, because the number of flies that cleared their infection were relatively low (see results section 3.5), nonetheless our results indicate that a persistent infection is not inevitable. These findings are supported by the observation that seven to ten days post injection, a "small number" of *D. melanogaster* had cleared infections with *Pr. burhodogranariea* (Galac and Lazzaro 2011), although Duneau *et al.* (2017) did not find clearance of the same bacterium. Similarly to *E. cloacae*, inoculation dose may play a role in clearance given that we predominantly observed clearance in the lowest inoculation dose. Duneau *et al.* (2017) injected around 30,000 *Pr. burhodogranariea* CFUs per fly, which lies between our two highest doses, for which we saw clearance in just three flies. It is also worth noting that our study used females, whereas Duneau *et al.* (2017) used males. Similarly to the current study, Kutzer &

Armitage (2016) also found that a few female flies, inoculated with a dose of *L. lactis* in common with this study (1,840 CFU), cleared the infection (3 out of 141; 2.1 %).

Lastly, we predicted that there may be selection for a fast and efficient early clearance of infection by the bacterium with high virulence, *Ps. entomophila*. The high level of pathogenesis caused by this bacterium is explained by the production of a pore-forming toxin called Monalysin (Opota et al. 2011) in association with activation of stress-induced pathways and an increase in oxidative stress (Chakrabarti 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, and Lemaitre 2013). If similar pathologies are induced in the haemocoel after infection, contrarily to other bacterial species, sustaining a chronic bacterial load might not be a viable option in the face of high levels of tissue damage. Instead the fly host might activate a stronger immune response that ideally clears the infection (Lazzaro and Rolff 2011; Moreno-García et al. 2014). Liehl *et al.* (2006) demonstrated the immune response to *Ps. entomophila* is mostly local, suggesting that the fly might invest in a stronger and targeted response, but also try to limit the immunopathology to the smallest area possible. Although clearance of this bacterium was possible, the majority of survivors remained infected (just under 80 %), and mortality was too high to assess clearance for longer than four days post injection. Interestingly, selected individuals from the ancestral population to the one used in this study, also showed clearance of *Ps. entomophila* five days after infection (Martins et al. 2013). The ability to resist *Ps. entomophila* infection showed genotypic variation (Kutzer, Kurtz, and Armitage 2018), and so may the ability to clear infections: for example, individuals from one outbred, and three out of nine inbred, fly lines contained a proportion of flies that cleared a *Ps. entomophila* infection one day post injection, whereas none of the remaining six inbred lines had flies with zero infection load (Kutzer, Kurtz, and Armitage 2018).

Even though dead flies were sampled for a longer period post-inoculation (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: dead flies showed dose dependency in clearance for those species that we could statistically test, whereby lower inoculation doses of *E. cloacae* and *P. burhodogranariea* were more likely to be cleared before death than higher doses. Once again, comparatively few dead individuals had cleared *L. lactis* infections, whereas proportionally more had cleared *Ps. entomophila.* 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) remains constant even after many weeks of infection (Duneau et al. 2017). 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 S1) 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. The generalisability of the observation that infection probability of dead individuals predicts that of live individuals, needs more investigation, in particular the infection status of individuals that died in the acute phase.

4.6. Differences in virulence are due to variation in parasite exploitation and per-parasite pathogenicity

Our survival data show that the infecting bacteria cover a range of virulence, from low to high. Therefore, in a final step, we aimed to decompose the causes of variation in virulence. We took the perspective of the pathogen, where variation in virulence across different genotypes (in this case bacterial species) infecting the same host, can be disentangled into two components: host exploitation and per-parasite pathogenicity (Råberg 2014; Råberg and Stjernman 2012).

Host 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 host exploitation varies across parasite genotypes (de Roode and Altizer 2010; e.g., monarch butterflies and their protozoan parasites: de Roode et al. 2008; *Daphnia magna* infected with the bacterium *Pasteuria ramosa*: Clerc, Ebert, and Hall 2015), and also, unsurprisingly, that it varies across parasite species infecting the same host genotype (Kutzer and Armitage 2016; Duneau et al. 2017; Chambers et al. 2019). Indeed, in the current study, all bacterial species tested showed significant differences in host exploitation, where bacterial load increased as virulence increased. Chambers 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, had we only examined host exploitation as a source of variation, we might have concluded that load alone explains the differences that we found in virulence.

Virulence is not only determined by the load that a pathogen attains: Råberg & Stjernman (2012) proposed that pathogen genotypes may also vary in per-parasite pathogenicity i.e., the

harm or damage caused per-parasite (Råberg 2014; Råberg and Stjernman 2012). Variation in per-parasite pathogenicity 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). For example, infection of rats with three different clones of the rodent malaria *Plasmodium chabaudi* uncovered variation in per-parasite pathogenicity (Råberg and Stjernman 2012), and De Roode & Altizer (2010) found that protozoan parasite strains infecting monarch butterflies, differed in per-parasite pathogenicity. Here we found a significant overall effect of per-parasite pathogenicity, whereby *Pr. burhodogranariea* and *L. lactis* had significantly more negative slopes compared to *E. cloacae*. This finding, combined with the host exploitation results, implies that *E. cloacae* is less virulent towards its host because of a combination of lower per-parasite pathogenicity and less exploitation. On the other hand, given that *Pr. burhodogranariea* and *L. lactis* showed similarly negative slopes for the relationship between parasite load and maximum hazard (our survival-related measure of the impact of the parasite on host health), it suggests that the variation in virulence between these two species is due to higher exploitation by *L. lactis*, rather than differences in per-parasite pathogenicity. These two sources of variation, host exploitation and per-parasite pathogenicity, have not frequently been explored in the same study, so it is generally difficult to ascertain the relative importance of the two sources of genetic variation. However, variation in per-parasite pathogenicity was demonstrated to explain more of the variance in virulence across HIV genotypes than did set point viral load (Bertels et al. 2018). Ours, and the results of others, suggest that per-parasite pathogenicity is an important component driving variation in virulence, and that disentangling its contribution towards virulence, in combination with the contribution of host exploitation, will undoubtedly help our mechanistic and evolutionary understanding of host-pathogen interactions. Future research will be needed to test the generality of our observation that the bacterium that had a higher prevalence of persistence (*L. lactis*), had higher virulence, higher PPP and higher host exploitation, whereas the bacterium that had lower persistence (*E. cloacae*) showed the opposite pattern.

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Tables and figures

Table 1. The effect of bacterial injection on survival. Model 1A includes all treatment groups and Model 1B includes only the flies injected with one of the 5 doses of bacteria. The person performing the injection and the experimental replicate were included as factors in the models. Statistically significant factors are in bold.

Injected bacterium	*Model*	*Tested effect*	*df*	*Chisq*	*p*
E. cloacae	*1A*	Treatment (with controls)	6	26.51	**0.00018**
		Person	1	1.89	0.17
		Replicate	2	32.60	**< 0.0001**
	1B	Treatment (without controls)	4	9.25	0.055
		Person	1	1.38	0.24
		Replicate	2	21.88	**< 0.0001**
Pr. burhodogranariea	*1A*	Treatment (with controls)	6	227.55	**< 0.0001**
		Person	1	7.64	**0.0057**
		Replicate	2	11.31	**0.0035**
	1B	Treatment (without controls)	4	27.50	**< 0.0001**
		Person	1	8.66	**0.0033**
		Replicate	2	8.12	**0.017**
L. lactis	*1A*	Treatment (with controls)	6	726.14	**< 0.0001**
		Person	1	3.98	**0.046**
		Replicate	2	0.21	0.90
	1B	Treatment (without controls)	4	100.51	**< 0.0001**
		Person	1	5.76	**0.016**
		Replicate	2	0.41	0.81
Ps. entomophila	*1A*	Treatment (with controls)	6	1687.88	**< 0.0001**
		Person	1	6.65	**0.0099**
		Replicate	2	11.37	**0.0034**
	1B	Treatment (without controls)	4	19.24	**0.00071**
		Person	1	5.09	**0.024**
		Replicate	2	13.54	**0.0012**

Table 2. Tests for whether there is a set point bacterial load. The effects of day post injection and injection doses 1840 and 9200 on bacterial load up to 14 days post injection were tested (Model 2). 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	DPI	1,90	3.08	0.083
	Injection dose	1,90	4.68	**0.033**
	DPI × Injection dose	1,90	0.13	0.72
	Person	1,90	0.13	0.72
	Replicate	2,90	2.99	0.055
Pr. burhodogranariea	DPI	1,132	9.22	**0.0029**
	Injection dose	1,132	2.18	0.14
	DPI × Injection dose	1,132	0.030	0.86
	Person	1,132	0.015	0.90
	Replicate	2,132	0.065	0.94
L. lactis	DPI	1,106	1.71	0.19
	Injection dose	1,106	0.23	0.63
	DPI × Injection dose	1,106	2.02	0.16
	Person	1,106	1.04	0.31
	Replicate	2,106	4.69	**0.011**

Table 3. The effect of initial injection dose on bacterial load at four and seven days post injection (Model 3). 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.

		Day 4			*Day 7*		
Injected bacterium	*Tested effect*	*df*	*F*	*p*	*df*	*F*	*p*
E. cloacae	Log(Injection dose)	1,28	27.08	**<0.0001**	1,25	26.41	**<0.0001**
	Person	1,28	0.85	0.36	1,25	0.16	0.69
	Replicate	2,28	2.70	0.085	2,25	1.78	0.19
Pr. burhodogranariea	Log(Injection dose)	1,50	39.24	**<0.0001**	1,47	37.33	**<0.0001**
	Person	1,50	1.64	0.21	1,47	0.23	0.63
	Replicate	2,50	0.45	0.64	2,47	2.11	0.13
L. lactis	Log(Injection dose)	1,46	3.94	0.053	1,37	3.81	0.058
	Person	1,46	0.42	0.52	1,37	0.71	0.40
	Replicate	2,46	5.65	**0.0064**	2,37	1.98	0.15

Table 4. The effect of day post injection (DPI) and injection dose on presence/absence of *E. cloacae* in live flies (Model 4). Experimental replicate and person performing the injection were also included as factors in the models. Statistically significant factors are in bold.

Tested effect	*df*	*Chisq*	*P*
DPI	8	15.26	0.054
Injection dose	1	5.00	**0.025**
DPI × Injection dose	8	18.45	**0.018**
Person	1	1.34	0.25
Replicate	2	14.14	**0.00085**

Table 5. The effect of injection dose on presence/absence of infection in dead flies (Model 5). 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	3.86	**0.049**
	Person	1	1.21	0.27
Pr. burhodogranariea	Log(Injection dose)	1	16.73	**<0.0001**
	Person	1	2.18	0.14
	Replicate	1	0.030	0.86

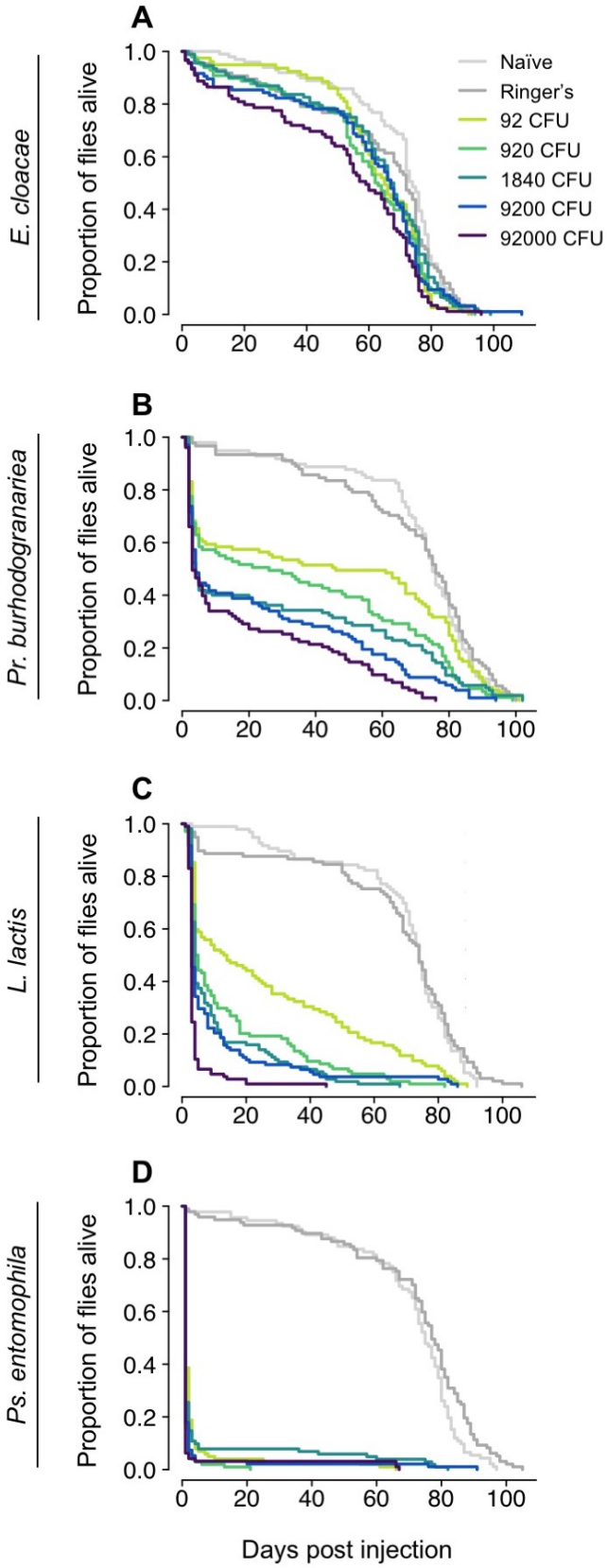


Figure 1. Fly survival after injection with one of four bacterial species **(A – D).** Controls were either injected with Ringer's solution, or received no injection (naïve). Each survival curve is from n = 79 to 108 flies. The injection dose legend for all panels is shown in **A**.

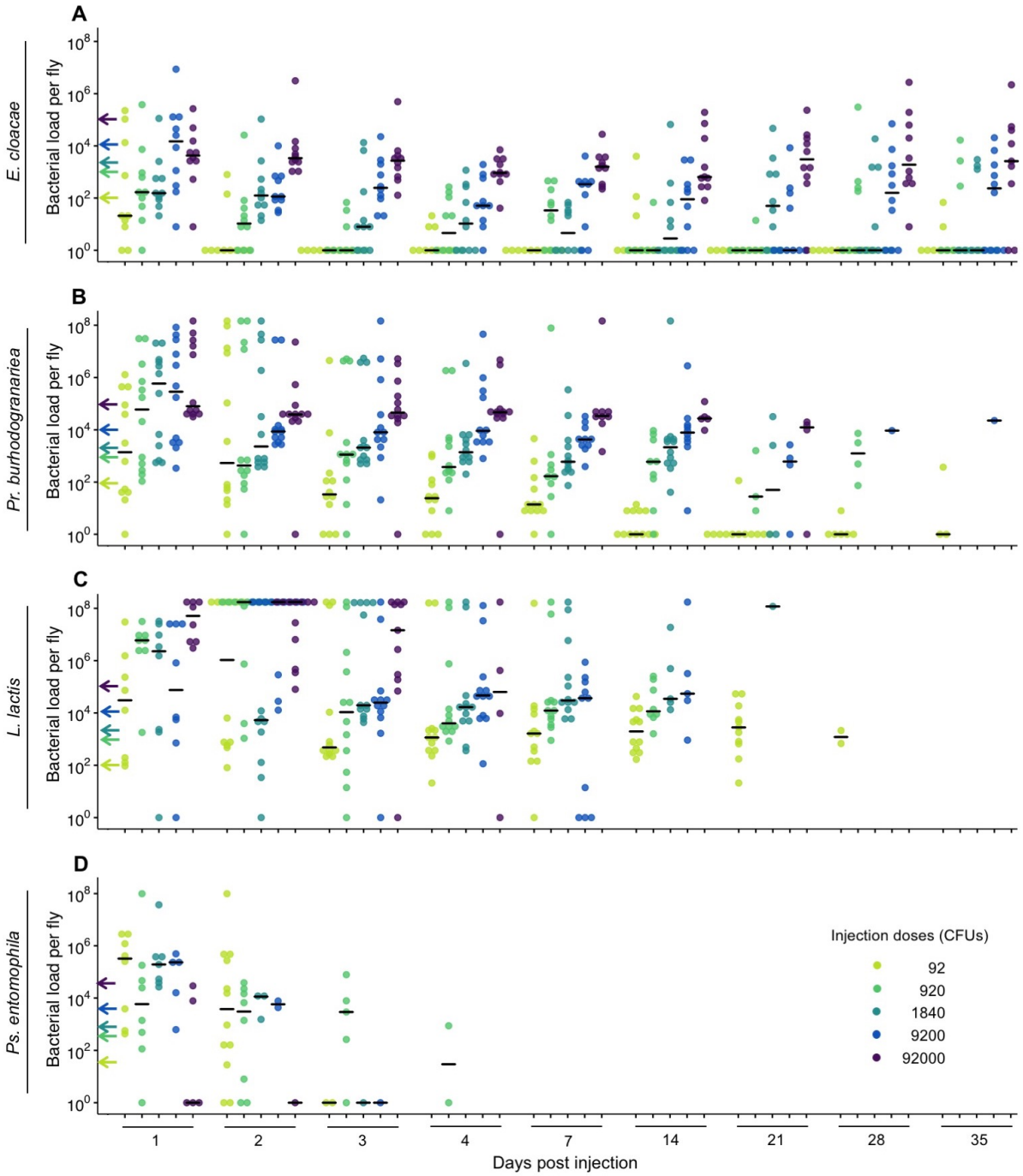


Figure 2. Bacterial load per living fly, after injection with one of four bacterial species (**A** – **D**)**.** Flies were homogenized at between 1 and 35 days post-injection. The injection dose legend for all panels is shown in **D**. The arrows on the y-axis indicate the approximate injection doses.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

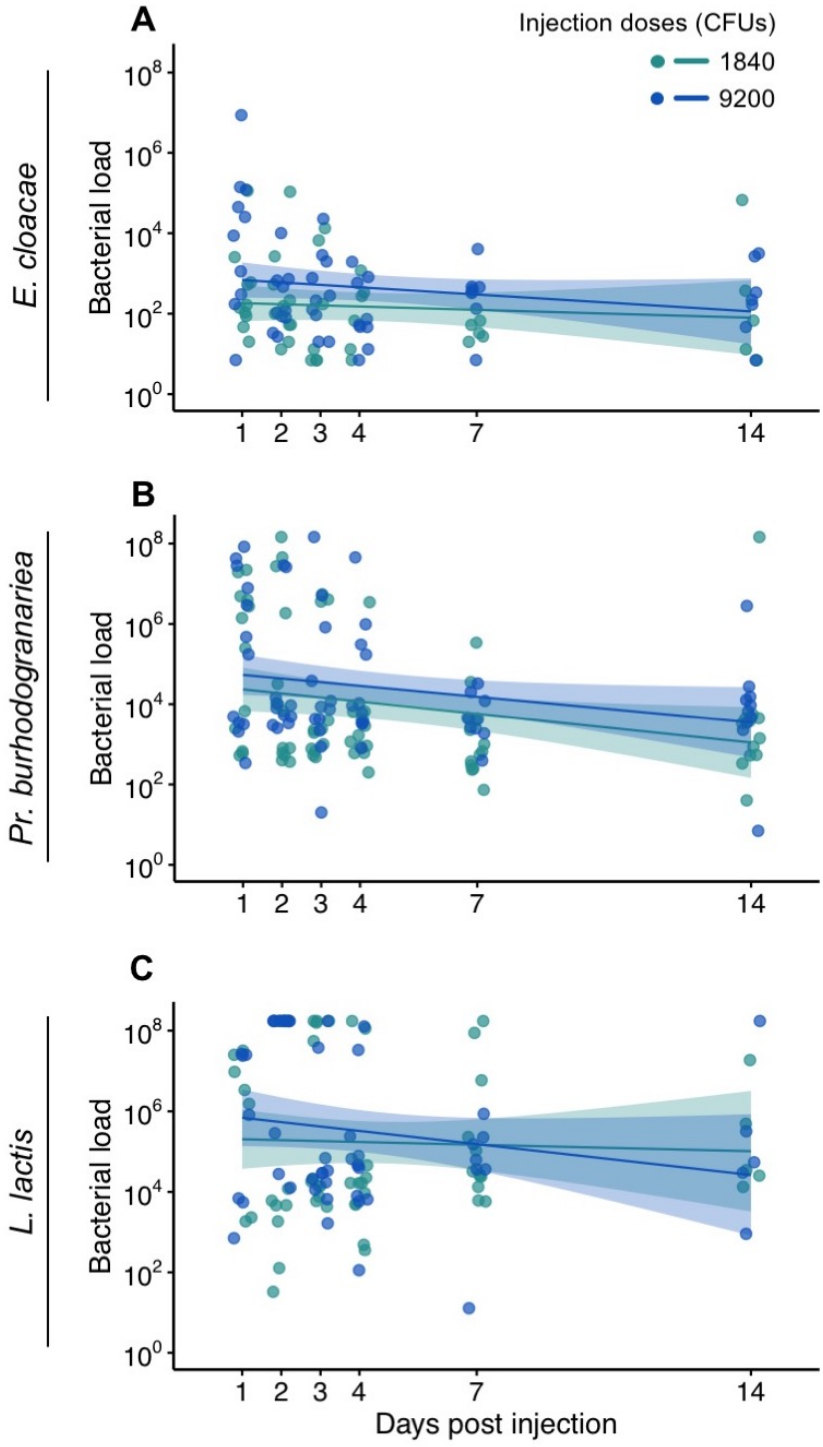


Figure 3. The relationship between bacterial load and day post injection for two injection doses (1840 and 9200 CFU) for three bacterial species (**A** – **C**). The injection dose legend for all panels is shown in **A.** Each circle is the bacterial load of one fly, and they are jittered along the x-axis to aid visualisation of overlapping data points. Flies with zero bacterial load are not shown (see methods). Linear regression lines are shown with 95 % confidence intervals.

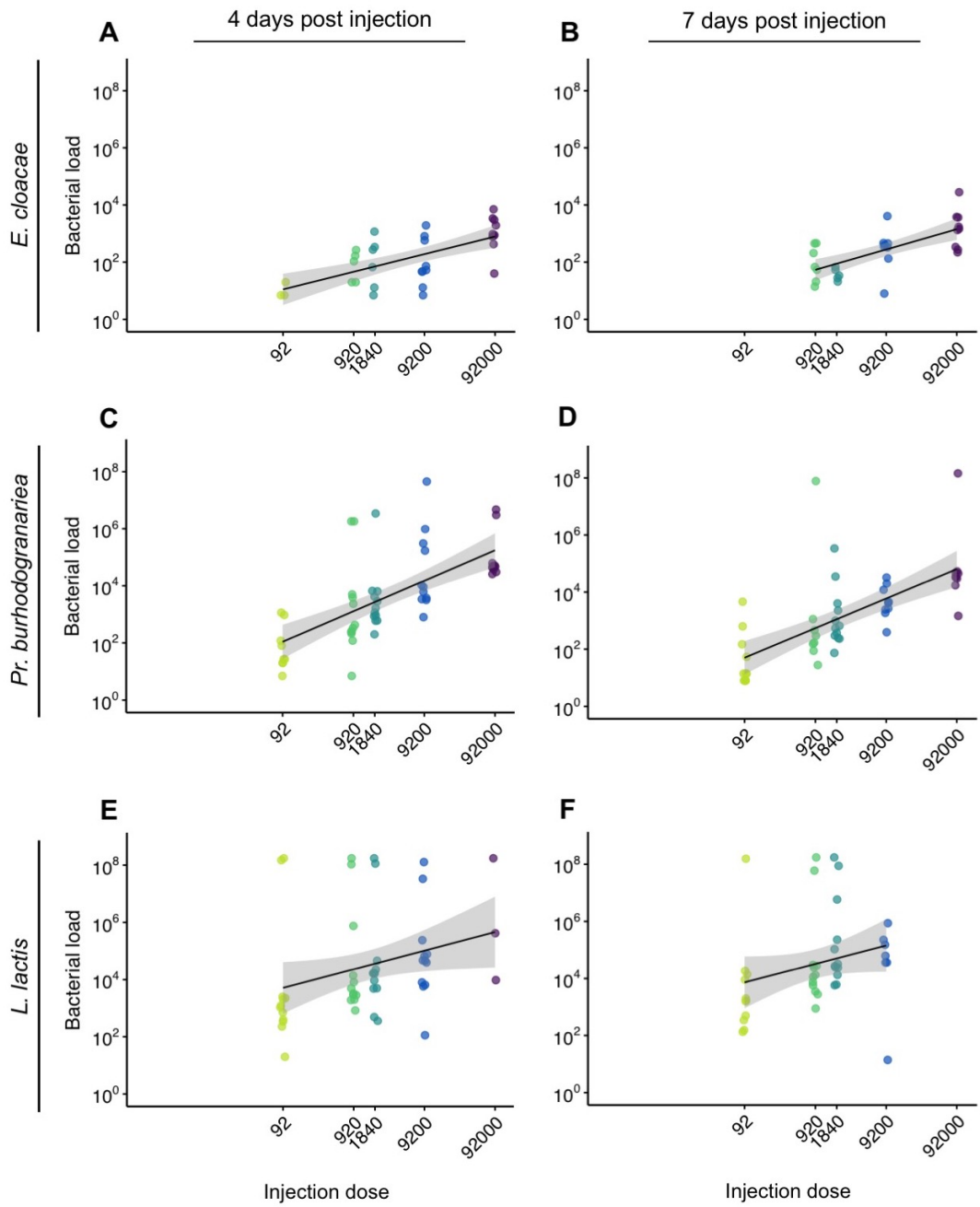


Figure 4. The relationship between bacterial load and the initial injection doses for three of the four injected bacterial species. Each row shows data from one bacterial species. The left- and right-hand columns show the bacterial load at four, and seven, days post injection, respectively. Panel **B** contains no flies injected with 92 CFUs because all flies had a bacterial load of zero at day seven; **F** 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.

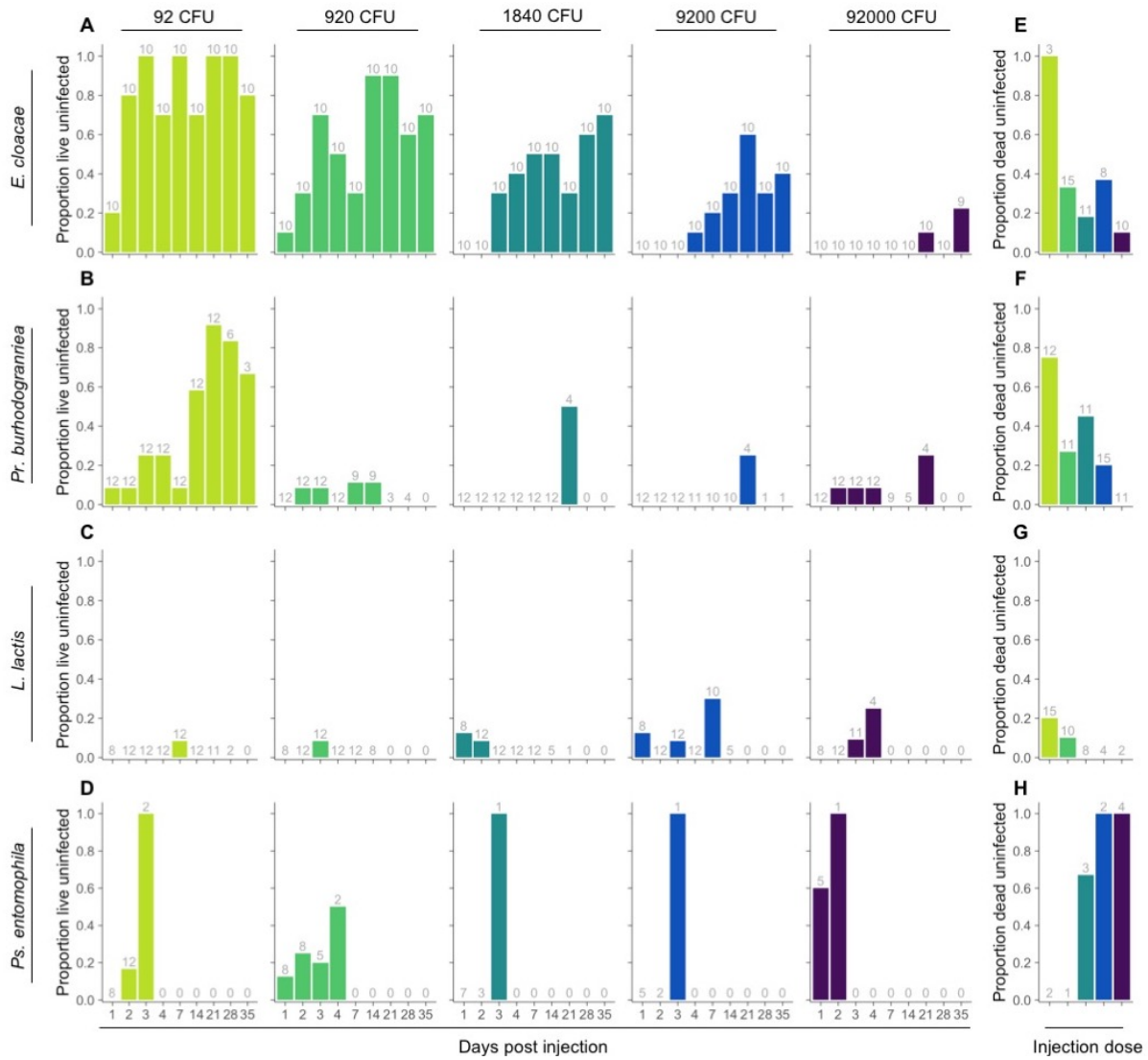


Figure 5. Bacterial clearance by living and dead flies. Each row shows flies that had been injected with one of four bacterial species. A-D The proportion of live flies that were uninfected. Each column shows a different injection dose. E-H The proportion of dead flies that were uninfected. The injection doses follow the same colours as shown in A-D. Dead flies were homogenised at between 14 and 35, and 56 and 78, days post injection. 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.

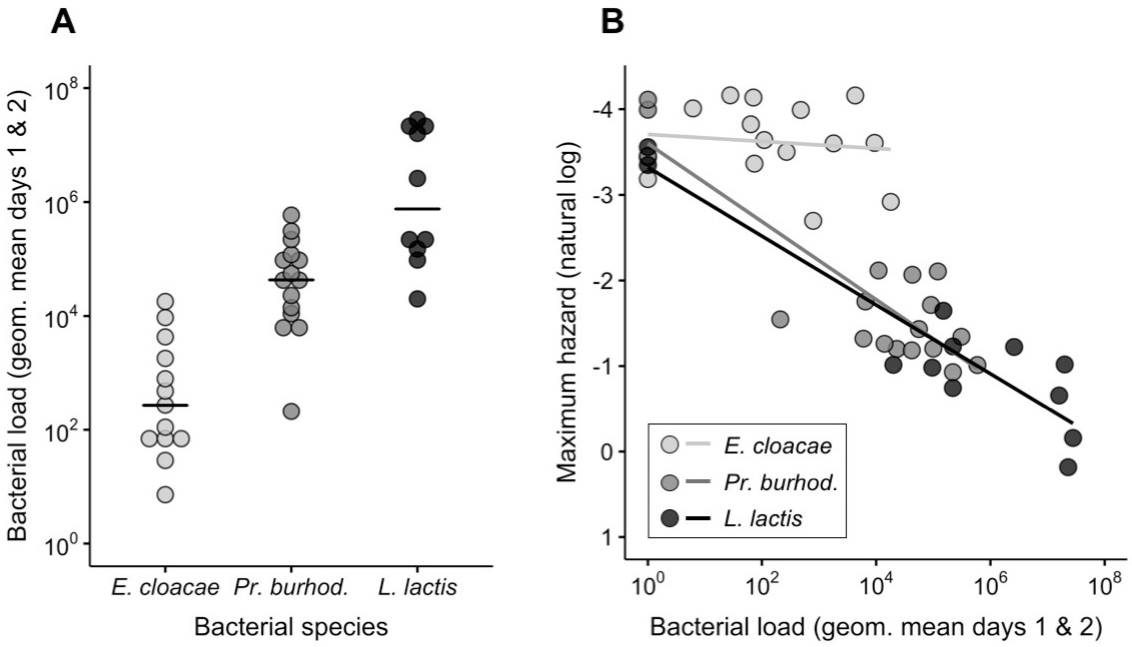


Figure 6. Virulence decomposition. **A**. Parasite exploitation given as infection intensity/bacteria load across bacterial species. Each data point is from one injection dose per bacteria, per experimental replicate, and gives the geometric mean of bacterial load for days 1 and 2 post injection. The circles are jittered along the x-axis to aid visualisation of overlapping data points. Black lines show medians. **B**. Per-parasite pathogenicity 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 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.

Supporting information

Table S1. The effect of bacterial injection on survival. Comparisons are shown between the Ringer's injected flies and all other treatment groups (Model 1A). Statistically significant comparisons are in bold.

Injected bacterium	*Treatment*	*z*	*P*
E. cloacae	Naïve	0.57	0.57
	92 CFU	-1.72	0.086
	920 CFU	-2.63	**0.0086**
	1840 CFU	-0.60	0.55
	9200 CFU	-1.75	0.081
	92000 CFU	-3.58	**0.00035**
Pr. burhodogranariea	Naïve	0.2	0.84
	92 CFU	-6.14	**< 0.0001**
	920 CFU	-7.13	**< 0.0001**
	1840 CFU	-9.7	**< 0.0001**
	9200 CFU	-9.96	**< 0.0001**
	92000 CFU	-11.35	**< 0.0001**
L. lactis	Naïve	0.41	0.68
	92 CFU	-11.6	**< 0.0001**
	920 CFU	-19.31	**< 0.0001**
	1840 CFU	-22.06	**< 0.0001**
	9200 CFU	-23.94	**< 0.0001**
	92000 CFU	-28.55	**< 0.0001**
Ps. entomophila	Naïve	-0.45	0.65
	92 CFU	-60.21	**< 0.0001**
	920 CFU	-69.20	**< 0.0001**
	1840 CFU	-64.92	**< 0.0001**
	9200 CFU	-67.42	**< 0.0001**
	92000 CFU	-70.60	**< 0.0001**

Table S2. The effect of *Pr. burhodogranariea* injection on survival. P-values are given from statistical comparisons between all bacteria-injected treatment groups. The top half of the table shows the original p-values, and the bottom half of the table shows the p-values after correcting for multiple testing using the false discovery rate (fdr). Statistically significant comparisons are in bold.

P-values		*92*	*920*	*1840*	*9200*
Original p-values	*92*				
	920	0.277			
	1840	**1.48×10^{-3}**	**0.048**		
	9200	**5.80×10^{-4}**	**0.025**	0.779	
	92000	**2.40×10^{-6}**	**5.20×10^{-4}**	0.117	0.200
fdr-corrected p-values	*92*				
	920	0.308			
	1840	**3.70×10^{-3}**	0.080		
	9200	**1.93×10^{-3}**	**0.050**	0.779	
	92000	**2.40×10^{-5}**	**1.93×10^{-3}**	0.167	0.250

Table S3. The effect of *L. lactis* injection on survival. P-values are given from statistical comparisons between all bacteria-injected treatment groups. The top half of the table shows the original p-values, and the bottom half of the table shows the p-values after correcting for multiple testing using the false discovery rate (fdr). Statistically significant comparisons are in bold.

P-values		*92*	*920*	*1840*	*9200*
Original p-values	*92*				
	920	**1.10×10^{-5}**			
	1840	**3.50×10^{-9}**	0.118		
	9200	**1.30×10^{-12}**	**4.10×10^{-3}**	0.179	
	92000	**$< 2 \times 10^{-16}$**	**6.9×10^{-9}**	**1.40×10^{-5}**	**2.90×10^{-3}**
fdr-corrected p-values	*92*				
	920	**2.20×10^{-5}**			
	1840	**1.17×10^{-8}**	0.131		
	9200	**6.50×10^{-12}**	**5.13×10^{-3}**	0.179	
	92000	**$< 2 \times 10^{-16}$**	**1.73×10^{-8}**	**2.33×10^{-5}**	**4.14×10^{-3}**

Table S4. The effect of *Ps. entomophila* injection on survival. P-values are given from statistical comparisons between all bacteria-injected treatment groups. The top half of the table shows the original p-values, and the bottom half of the table shows the p-values after correcting for multiple testing using the false discovery rate (fdr). Statistically significant comparisons are in bold.

P-values		*92*	*920*	*1840*	*9200*
Original p-values	*92*				
	920	**1.15×10^{-3}**			
	1840	0.0721	0.147		
	9200	**4.31×10^{-3}**	0.711	0.285	
	92000	**4.90×10^{-5}**	0.388	**0.0222**	0.223
fdr-corrected p-values	*92*				
	920	**5.75×10^{-3}**			
	1840	0.144	0.245		
	9200	**0.0144**	0.711	0.356	
	92000	**4.90×10^{-4}**	0.431	0.0554	0.318

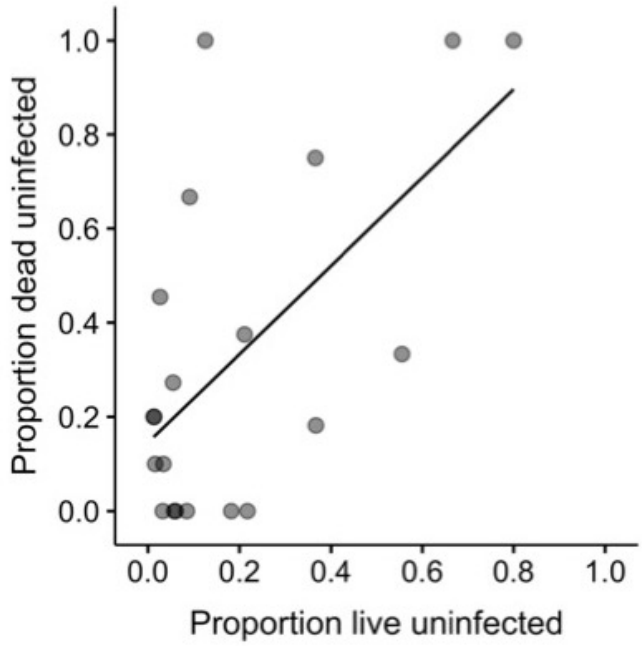
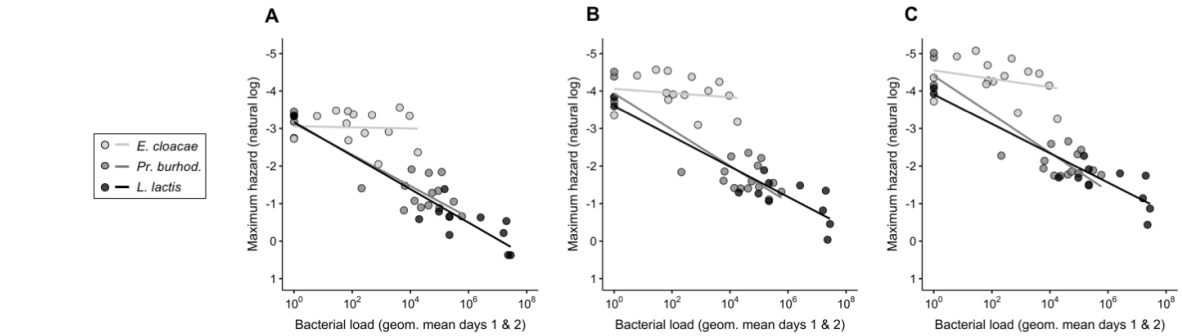


Figure S1. Proportion of live and dead flies that were uninfected across bacterial species and doses. Each data point is the proportion for one bacterial species and dose. Darker circles are due to overlapping data points. The black line shows the linear regression.



	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 S2. 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 for the smoothing parameter (1, 2, 3 and 5) as specified using"bw.grid". Shown above are **A.** bw.grid=1, **B.** bw.grid=3, **C.** bw.grid=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.

Chapter 4

Host tolerance and resistance dynamics using a branching model

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Abstract

Both resistance and tolerance contribute to the host health improvement through parasite reduction and damage control, respectively. Although the population interchange between the two strategies has been shown, how this kinetics affects the likelihood of the host to survive is poorly understood. Here, adapting a predictive model of infection outcome, we assessed how resistance and fecundity-tolerance differ across *Drosophila melanogaster* throughout infection phases and bacterial burden levels when infected with *Providencia burhodogranariea* or *Lactococcus lactis*. Our results indicate that resistance differs from acute to 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 differences in immune strategies, and particularly 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

A host can handle an infection by either limiting parasite burden, classified as resistance, or reduce its direct or indirect detrimental effects, named tolerance (Kutzer and Armitage 2016a; Råberg, Sim, and Read 2007). Resistance encompasses avoidance and reduction of the parasite burden (Restif and Koella 2004; Råberg, Graham, and Read 2009). Throughout the last decade, understanding the dynamics of these immune strategies has become of being importance. Resistance mechanisms present a production cost as they are often based on inducible components of the immune system (Moret and Schmid-Hempel 2000; Schmid-Hempel 2005; Alves et al. 2019; I. F. Miller and Metcalf 2019). These costs are recurrently evident in trade-offs with other life-history traits (Lawniczak et al. 2007), such as reproductive fitness (Short and Lazzaro 2010; Fedorka et al. 2007; Naim et al. 2020; Gwynn et al. 2005). Differently from resistance, tolerance is expected to be energetically less costly because it carries instead functional costs, as the evolution of tolerance mechanisms is often dependent on pre-existent elements that can be co-opted for a given infection (Huen et al. 2020; M. R. Miller, White, and Boots 2006). Therefore the host with the strongest immune response is not necessarily the fittest (Ayres and Schneider 2012; M Boots and Begon 1993). Examples in literature have shown that in some cases disease severity can be buffered and survival increased through tolerance mechanisms (Seixas et al. 2009; Silva et al. 2020; Schofield et al. 2002). Moreover, tolerance might impose more relaxed constraints than resistance on parasite growth (Roy and Kirchner 2000; Michael Boots and Bowers 1999; M. R. Miller, White, and Boots 2006). Research into this immune strategy might bear great implications for biomedicine, namely on the upraise of antibiotic-resistance crisis and persistent infections (Medzhitov, Schneider, and Soares 2012; Soares, Teixeira, and Moita 2017; Mok et al. 2020; Vale et al. 2016).

Nevertheless, what we observe in nature is rather a mosaic of resistance and tolerance mechanisms within an individual (Hayward et al. 2014). In literature, these immune strategies can be found positively (Zeller and Koella 2016; Howick and Lazzaro 2014), negatively (Råberg, Sim, and Read 2007; Vincent and Sharp 2014; Balard et al. 2020) or not even correlated (Lefèvre, Williams, and de Roode 2010; Sternberg et al. 2012; Mazé-Guilmo et al. 2014; Decker, de Roode, and Hunter 2018), suggesting their relationship might be infection model specific and dependent on the underlining immune mechanisms. For instance, resistance and tolerance mechanisms that hypothetically depend on the same pathway would be expected to be jointly selected and thus, positively correlated.

Infection is intrinsically a dynamic process with distinct phases of infection and thus, host immune strategies should vary accordingly to which is more beneficial in a given context (Howick and Lazzaro 2014; Kutzer and Armitage 2016b; Hayward et al. 2014; Lough et al. 2015). For instance, a study from Lough *et al.* (Lough et al. 2015) has shown that individual mice that survive or not survive infection differ in their resistance and tolerance, depending on their genotype. Moreover, Howick and Lazzaro (2014), the acute phase is characterized by a high mortality, high bacterial burden and low fecundity, whereas chronic phase exhibits constant levels of bacteria, mortality and fecundity. Considering the latter, we hereby classify acute phase from one to three days post infection and chronic phase the following ones. Furthermore, they unveiled that in flies infected with *Providencia rettgeri* different forms of tolerance (i.e. mortality-tolerance and fecundity-tolerance) are only present in the acute phase of infection, opposed to resistance that prevails throughout the beginning of the chronic phase (Howick and Lazzaro 2014). However, both previous studies (Howick and Lazzaro 2014; Lough et al. 2015) assessed point-tolerance, opposed to range-tolerance in which we focus in this study. While in the former host tolerance is based on one parasite load, in the latter it is based on the parasite load of a group of individuals (Kutzer and Armitage 2016a). Therefore, our conclusions on tolerance will greatly differ between using one point or a population reaction norm, as it has been discussed by Little *et al.* (Little et al. 2010) and shown in a study with house finches infected with a bacterial parasite (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 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.

Parasite load in the early stages of infection has been shown to bifurcate, meaning that infections can result in a bimodal outcome (Duneau et al. 2017). In a model by Duneau and colleagues, individuals could fall within one of these populations: i) host death due to bacterial proliferation until a host-bacteria specific burden, coined bacterial load upon death (BLUD); ii) host survival with a persisting infection at a set-point bacterial load (SPBL). 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 the authors, each bacterial species has a time interval until which an infection can be controlled, small variations in this time can predict if the individuals are fated to survive or die. While in general the bacterial

within-host growth during the first hours is a strong indicator of bacterial virulence and survival of an infection (Faucher et al. 2020), if it is an intermediate virulence infection the host t_c is the solo main predictor (Duneau et al. 2017). Given the distinct outcomes from each of the populations, it is expectable individuals between each of the populations might differ between in how they respond to the infection. For instance, individuals in the higher burden population 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 and, taking a novel approach, how does categorization into high load, individuals assumable fated to die, and low load, individuals fated to survive, explain the population response. We predict individuals fated to survive might exhibit distinct resistance and tolerance signatures comparatively to the ones fated to die. In order to assess if these signatures are parasite-specific, we infected mated female *D. melanogaster* with one of two opportunistic bacteria species extracted from wild-caught flies, *P. burhodogranariea* and *L. lactis*. We chose these species due to: i) their ability to persist within the fly for at least the duration of this experiment; ii) their intermediate degree of virulence with *L. lactis* being more virulent than *P. burhodogranariea* in this specific fly population due to its host exploitation (Acuña-Hidalgo et al., n.d.). At an intermediate level of virulence, as the one in this study, the host t_c is expected to dictate infection outcome (Duneau et al. 2017). Taking into account the higher host exploitation in *L. lactis* infection and if this fallouts in reproductive resources drainage 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 2004, 2003). We measured the dynamics of resistance and fecundity-tolerance during acute and early chronic phase 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 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 as in (Råberg, Graham, and Read 2009; Råberg, Sim, and Read 2007; Kutzer and Armitage 2016b; Graham et al. 2011; Sternberg et al. 2012; 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. Individuals were stored 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.

Experimental flies were reared at constant larval density: 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 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 when the females were allocated to treatment groups.

2.2. Bacterial culturing and preparation

We used the Gram-positive *Lactococcus lactis* (gift from Brian Lazzaro) and Gram-negative *Providencia burhodogranariea* strain B (gift from Brian Lazzaro, DSMZ; type strain: DSM-19968). Both were isolated from wild-collected *D. melanogaster* and can be considered as opportunistic pathogens. Bacterial preparation was performed as in Kutzer and Armitage (2016). In brief, bacterial aliquots were stored in 34.4 % glycerol at -80 ºC. First, the species were plated on lysogeny broth (LB) agar and incubated for 24 hours at 30 ºC. Per bacterial species, four colony forming units (CFUs) were added to 100 ml of sterile LB medium for each of two 500 ml Erlenmeyer flasks and incubated overnight (approximately 15 hours) at 30 ºC and 200 rpm. The two liquid 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} $CaCl_2$; 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 Ultrospec 10 classic (Amersham) at 600 nm. The concentration of the bacterial solution was adjusted to $5x10^7$ CFU/ml. To confirm *post hoc* the

concentration estimated by the OD, we serially diluted the solution to 1:10^7 and plated eight droplets of 5 µl of the bacterial solution on three LB agar plates, and counted the number of colony forming units (CFUs).

2.3. Infection assays

The experiment had four treatments: 1) injection with *L. lactis*; 2) injection with *P. burhodogranariea*; 3) injection control inoculated with Ringer's solution; 4) Naive, or non-injected treatment. This was executed in two replicates in two different days. In each replicate/day, 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 Naive. A fraction of the flies were sacrificed for bacterial load estimation at day two (*L. lactis*: 69, *P. burhodogranariea*: 69, Ringer's: 6, Naive: 8) and day four (*L. lactis*: 69, *P. burhodogranariea*: 71, Ringer's: 8, Naive: 8), whereas the remaining ones had their fecundity assessed for the following days (See section 2.4 below) and the survival flies by day ten then also sacrificed (*L. lactis*: 17, *P. burhodogranariea*: 49). The injections were performed on four to five day old female flies randomly allocated throughout the treatments. Females were anesthetized with CO_2 for a maximum of five minutes in groups of 8 or 9 flies. Flies 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 CFU. 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 assure there was no discrepancies in the concentration of the inoculum 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 ten days in the same order as they were processed on injection day. Fecundity was assayed as the number of adult offspring at day 12 post oviposition. The vials were frozen upside down at this time-point and later counted. Due to a technical error, we do not possess fecundity data for day six and ten post injection. Therefore, in our daily fecundity analysis we only considered flies that survived until day nine (*L. lactis*: 18, *P. burhodogranariea*: 52, Ringer's: 32, Naive: 32).

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. In a randomized manner, surviving flies from each treatment were 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 at 420 rcf for one minute at 4 ºC. After resuspending the solution, 80 microliters 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. Preliminary tests showed that three droplets gave a similar mean value to eight droplets per fly. Additional tests on the detection of bacteria in homogenised flies, we found 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 were 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. We hardly retrieved foreign CFUs after homogenising flies injected with Ringer's solution (n = 0 cases out of 47 flies) or non-injected (n = 1 cases out of 48 flies) and only 3 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 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 levels) and 'person' (two levels), meant that we fitted them as fixed effect, rather than random effect, factors (Crawley 2007).

2.6.1 Fecundity

We tested whether fecundity, measured as the number of adult offspring produced by each female in a 24-hour period, was affected by treatment (i.e. "Naive", "Ringer's ", "*L. lactis*" and "*P. burhodogranariea*"), replicate or person. Due a technical issue, we were not able to measure fecundity for days six and ten post injection. We only considered flies that survived until day nine to assure the same flies are represented across all days. Each day (i.e. one, two, three, four, five, seven, eight and nine) was tested individually using generalized linear models using the package "lme4" with Quasi-Poisson error structure. The resulting *p*-values were adjusted using the Benjamini-Hochberg (1995) correction for multiple testing.

2.6.2 Resistance

To analyse the data on bacterial load we aimed for an approach that was able to appropriately account for the bimodal pattern of the load distributions. For a similar kind of data Duneau *et al.* (Duneau et al. 2017) applied a mixture model, which accounts for a branching in bacterial loads during the first hours after infection. We adapted this approach to our data and our specific research question related to the longer-term dynamics in bacterial loads. Because our data collection started after two days past infection, we were not able to capture the initial branching in loads. Instead, we assumed that after these two days the main part of the branching process was already completed. Accordingly, we followed the idea of Duneau *et al.* that in the upper load category a state is reached in which loads are constant over time, which captures the BLUD. For the lower load category, we allowed the bacterial load to change linearly over time, which we assumed would indicate changes in host resistance.

Following Duneau *et al.* (Duneau et al. 2017) we fitted a model with a log-normal error distribution. In addition, we included for both load categories the predictors 'person' and 'experimental replicate'. For each species we ran a separate model that simultaneously captures the dynamics in the lower and upper load category, as follows:

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

We implemented this model in R and estimated the parameters using the 'optim' function. To assess the statistical significance of DPI in the lower load category we used a likelihood ratio test that compares the full model to the reduced model without DPI.

Finally, based on the estimated model, we calculated for each data point the probability of belonging to the lower burden category. This information was then used to categorise each data point as belonging either to the lower or upper category (based on a 50% cut-off). We later used this 'category' variable as a predictor in the tolerance analysis.

2.6.3 Tolerance

Here we asked whether fecundity-tolerance differed (1) between subpopulations/categories of flies having either a high or a low load (see previous section) and (2) between different days post infection (i.e. day 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 category (i.e. high or low, see previous section), '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, as follows:

$$Fecundity_{Infected} \sim Bacterial\ load * DPI * Burden + Replicate + Person$$

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 data set of *L. lactis*, an influential data point belonging to DPI two and the lower load category was detected based on Cook's distance and was removed from the analysis.

3. Results

3.1. Infection does not affect fecundity

After p-value adjustment, there was no significant effect of infection on fecundity for the flies that survived to ten days post treatment (Table S1). There was a gradual decline in fecundity over time (Figure 1), although this relationship was not tested statistically.

3.2. Reduction in resistance for L. lactis

The bacterial load after infection with both bacterial species resolved into a lower and upper category, 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 this category so that it was not allowed to vary over time, and tested for changes in the low load group only (Figure 2). In this lower group, flies infected with *L. lactis* showed a significant increase in bacterial load over time (Figure 2A), i.e., decrease in resistance from two to four days post-infection. Under similar conditions, *P. burhodogranariea* infected flies did not exhibit any change in bacterial load (Figure 2B).

In agreement with our previous result, when we allowed the upper group to vary with time, there was a similar result with an increase in bacterial load with time for *L. lactis* (Figure S1A), meaning reduction in resistance within this time interval. Similarly, there was no change for *P. burhodogranariea* between these two time-points (Figure S1B)

These results remain qualitatively the same when allowing the upper category to change over time, but results change a lot when including data points of day 10 (Figure S2 and S3). However, the latter results have to be interpreted cautiously due to the limited number of data points.

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

Although there was no cost of infection on fecundity (Table S1), *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 burden category, whereby the hosts categorised with higher loads were significantly less fecundity tolerant, than the hosts with lower loads (significant interaction between bacterial load and category; 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 burden category, whereby flies from the lower category were less fecund than the high load counterparts (Table 1). There was also a significant effect of the experimenter (Table 1).

Unfortunately, a three-way interaction between CFU, DPI and burden category was not observed and thus we must only look at difference in average slopes within DPI or burden category separately (Figure 3E, F). Despite the fact *P. burhodogranariea* infection did not lead to the same significant interactions, the average slopes, per DPI and burden category, still follow the same trend (Figure 3B, C).

4. Discussion

Here we show a novel multi-level approach to infection dynamic studies through the inclusion of a binary outcome mixture model proposed by Duneau and colleagues (Duneau et al. 2017). Our results show that both immune strategies can differ throughout a temporal scale but also between subgroups within a burden category based on their likelihood of survival. We also show evidence for terminal investment in individuals expected to survive or die, suggesting it might be a common response within a specific host-parasite interaction and not a consequence of disease severity. Altogether, we emphasize the need to consider population structure and infection stage when processing parasite dynamics and immune response within an infection model.

4.1. No cost of infection for daily fecundity

Infections can be costly for a host, for instance due to induction of immune effectors or direct tissue damage (Moret and Schmid-Hempel 2000), and infections have been shown to be costly in terms of reduce fecundity (Gwynn et al. 2005). Given that infection with *L. lactis* and *P. burhodogranariea* results in reduced survival over a four-day window, infection with these species have negative fitness consequences, and we predicted there to be a cost in terms of reduced fecundity. We assayed fecundity into the chronic infection phase in case fecundity costs were expressed later during the infection, considerable energetic costs to trade-off with other physiological and reproductive fitness elements, as shown in literature (Lawniczak et al. 2007; Fedorka et al. 2007; Naim et al. 2020; Gwynn et al. 2005; Short and Lazzaro 2010). Moreover, if we consider virulence a function of disease severity (Read 1994) and host

exploitation (Råberg and Stjernman 2012; Acuña-Hidalgo et al., n.d.), then we would classify *L. lactis* as more virulent than *P. burhodogranariea* and potentially carrying a heavier cost. However, we did not observe any clear cost of infection on our reproductive fitness measure, daily fecundity during the first nine days of infection (Table S1). This is not entirely atypical as studies with bacterial infections in *D. melanogaster* have previously reported this result for *L. lactis* and *Escherichia coli* species during the first three days of infection (Kutzer and Armitage 2016b). As trade-offs between immunity and life-history traits is not one of the focuses of this study we did not use any other measures. Additionally, our daily fecundity measure was assessed using only flies that survive until day nine post infection and therefore might be inadvertently selecting for a response from a more robust group of flies. Nonetheless, we still believe there are costs outside the scope of this study, namely on tissue damage.

4.2. Resistance and fecundity-tolerance to infection

Immune strategies can fluctuate throughout infection (Kutzer and Armitage 2016b; Howick and Lazzaro 2014). Based on Duneau *et al.* model (Duneau et al. 2017), we can anticipate if individual flies will succumb or survive an infection given their high or low bacterial load after a given time, defined by the authors as t_c (e.g. approximately 2 hours post infection for *P. burhodogranariea*). In here we focused on post-branching variations in immune strategies. Flies in the upper category have assumedly reached a plateau burden that eventually will lead to host death, defined BLUD. For this reason, we mainly measured changes in tolerance for this category and not resistance, as it should be linear and therefore constant. However, because there is no evidence BLUD remains constant after 48 hours, we also presented the results with the possibility of variation within this high burden category. The reasoning behind the latter is that flies that survive later in infection might die with a lower bacterial load than the one described as BLUD and potentially less tolerant as well. Tolerance depends on a fitness measure besides the bacterial burden, therefore changes in this category would still be possible within this framework. Therefore, we also analysed the upper category resistance and can be found in the supplementary information (Figure S1 and S3).

In *L. lactis* infected flies we observed an increased bacterial load from day two to four post infection (Figure 2), showing a reduction in resistance. Complementary, average tolerance does also decrease with time (Figure 3E). This fecundity shift between day two and four might support terminal investment hypothesis (Williams 1966; Clutton-Brock 1984). When infected

with *L. lactis*, female flies increase their investment towards early reproduction (first 48 hours) to potentially maximize their reproductive success. This resource allocation towards fecundity is particularly relevant for the high load category of flies, as they are unlikely to mitigate this cost of infection during their short lifetime. On the other hand, flies in the low burden category bear a strong early immune response that successfully allows them to control infection, as well as a considerable high reproductive effort. These accumulated costs are likely to drive the reduction in resistance and fecundity-tolerance between day 2 and 4 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 strong resistance response might lead to an uncontrollable infection as seen in the high burden category. As this bacterial species is known to persist at least until day 10 (Acuña-Hidalgo et al., n.d.), we measured the bacterial load at this point. In a long-term window, survivors seem to have their bacterial burden persist (Figure S1A) by day 10 post infection, in agreement with the SPBL hypothesis (Duneau et al. 2017) and previous data from our group (Acuña-Hidalgo et al., n.d.). In contradiction to the BLUD hypothesis, we observe a reduction in the bacterial load with time when we allow the high burden category is able to vary with time (Figure S3). This suggests that either the flies in the high burden category are able to control the infection later on or flies in the low burden category are unable to do so probably due to accumulated costs of infection and immunopathology. In either of the scenarios, there is an indication that these categories might not be restricted and flies might interchange between them according to individual infection dynamics, specially later on into the infection.

For *P. burhodogranariea* we note an overall different pattern (Figure 2B and 3D-F). We do not observe any difference between day two and four either on resistance (Figure 2B and S1B) or fecundity-tolerance (Figure 3D-F). Despite this, (Figure 3), we do have a significant difference in fecundity between high and low categories (Table 1). This result suggests flies fated to die might have too much of a burden compromising fly homeostasis and possibly leading to a cut in reproduction. Similarly to *L. lactis*, *P. burhodogranariea* also persists for at least 10 days of infection. In this time-scale we do observe a decreased bacterial load in the low burden category for this bacterial specie, indicating an increased resistance in the long term (Figure S2). More interestingly, flies in the low burden category, likely to survive, do not seem to pay a price in

reproductive fitness for their long-term increase in resistance, comparatively to flies in the high burden category, likely to die. Taking in account the early branching between survivors and succumbers (Duneau et al. 2017; Acuña-Hidalgo et al., n.d.), it is possible that for this species we are in the presence of a different infection dynamics and 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 very early stages of infection. In agreement with Howick and Lazzaro, during chronic phases of infection there is not a clear mark of fecundity-tolerance (Howick and Lazzaro 2014). Nevertheless, we have to be careful with our assumptions due to the limited sample size in the high burden category and day 4 post infection. This increased resistance is also present if we allow the high burden category to vary (Figure S3). Interestingly, in the latter we now see a significant increase in resistance in the high burden category as well. Unfortunately, we have very few flies at this time-point and this might be dragging this result.

Moreover, our data proposes the hypothetical low survival ability of the high burden category individuals might be due to a reduced average tolerance comparatively to their low load counterparts (Figure 3F), in agreement with a study performed in mice (Lough et al. 2015). In the latter authors observed surviving mice would exhibit less tolerance or resistance comparatively to surviving mice. This result suggest surviving flies might handle better this infection through and early and bolder investment in tolerance. The underlying cause for this disparity is unknown to us. Although Duneau *et al.* (Duneau et al. 2017) has hypothesized t_c is the most decisive host parameter to explain the binary outcome and disease severity, given the outbred nature of the categories 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*. Therefore, it is possible we do not have an optimal time-window to detect strong changes in *P. burhodogranariea*, as they might have happened earlier than for *L. lactis*. The latter reinforces the need to study infection host-parasite model dynamics and the danger of universality in temporal dynamic studies.

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Tables and figures

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 x DPI	1	0.41	0.523	1	7.39	**0.007**
Bacterial load x Burden	1	0.00	0.962	1	5.53	**0.019**
Burden x DPI	1	0.35	0.553	1	3.32	0.069
Bacterial load x DPI x Burden	1	0.30	0.583	1	1.62	0.203

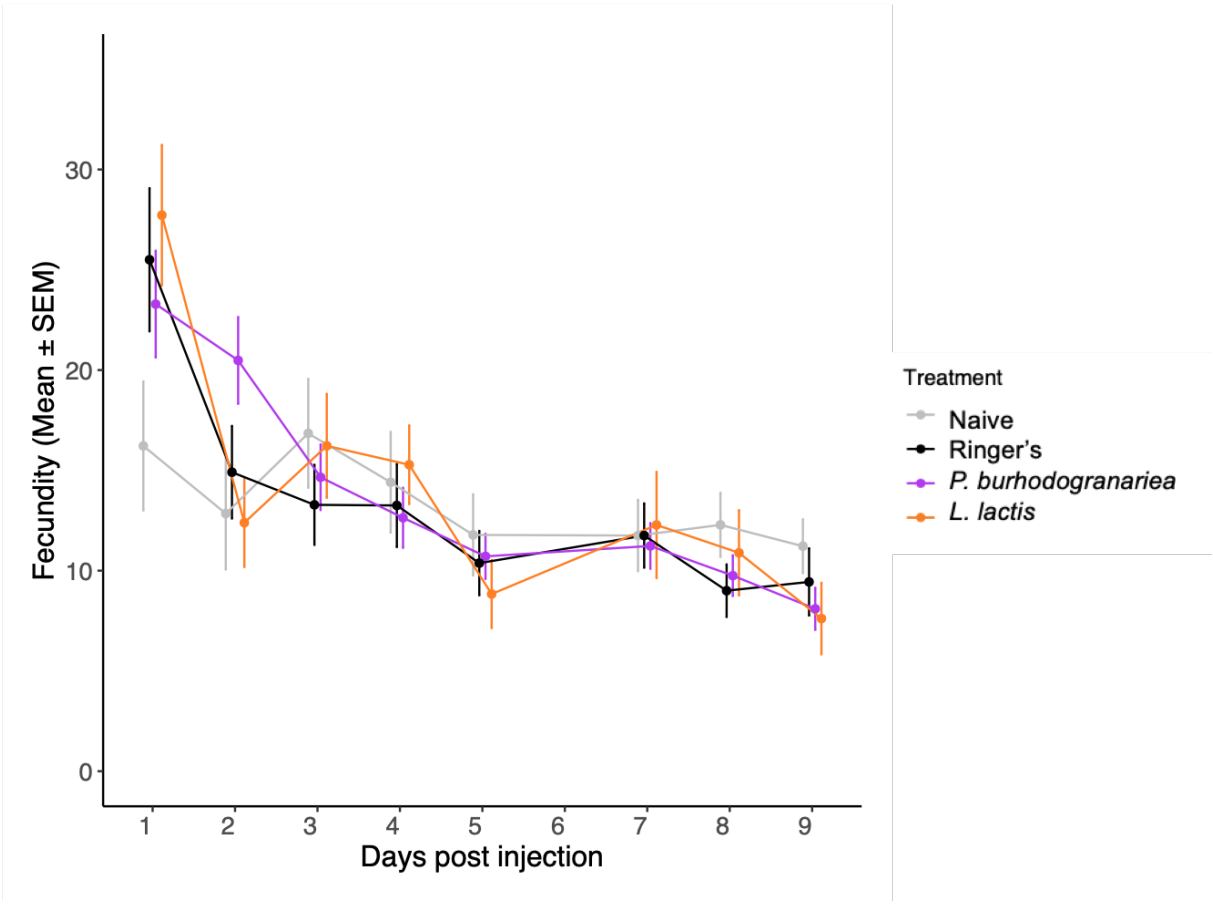

Figure 1. Mean fecundity of female flies that survived to ten days post-treatment. Flies were injected with one of two bacterial species, a control injection with Ringer's, or received no injection (naive). The sample sizes are the following: Naive n = 32, Ringer's n = 32, *L. lactis* n = 18, *P. burhodogranariea* n = 52 . For statistics see Table S1.

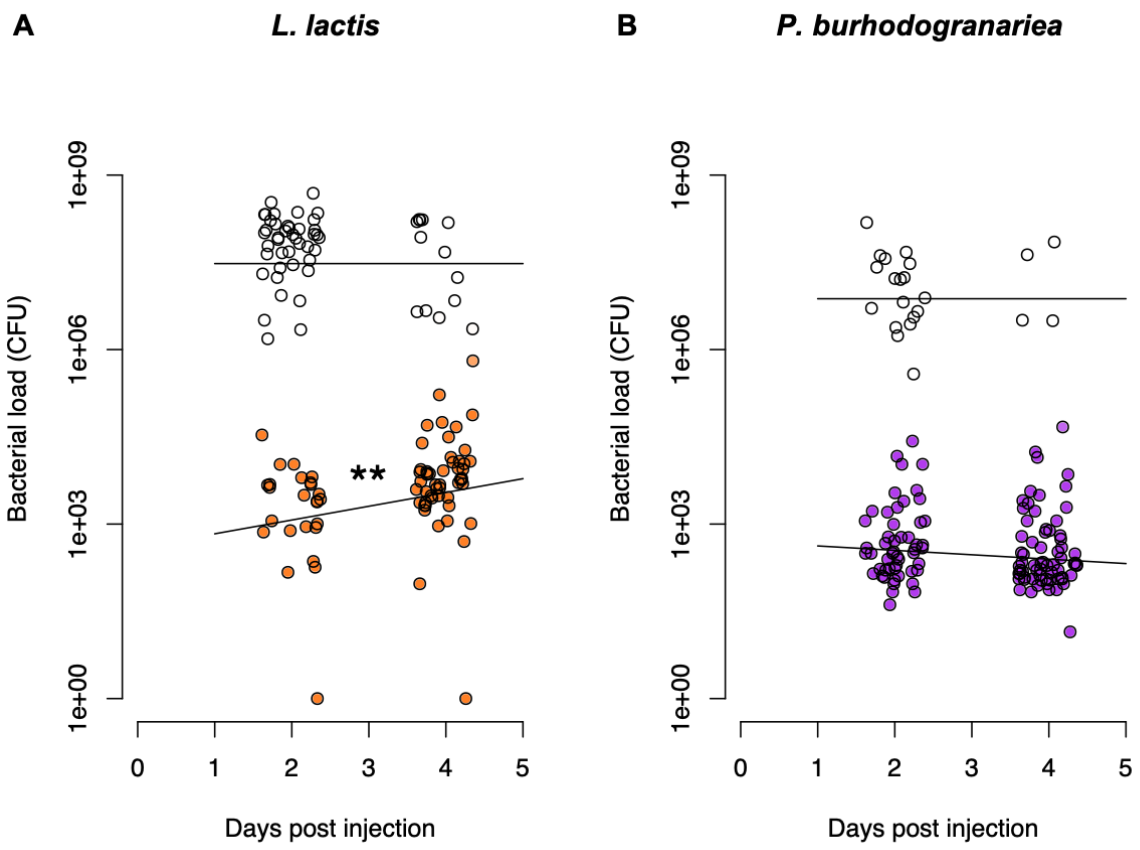

Figure 2. Bacterial load per living fly after injection with the respective bacterial species. Only flies homogenized at 2, 4 were analysed. Flies were assigned to higher or lower burden category and only the linear relationship of the latter was allowed to vary. The lighter the colour tone of a given individual datapoint, the higher the likelihood of belonging to the high burden category. The * asterisk symbol shows a significant increase in bacterial load across time for lower burden category of *L. lactis* (p-value = 0.0098).

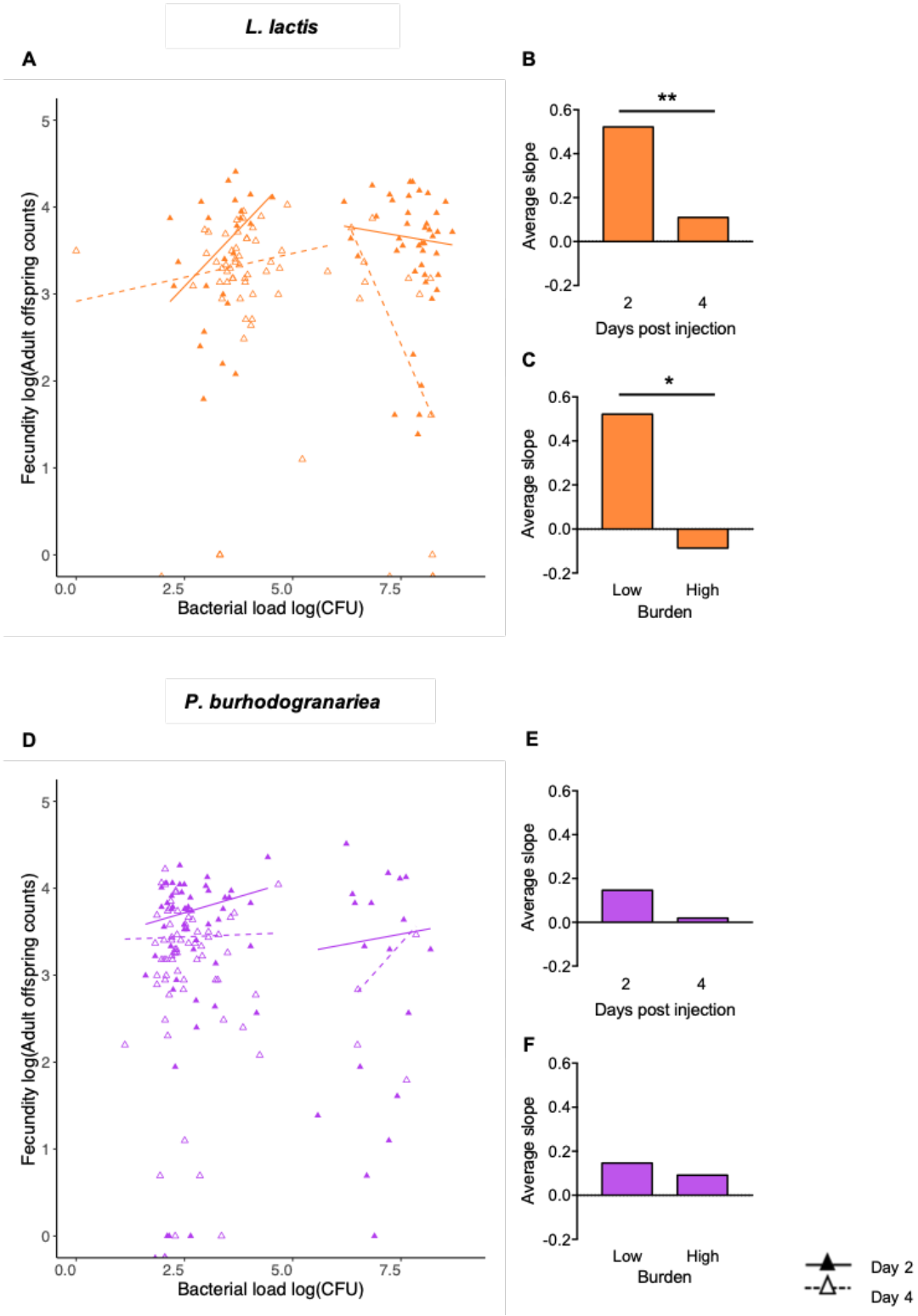

Figure 3. Fecundity-tolerance reaction norms are plotted for each bacterial species across different days post injection and burden categories. (A) *L. lactis* and (D) *P. burhodogranariea* show fecundity-tolerance with symbols and lines denoting days two and four. (B) and (E) illustrate the average slope for each of the days, while (C) and (F) represent the average for each burden category. The asterisks show a significant difference in the slopes. For statistics, see Table 1.

Supplementary information

Table S1. The effects of treatment (*L. lactis, P. burhodogranariea*, Ringer's and naive), experimental replicate, and person on the response variable fecundity. p^{BH} indicates the *p*-values after adjustment for multiple testing using the Benjamini–Hochberg correction. Statistically significant factors are shown in bold.

	Effects on fecundity			
Tested effect	***df***	χ^2	***p***	p^{BH}
Day 1				
Treatment	3	95.90	0.117	0.350
Replicate	1	7.50	0.497	0.636
Person	1	3.64	0.636	0.636
Day 2				
Treatment	3	99.17	**0.049**	0.098
Replicate	1	71.58	**0.017**	0.069
Person	1	26.42	0.148	0.160
Day 3				
Treatment	3	15.66	0.683	0.975
Replicate	1	0.01	0.975	0.975
Person	1	3.37	0.570	0.975
Day 4				
Treatment	3	9.01	0.830	0.910
Replicate	1	36.69	0.058	0.172
Person	1	0.13	0.910	0.910
Day 5				
Treatment	3	9.89	0.734	0.734
Replicate	1	2.41	0.577	0.734
Person	1	1.04	0.714	0.734
Day 7				
Treatment	3	1.44	0.978	0.978
Replicate	1	34.76	**0.030**	0.090
Person	1	18.92	0.109	0.164
Day 8				
Treatment	3	19.11	0.376	0.563
Replicate	1	1.87	0.581	0.581
Person	1	12.91	0.147	0.442
Day 9				
Treatment	3	25.71	0.304	0.455
Replicate	1	0.62	0.768	0.768
Person	1	11.51	0.202	0.455

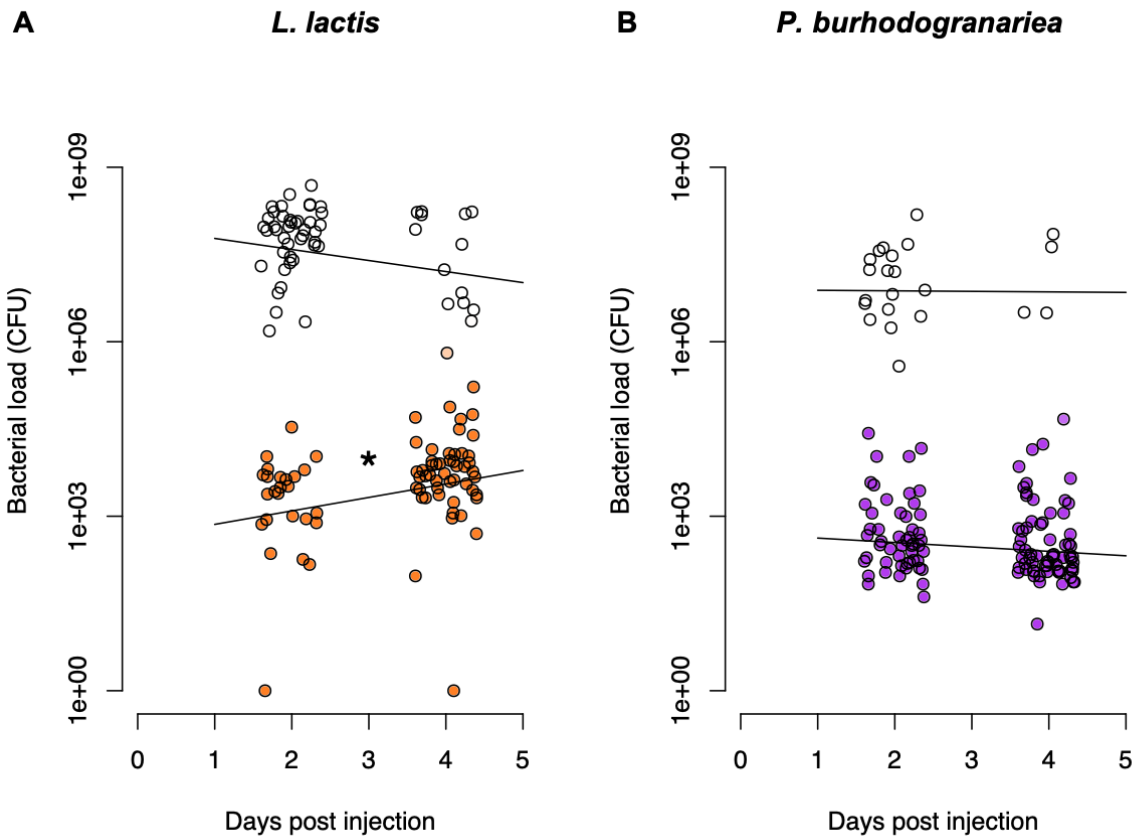

Figure S1. Bacterial load per living fly after injection with the respective bacterial species. Only flies homogenized at 2, 4 were analysed. Flies were assigned to higher or lower burden category and the linear relationship of the both categories were allowed to vary. The lighter the colour tone of a given individual datapoint, the higher the likelihood of belonging to the high burden category. The * asterisk symbol shows a significant increase in bacterial load across time for lower burden category of *L. lactis* (p-value = 0.0109).

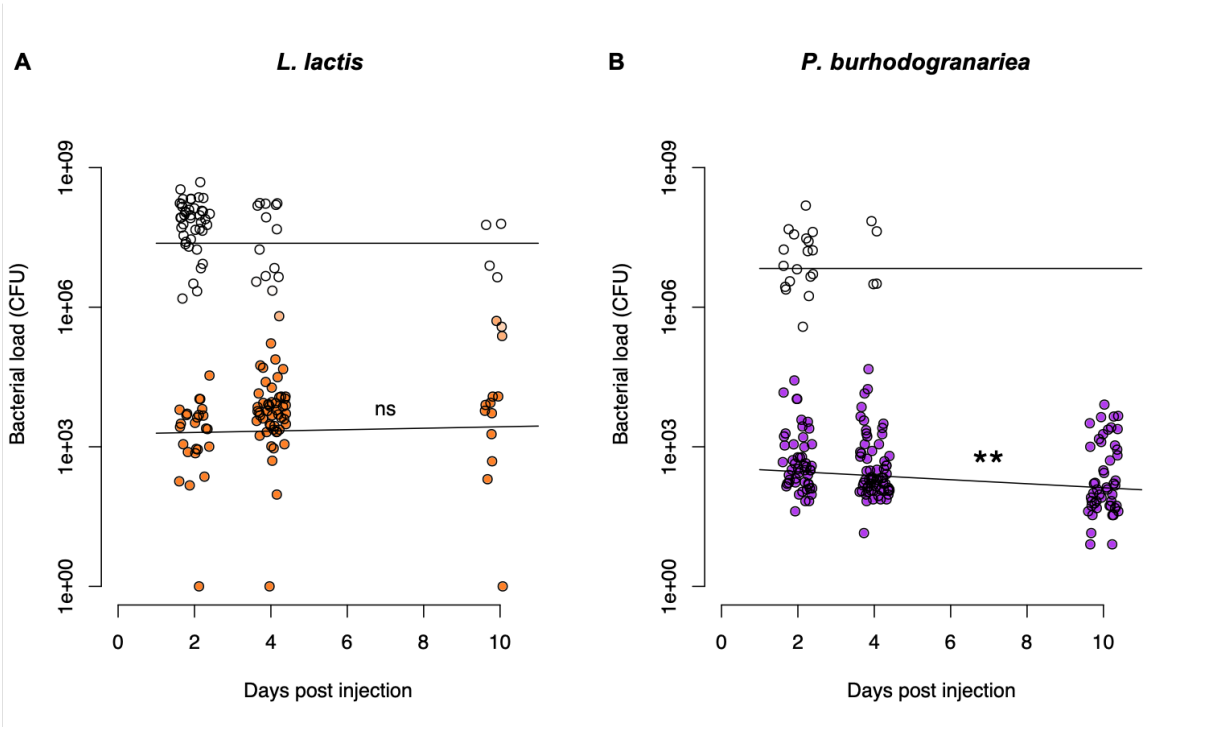

Figure S2. Bacterial load per individual fly after injection with the respective bacterial species. Flies were homogenised at two, four and ten days post injection. The bacterial loads were then statistically assigned to a higher (empty circles) or lower (filled circles) burden category, and only in the latter was the linear relationship allowed to vary with time. The lighter the colour tone of a given individual datapoint, the higher the likelihood of belonging to the high burden category. The ** asterisks indicate a significant decrease in bacterial load across time (*p*-value = 0.0065).

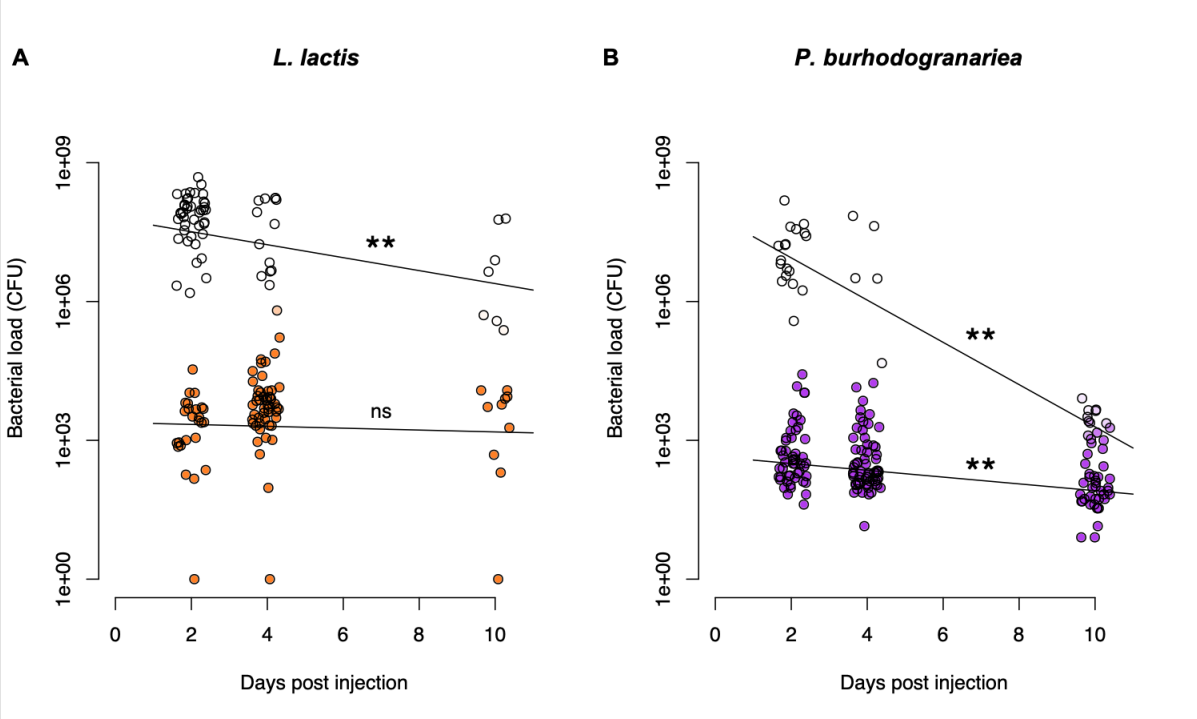

Figure S3. Bacterial load per living fly after injection with the respective bacterial species. Flies were homogenized at 2, 4 and 10 days post injection. Flies were then assigned to higher or lower burden category in the both the linear relationship was allowed to vary. The lighter the colour tone of a given individual datapoint, the higher the likelihood of belonging to the high burden category. The * asterisk symbol shows a significant difference in bacterial load across time. The *p*-values are the following: *P. burhodogranariea*, higher burden category *p*-value < 0.001, lower burden category *p*-value = 0.0065; *L. lactis*, higher burden category *p*-value = 0.0028.

Chapter 5

Effect of selection for host tolerance and resistance on a host-pathogen interaction and infection outcome

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Abstract

Hosts can defend themselves by either limiting parasite burden, defined as resistance, or reduce the fitness costs due to infection or host immune response, named tolerance. Besides the clear ecological properties between the two strategies, important evolutionary predictions have been proposed. Unfortunately, few studies aimed to provide empirical proof or support to these hypotheses. Here, using *Drosophila melanogaster* as a host and *Lactococcus lactis* as an opportunistic pathogen, we selected flies for increased fecundity-tolerance (i.e. reduction of infection cost in reproductive fitness) or quantitative resistance for eight generations. *L. lactis* exhibited costs in reproductive fitness in both acute and chronic phase of the infection. In this study we present on-going, mid- and post-selection readouts. Our results indicate tolerance might be a preferential immune strategy under this setting and for this host-parasite interaction, as it was selected in both Resistance and Tolerance-selection regimes. In contrast, there was no change in resistance across any of the selection regimes. Furthermore, after evolving *L. lactis* within the different experimentally evolved hosts, we did not an exhibit any selection regime difference effect on the intra-host evolved bacteria but we note an overall increase in virulence, early growth and persistence in bacteria that underwent the fly's immune system, comparatively to the ones that did not. Altogether, out studies places tolerance as a major immune mechanism with critical evolutionary implications in only a few generations of exposure to a parasite.

1. Introduction

When threatened with an infection, a host can elicit an immune response. The latter can be based on either resistance or tolerance mechanisms. Resistance acts by controlling or clearing the parasite load, subclassified into quantitative and qualitative resistance respectively (Restif and Koella 2004; de Roode and Lefèvre 2012; Råberg, Graham, and Read 2009; Graham et al. 2011). Alternatively, tolerance does not affect the parasite load but instead reduces the detrimental effects of infection (e.g. tissue damage) (Kutzer and Armitage 2016a). Although both defence categories lead to an increased host fitness, their intrinsic properties have very distinct implications on the physiology, ecology and evolution of host-parasite interactions.

First off, resistance mechanisms are often based in induction immunity with heavy and, frequently, long-lasting costs to the host (Moret and Schmid-Hempel 2000; Alves et al. 2019; I. F. Miller and Metcalf 2019). These costs might become apparent as trade-offs with some life-history traits (Lawniczak et al. 2007), namely reproductive fitness (Short and Lazzaro 2010; Fedorka et al. 2007; Naim et al. 2020; Gwynn et al. 2005). Although few studies have addressed the same in regard of tolerance, they do show diet and resource availability distinctly favour resistance and tolerance (Kutzer and Armitage 2016b; Zeller and Koella 2017). In this matter, tolerance is commonly seen as low-cost option for the host to handle an infection. However, this is not necessarily, and universally, true. Each parasite invades a specific set of tissues that consequently will possess distinct regeneration abilities, as well as different effects on host fitness (Medzhitov, Schneider, and Soares 2012). Therefore, each infection scenario will favour a particular toolset of tolerance mechanisms with contingent costs. Hosts naturally exhibit a mixture of both immune strategies to a given parasite infection and all of the above singular costs are only expected to be presented as such if the strategies are independent from each other. Interestingly, in literature this strategies can be positively (Howick and Lazzaro 2014; Zeller and Koella 2017), negatively (Råberg, Sim, and Read 2007; Vincent and Sharp 2014; Balard et al. 2020) and even not correlated (Lefèvre, Williams, and de Roode 2010; Sternberg et al. 2012; Mazé-Guilmo et al. 2014a; Decker, de Roode, and Hunter 2018). If both strategies assent on the same pathways then selection will likely favour evolution of both and their addictive costs will be smaller than the sum of the singular costs. Contrarily, if tolerance mechanisms use the same resources as resistance mechanisms we might see the increase of one with the reduction of the other through time (Restif and Koella 2004).

On the other side of the interaction, resistance and tolerance evolution do also have distinct effects on parasite dynamics and evolution (Roy and Kirchner 2000). In a resistance scenario, we observe an antagonistic coevolution between host and parasite, often referred to as Red Queen dynamics (Van Valen 1973; Lively and Apanius 1995). Within this evolutionary arms race, host alleles that favour resistance against the parasite are selected and increase in the population, leading to their fixation. Given the negative effect of parasite number, we observe negative frequency dependent selection acting on the parasite genetic pool and eventually leading to selection of alleles that favour infection to the new host. In the end, this series of selective sweeps from both host and parasite will lead to oscillations in host and parasite genotypes through evolutionary time (Roy and Kirchner 2000; Woolhouse et al. 2002; Restif and Koella 2004). However, it is important to note the latter is a classic an ideal example where both host and parasite are constantly exposed to each other and their coevolution is inevitable and stronger than other selective forces, such as intra-specific selection within the host and parasite population. Both host resistance and parasite virulence come at costs. Often these costs might be extinguished with time (Lenski 1988a, 1988b; McKenzie, Whitten, and Adena 1982), but in the short term both host and parasite need to balance the benefit and costs of their increased resistance/virulence. More often, what we observe is a mixture of resistant/virulent and susceptible/mildly-virulent parasites in each of the populations (Roy and Kirchner 2000; Woolhouse et al. 2002). Tolerance on the other hand does not lead to an antagonistic interaction between host and parasite since it does not act on the parasite load (Kutzer and Armitage 2016a). Instead, depending on which fitness measure tolerance is acting on, we expect it to have a neutral, or even positive, effect on the parasite prevalence (Restif and Koella 2003, 2004; Best, White, and Boots 2008). Curiously, tolerance has even been proposed as a possible evolutionary bridge between pathogenicity and commensalism for some host-parasite interactions (Little et al. 2010). In the host population, studies have hypothesized fixation should occur quite quickly as a higher fixation of tolerance in a population will lead to a high prevalence of the parasite and, therefore, a very high cost to non-tolerant or migrant individuals (Roy and Kirchner 2000). Nevertheless, further studies in the topic have shown that this is mostly true for mortality-tolerance, i.e. the ability to reduce the negative impact of infection on survival, and not necessarily in other forms of tolerance (Best, White, and Boots 2008; M. R. Miller, White, and Boots 2006). One of these forms of tolerance is fecundity-tolerance, i.e. the ability to reduce the negative impact of infection on reproductive fitness. Although, very few is known about the latter, models have predicted the evolution of this form can lead to branching of the population due to increasing costs with other traits, such as survival (Best,

White, and Boots 2008). In this model, fecundity-tolerance is supposed to evolve until a certain extent when further increase will present a strong cost against survival. At this point, the population is expected to branch into two subpopulations, one with high fecundity-tolerance and one with low fecundity-tolerance (Best, White, and Boots 2008).

The implications of resistance and tolerance have become increasingly relevant with the growing literature into them. Particularly tolerance, it has been proposed as a key factor in evolution and ecology, as well as a solution for many of the medical crisis we face nowadays and hence, contribute for the advance of immunology and epidemiology (Vale, Fenton, and Brown 2014; M. R. Miller, White, and Boots 2006; Soares, Gozzelino, and Weis 2014; Vale 2018; Ayres and Schneider 2012). Unfortunately, as previously mentioned most of the contrasting inferences made regarding ecology and evolution of tolerance come from theoretical models and not from experimental studies. Hence, in this study we aimed to provide experimental information regarding tolerance and resistance evolution. Here, we exposed an outbred population of *Drosophila melanogaster* to an infection with the opportunistic pathogen found in wild fruit flies, *Lactococcus lactis*. Prior studies have shown this pathogen successfully infects this host population resulting in an infection with intermediate virulence (Acuña-Hidalgo et al., n.d.). Moreover, this population is known to vary in fecundity-tolerance through time to this infection (Silva et al., n.d.) and therefore, we predict it to be likely to respond to selection for this trait. Artificial selection was performed on this model, selecting for fecundity-tolerance and quantitative resistance over eight generations. With this approach we aimed to answer a set of questions: (i) Does infection come with a fitness cost? (ii) Are resistance and tolerance evolvable in this model? (iii) How do they relate to each other? (iv) How do *L. lactis* evolved within different selection regimes hosts differ between them and to an ancestral state? In sum, with this set of questions we aimed to better understand the part of resistance and tolerance in a host-parasite interaction.

2. Materials and Methods

2.1 Fly maintenance and production of experimental animals

We used an outbred population of *Drosophila 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. For at least 30 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 et al. 2007). To avoid inbreeding, flies were kept in population cage with at least 5,000 flies and non-overlapping generations of 15 days. Both mother population and experimental flies were maintained at 24.3 ± 0.2°C, on a 12:12 hours light-dark cycle, at 60-80 % relative humidity. All the lines originated from the same mother population (three replicate lines per treatment). Prior to the beginning of the selection experiment, we created three subpopulations. Each subpopulation was kept in a cage with a controlled density of 4800 larvae (1 larva per 0,21 ml of food) and a generation time of 14 days. After at least two generations of density control, each subpopulation established one replicate of all treatments, founding in total nine lines (three treatments x three replicates explained below).

2.2 Bacterial culturing and preparation

All experiments were performed using the Gram-positive *Lactococcus lactis* (gift from Brian Lazzaro). This strain was isolated from a wild-collected *D. melanogaster* (Lazzaro 2002) and we consider it an opportunistic entomopathogen. Bacterial preparation was adapted from (Kutzer and Armitage 2016b; Acuña-Hidalgo et al., n.d.). In short, bacterial aliquots were stored in 34.4 % glycerol at -80 ºC. Prior to infection day, *L. lactis* aliquots were plated on lysogeny broth (LB) agar and incubated for 24 hours at 30 ºC. From the latter, four colony forming units (CFUs) were added to 100 ml of sterile LB medium for each of two 500 ml Erlenmeyer flasks and incubated overnight (approximately 15 hours) at 30 ºC and 200 rpm. The two liquid cultures were centrifuged at 2880 rcf at 4 ºC for 10 minutes and the supernatant removed. The bacteria were washed in 40 ml sterile *Drosophila* Ringer's solution (182 mmol·L-1 KCl; 46 382 mol·L-1 NaCl; 3 mmol·L-1 CaCl2; 10 mmol·L-1 Tris·HCl) (Werner et al. 2000) through centrifugation at 2880 rcf at 4 ºC for 10 minutes. 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 Ultrospec 10 classic (Amersham) at 600 nm. The concentration of the solution was adjusted to that required for each 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 $1{:}10^6$ and plated the bacterial solution two times and counted the number of CFUs.

2.3 Experimental evolution lines

The three selection regimes were the following: Resistance, Tolerance and Control. In Resistance and Tolerance regimes, flies were pricked in the lateral side of the thorax with *L. lactis*, while in the Control they were pricked with Ringer's solution. Flies were anesthetized with CO_2 for a maximum of 2 minutes in groups of 10 flies. Prior to injections, the needles were bent approximately 0,4 mm from the end, adapted from (Longdon et al. 2013), in order to reduce potential variation from the pricking methodology. A preliminary assay (Figure S1) compared the variation produced by this pricking method and different inoculum volumes using Nanoject II™ injector (Drummond). The dose of *L. lactis* was determined to cause an average mortality of 40 % at day 4 in the mother population (Figure S2), which corresponds to a concentration of $1x10^9$ CFU/ml (OD_{600} of the 1:10 dilution = 0.16±1). Given the high mortality across all bacteria-pricked lines upon generation F5, this dose was changed to $1x10^8$ CFU/ml in the next generations (OD_{600} = 0.16±1). At the end of each injection day all the remaining volume from all the *L. lactis* and Ringer's solution aliquots were plated to assure there was no contaminations. There were no signs of contamination in any of the solutions. After injection, flies were kept individually in vials with 7 ml of fly medium. At day two, all the alive flies were flipped and kept for two more days. In the Resistance and Tolerance regimes, all the flies that survived till day four post injection were sacrificed and their bacterial load was estimated (see below). We chose this time-point to assess the bacterial load because it is directly after the resolution phase (Duneau et al. 2017) and therefore allowed us to select on flies' bacterial control mechanisms. In the Tolerance regime we additionally measured the number of pupae each fly produced during day three and four post injection. The pupae counts were performed eight days after the end of the oviposition period since a preliminary assay showed that at this time-point 99 % of the larvae have pupated (Figure S3A). Resistance can be defined as the inverse of the bacterial load, therefore, the fewer the CFUs (including zero), the higher the resistance value. In the Tolerance regime, we calculated the residuals from the linear regression between pupae counts from day three and four post injection (fecundity as our fitness measure) and the bacterial load at day four. The higher the residual value for a given fly, the higher the deviation of that individual from the average fecundity-tolerance of the respective Tolerance line, and therefore the higher the Tolerance value. This concept was adapted from (Howick and Lazzaro 2017). At each generation, 200 four-to-five days old female flies were processed per selection regime and replicate. Flies were ranked according to the highest trait value in Resistance and Tolerance regimes, and randomly in the Control regime. From these 200 flies, only 100 with the highest ranking found the next generation. The flies

that formed the following generation were the flies produced during day three or four post injection. Since, we are artificially selecting at individual level, all the flies that did not produce offspring during this interval were replaced by the next one in the ranking, assuring every generation is formed by the genetic contribution of 100 females. Moreover, to assure few genotypes would not overtake the population we also controlled for every female's contribution. Every selected female was allowed to contribute a maximum of three males and three females. The offspring was collected once during the first 24 hours emergence period, approximately 10 to 11 days after oviposition, since our data shows that only a maximum of 12 % of total flies would mate before that (Figure S3B). For each line, we had six big vials, each with 50 males and 50 females, with 40 ml of fly medium. Flies were orderly split between the vials in a manner that avoids brother-sister mating, promoting genetic recombination and avoid inbreeding. Flies were allowed to mate for four days until injection day, where they were removed and processed according to their regime. The experiment ran for 8 generations with only one generation without selection (F6). At every generation we measured all lines' survival at day two and four post injection. As a consequence of the selection protocol, in Resistance and Tolerance regimes we also assessed qualitative/quantitative resistance and fecundity-tolerance, respectively. Ongoing and final response to selection readouts are described in the respective section below.

2.4 Bacterial load assay

L. lactis quantification was adapted from (Acuña-Hidalgo et al., n.d.). Flies were anesthetized 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 previously chilled at 4 ºC for at least 30 minutes to reduce further growth of the bacteria. The holders were placed in a Retsch Mill (MM300) and the flies homogenized 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 microliters of the homogenate from each fly were pipetted into a 96-well plate and then serially diluted from 1:1 to $1:10^6$. Per fly, two droplets of 5 µL of 1:1, $1:10^2$, $1:10^4$ and $1:10^6$ dilutions were plated onto LB agar. Preliminary *in silico* test showed that two droplets gave a similar mean value to higher numbers of droplets per fly (Figure S4). The lower detection limit of this protocol is 5 CFUs per fly, tested in (Acuña-Hidalgo et al., n.d.). The plates were incubated at 30ºC and the number of CFUs were counted after ~20 hours. Individual bacterial loads per fly

were back-calculated using the average of two droplets from the lowest countable dilution in the plate.

2.5 On-going and final response to selection readouts

Upon generation F5, an additional subset of 100 flies per regime and replicate (nine lines) were processed to monitor changes in resistance and fecundity-tolerance across all the regimes. All of the flies in these subsets were pricked with a concentration of $1x10^9$ CFU/ml of *L. lactis* in parallel with the selection lines' flies and processed as described above.

Regarding the final readouts, these were performed after three generations without selection to reduce potential maternal effects. Due to technical issues we lost the third replicate of the experiment during generation F9 and, therefore, these final readouts were only conducted in the first two replicates. This experiment was conducted in a full combinatorial setup with all the replicates and regimes with infected and Ringer-injected treatments, totalizing in 12 combinatorial treatments (3 regimes x 2 replicates x 2 infection treatments). Injection days were performed as described previously. In each combinatorial treatment we processed a subset for survival and fecundity assessment and a subset sacrificed at day four post infection to assess resistance, fecundity-tolerance, proteomics and CFUs extraction. In the first, we followed survival for 16 days post injection for 80 flies per combinatorial treatment and assessed their fecundity by counting the pupae produced in the following 48 hours oviposition periods: days 0-2, 2-4, 8-10 and 14-16 post injection. In the second, we measured fecundity during day 2-4 and quantified bacterial load at day four post injection. For every bacteria-injected combinatorial treatment we had 120 flies, while for Ringer-injected treatments we had 40 flies. All the alive flies at day four were plated regardless of the treatment. Fecundity and bacteria quantification were performed as described above. Additionally, we also extracted individual host and bacterial samples for proteomics and in the latter, also for re-infection purposes. Both methodologies and respective experiments are described below in their respective sections.

2.6 Bacterial re-infection experiment

Following incubation in LB agar plates, entire droplets of the lowest countable dilution in the LB agar plate were extracted with the help of a 1000 μL with the extremity cut. Each droplet was pipetted into a 1.5 ml centrifuge tube containing 1 ml 10% Ringer's-trehalose mixture. The tubes were then vortexed for approximately 1 minute to detach the bacteria from the agar and into the solution. Then, the supernatant was transferred to a second empty tube in dry ice until

storage at -80ºC. Here, we used trehalose as a cryopreserving agent because it is a disaccharide known to protect cells from freezing and it is commonly present in Drosophila haemolymph (Chapman 1998).

Prior to the experiment, flies were reared at controlled larval density: (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 mother 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 control for density during development. This was performed for two generations. One day after the start of adult eclosion, the flies were placed in fresh food big vials in groups of fifty males and fifty females and allowed to mate for four days. Upon injection day, female flies were separated and processed depending on their treatment. In this experiment we had: *L. lactis* retrieved from flies from each of the six combinatorial treatments (3 regimes x 2 replicates); stock *L. lactis* also retrieved from the agar plate and frozen at -80 ºC in Ringer's-trehalose mixture but without passing by the fly environment; and an empty agar droplet processed in a similar manner but without bacteria as a negative control. Therefore, it totalizes in eight bacterial treatments. For each bacterial treatment, a set of bacteria retrieved from 24 distinct flies were defrosted in a 37º C water bath for 90 seconds and then left at room temperature for one hour for a slow acclimatization. The samples were then pooled according to treatment. Each resulting mix was then washed three times through centrifugation at 2880 rcf for 10 minutes and replacing the supernatant by 100 µL of Ringer's solution. Lastly, the bacterial concentration was adjusted to $1x10^8$ CFU/ml in all treatments through OD. Two serial dilutions from each mix were prepared and the following ones were plated: $1:10^4$, $1:10^5$.

The experiment was split into two replicates separated by two days. For each treatment we had the following subsets: an acute phase subset, where 60 flies were pricked with the respective solution and the alive flies by day one post injection were sacrificed to assess bacterial growth during this phase of infection; and a chronic phase subset, in which 120 flies were pricked, their survival was tracked for two weeks and at the 14^{th} day of infection they were sacrificed to assess the set-point bacterial load for each treatment, discussed in (Duneau et al. 2017). Flies from the last subset were flipped to new vials every four days. Bacterial load was quantified through the protocol measured above but this time we platted all the dilutions from 1:1 to $1:10^{11}$ in case there was an increase in bacterial load due to the treatment.

2.7 Statistical analysis

Statistical analyses and figures were performed in RStudio version 1.3.1073 (R Core Team, 2019). We used the following packages in our statistical analyses "lme4" (Bates et al. 2014) , "glmmTMB" (Brooks et al. 2017), "car" (Fox and Weisberg 2018), "pscl" (Jackman et al. 2015), "lawstat" (Hui, Gel, and Gastwirth 2008), "survival" (T. M. Therneau and Lumley 2014) 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 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 levels) and 'person' (two levels), meant that we fitted them as fixed effect, rather than random effect, factors (Crawley 2007). In general, we employed a Wald χ^2 test (Bolker et al. 2009) to test for statistical significance, either by using the Anova function in "car" package or anova function in "stats" package, accordingly. Moreover, for the main effects we used a type II and for the interactions a type III Anova.

2.8.1 On-going and F5 response to selection readouts

During selection we obtained the following readouts: i) survival for all the regimes; ii) resistance for Tolerance and Resistance regimes; iii) fecundity for Tolerance regime; iv) fecundity-tolerance for Tolerance regime. Therefore, we tested for effect of generation, regime and replicate on survival and resistance readouts, whereas for fecundity and fecundity-tolerance we only tested for generation and replicate. Survival was measured as the proportion of flies alive by day four in each generation x regime x replicate. Individual resistance was estimated as the inverse of the median bacterial load at day four post infection for each fly. For changes in reproductive dynamics we measured the average and maximum population fecundity for each generation x replicate. Fecundity-tolerance was assessed as the population slope between the fecundity (i.e. pupae counts for day three and four post infection for each mother) and bacterial load (i.e. individual CFU counts four days post infection). On the other hand, for F5 generation only resistance, average fecundity and fecundity-tolerance were measured but across all regimes. In both on-going and F5 response to selection tests we analysed the readouts fitting a generalized linear model using the package "lme4" with a Quasi-Poisson error structure.

2.8.2 Post-selection readouts

In this experiment we assessed resistance, fecundity, fecundity-tolerance, survival in the presence and absence of infection with *L. lactis* across different selection regimes. To start with, we used bacterial load at day four post infection as a proxy for resistance, our dependent variable. As predictors we included regime (i.e. Control, Resistance or Tolerance) and replicate (i.e. one or two). Given the proportion of zeros (≥ 25%), we applied a Hurdle model with a truncated probability distribution for zero and non-zero values using "hurdle" function in "pscl" package. For values of zero CFU we used a binomial with logit link, whereas for non-zero values we used a negative binomial with log link function. The latter was decided based on the output from likelihood ratio and Akaike information criterion tests.

As a proxy for the response variable fecundity we used the number of pupae offspring produced by each individual female during day three and four post injection. We fitted distinct generalized linear model with a Quasi-Poisson error structure to test for: a) cost of infection, as the effect of injection treatment with Ringer's solution or *L. lactis* on fecundity; b) differences in fecundity across regimes infected with *L. lactis*; c) changes in fecundity across regimes injected with Ringer's solution. The different models are described below:

a) Fecundity ~ Infected/non-infected * Regime * Replicate

b) Fecundity $_{\text{Ringer's}}$ ~ Regime * Replicate

c) Fecundity $_{\textit{L. lactis}}$ ~ Regime * Replicate

In the case of fecundity-tolerance, we fitted a generalized linear model with negative binomial error structure using the "glmmTMB" package. Our previous models shown evidence of a cost of infection on fecundity, our response variable. For this reason, we adjusted the fecundity of individual infected fly to the average fecundity of non-infected flies, as in (Kutzer and Armitage 2016b; Graham et al. 2011). In detail, adjusted fecundity was calculated by subtracting pupae offspring counts from infected flies (infected fecundity, ω_i) from mean pupae offspring counts in our Ringer's control (uninfected fecundity, ω_o), for each regime x replicate, and dividing the resulting value by ω_o and multiplying by 100. As predictors we included individual $\log_{10}$-transformed bacterial load, regime and replicate. The resulting model is the following:

Adjusted fecundity ~ Bacterial load * Regime + Bacterial load * Replicate + Regime * Replicate

Lastly, 16-day survival was analysed using a Cox model with the four fecundity time windows as a time dependent covariate (Fisher and Lin 1999; T. Therneau, Crowson, and Atkinson 2017; Murphy and Sen 1991) using the function "coxph" of the package "survival" (T. M. Therneau and Lumley 2014). Only flies that survived past the fecundity time-window would contribute with their fecundity for the survival analysis. For instance, if a fly would die would die during day two post infection would not be contributed its fecundity for the analysis, but a fly dying on day three would as the latter was alive through the whole fecundity window measured. We selected this model based on the cox proportional hazards. The model used is the following:

Survival ~ Regime * Infected/non-infected * Replicate

Moreover, the fecundity windows were also individually analysed using generalized linear models with a Quasi-Poisson error structure using the package "lme4". The resulting *p*-values were adjusted using the Benjamini-Hochberg (1995) correction for multiple testing. For each of the fecundity windows we used the following model:

Fecundity ~ Regime * Infected/non-infected * Replicate

2.8.3 Parasite evolution

In this experiment we considered bacterial load at time-points 1- and 14-days post infection, as well as 14-days survival, as data output. Both bacterial load days were firstly analysed using a Levene test to assess the equal variance across treatments using the function "levene.test" in the package "lawstat" (Hui, Gel, and Gastwirth 2008). Later, we used a Hurdle model, as described above (Section 2.8.2 of Materials and Methods) given the high proportion of zeros, with regime, selection replicate and experimental replicate as predictors.

Survival was analysed with cox proportional hazard using the function "coxph" in the package "survival" (T. M. Therneau and Lumley 2014) with treatmente (i.e. regime and selection replicate, Ringer's or ancestral stock of *L. lactis*), and experimental regime as predictors. We ran the model including and excluding Ringer's treatment. The model fulfilled all the proportional hazard assumptions.

3. Results

3.1 On-going and F5 response to selection readouts

Survival at day four post injection differed according to regime and generation (Table S1) throughout the selection lines experiment (Figure S5). Bacterial load, as proxy for resistance, was not affected by neither of the infected evolved regimes (i.e. Resistance and Tolerance), replicate or generation (Table S2, Figure S6). Both maximum and average fecundity of tolerance regime lines exhibit a significant effect of generation (Table S3, Figure S7). Lastly, the slope of the regression line between fecundity and bacterial load, indicator of tolerance, did not significantly vary according to generation within the Tolerance regime (Table S4, Figure S8).

Upon F5 generation, a set of readouts were measured across all regimes, as mentioned above (Section 2.8.1 Materials and Methods). Populations fecundity was strongly affected by bacterial load and replicate but there was no evidence for an interaction between bacterial load and regime, meaning the different lines did not differ in fecundity-tolerance at this point (Table S5, Figure S9). A similar result was observed for resistance with no significant effect of either regime or replicate on bacterial load (Table S6, Figure S10). In the case of average line fecundity there was a significantly effect of replicate but not regime (Table S7, Figure S11).

3.2 Post-selection readouts

After three generations without a selection pressure, we measured the readouts described above (Section 2.8.2. Materials and Methods). As expected, flies injected with Ringer's solution had a higher survival than ones infected with *L. lactis* independently of their selection regime (Table 1, Figure 1A). Similarly, the time-dependent variable fecundity seems to have an effect on flies' survival, with an overall higher survival and fecundity for Ringer's injected flies, as indicated by an interaction between infection treatment and fecundity. An interaction between regime and replicate was also observed, with replicate 1 of the Tolerance and replicate 2 of the Control regimes exhibiting higher survival than their counterparts.

Fecundity exhibits a complex dynamic throughout the first 16 days post injection. During the first two days of infection there is only a marginal interaction between regime and replicate,

whereas during day 3 to 4 we observe an effect of infection treatment with Ringer's injected flies having higher offspring counts than bacteria infected ones (Table 2, Figure 1B). Into the chronic phase of infection, on day 8 and 10 there is no difference in fecundity. However, later on we do observe an effect of infection treatment, replicate and regime on day 14 to 16 after injection. The latter reflects a higher offspring counts for Ringer's injected flies, replicate 1, and Resistance regime overall (Table 2, Figure 1B).

In regard to fecundity-tolerance and resistance we see an interesting pattern. Our data indicates Resistance and Tolerance regimes both differ in tolerance comparatively to Control (Table 3) but not in qualitative resistance or clearance (Table 4). Due to the fact we observed a cost of infection in fecundity for these flies (Table S8) and for the subset we measured survival and fecundity (Table 2), we normalized infected flies' fecundity with the respective Ringer's injected ones, as described above. While for Control regime we spot a negative slope, representing a decreased fecundity with the increased bacterial burden, for Resistance and Tolerance regimes we have a highly positive slope, meaning that with the increase in bacterial load there is an increase in fecundity, comparatively to the respective Ringer's injected flies (Figure 1C). Although not tested, Resistance and Tolerance do not seem to differ in their slope but rather in their vigour, or fecundity baseline in the absence of infection. Flies from different regimes do not differ in either qualitative resistance or clearance at day four post infection (Table 4, Figure 1D and E). In sum, selection for fecundity-tolerance and resistance seem to have favoured the evolution of tolerance in opposition to resistance.

3.3 Parasite evolution

Bacterial parameters of *L. lactis* that underwent differently selected hosts immune systems were assessed during the first fourteenth days of infection and their response compared to the ancestral bacterial stock or absence of infection, Ringer's injected flies. Virulence has been described as a function of survival, the higher the virulence the lower the survival (Read 1994; Schmid-Hempel and Ebert 2011). Within this framework, we observed an effect of treatment, when including or excluding Ringer's (Table 5, Figure 2A). These results mean that first, as expected flies injected with *L. lactis* lead to a higher mortality than the saline independently of the source of the bacteria, and second, bacteria that were exposed to the inner fly environment are selected to kill more and therefore, are more virulent. However, regime does not seem to have an effect on the virulence evolution.

After 24h within a naive host, we observe a higher bacterial burden within evolved treatments of *L. lactis*, comparatively with the ancestral stock (Table 6 and S9, Figure 2B). Interestingly, this result is in part due to the increased number of successful infections established at day one in evolved treatments comparatively to the ancestral stock (Table S8). This result agrees with Levene's test that indicated a high variance for evolved bacteria, comparatively to ancestral stock (df = 3, F-value = 3.29, p-value = 0.021). Despite this difference, within evolved treatments there is no difference in the bacterial load either in median burden or variance (df = 2, F-value = 1.96, p-value = 0.143). Notably, there is also an effect of experimental replicate across this day (Table 6 and S8).

Into the chronic phase, 14 days post infection, we observe a similar pattern to the 24h with most evolved treatments presenting a higher number of persisting bacteria (Table 7, Figure 2C), except for Control 1 and Tolerance 1. The level of clearance at this stage is also variable only Control 2 and Tolerance 2 differing from the ancestral stock (Table S10). Both these results are shown by the strong effect from the interaction between treatment and replicate (Table 7). Furthermore, they do not differ in variance at this stage, either when including (df = 3, F-value = 0.97, p-value = 0.409) or excluding the ancestral stock in the analysis (df = 2, F-value = 0.52, p-value = 0.598).

4. Discussion

Here, we report the first study using experimental evolution to select for increased tolerance to infection. Altogether, our results exhibit fecundity-tolerance to *L. lactis* is evolvable within eight generations of exposure and selection. Moreover, we emphasize the importance of taking in consideration tolerance evolution when selecting for resistance, largely overlooked in the past decades, as we show selection for resistance can also collaterally select for fecundity-tolerance. Lastly, we show four-days intra-host pathogen evolution selects for higher virulence and infection success independently of the host selection regime. In sum, our study sets tolerance as a fundamental immune strategy that needs to be included in upcoming eco-evo-immunology studies.
selection.

4.1 Increase in fecundity throughout selection

During the eight generations of selection we observed a clear difference between non-infected (i.e. Control) and infected regimes (i.e. Resistance and Tolerance) in survival (Table S1, Figure S5). There was also a statistical effect of generation due to the steady decrease in survival along the first five generations and later increase in survival during generation seven and eight for infected regimes (Table S1, Figure S5). This result is likely due to an excessive cost of infection at this inoculation concentration ($1x10^9$ CFU/ml) that was passed into following generations. After a generation without selection and a reduction in the concentration ($1x10^8$ CFU/ml) we observe a quick increase in the survival (Figure S5) presumably because of the lightening of the costs, allowing the population to evolve and supporting the previous assumption.

In regard to resistance, although we do not observe differences between regimes at F5 generation (Table S6, Figure S10), we do detect an overall significant effect of regime throughout selection generations (Table S2). Nevertheless, generation is a stronger predictor of resistance and it seems to interact with regime, indicating resistance differs between generations and each of the regimes (i.e. Resistance and Tolerance) behave distinctively (Table S2, Figure S6). F5 response to selection only reflects changes up to this point and therefore does not consider changes under a lighter selective pressure during generations six and seven. Moreover, a prior study from Martins and colleagues in this fly population has shown can take up to 12 generations of selection to evolve resistance from systemic infection with *Pseudomonas entomophila* (Martins et al. 2013), implying the number of generations in this study might not be enough.

For fecundity-tolerance, we do not observe any change in the slope of the reaction norm between fecundity and bacterial load, our proxy for tolerance, across the different generations of selection (Table S4, Figure S8) or between regimes upon F5 generation response to selection (Table S5, Figure S9). In the latter, we observed a strong effect of bacterial load and replicate on fecundity, indicating individual fecundity varies according to flies' bacterial burden and that replicates might be starting to diverge in tolerance at this point.

Interestingly, we spot a striking effect of selection on fecundity. Upon F5 generation we only note a difference between replicate in fecundity, in agreement with the previous results for fecundity-tolerance. Intriguingly, during selection we observe an increase in both maximum and average fecundity for each of the Tolerance replicate lines, prominently during generation

six and seven (Figure S7), supported up by a statistically significant effect of generation for both measures (Table S3). Altogether, this result provides more support to our hypothesis that selective pressure was too high during the first five generations, not allowing population to evolve. Moreover, this fecundity increase during day three and four post infection might be a result of selection for early fecundity and therefore a fitness shift for this populations.

4.2 Selection for higher tolerance in both infection regimes

After three generations without selection, we assessed the response to selection throughout the different regimes and replicates in the presence and absence of infection. Contrarily to what previously observed from our group, when using the same host-parasite model (Silva et al., n.d.), we observed a cost of infection on fecundity during day three and four (Table 2, Figure 1B). As evidently indicated in Figure 3, but not statistically tested, there is a dramatic decrease in offspring number between the first days and sixteen days post infection (Figure 1B). The selection protocol seems to have shifted reproduction to first days after injection, regardless of the injection treatment or regime. This shift led to higher offspring counts during the first four days after infection and, potentially, explains why we observe a cost of infection during the third and fourth day. Curiously, this cost is also found during day 14 and 16 post injection, indicated by a marginally significant effect of infection treatment on fecundity (Table 2).

As is well-known, life history traits are often entangled with each other, such is the case of survival, fecundity and immunity in this study. Fecundity seems to be a strong predictor of the regimes' survival, together and in mixture with infection treatment (Table 1). In this particular case, Ringer's injected flies both present a higher survival and fecundity across the sixteen days assessed (Figure 1A-B), comparatively to the flies inoculated with *L. lactis*. The latter treatment presents both costs in survival and fecundity due to immunity against infection, as it has been widely shown and discussed in literature (Flatt and Heyland 2011). More interestingly, we observe an effect of regime in survival (Table 1), as resistance regime seems to poorly handle the infection and therefore have lower resistance compared to its counterparts (Figure 1A). A potential explanation for this phenomenon is the deployment of tolerance mechanisms outside of the scope of our study that allow Control and Tolerance regimes to sustain a higher survival comparatively to Resistance one. This effect of regime also has an interaction with replicate, as both replicates of Control and Tolerance regimes behave differently within the regime (i.e. Control replicate 2 and Tolerance replicate 1 have higher survival). Together with the divergent fecundity between replicates observed during the on-going selection readouts (Figure S7), this

result might indicate different replicates have unique evolutionary history despite the same selection regime. Although we meticulously uniformized the selection protocol, we cannot discard genetic drift associated to the selection protocol or, an unlikely but possible, different standing genetic variation present in each of the replicates at the start of the selection lines that might be amplified into distinct evolutionary trajectories by the selection protocol. Moreover, due to our small number of generations and our large selective sieve (50% of the population selected at each generation) it is likely there is a lot of variation within the different lines and a few more generations of selection would trim this variation.

Despite the variation in survival across replicates of the same regime, the same was not found in resistance or fecundity-tolerance readouts (Table 3 and 4, Figure 1C-E). Higher tolerance was uniformly selected in both Resistance and Tolerance regimes in comparison to Control (Table 3, Figure 1C). On the other hand, the three regimes do not differ in quantitative resistance or proportion of clearance, suggesting there is no evolution for resistance after eight generations of selection (Table 4, Figure 1D and E). An important consideration is that to our knowledge, all the studies with *D. melanogaster* and bacterial species classify resistance evolution as an increase in survival after selection (Ma et al. 2012; Ye, Chenoweth, and McGraw 2009; Martins et al. 2013; Gupta et al. 2016). On the other hand, we classify resistance evolution as a reduction in the bacterial load (i.e. quantitative resistance) or increased infection clearance after selection. We argue that survival to infection reflects a combination of many immune and non-immune factors and, therefore, is an oversimplified measure of resistance. In this study we consider resistance as the inverse of bacterial load and within the fruit fly and within this framework only a study from Martins and colleagues have measured changes in bacterial load after selection for resistance (Martins et al. 2013). In this study, the same fly population and *P. entomophila*, as a pathogen, showed that although there is an increase survival after selection for resistance, differences in how the Control and Resistance-selected lines handle infection are only visible during at 14h and not at five days post infection (Martins et al. 2013). Taking this into account, we assessed bacterial load at day four post infection and it is conceivable that at this point we do not detect the fine differences in resistance between selected and non-selected regimes. Nevertheless, within the same time-window we observe a clear increase in fecundity-tolerance for both Resistance and Tolerance regimes (Figure 1C). This outcome suggests tolerance might be a faster, or even preferential, immune strategy to evolve, as it has been hypothesized (Roy and Kirchner 2000; Best, White, and Boots 2008). Moreover, the fact only one of these immune strategies evolved might represent in this host-

parasite interaction resistance and tolerance mechanisms might be independent and the evolution of one of them does not depend, in any way, on the other, as has been shown in a few cases throughout literature (Lefèvre, Williams, and de Roode 2010; Sternberg et al. 2012; Mazé-Guilmo et al. 2014b; Decker, de Roode, and Hunter 2018).

Nonetheless, there are caveats to the latter conclusions taken in this study. The first is the limited number of replicates in the post-selection readouts comparatively to the on-going and F5 generation ones. As in post-selection readouts we only possess two out of the three replicates, it is likely we do not observe a direct translation of the results prior to this time-point. Second, we focused on a set of fundamental readouts and therefore we do not address a wide range of physiological, developmental and other life history aspects that might being taking place in evolved populations, due to technical and model limitations. Within these readouts, an important consideration is the fact both resistance and tolerance are conceptually two distinct set of defence mechanisms that are based on the same individual bacterial load data. Therefore, it is possible resistance affects tolerance in our model. Lastly, as it has been shown (Howick and Lazzaro 2014; Lough et al. 2015; Kutzer and Armitage 2016b; Silva et al., n.d.), resistance and tolerance are intrinsically dynamic strategies that interchange and often throughout the course of infection. Our study aimed to select and detect changes in a small time-frame of the long infection process, therefore it is plausible variation in these, and other, immune strategies (e.g. immune priming) might be found outside of the time-frame studied.

Nevertheless, to our knowledge, only Zeller and Koella (Zeller and Koella 2017) have empirically measure both resistance and tolerance after experimental evolution, in this case for different infection and dietary settings. Although, several studies have hypothesized or presented signs of tolerance evolution (Vijendravarma, Kraaijeveld, and Godfray 2009; Ma et al. 2012; Martins et al. 2013; Kraaijeveld and Godfray 2008) after immune system evolution to pathogens we present the first evidence of active selection for tolerance in a host organism due to pathogen exposure. It is possible that we are able to observe this outcome because of the affinity of the host to this particular parasite. *L. lactis* used in this study was extracted from wild-caught *D. melanogaster* (Lazzaro 2002) and therefore it is likely they share a pre-existing coevolutionary history if not even part of this host microbiota. In this scenario, tolerance could be a strategy already in use by the host and easier to evolve compared to other ones.

4.3 Higher infection success after intra-host bacterial evolution

Heretofore, we analysed the different regimes as separate populations, but from in this section we will address every replicate of every regime as a separate population due to their distinct evolutionary outcomes discussed in the former sections. Here, we evolved a stock culture of *L. lactis* within six populations of hosts for four days before inoculating them into naive hosts. The six populations are a full factorial combination of three selection regimes and two selection replicates.

After inoculation with the different treatments, we observed all the bacterial treatments had a significant reduction in survival compared to the Ringer's control (Table 5), as expected. Across evolved and non-evolved *L. lactis* treatments, we measured virulence as a function of host survival. Hence, we observe that bacteria that underwent flies' immune system exhibit a higher virulence comparatively to the ancestral stock (Table 5), illustrated by the reduced host survival in these treatments (Figure 2A). However, there was no statistical effect of the different host evolved treatments (i.e. selection regime and replicate) on the bacterial virulence evolution. Our study falls in agreement with a study from Duneau and colleagues that has shown an increased virulence evolution of bacteria when previously exposed to the inner host environment for as few as 12h (Duneau et al. 2017).

In regard to early growth, measured as the bacterial burden at one day post infection, we observe that evolved treatments exhibit higher average and variance in bacterial load compared to the ancestral stock (Table 6 and S8, Figure 2B). This increased growth after intra-host parasite evolution has been observed by Haine and colleagues at different time intervals of incubation (Haine et al. 2008). To our surprise, we did not observe any difference between evolved treatments (Table 6 and S8, Figure 2B).

On the other hand, persistence, assessed as the bacterial load at 14 days post infection, exhibits a more distinct and complex pattern. First, the number of persistent infections is significantly higher in Control 2 and Tolerance 2 but rather similar to the ancestral stock for the other evolved treatments (Table 7 and S9, Figure 2C) reinforcing the idea that different selection replicates within each regime might have different starting points and/or evolutionary histories and consequently, selected different traits. Second, the persisting infections do vary in load across treatments (Table 7 and S9, Figure 2C), as Control 1 and Tolerance 1 do not differ from the ancestral stock. This result emphasizes the need to track several time-points during infection to understand the parasites dynamics and adaptation to the host immune system.

All in all, our intra-host evolution results seem to point out four days is enough to select for higher virulence and adaptation to the insect host but potentially not long enough to select a bacterial population for specific resistance or, more importantly, tolerance mechanisms. Particularly in the presence of tolerance, we would expect bacteria to require a longer time-period within a host to select for persistence, as this is likely to be a sum of adaptation to the host and clonal interference between different bacterial genotypes (Little et al. 2010).

Hence, our study has proposed tolerance as a potentially preferable immune strategy in this specific host-parasite interaction and experimental settings. Tolerance has long been found across different animal species and populations but our studies emphasizes that its evolvability might be possible. Moreover, we show short intra-host evolution does not select against immune strategies but does select for a better survival within a new host, both by increased early growth, increased persistence and overall higher parasite virulence. We hope with this study to shed a light on the importance of tolerance when studying immune responses evolution.

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Tables and figures

Table 1. The effect of regime, infection treatment (i.e. infected with *L. lactis* or injected with Ringer's), fecundity during the four time-windows and replicate. Statistically significant values are shown in bold.

Tested effect	*df*	χ^2	*p*
Regime	2	9.71	**0.008**
Infection treatment	1	458.24	**< 0.001**
Fecundity	1	498.97	**< 0.001**
Replicate	1	3.21	0.073
Regime x Infection treatment	2	1.30	0.523
Regime x Fecundity	2	0.13	0.936
Infection treatment x Fecundity	1	20.43	**< 0.001**
Regime x Replicate	2	29.13	**< 0.001**
Infection treatment x Replicate	1	1.62	0.203
Fecundity x Replicate	1	0.10	0.753
Regime x Infection treatment x Fecundity	2	1.35	0.509
Regime x Infection treatment x Replicate	2	1.85	0.396
Regime x Fecundity x Replicate	2	3.13	0.209
Infection treatment x Fecundity x Replicate	1	2.33	0.127
Regime x Infection treatment x Fecundity x Replicate	2	1.01	0.604

Table 2. Effect of regime, infection treatment and replicate on fecundity for each of the time-windows measured. p^{BH} indicates the *p*-values after adjustment for multiple testing using the Benjamini–Hochberg correction. Statistically significant factors are shown in bold.

	Effects on fecundity			
Tested effect	*df*	χ^2	*p*	p^{BH}
0 - 2 DPI				
Regime	2	7.71	**0.021**	0.074
Infection treatment	1	3.07	0.080	0.139
Replicate	1	4.46	**0.035**	0.081
Regime x Infection treatment	2	1.79	0.409	0.440
Regime x Replicate	2	11.83	**0.003**	**0.019**
Infection treatment x Replicate	1	0.60	0.440	0.440
Regime x Infection treatment x Replicate	2	4.44	0.108	0.152
2 - 4 DPI				
Regime	2	1.36	0.507	0.576
Infection treatment	1	15.46	**< 0.001**	**< 0.001**
Replicate	1	2.44	0.119	0.342
Regime x Infection treatment	2	2.48	0.290	0.406
Regime x Replicate	2	3.26	0.196	0.342
Infection treatment x Replicate	1	0.31	0.576	0.576
Regime x Infection treatment x Replicate	2	3.66	0.160	0.342
8 - 10 DPI				
Regime	2	4.14	0.126	0.220
Infection treatment	1	3.35	0.067	0.217
Replicate	1	7.09	**0.008**	0.054
Regime x Infection treatment	2	0.10	0.949	0.949
Regime x Replicate	2	1.45	0.484	0.564
Infection treatment x Replicate	1	0.58	0.446	0.564
Regime x Infection treatment x Replicate	2	4.75	0.093	0.217

14 - 16 DPI				
Regime	2	18.36	**> 0.001**	**> 0.001**
Infection treatment	1	5.92	**0.015**	**0.035**
Replicate	1	7.02	**> 0.001**	**0.028**
Regime x Infection treatment	2	1.11	0.573	0.801
Regime x Replicate	2	0.26	0.876	0.876
Infection treatment x Replicate	1	0.34	0.563	0.802
Regime x Infection treatment x Replicate	2	0.32	0.851	0.876

Table 3. The effects of bacterial load, regime and replicate on the response variable fecundity, measured as percentage difference in offspring counts. Statistically significant values are shown in bold.

Tested effect	*df*	χ^2	*p*
Bacterial load	1	0.51	0.475
Regime	2	5.89	0.054
Replicate	1	0.00	0.944
Bacterial load x Regime	2	10.65	**0.005**
Bacterial load x Replicate	1	0.79	0.375
Regime x Replicate	2	2.39	0.302

Table 4. The effect of regime and replicate on bacterial load (i.e. zero and non-zero CFU counts).

Tested effect	***df***	χ^2	***p***
Regime	2	2.09	0.351
Replicate	1	0.40	0.525
Regime x Replicate	2	0.07	0.965

Table 5. The effect of combinatorial treatment (e.g. regime x replicate), and replicate on survival. First, we compared to the Ringer's and secondly, to the ancestral stock. Statistically significant values are shown in bold.

Tested effect	*df*	χ^2	*p*
Compared to Ringer's			
Treatment	7	92.92	**< 0.001**
Replicate	1	1.04	0.307
Treatment x Replicate	7	13.67	0.057
Compared to Ancestral stock			
Treatment	6	25.04	**< 0.001**
Replicate	1	1.03	0.246
Treatment x Replicate	6	12.68	0.048

Table 6. The effect of combinatorial treatment (e.g. regime x replicate), and replicate on bacterial load (i.e. zero and non-zero values). Statistically significant values are shown in bold. For the full model and respective coefficients, see Table S9.

Tested effect	*df*	χ^2	*p*
Treatment	6	267.49	**< 0.001**
Replicate	1	8.65	0.003
Treatment x Replicate	6	13.78	0.032

Table 7. The effect of combinatorial treatment (e.g. regime x replicate), and replicate on bacterial load. Statistically significant values are shown in bold. For the full model and respective coefficients, see Table S10.

Tested effect	*df*	χ^2	*p*
Treatment	6	75.03	**< 0.001**
Replicate	1	0.11	0.745
Treatment x Replicate	6	119.81	**< 0.001**

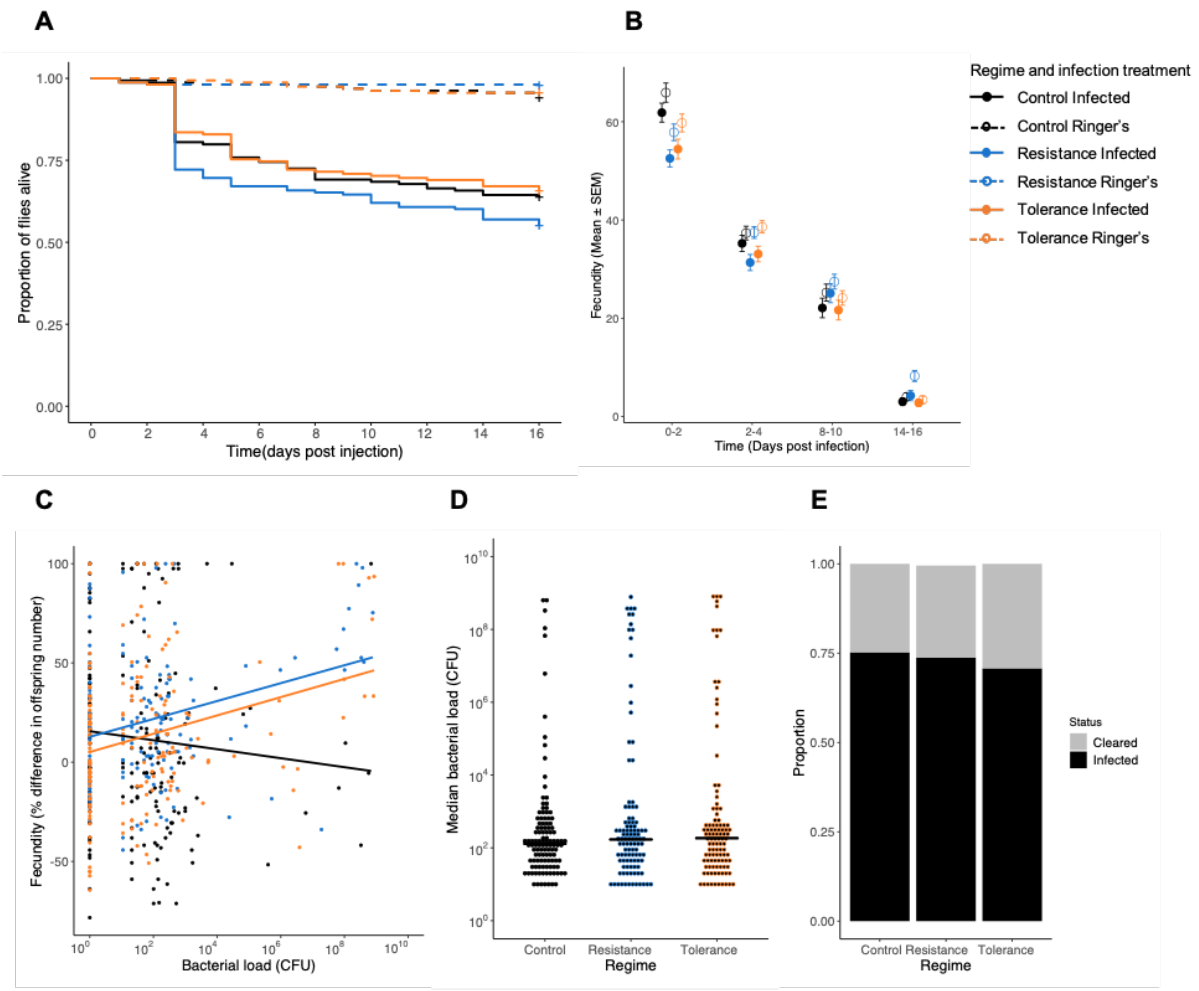

Figure 1. Response to selection for each of the evolving regimes and the respective injected control. (A) Survival of flies from each selection regime when infected with *L. lactis* or pricked with saline until day 16 post injection. There is a significant difference between infected and pricked with saline across regimes (Table 1) (B) Average fecundity for each regime when infected or pricked with saline for the four-fecundity time-windows post injection. (C) Fecundity-tolerance to infection with *L. lactis* for each of the evolved regimes at day four post infection. There is a significant difference of resistance and tolerance regimes comparatively to the control one (Table 3). (D) Quantitative resistance, as the median bacterial load for each regime for flies that did not clear infection. There is no evidence for a difference in bacterial load counts between regimes, meaning they share a similar level of resistance at this time-point (Table 4) (E) Bacterial clearance, as the proportion of flies with or without CFUs at day four post injection for each the regimes. Each regime is comprised of two replicates. Sample size for (A) and (B) is from 149 to 159 flies per combinatorial treatment, while (C), (D) and (E) ranges from 202 to 236 flies per infected regime. For statistics, see Table 1, 2, 3 and 4.

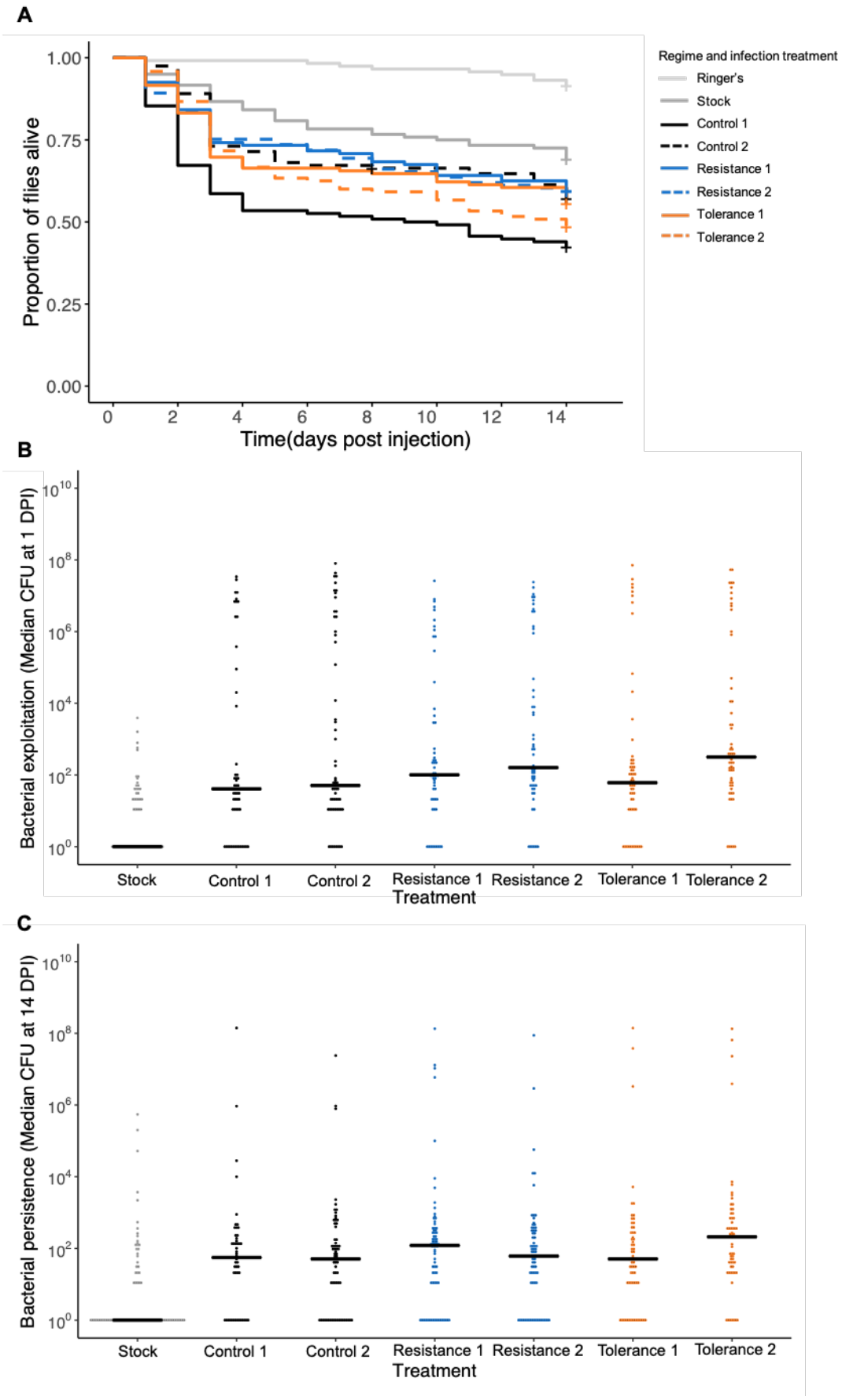
A
Proportion of flies alive
1.00
0.75
0.50
0.25
0.00
0
2
4
6
8
10
12
14
Time(days post injection)
Regime and infection treatment
Ringer's
Stock
Control 1
Control 2
Resistance 1
Resistance 2
Tolerance 1
Tolerance 2
B
Bacterial exploitation (Median CFU at 1 DPI)
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
10^{0}
Stock
Control 1
Control 2
Resistance 1
Resistance 2
Tolerance 1
Tolerance 2
Treatment
C
Bacterial persistence (Median CFU at 14 DPI)
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
10^{0}
Stock
Control 1
Control 2
Resistance 1
Resistance 2
Tolerance 1
Tolerance 2
Treatment

Figure 2. Bacterial virulence, exploitation and persistence after evolving inside different hosts. *L. lactis* was evolved within flies from different evolved regimes and replicates, in a combinatorial design and then injected in naive flies. As controls we have both flies injected with Ringer's or non-evolved stock of *L. lactis*. (A) Survival of flies from each selection regime and replicate when infected with the respective treatment until day 14 post injection. There is a significant difference between infected and pricked with saline across regimes (Table 5). (B) Bacterial growth in the first 24h as individual CFU counts for each evolved regime and stock control. There is a statistically significant difference between culture stock of *L. lactis* and evolved ones (Table 6). (C) Bacterial persistence in the chronic phase of infection, at day 14 post infection for each evolved regime and stock control. There is a statistically significant difference between stock *L. lactis* and previously evolved within hosts, as well as (Table 7). In (A) the sample size ranged from 116 to 121 flies per treatment, whereas in (B) from 55 to 60 flies and (C) 48 to 82 flies. For statistics, see Table 5, 6 and 7.

Supplementary information

Table S1. The effect of regime (i.e. Control, Resistance or Tolerance) and replicate (i.e. one, two or three) and generation on each line's survival. Survival was measured as the proportion of flies alive at day four at each generation. Statistically significant values are shown in bold.

Tested effect	*df*	χ^2	*p*
Regime	2	271.86	**< 0.001**
Replicate	2	0.17	0.9196
Generation	7	41.82	**< 0.001**
Regime x Generation	4	1.74	0.7841

Table S2. The effect of regime (i.e. Resistance or Tolerance) and replicate (i.e. one, two or three) and generation on each line's resistance. Resistance was measured as the inverse of the median bacterial load of flies alive at day four at each generation. Statistically significant values are shown in bold.

Tested effect	*df*	χ^2	*p*
Regime	1	7.19	**0.007**
Generation	6	0.17	**< 0.001**
Replicate	1	41.82	0.951
Regime x Generation	6	1.74	**0.027**

Table S3. Effect of generation and replicate (i.e. one, two or three) on both average and maximum fecundity for each of the replicate tolerance lines. Fecundity was measured as the number of pupae from egg laying during third- and fourth-day post-infection. Statistically significant values are shown in bold.

	Average fecundity			*Maximum fecundity*		
Tested effect	*df*	F	*p*	*df*	F	*p*
Generation	1	10.54	**0.004**	1	4.68	**0.043**
Replicate	2	0.19	0.823	2	0.74	0.492

Table S4. Effect of generation and replicate on fecundity-tolerance for the Tolerance-selected lines. Fecundity-tolerance was measured as the slope of the regression line between day three and four fecundity, and bacterial load at day four.

Tested effect	*df*	F	*p*
Generation	1	3.41	0.082
Replicate	2	1.28	0.302
Generation x Replicate	2	2.15	0.147

Table S5. The effect of bacterial load, regime and replicate on the response variable fecundity at generation F5, measured as offspring counts of egg laying at third and fourth day after infection. Statistically significant values are shown in bold.

Tested effect	*df*	χ^2	*p*
Bacterial load	1	35.52	**< 0.001**
Regime	2	1.43	0.490
Replicate	2	57.61	**< 0.001**
Bacterial load x Regime	2	0.84	0.660

Table S6. The effect of regime and replicate on the response variable bacterial load at day four after infection during generation F5.

Tested effect	*df*	χ^2	*p*
Regime	2	4.25	0.120
Replicate	2	1.51	0.470

Table S7. The effect of regime and replicate on the response variable fecundity during generation F5. The latter was measured as the average population fecundity during day three and four post infection.

Tested effect	*df*	χ^2	*p*
Regime	2	1.31	0.520
Replicate	2	65.59	**< 0.001**
Regime x Replicate	4	4.54	0.338

Table S8. Full model and coefficients representing the difference in zero and non-zero bacterial load across combinatorial treatments (e.g. regime x replicate) and replicate, when compared to the *L. lactis* stock one day post infection. Statistically significant values are shown in bold. For main effects, see Table 6.

Tested variable	Estimate	Std. error	*z*	*p*
Non-zero model coefficients (truncated negative binomial with log link)				
Control 1	11.445	1.165	9.823	**< 0.001**
Control 2	13.687	1.126	12.153	**< 0.001**
Resistance 1	13.814	1.106	10.681	**< 0.001**
Resistance 2	12.155	1.099	11.063	**< 0.001**
Tolerance 1	13.208	1.118	11.817	**< 0.001**
Tolerance 2	12.759	1.084	11.772	**< 0.001**
Experimental replicate 2	3.157	1.073	2.942	**0.003**
Control 1 x Experimental replicate 2	-1.608	1.721	-0.934	0.350
Control 2 x Experimental replicate 2	-4.789	1.623	-2.951	**0.003**
Resistance 1 x Experimental replicate 2	-3.775	1.645	-2.295	**0.021**
Resistance 2 x Experimental replicate 2	-3.366	1.611	-2.090	0.037
Tolerance 1 x Experimental replicate 2	-4.875	1.646	-2.963	**0.003**
Tolerance 2 x Experimental replicate 2	2.874	1.614	-1.781	0.075
Zero model coefficients (binomial with logit link)				
Control 1	0.868	0.547	1.586	0.113
Control 2	1.578	0.615	2.730	**0.006**
Resistance 1	2.554	0.824	3.098	**0.002**
Resistance 2	2.189	0.715	3.061	**0.002**
Tolerance 1	1.678	0.615	2.730	**0.006**
Tolerance 2	2.266	0.713	3.178	**0.001**
Experimental replicate 2	0.069	0.530	0.130	0.896
Control 1 x Experimental replicate 2	0.568	0.830	0.683	0.494
Control 2 x Experimental replicate 2	0.519	0.944	0.550	0.583
Resistance 1 x Experimental replicate 2	-1.409	1.005	-1.401	0.161
Resistance 2 x Experimental replicate 2	-0.030	1.013	-0.029	0.977
Tolerance 1 x Experimental replicate 2	-0.335	0.855	-0.391	0.695
Tolerance 2 x Experimental replicate 2	0.299	1.090	0.274	0.784

Table S9. Full model and coefficients representing the difference in zero and non-zero bacterial load across combinatorial treatments (e.g. regime x replicate) and replicate, when compared to the *L. lactis* stock 14 days post infection. For main effects, see Table 7.

Tested variable	Estimate	Std. error	*z*	*p*
Non-zero model coefficients (truncated negative binomial with log link)				
Control 1	1.057	1.451	0.729	0.466
Control 2	3.499	1.158	3.022	**0.003**
Resistance 1	5.987	1.337	4.477	**< 0.001**
Resistance 2	-2.244	1.140	-1.969	**0.049**
Tolerance 1	1.674	1.204	1.391	0.164
Tolerance 2	5.528	1.197	4.617	**< 0.001**
Experimental replicate 2	-0.4705	1.446	-0.325	0.745
Control 1 x Experimental replicate 2	5.035	2.077	2.425	**0.015**
Control 2 x Experimental replicate 2	-1.887	1.869	-1.010	0.313
Resistance 1 x Experimental replicate 2	-8.005	1.859	-4.307	**< 0.001**
Resistance 2 x Experimental replicate 2	7.523	1.800	4.180	**< 0.001**
Tolerance 1 x Experimental replicate 2	4.500	1.880	2.392	**0.017**
Tolerance 2 x Experimental replicate 2	-0.238	1.890	-0.126	0.900
Zero model coefficients (binomial with logit link)				
Control 1	0.013	0.524	0.025	0.980
Control 2	1.390	0.510	2.723	**0.006**
Resistance 1	0.647	0.494	1.310	0.190
Resistance 2	0.947	0.509	1.862	**0.063**
Tolerance 1	0.793	0.486	1.631	0.103
Tolerance 2	2.334	0.685	3.407	**< 0.001**
Experimental replicate 2	-1.063	0.481	-2.209	**0.027**
Control 1 x Experimental replicate 2	3.592	0.974	3.687	**< 0.001**
Control 2 x Experimental replicate 2	0.322	0.731	0.440	0.660
Resistance 1 x Experimental replicate 2	2.336	0.754	3.097	**0.002**
Resistance 2 x Experimental replicate 2	1.484	0.730	2.034	**0.042**
Tolerance 1 x Experimental replicate 2	2.280	0.810	2.817	**0.005**
Tolerance 2 x Experimental replicate 2	0.621	0.945	0.658	0.511

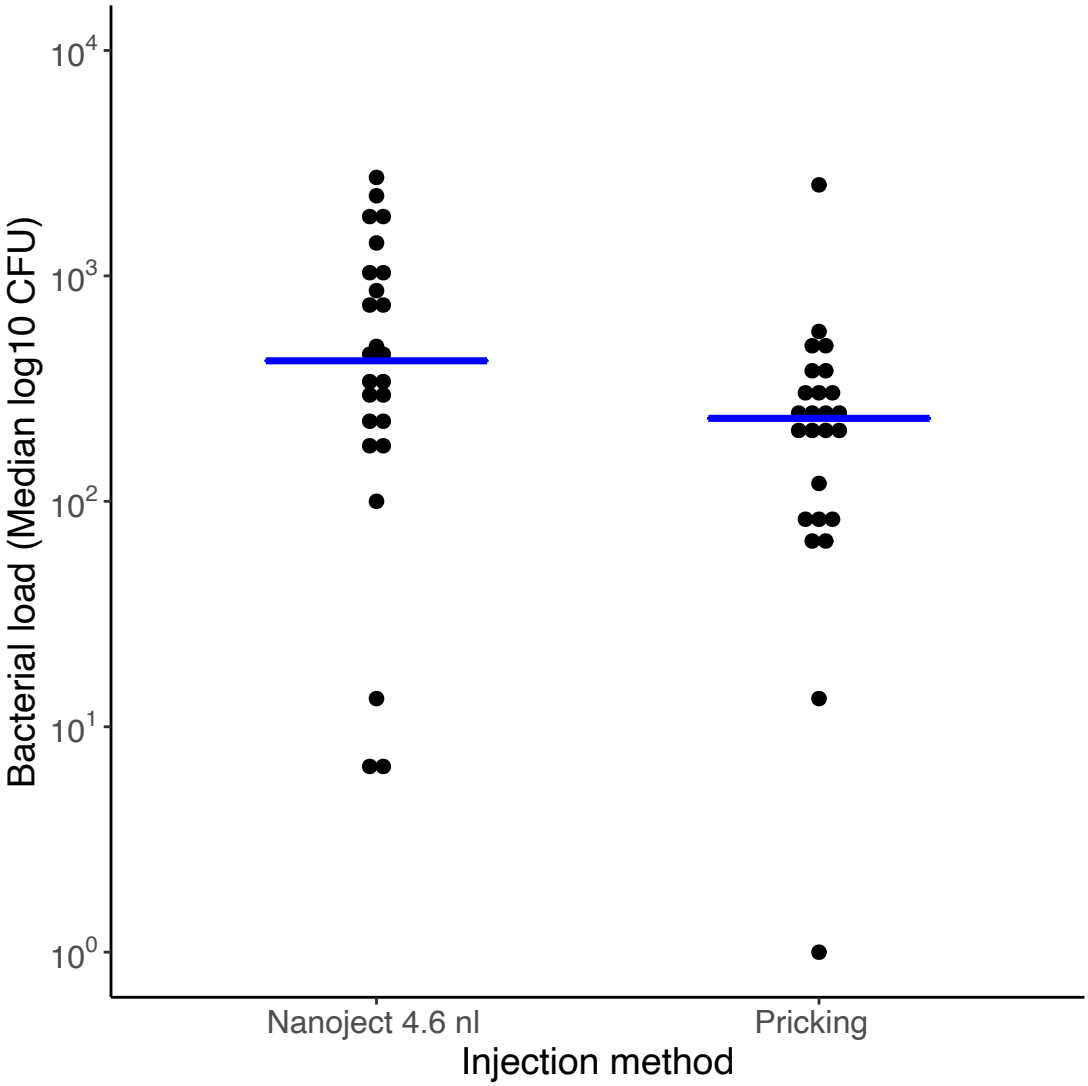

Figure S1. Comparison between variation produced by different bacterial injection methods. A bacterial solution of *L. lactis* at the concentration of $2x10^8$ CFU/ml was injected into female flies either through pricking or injection of 4.6 nl with the help of a Nanoject. Each treatment is composed of 25 female flies. After injection, each fly was placed into a pre-prepared 1.5 ml tube with LB agar and a steel bead and kept on ice for immediate homogenization. Bacterial load quantification was performed as described in the Materials and Methods. There was no significant difference in variation between the two injection methods.

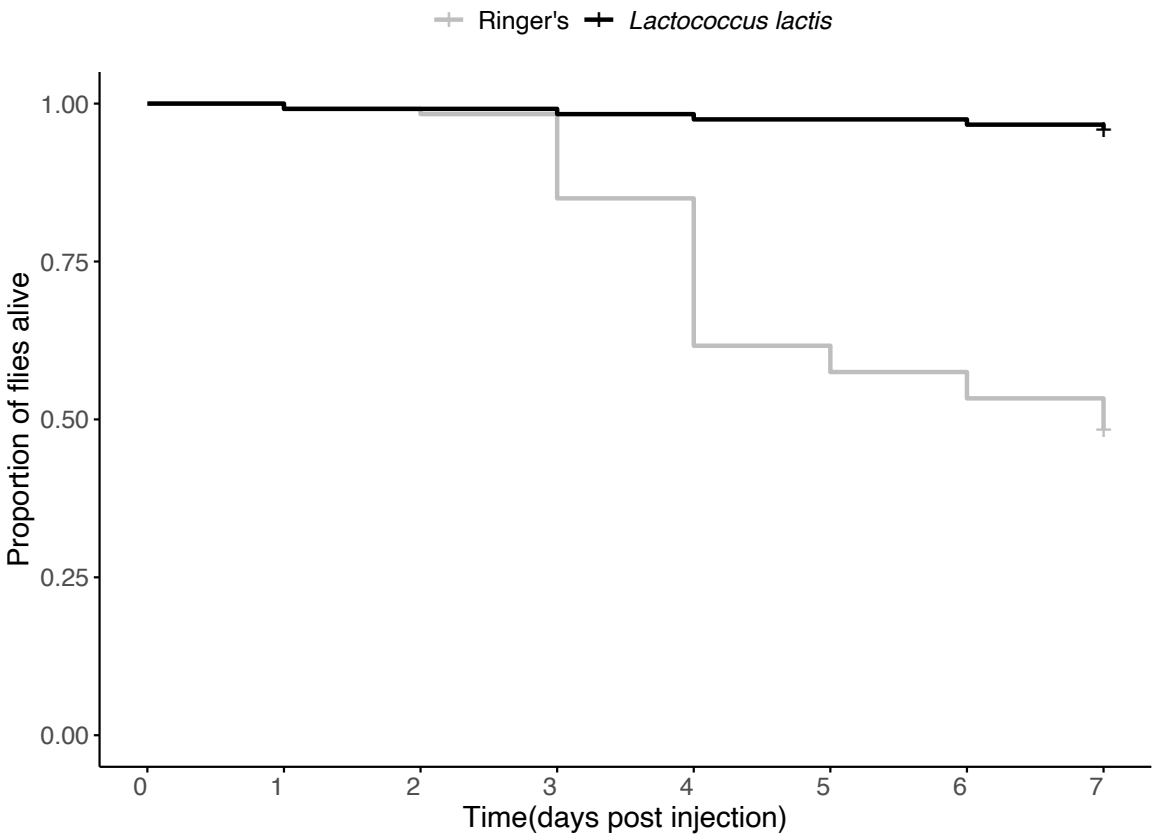

Figure S2. Seven-day survival after pricking with *L. lactis* at the concentration of $1x10^9$ CFU/ml. Five days after emergence, 120 female flies per treatment were pricked with *L. lactis* and their survival was followed by seven days after. At day four post infection, we have approximately 40 % mortality in the infected treatment. The two treatments differ significantly (Cox proportional-hazard, p-value < 0.001).

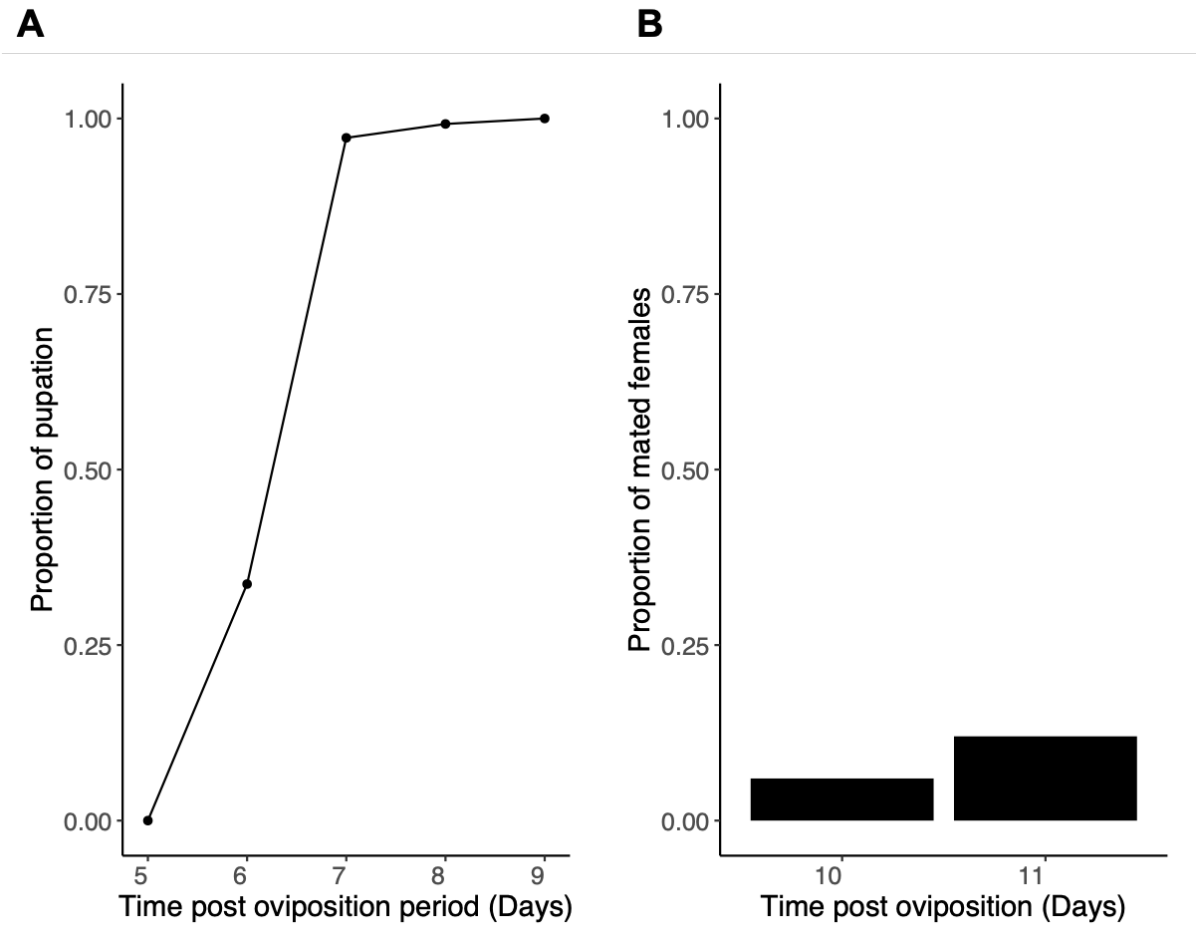

Figure S3. Preliminary assay on time to pupation and sperm prevalence. A group of 40 female flies were allowed to mate and to egg lay for 48 hours. (A) The pupation rate for the offspring of the latter female flies was followed until day 12 post oviposition. We are only representing until day 9 as all the larvae had developed to pupae until this day. (B) Female offspring was collected and individualized either only in the morning of the 10th or 11th day post oviposition and changed to new vials four days after to simulate selection lines mating protocol. Afterwards, vials were followed for offspring and female flies were classified into virgin or mated depending on the absence or presence of larval offspring. We illustrated here the proportion of mothers that did not mate depending on the time they were collected. As expected, emerging flies' collection on day 11 had a higher proportion of mated flies comparatively to day 10, 12% and 6%, respectively.

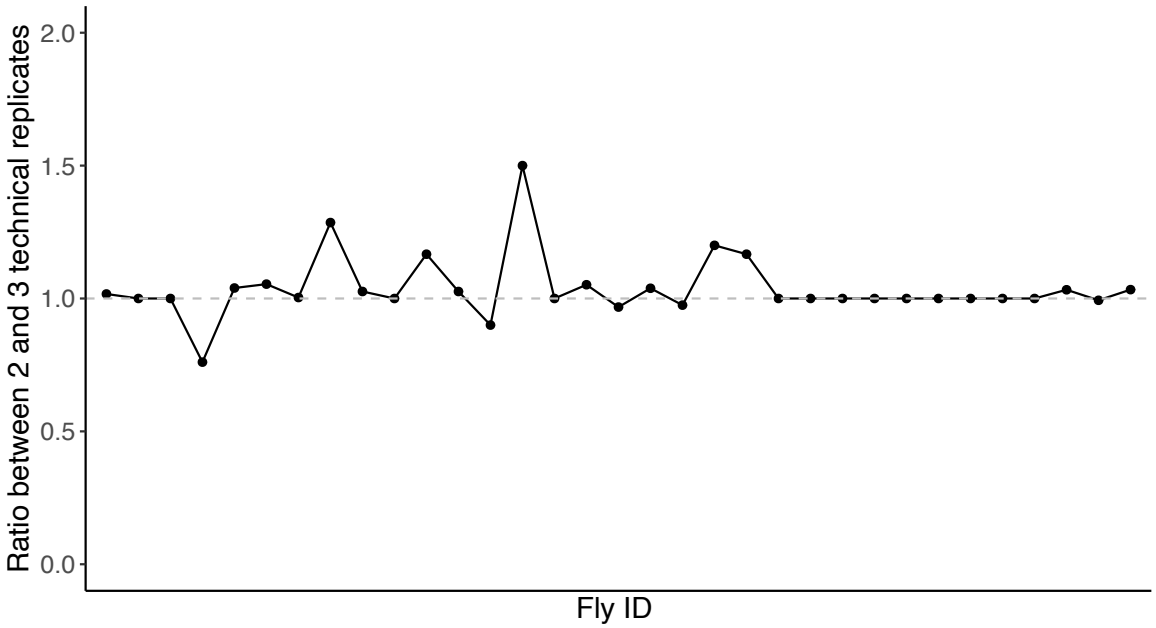

Figure S4. *In silico* test to estimate individual bacterial load with 2 or 3 technical replicate droplets. Using the data from Figure S1 we compared the CFU/fly calculation when using three droplets and when using only two, by randomly removing one of them. In the horizontal axis we have 20 different flies represented. The grey dashed line stands for a ratio of 1, meaning in a ratio with this value there is no difference between using either of the technical replicates. Apart from some occasional flies, most bacterial load estimates do not differ greatly between the two methods (e.g. biggest difference is 1.6x difference for one of the flies). Moreover, this plot represents proportional changes and coincidently, the flies with stronger deviations from y = 1 have a low bacterial load and therefore small changes in estimation methodology translate have a larger impact on their ratio.

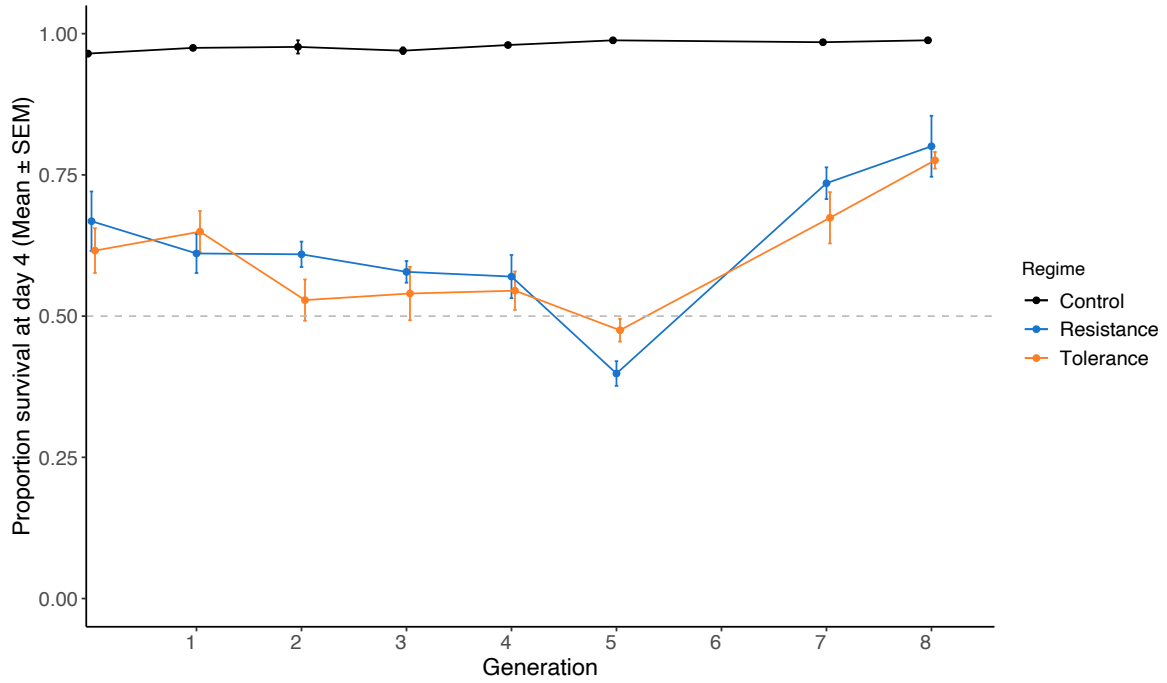

Figure S5. Survival at day four post injection of the flies from each selection regime when infected with *L. lactis* (Resistance and Tolerance) or injected with Ringer's solution (Control). Each regime is composed of three replicate lines with 200 female flies each. Generation 6 did not undergo selection and therefore there is no data for this time-point. Dashed line at y = 0.5 represents the selection threshold. There is a significant effect of both regime (p-value < 0.001) and generation (p-value < 0.001) on survival.

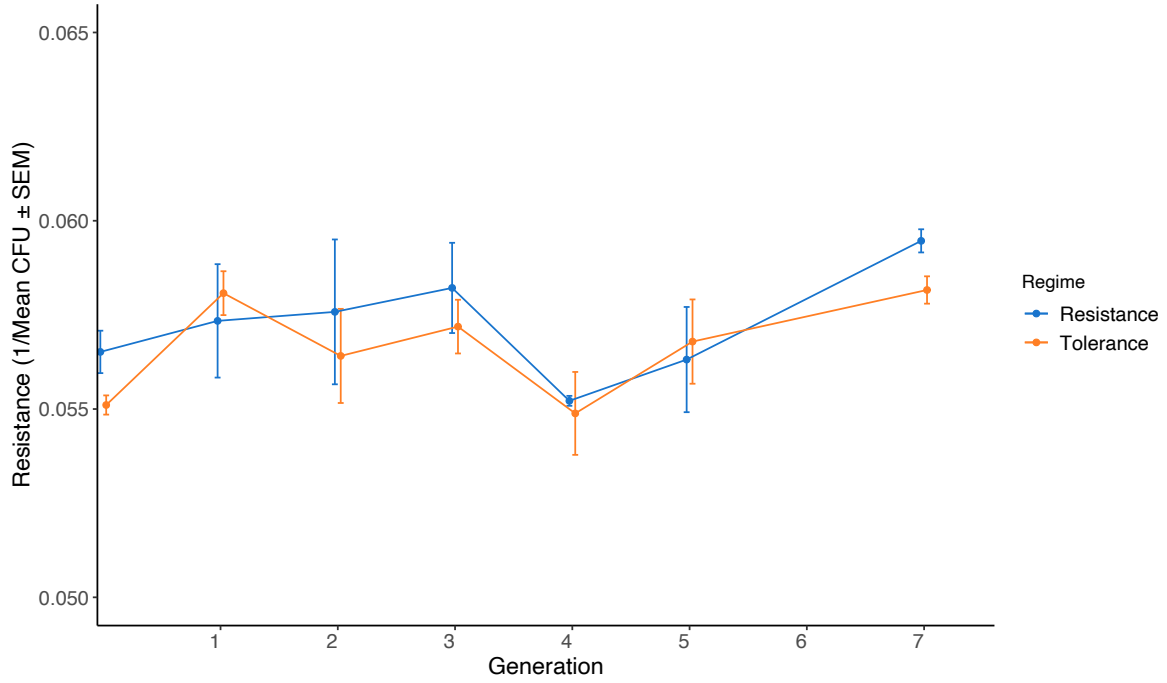

Figure S6. Resistance day four post injection with *L. lactis* for Resistance and Tolerance selection regimes. As a proxy for resistance we calculated the mean of the inverse of the bacterial load from each individual and the respective standard error. Each regime is composed of three replicate lines. Generation 6 did not undergo selection and therefore there is no data for this time-point. There is no significant effect of either regime, replicate or generation.

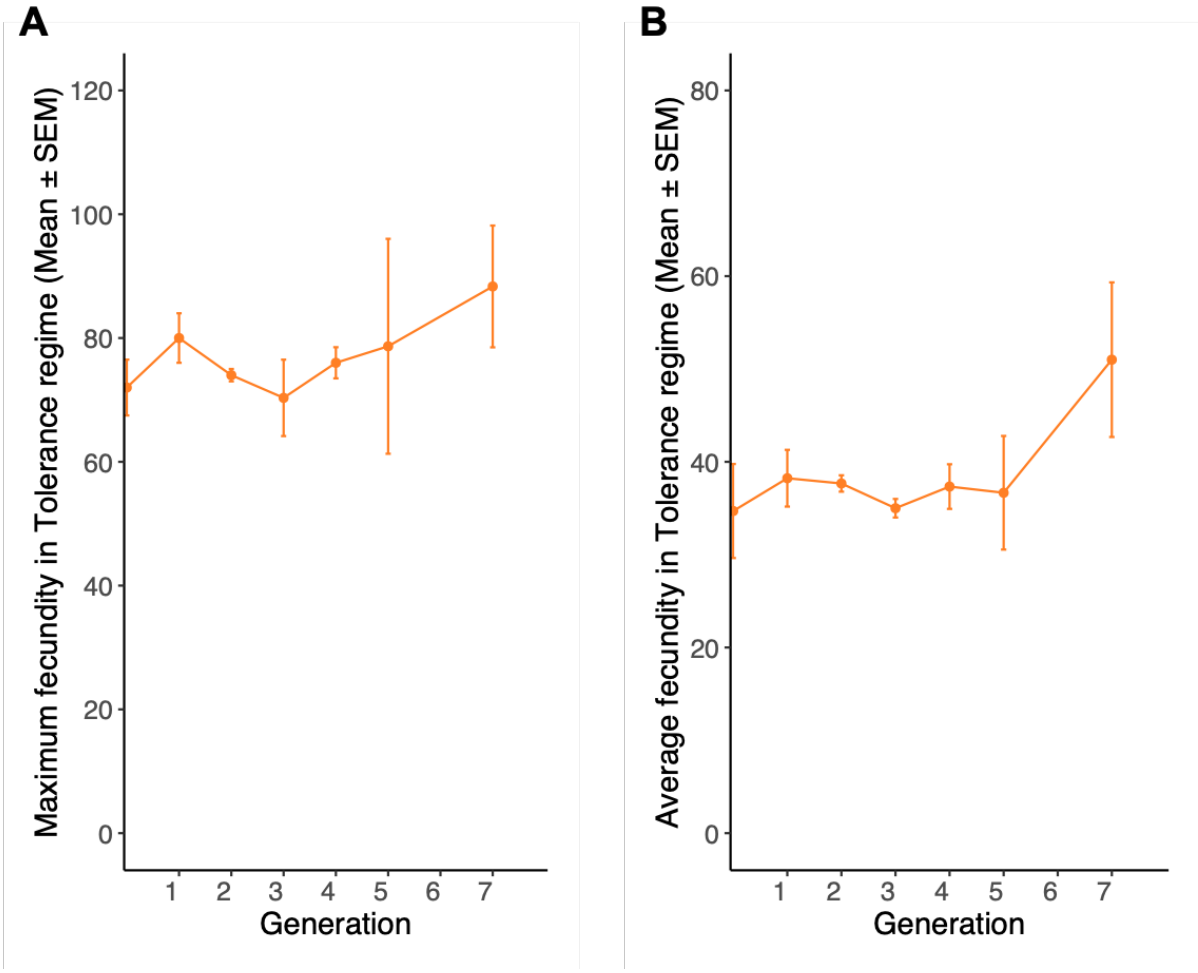

Figure S7. Fecundity of tolerance regime across generations. (A) Maximum fecundity represents mean of the highest individual offspring pupae count in each of the three replicates of this regime for each generation. Similarly (B) average fecundity represents the mean of the average offspring pupae counts from each replicate at each generation. Generation 6 did not undergo selection and therefore there is no data for this time-point. There is a significant effect of generation on both maximum (p-value = 0.0440) and average fecundity (p-value = 0.0075).

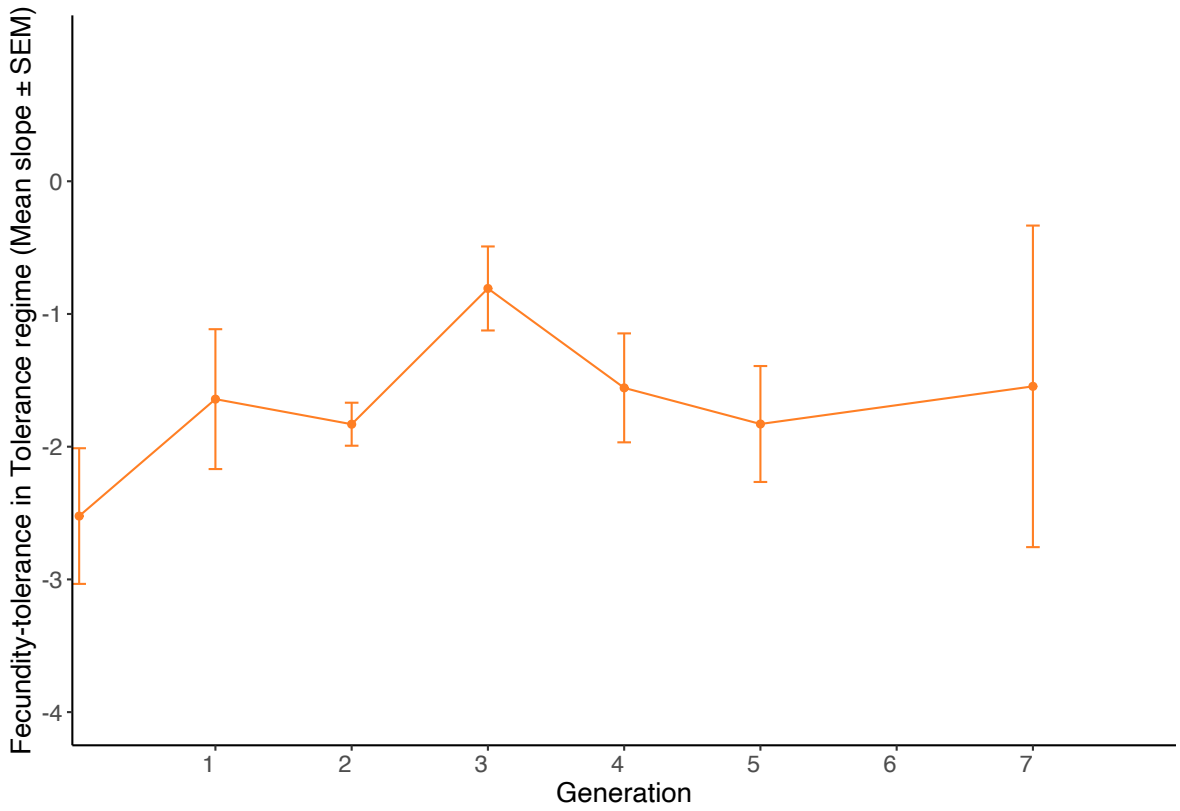

Figure S8. Fecundity-tolerance for tolerance regime across selection generations. Here we used slope of the regression norm between our reproductive fitness measure (i.e. individual pupae count during day three and four post infection) against bacterial load (i.e. CFU counts at day four post infection). Each time-point is comprised of three replicates to which we present the average with the standard error of the mean. Generation 6 did not undergo selection and therefore there is no data for this time-point. There is no significant effect of either replicate or generation.

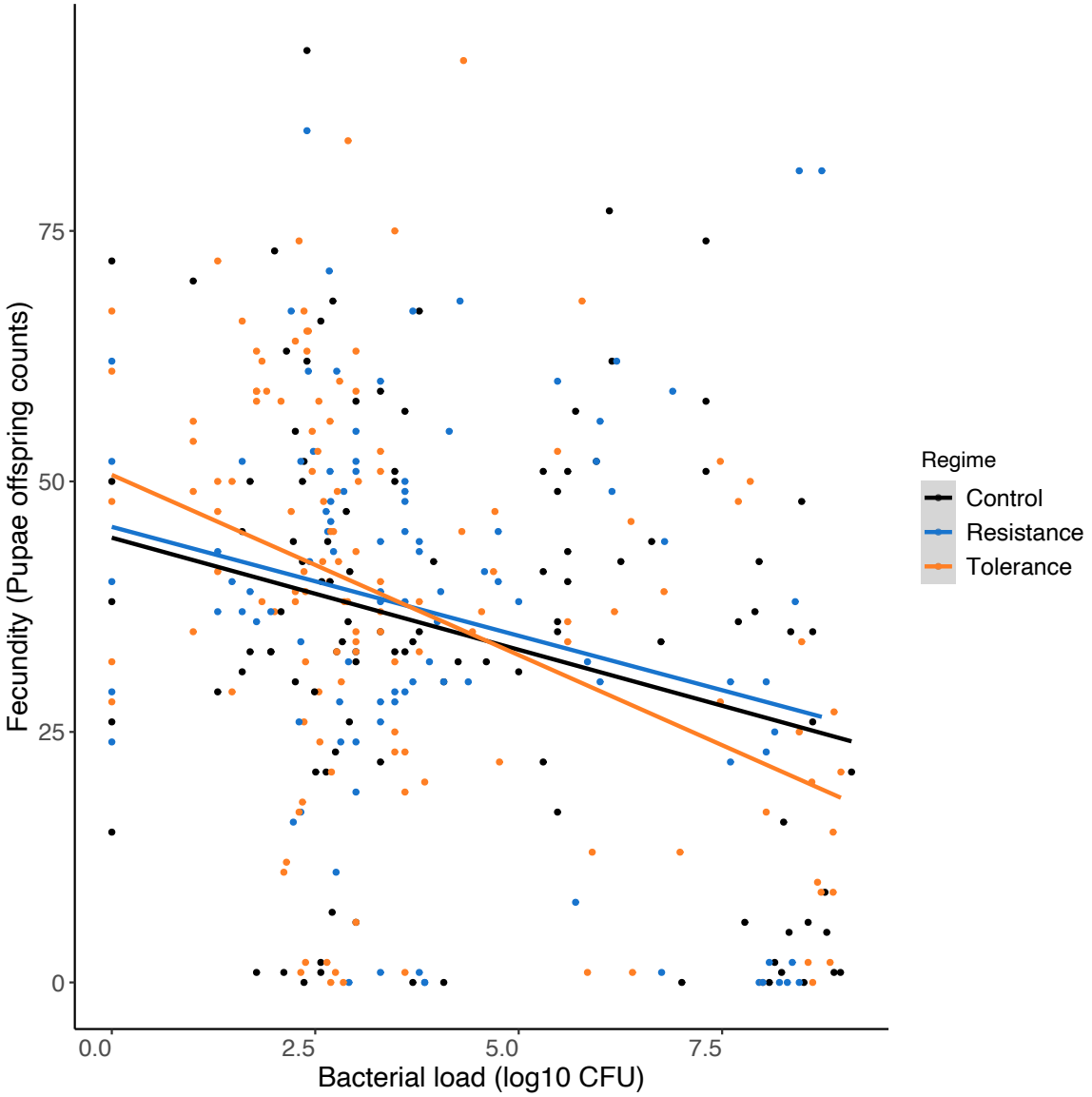

Figure S9. Fecundity-tolerance across all the regimes at F5 generation as the reaction norm between reproductive fitness and bacterial load at day four post infection with *L. lactis*. Each regime is comprised of 102 to 128 individuals split by three replicates. There is a significant effect of both bacterial load (p-value < 0.001) and replicate (p-value < 0.001) on our fecundity measure but no interaction was observed, meaning the different regimes do not differ in fecundity-tolerance between them.

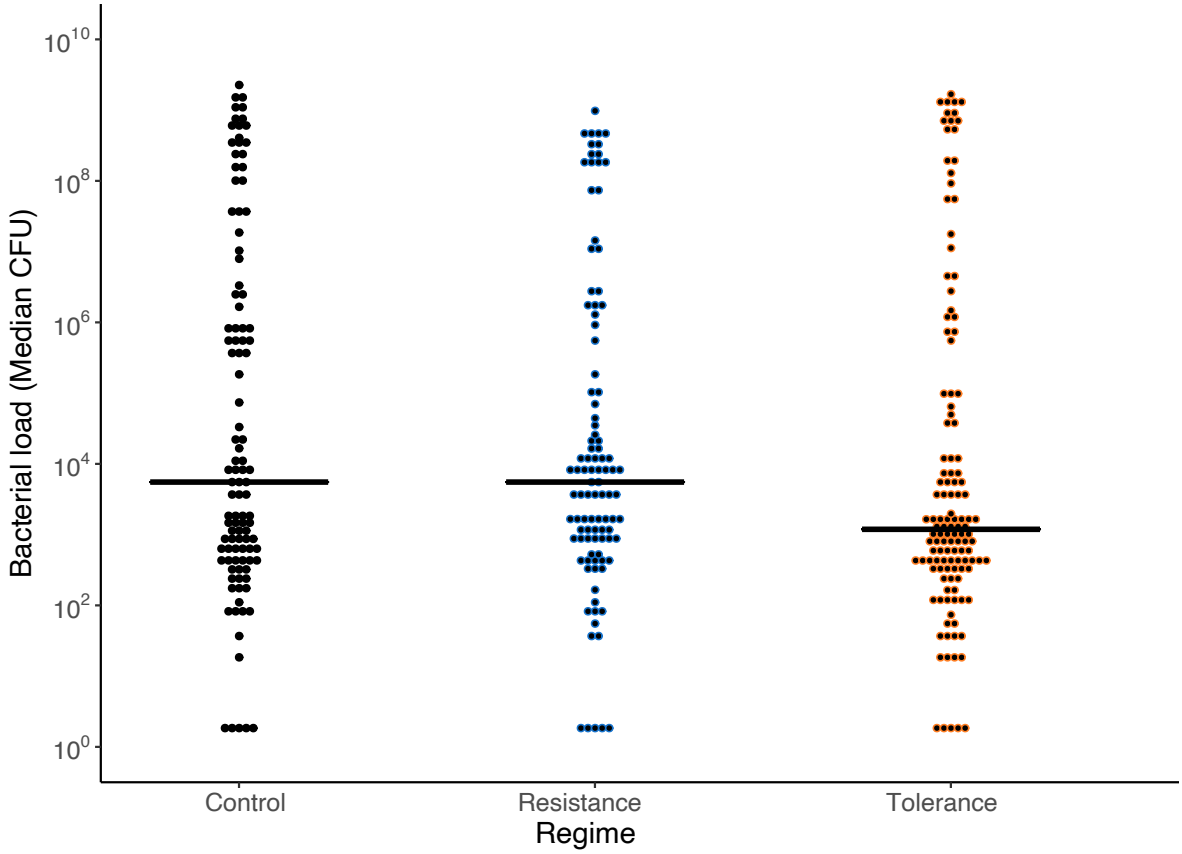

Figure S10. Resistance across all the regimes at F5 generation as median bacterial load at day four post infection with *L. lactis*. Each regime is comprised of 102 to 128 individuals split by three replicates. There is no significant effect of either regime or replicate.

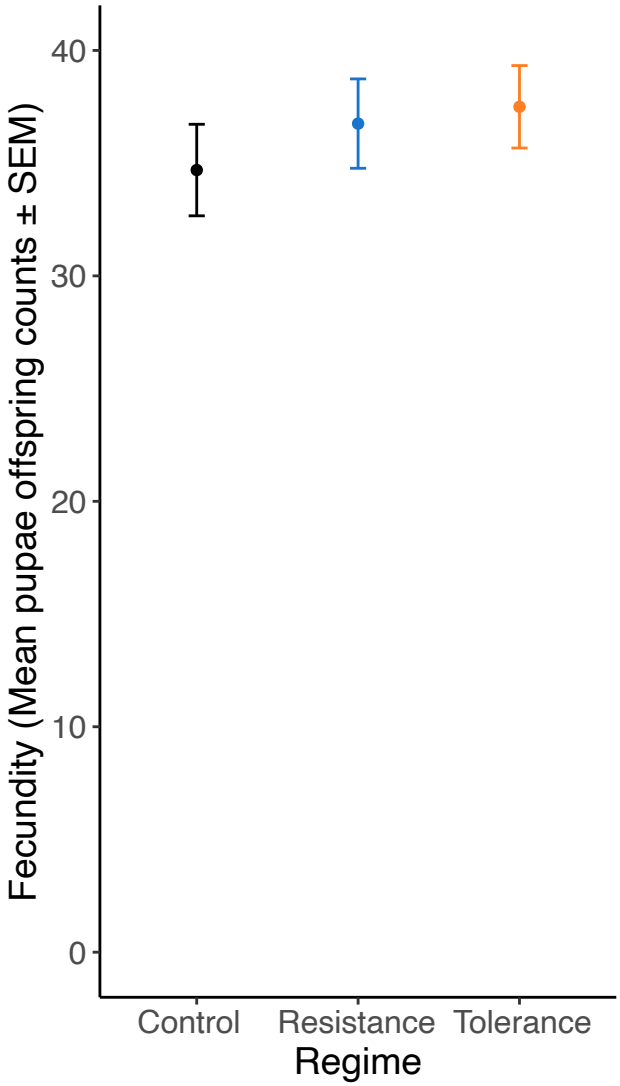

Figure S11. Average fecundity for each regime (i.e. Control, Resistance and Tolerance) at F5 generation as mean of the individual pupae counts during an overlaying period of 48 hours during day three and four post infection with *L. lactis*. Each regime is comprised of 102 to 128 individuals split by three replicates. There is a significant effect of replicate (p-value < 0.001) but not regime.

Chapter 6

General discussion and future perspectives

6.1 General discussion and concluding remarks

In the present thesis, we examined the contributions from the host and the pathogen to the host-parasite interaction and infection outcome, with main focus on host resistance and tolerance strategies. In this section, I will gather all the key points from the different experimental chapters and briefly discuss them by parasite and host perspective.

In Chapter 3, we demonstrated infection outcome is not restricted to two possible outcomes, survive with a persistent infection or die with a high bacterial burden, as described by Duneau and colleagues (Duneau et al. 2017). Certainly, some hosts did not control the infection and died within the acute phase presumably due to bacterial overgrow to a potential unsustainable bacterial load. This outcome is strongly affected by inoculation dose and bacterial virulence. However, distinctly to Duneau and colleagues, we focused on chronic phase, in which there was a variety of outcomes. Hosts that were able to control the infection past the acute phase did exhibit bacterial persistence up to 75 days of infection. However, bacterial species did not persist in all individuals as we observe evidence for bacterial clearance by the host throughout all stages of infection independently of bacterial species or inoculation dose. This infection outcome is particularly relevant for the most virulent of the bacterial species tested, *P. entomophila*, as the cost of tolerating an infection with high level of virulence could tend to infinite (Restif and Koella 2003, 2004). Moreover, bacteria did not necessarily persist at a constant bacteria burden. For instance, *P. burhodogranariea* actually showed evidence for a steady decrease throughout the chronic phase. The latter was also observed in the supplementary information of Chapter 4, suggesting it is not an universal phenomenon across bacterial species. We have evidence that some persistent infections follow individuals throughout their lifetime with some being cleared before death, while others not. Although not tested, it is possible both these group of flies died earlier due to a cost of a strong unsuccessful early immune response, a continuous response, a cost of the infection itself or a combination of the different factors.

We further decomposed virulence for three of the bacterial species tested in this thesis (i.e. *L. lactis*, *P. burhodogranariea* and *E. cloacae*). *L. lactis* and *P. burhodogranariea* seem to be more virulent due to their higher host exploitation and per-parasite pathogenicity (Råberg and Stjernman 2012). In particular, *L. lactis* seems to present the higher host exploitation out of the three bacterial species tested. Based on the latter result, we would expect to observe a

higher investment in resistance compared to tolerance. However, in Chapter 5 we did observe both strategies contribute to the infection dynamics during the acute phase of infection. Particularly fecundity-tolerance seems to be a fundamental strategy in this specific species interaction. Adapting Duneau *et al.* branching framework (Duneau et al. 2017), where flies can be split into two groups based on their bacterial load and consequent likelihood of surviving or succumbing to death, we assessed their fecundity-tolerance across branching categories and time-points. Our results show that flies likely to die show lower values of tolerance compared to the flies likely to survive. Moreover, tolerance seems to decrease between acute and early chronic phase, in agreement with literature in the field and reinforcing the idea that infection is dynamic due to variation within parasite properties but also within the host (Kutzer and Armitage 2016; Lough et al. 2015; Howick and Lazzaro 2014). For instance, in this Chapter we can hypothesize these reductions in both resistance and tolerance from acute to chronic phase might be due to an accumulation of costs from the immune response. Given that for tolerance we are using fecundity as a fitness measure, it is possible we are in the presence of terminal investment and that early tolerance might actually reflect an early focus in fecundity to assure the mother's contribution for next generation. However, we cannot say for sure which is the case as neither our experimental design nor questions were performed with that in mind.

Due to the intermediate level of virulence, discussed in Chapter 3, and clear variation and role of resistance and tolerance, discussed in Chapter 4, *L. lactis* represented the most logical bacterial species to test for the selection for host resistance and tolerance. In Chapter 5, we started by observing that the infection with *L. lactis* might actually carry heavier costs than previously expected. On light of all the data in this thesis, it is possible these costs might reflect the intense host exploitation *L. lactis* was demonstrated to possess, explored in Chapter 3, and indicated by the slow but steady reduction in survival during the first five generations of selection. Nevertheless, in this Chapter 5 we have evidence for the evolvability of fecundity-tolerance after either artificially selecting for it or resistance. This surprising result might suggest tolerance is more promptly available for selection, while resistance might demand more time to be selected in this model. Another interesting outcome of selection was the presence of a cost of infection in fecundity, in contradiction with what we observed in Chapter 4. While in Chapter 4 we assessed daily fecundity of survivors by day 9 post infection, in Chapter 5 we measured fecundity per time-window. Therefore, the two comparison are not really equivalent and might reinforce the importance of incorporating more complete analysis that include all individuals instead of sub-structuring them. Nevertheless, it is also likely that

the selection protocol selected for early fecundity and this shift exhibited a cost that was not present before or was too small to be detectable.

In addition to the number of factors that affect infection outcome and parasite success during infection addressed in Chapter 3, we demonstrated it is also important to consider previous adaptations to host system or their effectors, as shown in Chapter 5. Here, we demonstrated previous adaptation to the fly haemocoel for as long as four days is enough to select bacteria to be more virulent, grow faster in the acute phase and have a higher success in the chronic phase of infection, compared to the non-evolved bacterial counterparts. This result shows bacteria adapt very fast to the host environment, as previously shown by some authors. (Haine et al. 2008; Duneau et al. 2017). However, adaptation to specific mechanisms/strategies might take longer than the ones explored in this study. The latter might in part be due to the host dynamics in infection as shown in Chapter 4 in addition to the few time-points explored in Chapter 5. In either case, it would be important to address this question with a more extensive experiment than the one presented in here.

Altogether, this thesis shows evidence for the complexity of a host-parasite interaction. From the variety of factors that influence infection outcome to the temporal dynamics of both sides of the interaction, we highlight the need to perform a range of integrative experiments that take in consideration different levels of complexity in the infection process. In this study, we mostly explored host-parasite interaction from a descriptive broad populational level and therefore, our conclusions are in part limited due to it. We recognize the need to go deep into the mechanistic underpinnings of all these processes to fully understand its dynamics and that would be the next natural step for this project, but also field. In the next section we discuss in more detail potential routes for the further study of these questions.

6.2 Future perspectives

The work performed in this thesis was predominantly a representation of the host populational level response and how host tolerance and resistance vary within and across generations inside a given host population. Therefore, further steps into this project should take a more mechanistic approach. These are described and explored in the following sections.

6.2.1 Graphical and physiological bacterial characterization

In eco-immunology there is a very restricted number of *D. melanogaster* pathogens (Louie et al. 2016; Duneau et al. 2017), of which a fraction was explored in this thesis. From these, to our knowledge only *P. entomophila* has been studied in more detail with the identification of a toxin that explains part of its virulence (Opota et al. 2011) and *P. burhodogranariea* to which we have genomic data on the strain used here and a less-virulent one (Galac and Lazzaro 2012). In order to understand the infection process, it is important to not only comprehend how the host behaves but also what characterizes the pathogen and its pathogenicity, from infection site to the weapons in its toolset. For this, we can use both fluorescent microscopy after infection for detection of the path bacteria injected into a fly might take. For the second one, there are several tools from proteomics and eventual knockouts that would be fruitful to perform. An interesting starting point is a comparison between the two strains of *Providencia burhodogranariea* presently available, first a direct alignment of the two genomes against each other, second a knock-down or knock-out of possible virulent effectors in the more virulent strain. In addition, further questions can be made, for instance, does priming with a less virulent strain of *P. burhodogranariea* confers protection to challenge with the more virulent strain?

6.2.2 Host-parasite proteomic and metabolomic profiling

Insects are known to undergo severe metabolic changes during infection (Dionne et al. 2006; Schilder and Marden 2006). Based on the growing evidence for differential effectors and metabolic profiles for tolerance (Troha et al. 2018; Ganeshan et al. 2019; Wang and Medzhitov 2019; Mazé-Guilmo et al. 2014; Godwin et al. 2020), one of the clearest routes would be to take advantage of the selection lines developed in this study and test for differences in their proteomic or metabolomic profile. It has been shown that the expression of tolerance leads to change in metabolic profiles across a range of species (Ganeshan et al. 2019; Herms and Mattson 1992), namely with changes in glycolysis and, in insulin and insulin-like signalling (Cumnock et al. 2018; Flatt et al. 2008).

Although in this thesis we only addressed a few life history traits, there is the possibility that a number of other also vary, particularly physiological ones, as discussed above. If the selection protocol indeed interfered with some of them, it is possible this is reflected into the hormonal programme. If that is the case we would be able to see differences in hormones that balance reproductive, survival and physiological fitness, such as juvenile hormone 3 (Flatt and Heyland 2011; Schmid-Hempel 2005; Rolff and Siva-Jothy 2002; Flatt, Tu, and Tatar 2005).

This can be extended into the parasite side by analysing which elements are selected after undergoing resistance or tolerance-selected hosts. The results we obtained in Chapter 5 do not necessarily reflect the active bacteria within the host, therefore it might be more relevant to quantify active and growing bacteria through the measurement of replicating factors. Through host-parasite analysis we might uncover the differences in the selection outcome between different regimes but also within replicates of the same regime.

6.2.3 Tolerance as a function of age and energy availability

It has been hypothesized and partially demonstrated that immune strategies are differently expressed and/or available throughout a host lifetime (Harbeson, Francis, et al. 2018; Sheffield et al. 2020; Medzhitov, Schneider, and Soares 2012; Wang and Medzhitov 2019; Harbeson, Ben-Othman, et al. 2018). According to this theory, individuals might differ in their infection outcome not necessarily due to an immature or insufficient immunity but rather due to other constraints outside of the scope of this field. In this sense, tolerance might be a predominant strategy early and late in organisms' development because of the developmental and energetic constraints during earlier phases of life, and strong inflammatory responses late in life. Unfortunately, our field is biased to assess infection shortly after adult emergence and not outside of this time-window. However, a study from Sheffield and colleagues (Sheffield et al. 2020) has demonstrated that infection in older flies exhibits a lower tolerance than the younger counterparts after infection with Flock House Virus. This age-dependent pattern highlights the need for the characterization of infection in a further number of time-points throughout a host lifetime.

Moreover, one of the premises in which the previous hypothesis was based is the possibility that tolerance is a low-cost immune strategy and resources are allocated according to availability and host needs (Van Noordwijk and de Jong 1986; Flatt and Promislow 2007). To this date, we are aware tolerance is strongly affected by diet, with often an increase in tolerance in higher resource availability (Zeller and Koella 2017; Kutzer and Armitage 2016). Hence, these results actually suggest tolerance might be costlier than previously predicted and therefore, might be necessary to quantify the actual costs of infection. For this, an assessment of the individuals' reserves before and after infection would be possible through the quantification of lipids, such as triglycerides. This would allow us to quantify resistance, tolerance for each individual, or a group of individuals, and their energetic expenses/resource

allocation. Due to the destructive nature of the sampling in this host, for energetic, resistance and tolerance quantification, the experiment would need to be independently performed before and after infection in a high sample size to capture a populational response. Given the fact that we possess tolerance-evolved lines within this study, the most intuitive path would be to directly compare energetic responses between tolerance-evolved and non-evolved lines.

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Chapter 3: Beatriz Acuña Hidalgo, Luís M. Silva, Roland R. Regoes, Mathias Franz, Sophie A. O. Armitage, Decomposing virulence in persistent infections. In preparation.

SA conceived the idea; BAH, LS & SA designed the experiments, collected the data, and wrote the manuscript. MF, RRR & SA analysed the data. All authors contributed critically to the draft.

Chapter 4: Luís M. Silva, Beatriz Acuña Hidalgo, Mathias Franz, Sophie A. O. Armitage, Host tolerance and resistance dynamics using a branching model. In preparation.

BAH, LS and SA conceived and designed the experiments, BAH and LS collected the data, LS and MF performed the analysis and, LS and MF wrote the manuscript. All authors contributed critically to the draft.

Chapter 5: Luís M. Silva, Sophie A.O. Armitage, Effect of selection for host tolerance and resistance on a host-pathogen interaction and infection outcome. In preparation.

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Relevant meetings and workshops

Presentations

Silva *et al.* Effects of selection for host resistance and tolerance on a host-pathogen interaction. Oral presentation in the upcoming Ecological Immunology Workshop, Berlin, Germany. August 2020.

Silva, Acuña-Hidalgo & Armitage. Dynamics of insect tolerance to bacterial infections. Oral presentation in the AG Kurtz, AG Fricke and AG Armitage group workshop. Klingemühle, Germany. June 2019.

Silva, Acuña-Hidalgo & Armitage. Dynamics of insect tolerance to bacterial infections. Oral presentation in the Annual Ecology and Evolution PhD meeting, Bayreuth, Germany. March 2019.

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