The evolutionary ecology of reciprocal resistance in *Tenebrio molitor* and *Staphylococcus aureus*

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It's my tail,
and I'll chase it if I want to.

Oceansize, 2010.

Abstract

Hosts and infections can interact in potentially subtle and under-appreciated ways. These interactions are reciprocal and there is evidence that identical processes govern host defence and parasite pathogenicity. I demonstrate the benefits of approaching host and microbe in the same way by studying how costs and benefits of resistance in *Tenebrio molitor* and the opportunistic pathogen *Staphylococcus aureus* determine parasite persistence, pathogenicity, and collateral immunological self-damage.

Focusing first on bacteria, I divergently selected bacteria derived from one ancestral clone for resistance to an array of antimicrobial peptides (AMPs) to measure the genetic costs of evolving resistance to these immunological stressors, which has rarely been observed and was thought to be highly costly. Costs of resisting AMPs were comparable to those of resisting antibiotics against which resistance is common in environmental isolates, suggesting that intrinsic costs do not constrain natural AMP resistance. However the response to selection from AMPs in combination was much weaker, suggesting that the multiplicity of AMPs in antimicrobial immunity slows resistance evolution.

AMP-resistant bacteria showed significantly elevated resistance to *T. molitor's* immune response. My results therefore suggest that proposed medical use of these compounds could mediate pathogenesis.

I consider why autotoxic immunity has been conserved, and suggest autopathology is a secondary effect of a requirement for immune defence to remain potent against diverse infections. I found morphological self-damage after simulated infection, but fitness effects were unclear. I show that infected *T. molitor* transcribe numerous reactive enzymes that are predicted to damage self-tissue.

As a body of work these studies demonstrate that applying the same conceptual approach to hosts and their parasites can improve understanding of host-parasite coevolution. Costs of resistance can be classified identically in hosts and microparasites, likely generating identical consequences.

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Glossary

Term	*Description*
AMP(s)	Antimicrobial peptide(s). Small peptides synthesised by the innate immune system, with *in vitro* antimicrobial activity, and under development as new antibiotic drugs. Also referred to elsewhere as ribosomal AMPs (rAMPs) and cationic ribosomal AMPs (crAMPs).
Autopathology	Self-damage.
Immunopathology	a) An autopathological trait that is also associated with parasite killing. b) The proportion of post-infection fitness loss that can be directly attributed to collateral damage from endogenous immunological processes
Inoculum	Exogenous biological material introduced into a host's tissues.
Parasite	A functional classification for a symbiont that invades host tissues and gains energetic resources from a larger host. They do not necessarily cause pernicious harm to a host (see 'pathogen')
Pathogen	A sub-class of parasites, which gain additional fitness by causing pernicious damage to the host through activation of virulence factors, in addition to consuming a host's resources.
Pathology	a) The phenomenon of damage to a host after infection b) The proportion of host fitness loss that can be directly attributed to activation of a pathogen's virulence factors.
Pathogenicity	The property of pathology
PO	Phenoloxidase, an oxidative enzyme commonly assayed as a metric of 'immunocompetence' in

	invertebrates.
pro-PO	Pro-phenoloxidase, the inactive precursor to PO.
Selected culture	A bacterial culture after selection from a given stressor.
Symbiont	Any non-self organism living in a host's tissues, including the full range of functional interactions between mutualism and pathogen.
Tolerance	The condition of a host’s immune system permitting coexistence with a parasite.
Virulence	The sum of host damage caused by expression of immunopathology and parasite pathology
Virulence factor	A parasite trait that enhances that parasite’s fitness by damaging its host, e.g. secreted toxins that compromise the immune response by damaging host tissues.


resistance
AMPs
aureus
infection
bacteria
host
immune
response
responses
costs
selection
cultures
molitor
evolution
immunity
data
AMP
Figure
Resistance
antimicrobial
Antimicrobial
treatment
Chapter
parasite
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results
ability
Lee

Chapter One

Introduction

1.1. The conceptual framework of this thesis

This thesis aims to develop a more integrative understanding of how hosts and parasites resist one another, and to identify shared benefits and costs that determine these traits. There has been widespread recent recognition of the subtleties of how immunity evolves, which emphasizes that resisting a parasite may not always be the ideal strategy for a host. A reciprocal case is also made, arguing that there are some situations in which costs to a parasite of resisting host defence are too great for this strategy to be optimal. This is achieved by measuring costs of resistance in both a model host and model parasite. The approach is to try to understand the mechanistic bases of infection and immunity from an evolutionary biology perspective, and knowledge of the economic principles of modern evolutionary theory is assumed in the reader.

The body of the thesis is structured into two main parts, split over four chapters. Chapters 2 and 3 detail experiments regarding the costs and benefits to bacteria of resisting components of host immunity. Chapters 4 and 5 respectively aim to measure the costs to hosts of induced resistance to an infection; and to elucidate the mechanisms that might induce these costs of resistance. By studying the costs of resistance in both host and parasite, these experiments are intended as a case study in the advantages of applying a unifying conceptual framework to both components of the system. For continuity and comparability, the mealworm beetle *Tenebrio molitor* is used as a model host throughout, and the gram-positive opportunistically pathogenic bacterium *Staphylococcus aureus* is used as model parasite.

1.2. The power of parasites

Hosts and parasites impose powerful selection on one other (Frank, 2002; Dittmer and Kanost, 2010; Prasain et al., 2012). This selection is ancient and ubiquitous: any cellular host species from protozoa to

primates can be infected, and there is a greater diversity of parasites than any other functional group of organisms (Leclerc et al., 2006; Schmid-Hempel, 2011). The complicated and often fascinating effects of parasitism are thought to range between traits and processes as diverse as sex, speciation, lekking, and human body hair (Folstad and Karter, 1992; Kirkness et al., 2010; Brockhurst, 2011; Dean and Siva-Jothy, 2011).

Hosts have responded to selection from parasites by evolving sophisticated immune systems. The dominant paradigm in immunological research has long been to understand how and why hosts kill their parasites. However there has been a recent recognition that immune systems can behave in subtle and previously under-appreciated ways.

1.3. The sophistication of immunity

It has recently been recognised that there are two basic ways for a host to survive infection, by either resisting or tolerating infection (Lazzaro and Rolff, 2011). Resistance responses can be defined as mechanisms to reduce fitness of a parasite, killing it or externalising it from host tissues. This is the traditional view of animal immunity, and the focus of this thesis is to find common features of resistance responses in hosts and microbes. The second route to immunity is common between parasites and other symbionts inhabiting the host, which is to tolerate infection by controlling and minimising the damage caused by the symbiont. Resistance and tolerance are defined by Raberg et al., (2007); and Rowntree et al., (2011b), who base their definitions on animal and plant systems respectively, but converge in their conclusions.

Implicitly, resistance responses must harm parasites by killing, or terminally damaging cells, or blocking access to resources. Resistance is well defined, and its biological mediators are well understood. The classical view of immunity was that hosts should always resist infection

as strongly as possible, by avoiding parasitism or eradicating parasites after they have entered host tissue. As the mediators of resistance are well understood on a molecular and biochemical level, they are discussed later in this introduction, with specific respect to insect systems. On the other hand, the chemical mediators of tolerance are not clear, and there is scope to develop the phenomena that have to date been termed tolerance. Epidemiology-centric definitions of tolerance (e.g. Boots, 2008; Best et al., 2010) consider tolerance as any means of isolating the host from the effect of harm caused by the parasite, for example by detoxification; and resistance as direct control of the parasite by killing, externalising it from host tissue, or preventing growth. However reducing a parasite's metabolism does not necessarily render it inviable, and may simply delay the parasite's growth until the host dies or relaxes its tolerant control of the parasite. In this case slowing growth delays but does not reduce parasite fitness, and is therefore a tolerance mechanism.

1.4. The evolutionary ecology of resistance

The costs and benefits of resistance responses are clear: the host gains the benefit of reduced future parasitism and damage from the parasite, at the cost of evolving and activating resistance responses. Classic life-history theory predicts that trade-offs and therefore costs must operate on immunological resistance, because natural selection has not favoured universally perfect immunity (Siva-Jothy et al., 2005). These principles are well-established theoretically, supported empirically, and are the fundamental basis of evolutionary immunology (Sheldon and Verhulst, 1996; Graham et al., 2010). Costs of resistance can be classified as the costs of evolving an immune system, and the costs of initiating an immune response (Schmid-Hempel, 2011). Costs of evolving resistance responses can be sub-categorised as genetic and physiological; and costs of activating an immune response are sub-categorised as genetic, physiological and auto-reactive. Each class of costs is briefly discussed below, with examples. To maintain consistency

with the source of these definitions (Schmid-Hempel, 2011) these classes of cost are termed costs of immunity, but they are implicitly costs of resistance:

- *Genetic costs of evolving immunity* are described by negative genetic correlations between the ability to survive an infection and other life-history components, which has often been described between parasite resistance and fecundity. For example, *Biomphalaria glabrata* snails selected for parasite resistance are less fecund than susceptible lines (Webster and Woolhouse, 1999); and, conversely, sexual competitiveness is negatively related to immune function and resistance to bacterial infection in male *Drosophila melanogaster* (McKean and Nunney, 2008; McKean et al., 2008). Recent work has shown that antiviral defence and growth rate are negatively related in a moth (Boots, 2011).

- *Physiological costs of evolving immunity* are caused by maintaining immunological machinery to ward off potential infection. For example, constitutive expression of resistance traits to infer prophylactic resistance to potential parasites from the environment (e.g. Gross et al., 2008) will come at an energetic cost. As evidence of such costs, (Schmid-Hempel, 2011) cites examples of regulation of wild mammalian immune-related genes in response to seasonal variation in the risk of parasitism.

- *Genetic costs of activating an immune response* are similar to the life-history trade-offs caused by evolving immunity, with the essential difference that they are inducible upon activation of the immune system, rather than being constitutively expressed as 'overheads'. For example, fever caused by a simulated bacterial infection leads to a greater risk of predation in crickets (Otti et al., 2012). Interestingly this trade-off may be bi-directional, for

instance copulation interferes with subsequent immunity in damselflies and beetles (Siva-Jothy et al., 1998; Rolff and Siva-Jothy, 2002).

- *Physiological costs of activating an immune response* are the costs of using deploying mediators of immunity. Numerous negative relationships between immunity and fecundity have also been shown on this level. (Moret and Schmid-Hempel, 2000) showed that stimulating an immune response by using sterile, non-pathological material caused a dramatic reduction in the lifespan of bumblebee workers, as a consequence of the energetic cost of immunity. There are also strong negative relationships between immunity and indicators of general condition, such as anorexia and body fat content (Rolff and Reynolds, 2009).

- *Auto-reactive costs of activating an immune response* are of particular relevance to this thesis, specifically in Chapter 4. These costs can be considered a sub-class of the physiological costs of activating an immune response, but with a more specific outcome in which the active immune system causes collateral damage to the host's own tissues (Long and Boots, 2011). The clearest example of this phenomenon is detailed by (Sadd and Siva-Jothy, 2006), who showed a decline in essential physiological function of *Tenebrio molitor* resulting from the immune system attacking self-tissue. A more recent study connects this process in early life with reduced adult mortality (Pursall and Rolff, 2011). In the case of *T. molitor* it is thought that these effects are mediated by the cytotoxic intermediates of the phenoloxidase (PO) system (Nappi and Ottaviani, 2000; Christensen et al., 2005). In plants, parasitic attack often results in dramatic occlusion and death of host tissues (Cameron et al., 2006). In malaria-infected mice, inhibiting interleukin signaling strongly improved host survival and

decreased parasite density independent of parasite genotype, demonstrating powerful auto-pathology associated with this signaling pathway (Long et al., 2008). These studies demonstrate that the phenomena of host sickness and death, termed virulence, are the sum of parasite and host pathologies (Graham et al., 2010).

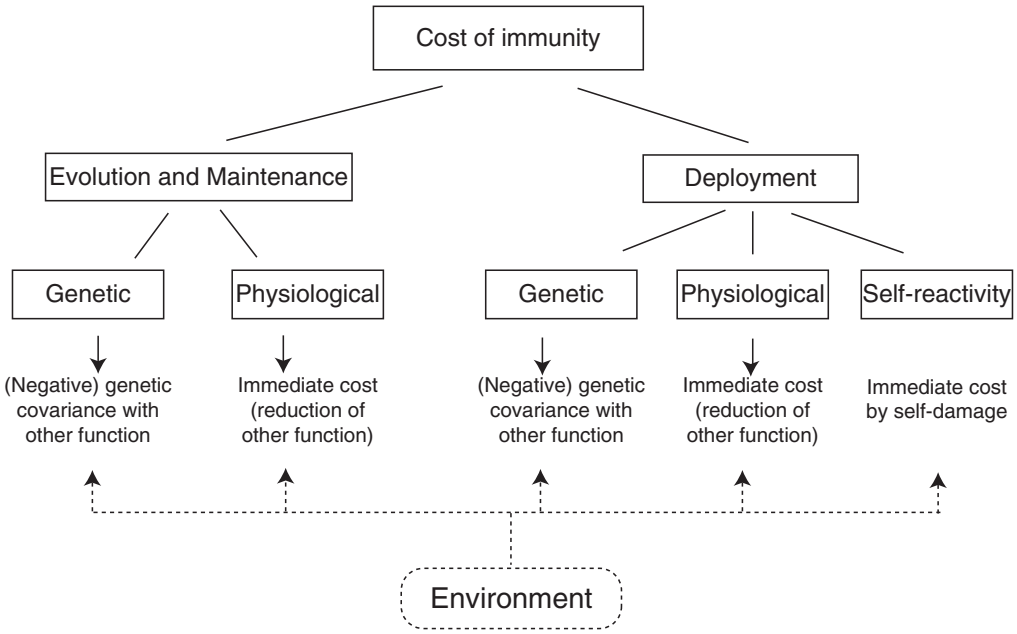


Figure 1.4.1. Costs of immunity/resistance (From Schmid-Hempel, 2011)

Costs of immunity are the trade-offs involved with the evolution and maintenance or deployment of immune traits. Costs of evolution and maintenance are standing costs associated with having a certain trait at the expense of another (genetic), and maintaining its machinery (physiological). Costs of deployment relate to use of a trait, and can also manifest as a negative correlation with another trait (genetic), costs of using the trait (physiological) or host self-damage caused by the immune system (self-reactivity). The net costs of traits are environment-dependent.

These are the principles that have guided the evolution of immunity, and the trade-offs inherent in host management of symbiotic organisms of all descriptions. Although there are clear benefits to resistance, there are numerous associated costs and negative feedbacks, evidenced by the fact that hosts are not immune to all parasites.

1.5. The evolutionary ecology of tolerance

Although parasite tolerance has been observed in both animal and plant systems, there is no clear suggestion of why it should evolve. This thesis experimentally parameterises costs of resistance responses, so there is value in considering whether these costs could be related to tolerance. Theory (Stowe et al., 2000) suggests that resistance and tolerance are negatively correlated, which has received some support in animal and plant systems (but see also examples in Koskela et al., 2002; Rodenburg et al., 2006; Raberg et al., 2007; Ayres and Schneider, 2008; Stjernman et al., 2008; Vale et al., 2011; Rowntree et al., 2011a). Assuming this negative correlation, then the costs and benefits of each strategy must be related, and each is a stable strategy when the other is not. Since the expression of resistant phenotypes is determined by condition (Fellous and Lazzaro, 2010; Boots, 2011), it seems likely that the optimality of tolerance is determined by the relationship between condition and costs of resistance. This is discussed further in Chapter 4.

1.6. A natural history of insect immunity

The experiments in Chapters 3, 4 and 5 use the mealworm beetle *Tenebrio molitor* as a model host, so the mechanistic bases of insect immunity merit some introduction. Although the mechanisms described are general, specific mention is made of *T. molitor* to introduce the immune system of this host.

The arthropod immune system consists of cellular and humoral components, which can be sub-classified as constitutive or induced (Nappi and Ottaviani, 2000). “Cellular immunity” refers to haemocytes, which phagocytose or encapsulate parasites (Strand, 2008), and are responsible for coagulation and wound healing (Haine et al., 2007). “Humoral” components comprise soluble proteins such as antimicrobial peptides (AMPs) (Bulet et al., 2004) and lysozymes; and a range of reactive cytotoxins e.g. reactive oxygen species (ROS), quinones and lectins (Siva-Jothy et al., 2005). The specifics of these components are discussed below.

1.6.1. Haemocytes

The functional significance of haemocytes is clear (see Rosales, 2011 for review). Upon inoculation with foreign microbes, phagocytes rapidly surround the alien matter, isolating them from the haemocoel (Lavine and Strand, 2002). Parasites are therefore rapidly externalised from the haemocoel. After phagocytosis, haemocytes aggregate on other tissues, forming ‘nodules’ (Gillespie and et al., 1997). Larger foreign bodies – such as parasitoids, nylon filament or polystyrene beads - are encapsulated by a layer of haemocytes (Siva-Jothy, 2000). After encapsulation or nodulation, haemocytes enter a program of cell death and melanise (Gillespie and et al., 1997). Pathogens are thus rapidly removed from circulation, starved of oxygen and nutrients, and bombarded with the toxic products of melanisation.

Genetic ablation of haemocytes has shown them to play a pivotal role in *Drosophila* resistance to *S. aureus* infection (Defaye et al., 2009). Parasites are nearly always removed by haemocytes (Lavine and Strand, 2002), so their function is under strong positive selection, leading to, for instance, the evolution of 'hyperphagocytes' in *Manduca sexta* (Dean et al., 2004). It is thought that the action of haemocytes is responsible for a large proportion of the clearance of infectious bacteria from the haemocoel of *T. molitor* (Haine et al., 2008a). Furthermore,

they play a role in clearing recalcitrant infection and are the agents of immunological 'priming' against a previously cleared parasite in *Drosophila* (Pham et al., 2007).

Immunological haemocyte function is tightly integrated with other immune effectors (Nehme et al., 2011). The externalisation of both micro- and macro-parasites is dependent on oxidative enzymes involved in melanin production (Zhao et al., 2007; Jiang, 2008), and they produce soluble antimicrobial peptides (Lavine et al., 2005).

1.6.2. Cytotoxins and oxidative enzymes

A crucial constitutive immune defence is the proteolytic casecade of tyrosinase reactions resulting in the eventual production of phenoloxidase (PO) and melanin, known as the PO cascade (Sugumaran, 2002; Laughton et al., 2011a). The precursor compound proPO (Nigam et al., 1997) is activated by stimuli indicating infection, including mechanical wounding, detection of pathogen-associated molecular patterns (PAMPs) (Cerenius et al., 2008; 2010); or disruption of the basement membrane (Brennan et al., 2007). The PO cascade is often assayed as a proxy for general immune activity and "immunocompetence" (Mucklow and Ebert, 2003; Lee et al., 2008), although the associated phenotypes are probably the sum of the activities of a diverse range of oxidative enzymes (Dittmer and Kanost, 2010; Prasain et al., 2012)

PO is involved in melanin and sclerotin production (Sugumaran, 2002), and is thus pivotal in externalising phagocytosed or encapsulated parasites. PO has also been implicated in producing compounds that aid phagocytosis (Nigam et al., 1997; Cerenius et al., 2008; 2010). Killing is probably caused by production of toxic intermediates such as lectins, quinones and reactive oxygen species (ROS) (Christensen et al., 2005; Zhao et al., 2007; Jiang, 2008). This hypothesis is supported by the finding that PO-deficient *Drosophila* mutants are not

immunocompromised relative to wild-type strains (Leclerc et al., 2006) suggesting that toxicity is mediated by up-stream compounds. However this need not invalidate measurement of PO as a proxy for the production of these compounds and therefore immune activity.

The PO cascade and activity of other oxidative enzymes such as laccases and multicopper oxidases are known to be autopathological (An and Kanost, 2010; Dittmer and Kanost, 2010; Prasain et al., 2012). Sadd and Siva-Jothy, (2006) showed a mechanistic link in *T. molitor* between the induction of phenoloxidase activity by nylon implant, and the melanisation and impairment of a fundamental physiological function (malpighian tubule activity). This is likely mediated by the release of toxic by-products into the open insect haemocoel. Despite some ongoing debate caused by sometimes equivocal functional evidence for a role of PO in antimicrobial defence - which quite often comes from a small selection of research groups working on *Drosophila* - there is a strong evolutionary argument that these self-damaging processes would be strongly selected against unless they had a direct or pleiotropic role in immunity. On this basis, the antimicrobial and autopathological roles of melanin are central to the arguments presented in Chapters 4 and 5.

1.6.3. Antimicrobial peptides

Antimicrobial peptides (AMPs) are components of the induced immune response. They are present at low concentrations in unwounded insects, but their production by the insect fat body and haemocytes is greatly up-regulated after inoculation with microbes or pathogen-associated molecular patterns (Iwanaga and Lee, 2005; Lavine et al., 2005; Haine et al., 2008b). AMPs can be detected in the haemolymph of *Drosophila* as quickly as two hours post-infection (Meister et al., 1997; Lehrer and Ganz, 1999). Typically, AMPs are composed by fewer than 100 amino acid residues (Huang, 2000), with molecular masses below 25-30kDa. They are hydrophobic and cationic (Zasloff, 2002), and demonstrate amphipathic structures crucial to their bactericidal action. Structurally,

AMPs consist of α-helices, β-sheets, or combinations of the two (Bulet et al., 1999). The diversity of AMPs is so great that any classification by any system other than structure is difficult (Zasloff, 2002). AMPs have been extensively studied in *Drosophila*, in which they have been shown to be powerful predictors of resistance to infection, and wild-type fitness can be restored in AMP-deficient mutants by re-expression of just one AMP (Tzou et al., 2002). There is published data on five AMPs from *T. molitor*, and unpublished transcriptomic data predicts the existence of an additional two (P. Johnston, Pers. Comm.).

AMPs damage microorganisms in two ways. The cationically-charged AMPs exploit the uniquely negative charges on the outer cell membranes of microorganisms, then disrupt the membrane structure and lyse the cell (The Shai-Matsuzaki-Huang model) (Boman, 1995; Bulet et al, 1999; Zasloff, 2002; Ganz, 2003; Zanetti, 2005). Others penetrate the bacterial cell membrane and act on intracellular structures (Brogden, 2005). By acting on highly conserved features of microbial structures, AMPs exhibit extraordinarily broad-spectrum activity (Zanetti, 2005). Eukaryote cell membranes do not attract AMPs, in contrast to those of prokaryotes, so AMPs are not toxic to hosts.

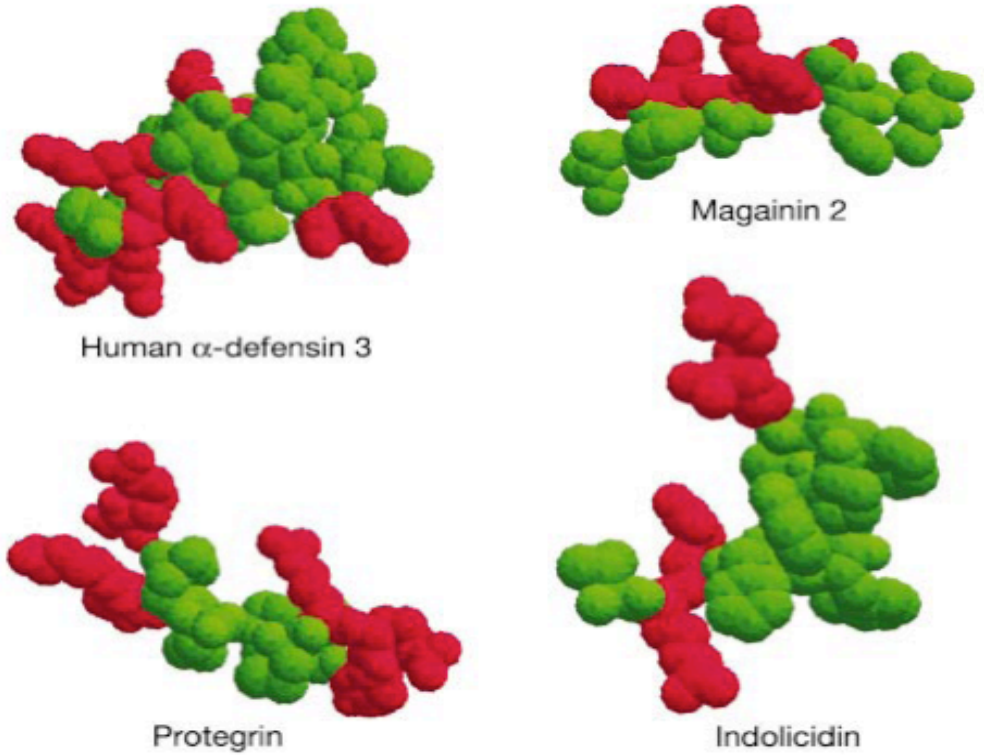


Figure 1.6.3.1. Examples of amphipathic AMP structure.

The amphipathic structures of AMPs are fundamental for their tertiary structure and bactericidal activity. This Figure shows the positions of basic, positive-charged amino acids (red); and green, hydrophobic amino acids (green). Figure from (Zasloff, 2002)

Whilst conservation of homologs is rare across higher taxa, there is frequent analogy: for example tenecin 1 (from *T. molitor*) and holotricin 1 (from a scarabid beetle) show 60% sequence homology, disulphide bridges on homologous amino acids, and are both effective against gram-negatives but not gram-positives (Moon et al., 1994b; Lee et al., 1995) (Figure 1.6.3.2). On a broader scale, analogues of peptides originally identified in the moth *Hyalophoria cecropia* (cecropins) have been found in animals as evolutionarily distant as vertebrates (Moore et al, 1996); but defensin family peptides show a greater molecular distance within dipterans than between odonates and arachnids (Bulet et al, 1999). Figure 1.6.3.3. (from Altincicek et al., 2008a) shows the molecular conservation of AMP protein sequence across insect orders. Based on the contrast between genomic evolution and AMP evolution in *Drosophila*, Juneja & Lazzaro (in Rolff and Reynolds, 2009) suggest that the lack of peptide evolution within *Drosophila*, contrasted with genomic evolution across higher taxonomic levels, implies that AMPs are effective in specific ecologies, but adaptation is required in different environments. The extraordinary functional and structural conservation of AMPs (Figures 1.6.3.2. & 1.6.3.3) suggests an important adaptive role for these immune effectors.

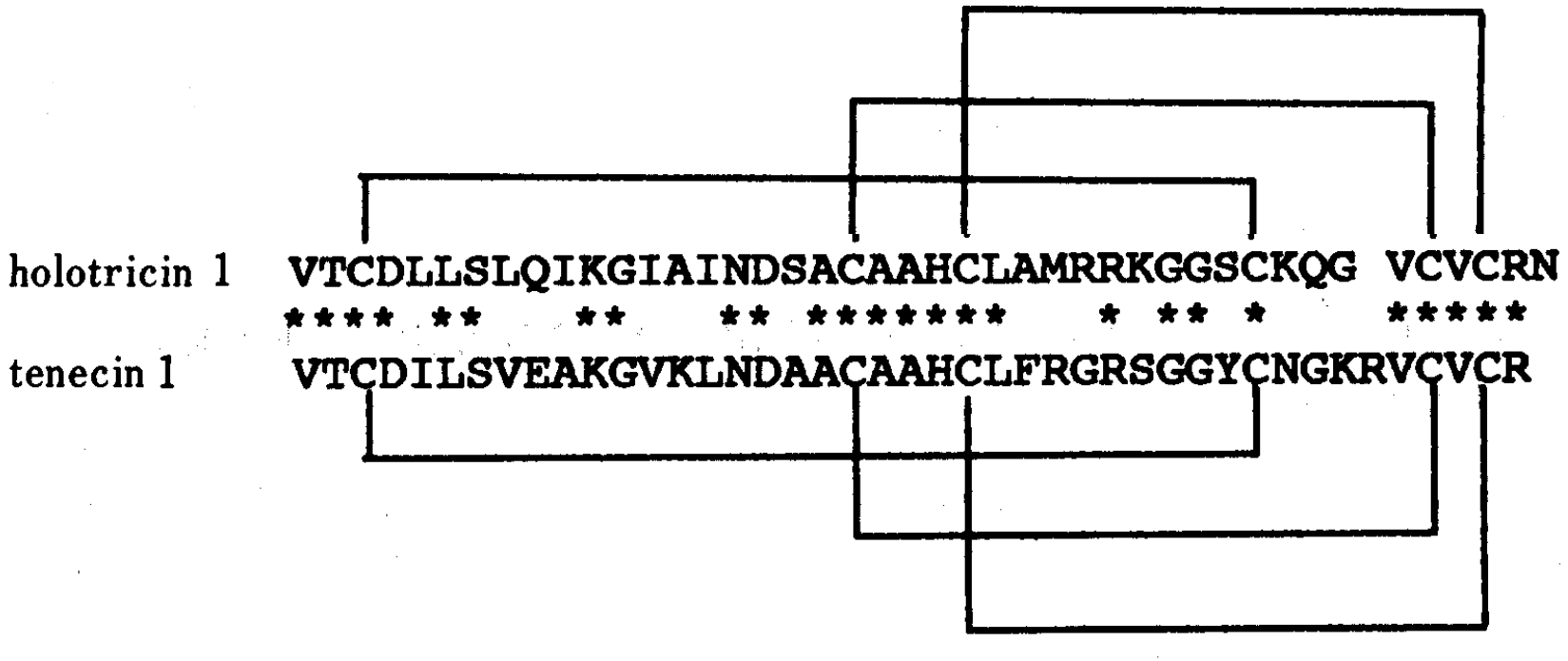


Figure 1.6.3.2. Amino acid sequence conservation between beetle antimicrobial peptides, from (Lee et al., 1995)

Holotricin-1, from the scarabid beetle *Holotrichia diomphalia*, and tenecin-1, from the tenebrionid *Tenebrio molitor*, show 60% conservation of amino acid sequence (shared residues are starred), disulphide bridges to identical residues (shown by large brackets), and identical spectra of microbicidal activity.

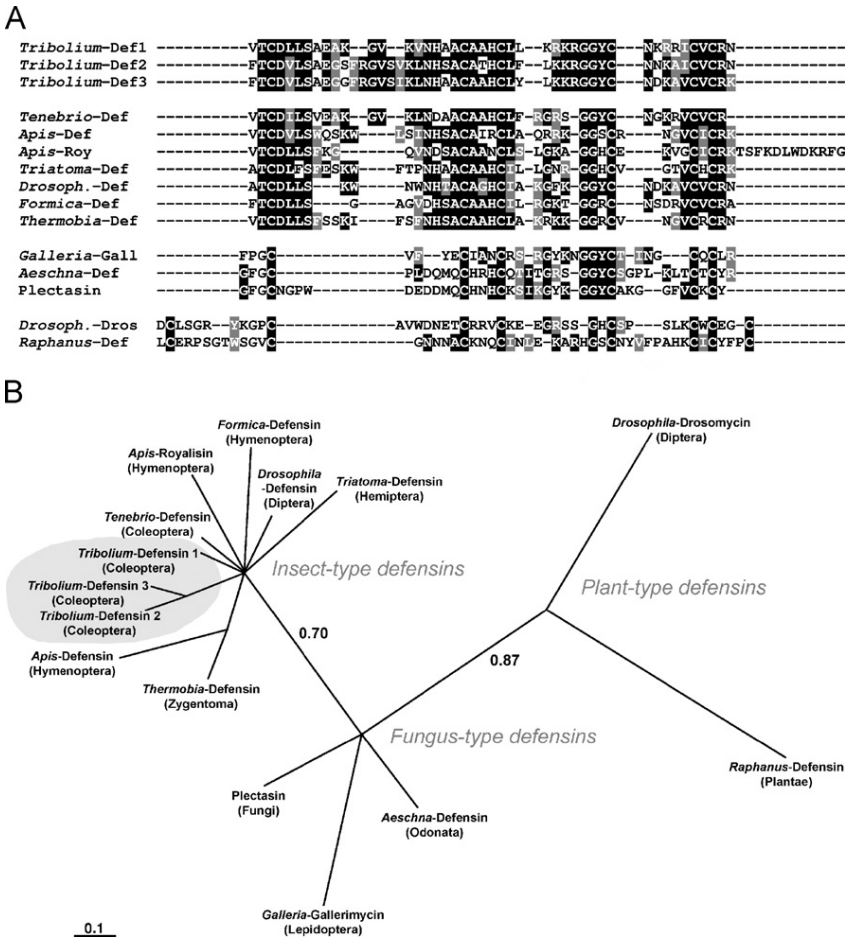


Figure 1.6.3.3. Molecular conservation of defensins across insect orders and higher taxa, from (Altincicek et al., 2008a))

Antimicrobial peptides are highly conserved across taxa. **A** shows amino acid conservation of defensins within the beetle *Tribolium castaneum*; compared to the confamilial beetle *Tenebrio molitor*, the honeybee *Apis mellifera*, the kissing bug *Triatoma brasiliensis*, the fruitfly *Drosophila melanogaster*, the ant *Formica aquilonia*, the firebrat *Thermobia domestica*, the moth *Galleria melonella*, and the dragonfly *Aeschna cyanea*. **A** also demonstrates how these insect defensins compare to defensins from organisms in separate kingdoms, including the plant *Raphanus sativus*, and the fungus *Pseudoplectania nigrella.* **B** shows clustering of defensins by species. The scale bar represents amino acid substitutions per site.

There is scope for interesting work on the evolution of resistance with particular respect to AMPs in *T. molitor*. Haine and colleagues (Haine et al., 2008a) found that inoculating *T. molitor* with a high dose of *Staphylococcus aureus* led to a low level of persistent infection for up to four weeks after initial infection (but see Appendix 2). Beetles coincidentally up-regulated humoral antimicrobial activity, measured as the bactericidal activity of crude haemolymph samples, which the authors proposed was likely mediated by AMPs. These data complemented earlier work that showed a long-lasting induction of antimicrobial activity after inoculation with a range of microbial elicitors (Haine et al., 2008b). Haine et al. (2008a) proposed that this phenomenon of long-lasting immunity serves to control persistent bacteria that have evolved resistance to constitutive defences, rather than as a prophylactic defence against reinfection from environmental bacteria. There is an argument that this distinction is unnecessary, because defending against a new infection from environmental bacteria or secondary infection from persistent cells living *in vivo* is fundamentally the same process, but that need not violate the notion that long-lasting immunity defends against the haemocoel being re-invaded with bacteria. Chapter 3 shows results from an experiment on the dynamic interplay between *T. molitor*'s long-lasting immune response and AMP-resistant *S. aureus*. Chapter 4 contains data that suggest an additional reason for hosts to invest in long-lasting induced humoral immune responses.

1.6.4. Other responses to infection

Specific mention has been made of the role in insects of haemocyte-mediated defence, cytotoxic and oxidative resistance mechanisms, and antimicrobial peptides; because these are the best-understood and arguably most potent defence mechanisms, and moreover because they are most pertinent to the data presented in Chapters 2-5. It is worth noting that there is a great diversity of other parasite resistance mechanisms in insects. The ecology and evolution of the full diversity of

insect defence is reviewed by (Rolff and Siva-Jothy, 2003; Siva-Jothy et al., 2005), and thorough mechanistic accounts of the immunological transcriptomes of two coleopterans are provided by (Altincicek et al., 2008a; Vogel et al., 2011a)

1.7. The evolution of bacterial resistance

Microparasites derive many benefits from living in a host, such as the provision of effectively infinite resources, and a stable physiological environment. To be able to colonise hosts, would-be parasites must have means to avoid being killed by the immune system. This section will discuss the mechanisms by which infectious bacteria resist being killed by immune effectors. In particular, I focus on AMPs and *S. aureus*, since they are the main components of the study system used in Chapters 2 & 3. A case is made that costs of resistance to the immune system can be studied in the same evolutionary ecology framework as costs of host resistance to parasites. Just as hosts can avoid or fight infection, so can bacteria directly resist and subvert host immunity, or evade and tolerate it.

1.7.1. Bacterial resistance to (and tolerance of) host immunity

Well-established theory stipulates that parasite damage to a host - referred to hereafter as "virulence" (as discussed by Alizon et al., 2009), or "pathology" to build cohesion with endogenous host damage (autopathology) - should only evolve if it enhances parasite fitness by increasing transmission rates (Anderson and May, 1982). Therefore, pathogen "virulence factors" might be more accurately termed "survival factors", although correlation between the molecular basis of traits that might be expected to confer virulence do not always correlate with disease (e.g. Tong et al., 2012). However a number of toxins secreted by pathogenic microorganisms are known to kill host cells responsible for antimicrobial immunity. A full review of secreted microbial toxins could be a thesis in itself, and a topical example is sufficiently

explanatory: *Staphylococcal* phenol-soluble modulins (PSMs) are small α-helical peptides secreted by inter- and intra-cellular infectious *S. aureus*, which attract and destroy mammalian neutrophils in a manner analogous to the action of AMPs on bacteria, thereby resisting a primary immune effector (Wang et al., 2007). However, PSMs also activate inflammatory responses, which may explain why their expression is tightly linked to bacterial cell densities by quorum-sensing, and therefore the probability of alerting the immune system to their presence (Wang et al., 2007). The β subclass of PSMs have also been shown to promote dissemination of cells from biofilms - protective agglomerations of cells bound in extracellular proteins, and a common microbial survival phenotype (Lewis, 2005) - thereby aiding colonisation of new regions of the host. Such secreted toxins are arguably the best-studied factors contributing to microbial survival in hosts, and since their expression is energetically costly and pleiotropic (Jarraud et al., 2002) their expression is associated with an inducible physiological cost of resisting the host. The loss of these exotoxins and their cell-bound counterparts from microbial genomes when they lose adaptive value is strongly suggestive of genetic evolutionary costs. For example, during host specialisation to horses, the pathogen *Streptococcus equi equi* has lost many of the exoproteins used by its ancestor *S. equi zooepidermicus* to infect a broader range of hosts (Kadioglu et al., 2008; Holden et al., 2009),

Other infectious phenotypes are subtler in their contribution to microbial fitness. The immune response can be avoided either passively or actively (Boman and Hultmark, 1987). Passive resistance tends to involve antigenic modification to avoid alerting the immune system of the microbe's presence. *Spiroplasma poulsonii*, for example, lacks cell walls and therefore does not present many of the antigens that would ordinarily stimulate an immune response (Hurst et al., 2003). Active resistance responses tend to be associated with more pathological infection. Biofilms have commonly been considered a stress response and a survival phenotype, although considering that the bountiful and

stress-free conditions found in laboratory culture tubes are fairly rare in nature, it seems more likely that bacteria are more commonly found in biofilm-like states in nature than as exponentially growing plankton. Biofilms are associated with elevated resistance to stressors including antibiotics and salinity (Lewis, 2001; 2005). They have been estimated to be responsible for 60% of human infections (National Institutes of Health, USA, in Lewis (2001)) and are commonly associated with chronic human infections after surgery or on prostheses and implants (Otto, 2008). Biofilms aid in resistance to the immune system by surrounding living cells from the host with an outer layer of dead cellular material and an excreted protein matrix (Costerton et al., 2003), which defends against phagocytes and soluble antimicrobial proteins and peptides (Foschiatti et al., 2009). Cells within biofilms are often in a physiological "persistence" state, where low metabolism allows individual cells to survive in stasis for a long time without dividing, immune to the effects of any stressor whose function is proportional to microbial growth rate (Balaban, 2004). Persisters seem to have evolved as specialised survival cells, but little is known about the conditions required to activate this phenotypic switch (Keren et al., 2004). The net result of biofilm formation is effectively a bacterial fortress in which besieged cells can long persist in the face of a potent immune response. As they isolate infection from the immune system, these processes are ecologically comparable to host tolerance of parasites.

Bacterial resistance to AMPs is the main focus of Chapters 2 and 3, and is discussed further in these two Chapters. Bacterial resistance to AMPs has rarely been observed (Gillespie and et al., 1997; Hancock, 2000). However AMP resistance is not impossible (reviewed in Yeaman and Yount, 2003), and it can evolve in response to an individual peptide after only a few hundred generations under selection (Perron et al., 2006; Habets and Brockhurst, 2012). There are also data on some of the mechanisms of AMP resistance, discussed further in Chapter 2. The dichotomy between the evolutionary potential for AMP resistance and its

apparent rarity raises a perplexing question: why has AMP resistance not been commonly observed?

It is conceivable that the mechanisms by which AMPs exert their bactericidal effect prevent resistance. AMPs exploit the inherent properties of prokaryotic cell membranes, and so some have proposed that evolutionary change to avoid this force would be highly costly (Zasloff, 2002), particularly when infectious cells are faced by multiple and various molecules exerting this stress on cells. The costs of evolution of such fundamental traits would render resistant mutants weak competitors in an infection, in a similar fashion to the costs of cell wall and membrane evolution to vancomycin-resistant mutants (Weigel, 2003; Chambers and DeLeo, 2009). Others suggest that the deployment of multiple peptides accounts for the lack of natural resistance (Moon et al., 1994a; Lee et al., 1995; Rolff and Reynolds, 2009), because simultaneously evolving resistance to diverse peptides which each disrupt fundamental cytostructure is unlikely. However, it is important to emphasise that observations of rare AMP resistance are based largely on *in vitro* studies, and certain bacteria may be resistant to AMPs from natural hosts in ways that would not be revealed by lab study.

The multi-pronged assault exerted by a panel of AMPs may provide scope for further bactericidal benefits. (Bulet et al., 1999) suggest that multiple AMPs may interact synergistically. By way of analogy, models of antibiotic resistance evolution (Chait and Craney, 2007; Hegreness et al., 2008; Fischbach, 2011) suggest that the direction of functional interactions between antibiotics affect the resistance outcome. This prediction is borne out by data: of 119 isolates of drug resistant *Burkholderia cepacia* exposed to 10 to 15 antibiotics, 50% were resistant to individual antibiotic administration, 8% resisted two-drug combinations, but 0% could resist three-drug combinations (Aaron et al., 2000). Complementarily, antagonistic epistatic effects have been shown

by experimental evolution of streptomycin resistance in a rifampicin-resistant strain of *Pseudomonas flourescens*, in which the controls performed better than combination-selected lines (Ward et al., 2009). Considering that AMPs tend to act only on one class (e.g. gram-positive) of parasite, Bulet et al's (1999) hypothesis is an intriguing suggestion: the window of opportunity for resistance evolution is diminished if the sum of a system's bactericidal action is greater than its parts. Alternatively, if a pair of AMPs act antagonistically, evolving resistance to one will remove the suppressive effect on the other and thus help to prevent resistance to the immune system (Chait and Craney, 2007). In either case, investigating bactericidal action of peptides in isolation and combination may help elucidate the circumstances under which AMP resistance evolves. This issue is addressed experimentally in Chapter 2.

There is a potentially important applied biomedical angle to understanding the constraints on natural AMP resistance. The apparent stability of bacterial susceptibility to AMPs has generated strong interest in their potential as a novel class of therapeutic antibiotics or immunomodulatory drugs (Reddy et al., 2004; Wuerth and Hancock, 2011). The basic premise of this proposal is that if AMP resistance has rarely been observed, then there must be strong intrinsic factors constraining natural AMP resistance, and so they can be co-opted as powerful "resistance proof' clinical antibiotics. However there are potential dangers to using AMPs clinically. AMP resistance may be rare, but their bactericidal action is just one component of complicated and finely-tuned immune responses, and their use out of this context could select resistant strains. As AMP protein motifs are conserved, resistance to one AMP could in principle select for broad cross-resistance to many others, endowing resistant strains with defences against host immunity (Bell and Gouyon, 2003; Buckling and Brockhurst, 2005). Such cross-resistance has recently been shown *in vitro*, between *S. aureus* artificially selected for resistance to an AMP developed as a therapeutic antibiotic (pexiganan) and a human

cathelicidin (Habets and Brockhurst, 2012). Chapter 3 goes further, by testing the interaction between AMP-resistant cultures and immunity in a living host.

1.7.2. Evolutionary responses to antibiotics as a model of resistance evolution

Antibiotics are without a doubt one of the most important public health interventions in human history. Unfortunately, in just over seventy years since their introduction, their effectiveness has rapidly waned (Davies & Davies, 2010). A high-profile clinical problem of recent years is the increasing prevalence of antibiotic-resistant bacteria (Peterson and Dalhoff, 2004), and particularly 'superbugs' such as methicillin resistant *Staphylococcus aureus* (MRSA) (Enright et al., 2002). The emergence of antibiotic resistance surprised few evolutionary biologists (Margolis and Levin, 2008), since the rapid evolution of ubiquitous and easily transferred resistance genes is quite predictable in response to strong selection from solitary chemical stressors, on organisms with rapid generation times and capacity for horizontal gene transfer (Maisnier-Patin and Andersson, 2004; Martinez, 2008).

The rapid emergence of antibiotic resistance is arguably the largest experimental evolution experiment in history, albeit accidental and unfortunate. Nevertheless, it is an excellent model of the way that populations adapt to environmental chemical stresses, particularly when the populations are microbial and the stresses chemical. Consequently there is value in understanding how antibiotics have selected resistant bacteria, as a model of resistance evolution that can potentially be applied to host-microbe associations.

The most commonly studied way for bacteria to evolve resistance is through the acquisition of resistance genes, since this causes most clinically significant resistant infections (e.g. Fricke et al., 2008). Resistance genes could in principle arise through spontaneous

mutation, but the molecular complexity of many antibiotics means that this is highly unlikely (Wright, 2007). A far more commonplace means of acquiring resistance is through horizontal transfer of resistance genes (Martinez, 2008). Genes for antibiotic resistance are more ancient than the divergence between gram-positive and gram-negative bacteria (Hall and Barlow, 2004), and the compounds they produce are too complicated to have arisen since the advent of clinical antibiotics (Wright, 2007). Furthermore, since resistance is frequently mediated by plasmid DNA (Kruse and Sørum, 1994), genes for resistance are easily horizontally transferred.

In common with evolved responses to immune systems, microbial resistance to antibiotic stress can be active or passive. There are however numerous genetic and physiological costs (Levin et al., 2000), which typically manifest as compromised growth rate or greater sensitivity to environmental stress (Weigel, 2003; Chambers and DeLeo, 2009). However, these costs can be ameliorated by compensatory mutations, which are rapidly fixed in populations (Maisnier-Patin et al., 2002; 2005). Antibiotic tolerance can also evolve, expressed as a microbe's non-heritable ability to survive under antibiotic stress, but remaining sensitive to it (Levin and Rozen, 2006). The theory and cost structure from ecological immunology (Schmid-Hempel, 2011) can once again be applied to this antibiotic-microbe model. Conversely, findings from studies concerned with antibiotic resistance can be hypothetically applied to the evolution of infection and immunity. For example, bacterial 'charity work' by a few mutants leading to population-wide antibiotic resistance (Lee et al., 2010) may be also occur in infectious bacteria.

1.8. Summary and perspective

The framework of evolutionary immunology is well defined, and although the picture of the evolutionary costs of parasite resistance is not complete, its broad strokes are fairly clear. Costs can manifest both in

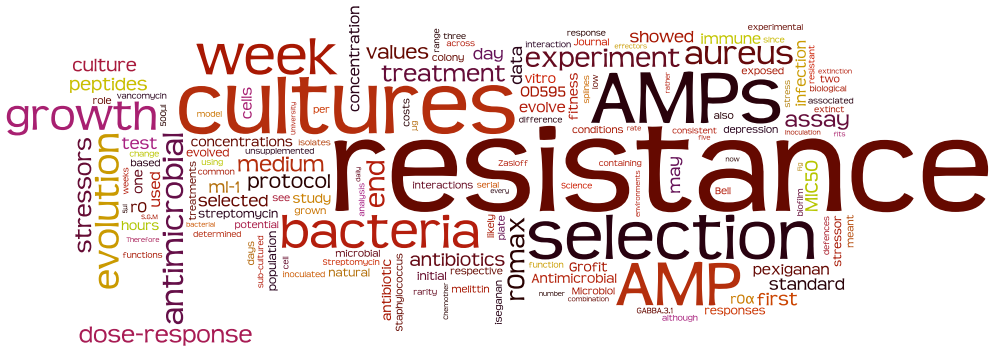
the evolution and maintenance of parasite resistance in terms of the use and maintenance of traits, or genetically determined life history trade-offs. This framework can also be explicitly and integratively applied to parasites in terms of the way they deal with host immunity. With specific reference to bacteria, there is abundant evidence that the cost framework determining how bacteria deal with stress from antibiotics and immune systems is the same as that which determines how immune systems resist parasites. Additionally, although the evolutionary drivers of tolerance are unknown and there is room for disagreement over its proper definition, this phenomenon is clearly common to both hosts and parasites: hosts tolerate parasites, and parasites tolerate host immunity. These parallels demonstrate the merit of applying the same evolutionary framework to both host and parasite as one intrinsically connected symbiotic unit, facilitating a richer and broader understanding of how they coevolve and selectively sculpt each other as part of their biotic environment.

The remainder of this thesis aims to use this proposed integrative framework by examining the costs and benefits of resistance in a host-microbe association, using the mealworm beetle *Tenebrio molitor* as a host, and the opportunistic pathogen *Staphylococcus aureus* as a parasite and pathogen. Chapter 2 describes an experiment to parameterise the genetic costs to *S. aureus* of evolving resistance to antimicrobial peptides. Chapter 3 follows 2 by using these resistant cultures as model parasites, to test whether genetic costs of resistance are traded against benefits of increased host persistence. There is a secondary but important applied biomedical angle to the experiments in Chapter 3, due to the potential for the pathogenic interactions it highlights should AMPs be developed as therapeutic drugs. The focus of the latter half of the thesis switches to *T. molitor* resistance to *S. aureus*. Chapter 4 is concerned with how costs of antimicrobial resistance, particularly autopathological immunity, could modify an individual's optimal immune strategy over time after infection. This is achieved through an experiment that attempts to measure the costs of

immunopathology relative to the costs of infection. Based on Chapter 4, Chapter 5 focuses on the transcriptomics of *T. molitor*'s resistance to *S. aureus*, to qualitatively assess the mechanisms used to fight the infection and how immunopathological they might be. These studies demonstrate reciprocal and parallel processes operating in host-microbe resistance and microbe-immunity resistance. The general discussion discusses the common conceptual threads in each Chapter, and what they likely mean for the coevolution of host-microbe interactions, be they resistant, tolerant, or mutualistic.

Chapter Two

Comparative experimental evolution of antimicrobial peptide resistance in Staphylococcus aureus



2.1. Abstract

Antimicrobial peptide (AMP) resistance has been demonstrated in experimental studies, but has rarely been observed in environmental bacterial isolates. By contrast, antibiotic resistance is extremely common in environmental bacteria. Since the selection imposed by antibiotics and AMPs is thought to be the same, why is there this disparity in resistance? I propose that it could be driven by different costs associated with AMP and antibiotic resistance, by the multiplicity and diversity of AMPs in immune responses, or by weak selection in the wild. This study uses an experimental evolution experiment to select for resistance to antibiotics and to preparations of single and combined AMPs, allowing comparison of the associated fitness costs in a fully controlled framework. The results of this experiment suggest that costs of resistance to single AMPs are in the same range of the costs of antibiotic resistance. The evolutionary response to selection from a combination of AMPs was weaker, which suggests that natural patterns of AMP resistance are constrained by the multiplicity of AMPs that hosts transcribe to fight infection.

2.2. Introduction

2.2.1. The AMP resistance paradox

Antimicrobial peptide (AMP) resistance has rarely been observed in environmental isolates of bacteria (Hancock, 2000; Loose et al., 2006). However, resistance to these molecules is possible (Yeaman and Yount, 2003). AMPs are ancient, occur in phyla spanning prokaryotes through to humans, and are commonly produced by infected hosts as part of an immune response (Zasloff, 2002; Reddy et al., 2004; Pränting et al., 2008). As such, AMPs are ancient and commonplace stressors of parasitic microorganisms. The rarity of AMP resistance contrasts starkly to the rapid and widespread evolution of resistance to antibiotics that has taken place over the last century. Ice-core data suggest that in

addition to being highly evolvable, antibiotic resistance is at least tens of thousands of years old (Miteva et al., 2004; D'Costa et al., 2011), in accordance with the observation that bacteria have been historically exposed to compounds that are now used as antibiotics, which are naturally produced by environmental microbes (Martinez, 2008). Analogously, the observation of AMPs in all taxonomic kingdoms suggests that microbes have been selected by these compounds for millennia, and bacteria exhibit similar phenotypes when stressed by AMPs and antibiotics (Zasloff, 2002; Bulet et al., 2004). So, the rarity of natural AMP resistance is a puzzling evolutionary paradox: why have bacteria, notorious for their speed and flexibility in evolving responses to environmental stresses, not commonly evolved AMP resistance?

This paradox is a fascinating problem for immunologists (and drug designers, as discussed in Chapter 3), since it may reveal how the immune system limits the *in vitro* emergence of resistant bacteria (Haine et al., 2008a). The most common initial hypothesis was that bacteria simply cannot evolve AMP resistance, because of the way that AMPs disrupt prokaryote cell membranes: it was thought that evolving resistance would involve enormous fundamental change to cell structure, and that these changes would be great enough to render resistant cells unviable (Zasloff, 2002). This idea has since been debunked by experimental evolution of AMP resistance (Perron et al., 2006). AMP resistance has been selected in numerous subsequent studies (Pränting et al., 2008; Sun et al., 2009; Pränting and Andersson, 2010; Habets and Brockhurst, 2012), and its mechanisms are beginning to become clear based on some few resistant environmental isolates (see Peschel and Sahl, 2006 for review). It is now known that AMP resistance can be mediated by four basic mechanisms (Fig 2.1.1). Additional to these physiological resistance mechanisms, there is evidence that pathogenic gram-negative bacteria can actively down-regulate host AMP expression (reviewed in Gruenheid and Le Moual, 2012).

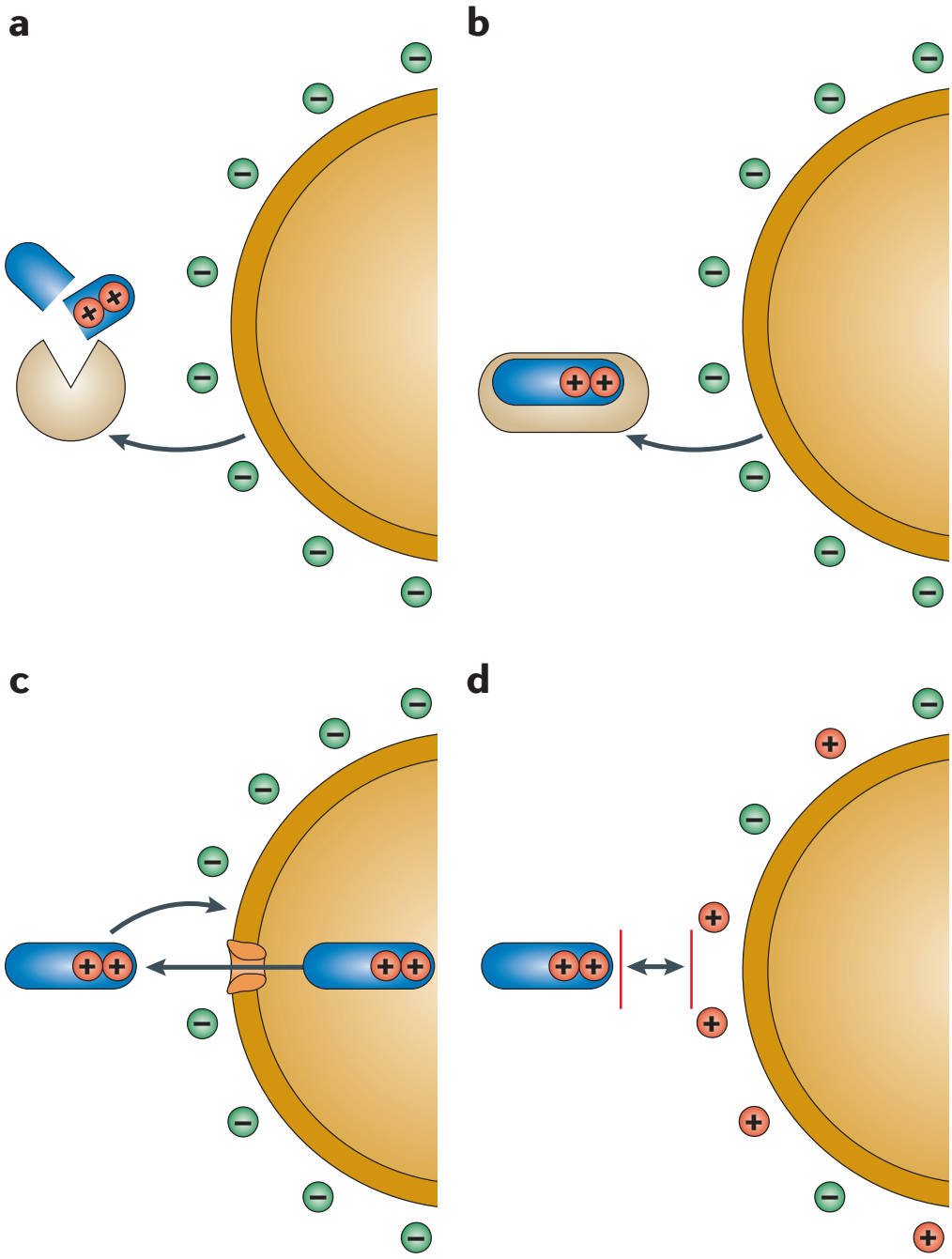


Figure 2.1.1. Mechanisms of bacterial resistance to antimicrobial peptides (AMPs) (from Peschel and Sahl, 2006)

AMP resistance can be mediated by a) extracellular cleavage of the AMP, b) inactivation of the AMP by binding it to another compound, c) active extrusion of AMPs by membrane-bound pumps after the AMP breaches the cell membrane, d) reducing the net anionic charge of the membrane to reduce its attractiveness to cationically-charged AMPs.

As the question of whether or not AMP resistance can evolve has been affirmatively answered several times, its rarity becomes more puzzling. Understanding what governs natural patterns of AMP resistance could reveal whether innate immune systems have means to subvert microbial evolution. The notion of a high intrinsic cost of AMP resistance has been frequently proposed (e.g. Hancock, 2001; Bulet et al., 2004), although it is not supported by extrapolation from experimental evidence (Habets and Brockhurst, 2012). Using a simple mathematical model, Bell and Gouyon (2003) demonstrate that the probability of resistance to a heterogeneously-distributed environmental stressor is determined by the frequency of exposure to the stressor, and the associated costs of resistance. This argument is predicated on costs of resistance, environmental heterogeneity, and the assumption that the compound or process in question stresses the initially susceptible population. This model is supported by recent surveys, which have shown that antibiotic-resistant clinical isolates tend to bear few costs of resistance, suggesting that only the most competitive strains survive, and so costs of resistance are a truly limiting factor in microbial resistance evolution (Ward et al., 2009). To properly address why AMP resistance is rare, the validity of these arguments with specific reference to AMPs needs to be evaluated. Thorough consideration of Bell & Gouyon's model generates a number of explanations other than the 'high cost' argument, which can be categorised as consequences of the multiplicity of immune defences, or as issues associated with the strength of selection for AMP resistance. These hypotheses are discussed in detail below.

2.2.2. Multiple effectors and the probability of resistance evolution

Animals transcribe a broad panel of AMPs after subcutaneous infection (reviewed in Bulet et al., 2004). This is a nonsensical strategy if one alone is sufficient. The deployment of multiple, varied and interacting immune effectors may limit the opportunity for bacteria to adapt against them: the probability of evolving resistance to a suite of stressors with different mechanisms should be inversely proportional to the number of

stressors. These effects could be exaggerated if the stressors interact functionally, or if their distributions vary spatially or temporally. Such effects have been modeled and to an extent empirically demonstrated for antibiotic regimes, (Yeh et al., 2006; Hegreness et al., 2008; Michel et al., 2008; Torella et al., 2010; Fischbach, 2011), the principles of which can be easily applied to immunological processes. Antagonistic epistasis between resistance loci has been shown in multidrug-resistant *Pseudomonas aeruginosa* (Ward et al., 2009). These studies all point towards a difficult evolutionary balancing act for infectious microbes faced with a diverse panel of immune effectors: bear the cost of susceptibility, or evolve resistance at the cost of weaker competitiveness?

2.2.3. How strong is selection for AMP resistance in nature?

An even more parsimonious explanation for the scarcity of AMP resistance is that natural selection for it is actually quite weak. This argument is predicated on the proposition that our understanding of the immunological functions of AMPs is incomplete, for which three cases are made below. The arguments are that microbial strategies other than resistance are likely to be more efficient; that relatively few bacteria are exposed to immunological AMPs; and that AMPs may not be bactericidal *in vivo*.

2.2.3.1 – The virtues of and evidence for alternative strategies to AMP resistance

Strategies other than resistance may be more straightforward solutions for microbes under stress from AMPs: tolerance may be less costly than resistance. In principle, phenotypically plastic responses are a more elegant way to deal with stress than evolved resistance, assuming that the trade-offs involved in evolved resistance are greater than the costs of plastic tolerance of the stressor. For example, biofilms are a common phenotype of stressed bacteria. Bacteria in biofilms are better able to

survive antibiotics, hypersalinity and unpredictable temperature than planktonic cells are (reviewed by Lewis, 2001; 2005). Biofilms have also been shown to improve the ability of constituent cells to survive AMPs (Otto, 2008; Foschiatti et al., 2009; Wang et al., 2011). It is even conceivable that the physical structure of a biofilm is not required to mediate these tolerance effects, and that they are mediated by the physiology of the cells that form them (Balaban, 2004). For example, small colony variants (SCVs), which are commonly associated with bacteria derived from biofilms or anoxic tissue, are more tolerant of AMP stress (Kahl et al., 1998; Sadowska et al., 2002; Besier et al., 2007). Bacteria can sense AMPs (Li et al., 2007), which may have evolved to modulate induction of tolerant phenotypes. If the physiologies associated with such phenotypes are commonly switched on in response to AMP stress, then bacteria have in-built mechanisms that minimise their stress and therefore mitigate effects of selection from AMPs.

2.2.3.2. The strength of selection from constitutive and induced defences

AMPs are often constitutively expressed in dermal tissues as a prophylactic defence against infection, e.g. defensins in mammal skin (Kulkarni et al., 2011). AMPs are not usually constitutively expressed in internal body tissues, but rather transcribed after infection is detected or dermal tissues are breached (Bulet et al., 2004; Zasloff, 2009). Consequently, infectious bacteria are exposed to constitutive defences before AMPs, which diminish the size of the viable population of infectious cells before it is exposed to AMPs. Therefore, there is a reduced probability of the presence of mutant alleles that could aid AMP resistance. For example, in invertebrates, inoculated bacteria are exposed to oxidative enzymes, phagocytes and numerous other lethal immune effectors for up to several hours before AMPs are detectable (Haine et al., 2008a). If AMPs merely prevent secondary infection by the few persistent cells that remain after constitutive defences have done

their work, only a small subset of the initial inoculum will be exposed to them.

2.2.3.3. Do AMPs have a clear role in immunity?

Our understanding of AMPs' bactericidal functions is based on *in vitro* tests. If these *in vitro* functions are not mirrored *in vivo*, then the rarity of AMP resistance could be explained as a consequence of this misunderstanding, and AMPs may not in fact select for resistance at all. The essence of what we believe to be the role of AMPs is based on the observation that they interact with prokaryote cell membranes, and kill these cells above a certain concentration. However this does not necessarily mean that AMPs are killers of infection *in vivo*, since interactions with membranes are crucial in a broad range of molecular and cellular processes other than killing. Concentration-dependent *in vitro* lethality of AMPs may be a secondary consequence of the interaction with cell membranes and physiologically unrealistic AMP titers in *in vitro* assays. Inference about the functions of AMPs based on immunodeficient subjects (e.g. *Drosophila* (Tzou et al., 2002); and human children (Gruenheid and Le Moual, 2012)) should be made cautiously, because results are confounded by compromised function of other immunological systems.

There is some reason to suspect that the *in vivo* role of AMPs is not entirely properly understood, or at least that they serve dual functions. They are known to have roles in intercellular signaling in metazoans, for instance in iron regulation and responses to inflammatory compounds in humans (Collins et al., 2008; Amatngalim et al., 2010). The immunomodulatory effects of AMPs have been extensively reviewed by Hancock and colleagues (Easton et al., 2009; Yeung et al., 2011). Immunomodulation by AMPs may be a conserved feature, and "AMPs" produced by microbes may serve similar functions in microbial communication, in common with other compounds of microbial origin that are now used as antibiotics (Martinez, 2008). It is even possible that

such small peptides are involved in communication with hosts, as recently demonstrated in *Pseudomonas* (Jarosz et al., 2011). Accordingly, correlational studies show that human deficiency in AMP production is associated with a diminished ability to manage gut microbiota (Gruenheid and Le Moual, 2012). Since AMPs have putative roles in metabolic regulation as well as in membrane poration (Brogden, 2005), a role in intercellular communication is easily conceivable.

There is recent exciting evidence suggesting a real role of AMPs in communication on an important ecological level. *Sitophilus* weevils are dependent on just one AMP to manage a bacterial symbiosis (Login et al., 2011). In plants, *Sinorhizobium-Medicago* symbiosis is dependent on just one bacterial gene to allow the bacteria to resist the defensins that the plant uses to protect against other infections, and thereby inhabit the host's roots (Haag et al., 2011). These results suggest that, rather than just being broad-spectrum antimicrobials, AMPs can also be exquisitely specific tools to manage interspecific associations. If these are general phenomena and occur in other organisms, then the lack of expected resistance to AMPs could be explained by a misunderstanding of their true function, and selection for AMP resistance is weak.

2.2.4. Role of the present study

The remainder of this chapter details an experiment designed to test two of the hypotheses detailed above through a comparative experimental evolution experiment. Bacteria derived from one original *S. aureus* clone are selected in parallel by a range of AMPs and antibiotics, and the response to selection is compared, to see if AMP resistance is more costly to bacteria than antibiotic resistance. One treatment group is selected by a 50:50 combination of two of the other AMPs, testing whether the combination is greater than the sum of its parts in terms of slowing resistance evolution. This allows parameterization and comparison of the fitness of these selected cultures. Since the frequency of exposure and intensity of stress was controlled, the

emergence of resistance is determined by cost or functional interactions.

2.3. Materials and methods

2.3.1 Bacteria and stressor selection

All bacteria were derived from one ancestral colony of *S. aureus* (JLA 513, SH1000 background; sourced from Simon Foster, University of Sheffield). This strain is tetracycline resistant, controlled by a chromosomal intergenic resistance cassette. This cassette does not affect transcription of surrounding genes or growth in nonselective medium (G. McVicker, Pers. Comm; Shaw et al., 2006) This label is useful in subsequent animal infection experiments (Chapter 3), and allowed a "belt-and-braces" approach to controlling contamination.

Five antimicrobial stressors were chosen as selective agents. Streptomycin sulphate (Sigma-Aldrich S9137-25G) is an antibiotic in clinical usage, with a history of use stretching back to the 1940s (D'Arcy Hart, 1999). In nature it is produced by *Streptomyces griseus* – a widespread soil bacteria – so *S. aureus* is likely to have a history of low-level association with streptomycin. In contrast, Vancomycin (Sigma-Aldrich V1130-1G) is an antibiotic which has only been in widespread since the 1980s, and environmental isolates of vancomycin-resistant *S. aureus* are less (albeit increasingly) common than streptomycin-resistant isolates (Weigel, 2003). Streptomycin and vancomycin were respectively used as 'soft selection' and 'hard selection' treatment controls.

Pexiganan was included as a stressor because it is the only AMP currently commercially available as an antibiotic. Several grams of pexiganan acetate were generously supplied by Michael Zasloff (Georgetown University, USA). The synthetic AMPs melittin and

iseganan were also used as stressors. Melittin was purchased from a commercial supplier (Sigma-Aldrich M2272), and expressed iseganan (expression protocol and data unpublished) was generously supplied by Wojciech Kamysz (University of Gdansk, Poland). Pexiganan and melittin were additionally combined in a 1:1 ratio, constituting a fourth treatment ("pgml") to test the effect of combinations of AMPs on bacterial evolution.

2.3.2. Pre-selection MIC_{50} determination

The concentration of each stressor sufficient to inhibit 50% population growth was determined by a protocol based on that of (Perron et al., 2006), who modified the United States' National Committee for Clinical Laboratory Standards broth macrodilution susceptibility assay guidelines. A single colony from the tetracycline-resistant SH1000 *S. aureus* clone JLA 513 (source: S. Foster, University of Sheffield) was inoculated into 5ml Müller-Hinton broth containing tetracycline at 5 μg ml^{-1} and amphotericin-B at 5.6 μg ml^{-1} [these conditions will henceforth be referred to as 'standard growth medium'], and grown for 48 hours at 30^{o}C in a shaking incubator at 120rpm. A sub-culture was then started by inoculating 500μl of this culture into 4.5ml of fresh medium and grown to exponential phase.

A concentration gradient of each antimicrobial stressor ranging from 64 μg ml^{-1} to 0.125 μg ml^{-1} in standard growth medium was constructed by two-fold serial dilution in 96-well microtitre plates in 100μl solution per well, with an additional series of unsupplemented controls on each plate. 10μl of the previously sub-cultured bacteria was added to each well, giving an OD_{595} of 0.046. Optical density at 595nm (OD_{595}) was measured immediately after inoculation and again once every hour for up to seven hours.

Maximal growth rates (μ) data from these data were used to estimate the concentration of each stressor causing 50% inhibition of maximum

growth (MIC_{50}) with the R package Grofit. The intensity of selection imposed by each stressor was therefore standardised.

2.3.3. Selection protocol.

Selection protocols were based on those of Perron et al. (2006), in which opportunities for growth and evolution are standardized between treatments, whilst providing time for evolution of varied trajectories.

Before selection began, a single colony of *S. aureus* JLA 513 was inoculated into standard growth medium, and grown for 24 hours at 30^{o}C in a shaking incubator at 120rpm. 50µl of this culture was then sub-cultured into 5ml standard growth medium and cultured under the same conditions. This process was repeated daily for 10 days to allow random mutation and accumulation of genetic diversity.

The ten-day serial culture was used to establish parallel selection lines that were exposed to each microbial stressor, and unselected controls. 5µl samples (c. 2.8 x 10^{7} colony forming units) of this bacterial culture were inoculated into 500µl preparations of standard growth medium, containing stressors at the estimated MIC_{50} for each respective treatment. Five parallel replicate cultures were established in each treatment. Unselected control cultures which were grown in 500µl standard growth medium. A further five dummy cultures containing 500µl standard growth medium were supplemented with 5µl sterile water as a sterility control. Once the sub-cultured selection lines were established, the parental culture was frozen at -90 ^{o}C in a 1:1 ratio with sterile glycerol, for future revival and comparison (Chapter 3), in which it is referred to as the Ancestor strain.

These cultures were grown for 24h in a shaking incubator at 30 ^{o}C, before 5µl was inoculated into a fresh 500µl of growth medium identical to the previous day for each respective treatment, i.e. standard growth medium plus stressor. Optical densities at 595nm (OD_{595}) of 100µl of the

24-hour cultures were determined in a 96-well plate reader (Molecular Devices Versamax). Co-ordinates for each culture on the plate were randomised daily. This procedure was repeated daily for 28 days. On days 7, 14 and 21 the concentration of antimicrobial compound in each respective treatment was doubled, in accordance with the protocol developed by Perron et al (2006). Consequently each stressor was suspended at its MIC_{50} during week 1, $2xMIC_{50}$ (week two), $4x\ MIC_{50}$ (week three), and $8x\ MIC_{50}$ (week four). Each 24-hour culture was frozen daily at -90 ^{o}C in 50% sterile glycerol.

Contamination was checked for bi-daily by either serial dilution of each culture in PBS and plating with beads, or by spreading a sample onto a plate with a sterile inoculation loop. In either case samples were plated LB containing 1.5% agar. Any colonies displaying abnormal colour or morphology were checked on Mannitol Salt Phenol Red Agar. These protocols revealed no contamination through the course of the experiment.

An incubator error meant that all lines, including unselected controls, dwindled between day 14 and day 21 at the first attempt. To revive the cultures from day 14, 10µl of an aliquot of a day 13 glycerol stock was sub-cultured into day 7-14 growth media, respective to each treatment. After 24h, these cultures were pelleted and re-suspended in standard growth medium to within 0.02 of the 24h OD of the original day 14 cultures. 5µl of each of these re-suspended cultures was then sub-cultured into day 15 stressor conditions and the selection protocol resumed as per normal. The data presented from day 15 onwards are from these restarted cultures.

2.3.4. Dose-response assays

The multiple levels of replication in this assay necessitate clear definitions of the working terms. The five biological replicate cultures in each treatment are referred to as populations, since after time growing

in serial passage they are unlikely to be monoclonal. Growth was assayed across 11 different concentrations (two-fold dilutions of 64-0.125μg ml^{-1} plus unsupplemented medium at the end of weeks one and two, and the same with an additional 128 μg ml^{-1} level in weeks three and 4) of their respective selective stressor, each of which is referred to as a test concentration. Each population/test concentration combination was technically replicated three times.

These assays were conducted in 96-well microtitre plates, in common with the initial MIC_{50} determination assay. 50μl of all surviving populations were sub-cultured into 5ml unsupplemented standard growth medium at the end of days 7, 14, 21 and 28. Standard (i.e. rather than selective) growth medium was used for these sub-cultures to relax selection and provide opportunity for expression of plastic phenotypic mechanisms of resistance to be down-regulated prior to the assay, increasing confidence in the assay's detection of heritable traits. These cultures were incubated at 30^{o}C, shaking at 120 rpm, until late exponential phase.

10μl of each 18-hour culture was added to its full range of technically replicated test concentrations. OD_{595} was determined every hour for six hours as per the initial MIC_{50} assay.

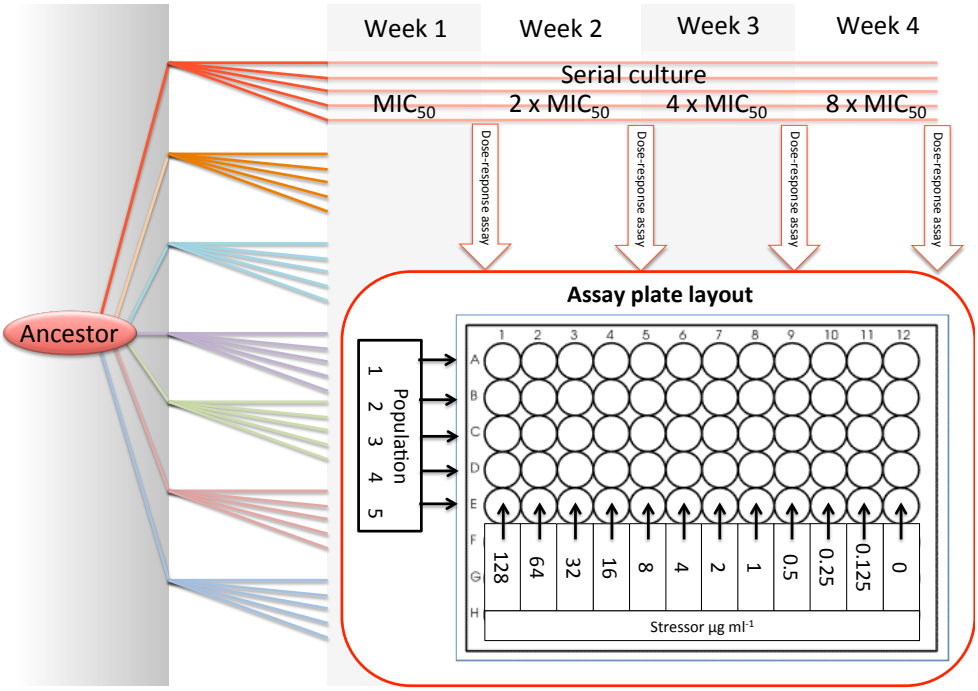


Figure 2.3.3. Schematic of the protocol for resistance selection and dose-response assay.

The original culture was grown by serial passage (10 days) to generate the ancestor. The ancestor was split between 7 treatments, containing 5 replicate populations. These cultures were serially cultured in their respective stressors, starting at MIC_{50}. Stressor concentration was doubled weekly. Dose-response assays were performed weekly (3 replicate plates per treatment). On each plate, each population was exposed to a two-fold 128 – 0.125 µg ml^{-1} dilution series of its stressor.

2.3.5. Data analysis

Data were exported from Softmax Pro as text files and manually assembled into data frames in Microsoft Excel for Mac (version 14.1.4). All analyses were conducted in R (version 2.14.1).

2.3.5.1. r0 calculation

Instantaneous intrinsic growth rates of each culture (i.e. three technical replicates/12 test concentrations/ 5 biological replicates/treatment/week) were calculated by logging the input OD data to calculate r0α, where α represents an unknown constant, according to the logic detailed in box 2.3.5.1 (see Appendix 1 for further information):

Box 2.3.5.1: calculating instantaneous r0

If N = OD and T = time, then each growth curve can be summarised as:

$$\frac{dN}{dT} = rN$$

$$\left(\frac{1}{N}\right)\left(\frac{dN}{dT}\right) = r0$$

$$\frac{dlogN}{dT} = \left(\frac{1}{N}\right)\left(\frac{dN}{dT}\right) = r0$$

To extend this to the data in the present study, including α as an unknown constant:

$$OD = N\alpha$$

$$log\ OD = \alpha(logN)$$

$$\frac{log\ OD}{T} = \left(\frac{1}{N}\right)\left(\frac{dN}{dT}\right) = \frac{r0}{T}$$

Slight variation in the starting ODs of cultures entering the dose-response assay and the technical impracticality of controlling the growth dynamics of up to 40 independent serial cultures meant that parametric models fitted to r0α data would likely have varied r^2 values. Therefore, the R library Grofit (Kahm et al., 2010) was used to fit smoothed splines to logged OD data in a similar analysis to that described in 2.3.2. Grofit fits splines by implementing the R function *smooth.spline()*, for which the user determines smoothing values. 0%, 25%, 50%, 75% and 100% smoothing of these splines was tested on the week 1 dataset, which revealed that 50% spline smoothing provided fits to the data that were smoothed but still sensitive to departures from linearity. Each growth curve was bootstrapped 100 times using Grofit's inbuilt bootstrapping features to increase the confidence of these spline fits.

As input OD_{595} values ranged between 0 and 1, calculated r0α values (log OD_{595}) were negative. Grofit requires positive input data, so splines were fitted to logOD+10: this process increased all y-intercepts to >0 but maintained slopes.

Grofit's implementation of the *smooth.spline()* R function fits splines determined as a sequence of cubic polynomials. The Grofit library takes the first derivative of this sequence and determines its maximum (M. Kahm, Pers. Comm.). This equals the maximum slope during the 6-hour assay, during which each culture should reach exponential phase. Consequently the outputs from the Grofit analysis were the max instantaneous r0α x three technical replicates x 12 test concentrations x five biological replicates x five treatments x four weeks (see Figure 2.3.3). These values were plotted with smooth geometric fits to each biological replicate by their respective test concentration, per treatment, per week, using the R library ggplot2 (Wickham, 2009). 0 μg ml^{-1} cannot be presented on the log scale used in this figure and so are presented separately in Figure 2.4.2.

These analyses produced dose-response curves by treatment and week, showing the range of stressor concentrations in which each selection line is growing well or badly. This allows an assessment of the trade-offs that each treatment group was subject to as the intensity of selection increased over the course of their evolution, for example an improvement of growth at high concentrations of stressor associated with deteriorating growth rate at low concentrations.

Absolute minimum inhibitory concentrations (MIC) provide a measure of a population's absolute resistance to a stressor. These values were calculated conservatively. There was some error in the assay, evidenced by negative growth values in certain treatments. To account for this error, the maximum negative growth value over the entire course of the 4-week experiment (-0.07) was taken as an estimate of the maximum potential error. On this basis growth values in the range 0 - 0.07 can be explained as error: any cultures showing growth rates <0.07 were therefore counted as inhibited. The lowest concentration of stressor from the inhibited subset was taken as the MIC. In some treatments inhibition was not observed in the range of stressor concentrations used in the assay, i.e. the MIC was greater than assayed for - such results are reported accordingly. This procedure was applied to each population, per week (including week 0). The statistical modes of MIC values were calculated per treatment per week. Fold-change of modal MIC over week 0 MIC was then calculated.

Change in the concentration of stressor in which a population shows maximum growth (maxG) provides a measure of the population's adaptation to that environment. As for MIC, these values were calculated for each population per week, followed by fold-change of the statistical mode relative to week 0.

2.3.4.2. Uninhibited growth analyses

A linear model was fitted to r0α values (means of technical replicates) of selected cultures growing in unsupplemented standard growth media in the dose-response assay. Week 0 data were excluded from this analysis because all cultures grew by serial passage in uninhibited S.G.M. between days -10 and 0, increases in growth rate between week 0 and week 1 are largely systemic and independent of treatment. Cultures showing r0α values <0.005 were excluded as noise from dead cultures generated by inaccuracies in initial OD_{595} measurement. A linear model with an interaction between week and treatment provided the best fit to the data ($R^2 = 0.82$)

2.4. Results

2.4.1. Initial MIC_{50}

Pre-selection MIC50 estimates and the maximum stressor concentrations in which strains survived are presented in Table 2.4.1.

Table 2.4.1. Estimates of antimicrobial concentration inhibiting 50% growth of the target population (MIC_{50})

Stressor	MIC_{50} before selection (μg ml^{-1})	Maximum tolerated stressor concentration (μg ml^{-1})
Iseganan	3	24
Melittin	1	8
Pexiganan	6	18**
PGML	5	10*
Streptomycin	3	24
Vancomycin	1	2*

*Extinct at week 3; **Functionally extinct at week 3.

The data in Table 2.4.1 show that iseganan-, melittin- and streptomycin-selected *S. aureus* survived in up to 800% MIC_{50}. PGML- and vancomycin-selected cultures grew up to 200% MIC_{50}, and pexiganan-selected cultures maintained functional populations up to 400% MIC_{50} (see 2.4.4. for more discussion of Pexiganan-selected cultures)

2.4.2. Evolution of dose-response curves

Dose-response curves evolved in a strongly treatment-dependent fashion, indicating that selection from different stressors acted on different traits (Fig. 2.4.1). Table 2.4.2 summarises minimum inhibitory concentration (MIC) and concentration in which maximum growth was observed (maxG), extrapolated from the dose-response data.

There was some consistency within some treatments over the progression of the experiment but not between them, and changes in y-intercepts of dose-response curves and inflection points after the end of week 1 were not linear. These results show that selection from different stressors acts on distinct physiological traits. Treatments are discussed by case below.

All cultures evolved greater r0 values across the full range of test concentrations (Fig. 2.4.1). This change was most pronounced between week 0 (selection day -10) and the end of week 1, likely as a consequence of the initial 10 days of uninhibited serial passage before selection began, which is predicted to select for fast growth. Streptomycin-selected cultures showed the strongest response to selection, and were almost completely indifferent to streptomycin concentration by the end of week 1. This pattern was consistent throughout the remainder of the experiment.

After the initial universal increase in r0 at the end of week 1, dose-response y-intercepts decreased at the end of week 2 across all treatments except streptomycin. Melittin-selected cultures showed an

increased r0 at 8μg ml^{-1} at the end of week 2, consistent with a tradeoff between resistance and fitness in low-stress environments. However there was little apparent increase in resistance in other treatments at this point.

Vancomycin- and pgml-selected cultures were not viable in the selection protocol beyond the end of week 2, and went extinct at the beginning of week 3. Extinction was confirmed by plating. Pexiganan-selected cultures showed a strong depression of r0 across all test concentrations at the end of week 3, in conjunction with a depressed OD in the main selection cultures. The low density of cells in the selection culture is likely to negatively affect the output of the dose-response assay (see Appendix 1), so these data from the end of week 4 are not shown for pexiganan-selected cultures.

Melittin and iseganan-selected cultures showed further increases in r0 at higher (≥8 μg ml^{-1}) concentrations of their stressors at the end of week 4. Indeed, iseganan-selected cultures showed an almost flat dose-response, similar to that of streptomycin-selected cultures. This was associated with a recovery of r0 at low concentrations of stressor, to levels approximating those at the end of week 1.

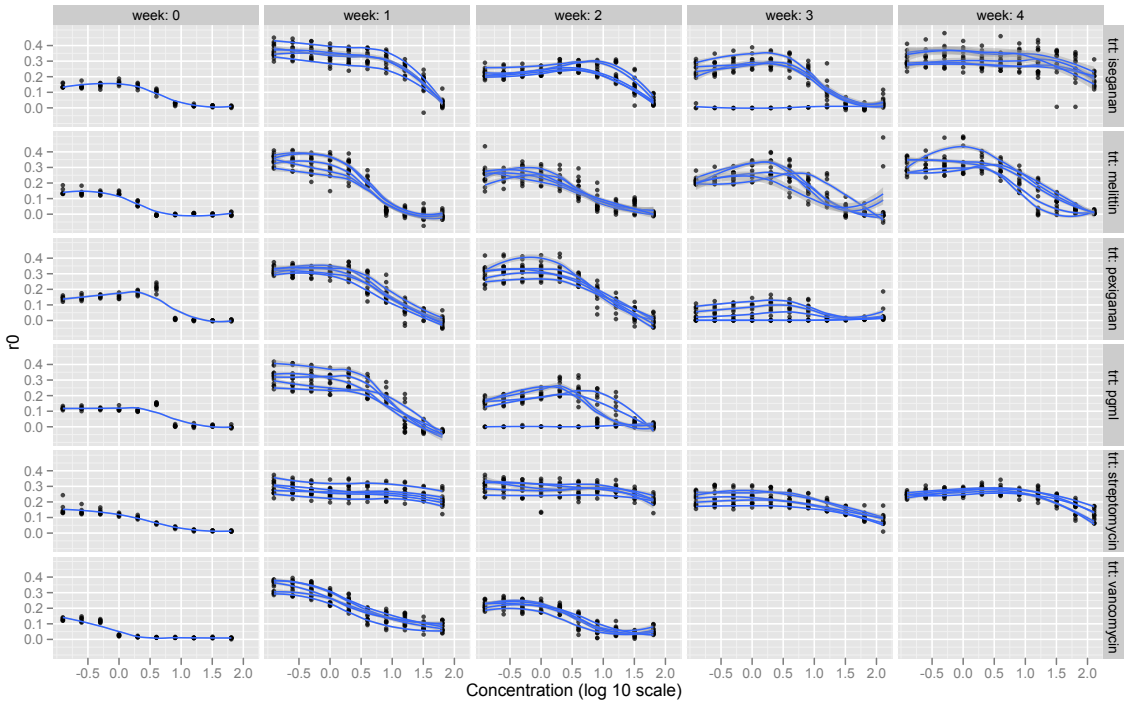


Figure 2.4.1. Evolution of dose-responses during selection from antimicrobial stressors

S. aureus cultures under standardised weekly-increasing selection from antimicrobial stressors were sub-cultured into unsupplemented media until late exponential phase and then grown for six hours in a dilution series of the stressor against which it had been selected. Optical density (595nm) was measured hourly, and used to calculate r0 of cultures at each concentration, from which dose-response curves are constructed. Dose-response assays were repeated weekly until cultures became functionally extinct or had been selected for four weeks. Some input cultures (iseganan-selected, week 3; and pgml-selected, week 2; pexiganan-selected, week 3) did not grow or barely grew before the assay.

Table 2.4.2. Evolution of *S. aureus* resistance to AMPs and antibiotics: Fold-change in modal minimum inhibitory concentration (MIC) & and concentration showing maximum growth (maxG) (both μg ml^{-1}) relative to week 0, under increasing selection from antimicrobial stressors, starting at MIC_{50}. Grey blocking denotes functional extinction.

** assay over range 0.125 - 64 μg ml^{-1}.** assay over range 0.125 - 128 μg ml^{-1}.*

	Weekly fold change (modal values) over day -10							
	W_1		*W_2*		*W_3*		*W_4*	
Treatment	*MIC**	*maxG**	*MIC**	*maxG**	*MIC***	*maxG***	*MIC***	*maxG***
Iseganan	8	0.5	8	2	4	2	>16	16
Melittin	2	0.25	4	0.5	8	1	4	0.5
Pexiganan	8	0.5	8	0.63				
PGML	2	0.32	4	0.5				
Streptomycin	>8	1	>8	1	>16	2	>16	32
Vancomycin	>64	0.5	32	1				

Selection intensity

High — Low

MIC_{50} | 2 x MIC_{50} | 4 x MIC_{50} | 8 x MIC_{50}

Table 2.4.2 summarises fold-change of MIC and maxG values extrapolated from the data presented in Figure 2.4.1. Consistent with Figure 2.4.1, the data in the table show a marked increase in resistance (MIC) across all treatments, particularly in the first week of selection. There is no evidence of a negative relationship between these parameters.

2.4.3 – Evolution of r0 in stress-free environments

r0 in stress-free conditions ("r0*max*") was strongly related to the interaction between treatment and week of selection (Linear model: F-ratio=23.04, df=18,72, $p<0.0001$, adjusted $r^2=0.82$) (Fig 2.3.2). An increasing background of selection corresponded to a depression of r0*max* across treatments for most of the selection protocol, apart from in week 4 when the surviving cultures (iseganan, melittin and streptomycin) showed a marginal recovery of r0*max*. Pexiganan-selected cultures maintained the same r0*max* throughout weeks one and two, but this was strongly depressed in week 3, prior to extinction. Conversely, streptomycin-selected cultures showed a slight depression of growth rate in the first week relative to the other cultures, but there was little change in this trait through the remainder of the experiment. The r0*max* of PGML and vancomycin-selected declined in week 2, before extinction in week 3.

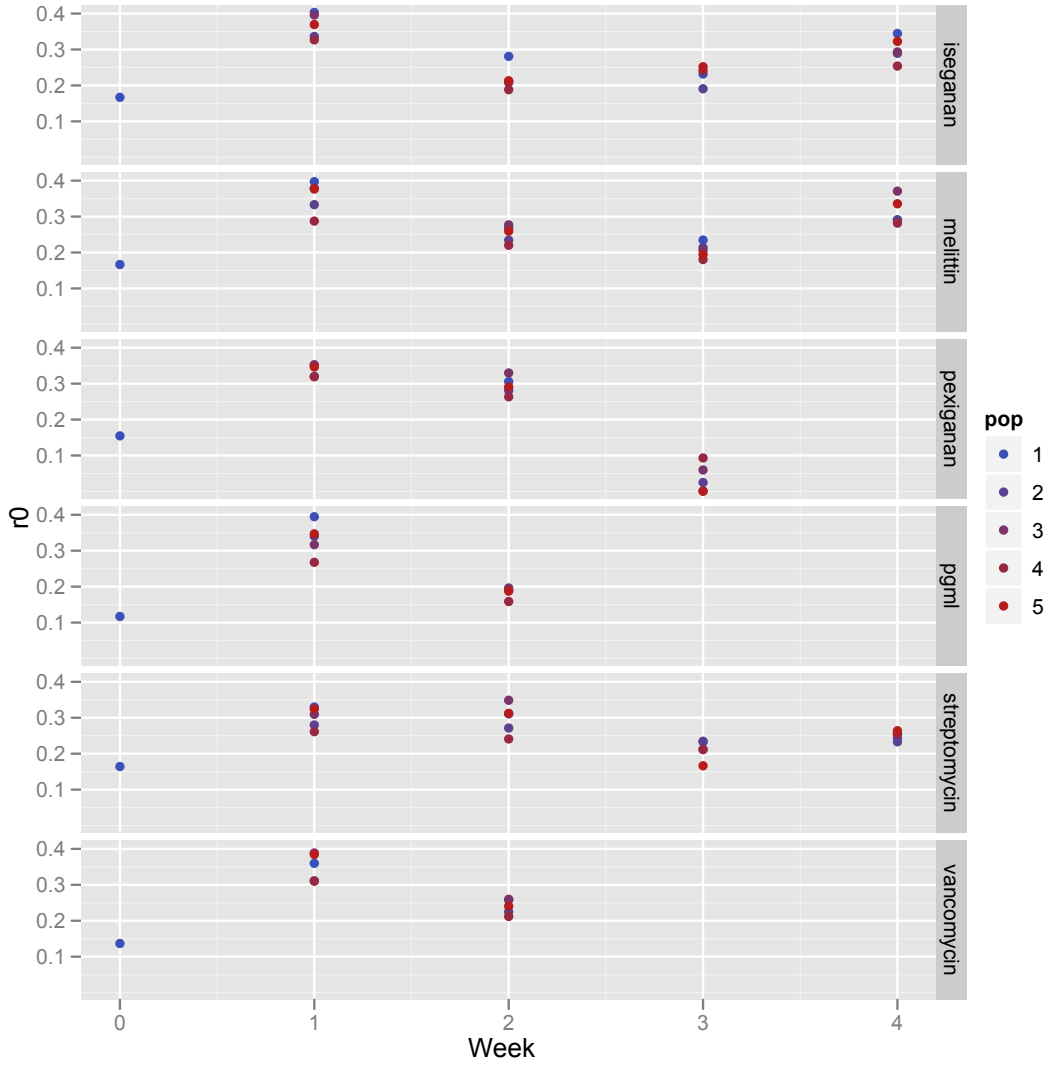


Figure 2.4.2 – Uninhibited r0α values (population means) by week

S. aureus cultures under standardised weekly-increasing selection from antimicrobial stressors were sub-cultured into unsupplemented media until late exponential phase. They were then sub-cultured into unsupplemented media again, optical density (595nm) was measured hourly for six hours, and the subsequent data were used to calculate r0. This assay was repeated weekly until the cultures under selection became functionally extinct, or until four weeks had passed.

2.4.4 – Population size

There are few universal trends in the daily 24h ODs of cultures between treatments, but ODs were consistent within treatments (Fig 2.4.3). 24h ODs were consistently equivalent across all treatments in week 1 apart from pgml selected cultures, in which populations were smaller. There was a steady systematic decline of 24h ODs in vancomycin- and pexiganan-selected cultures during the first few days of week 2. Pexiganan populations remained depressed, but vancomycin-selected populations recovered on day 9, suggesting simultaneous evolutionary innovation in vancomycin-selected cultures. Pgml-selected lines showed consistently low population size through this second week.

Unfortunately, the incubator fault described in 2.4.2 led to slower growth of all populations on day 15, despite the attempt to normalise OD to day 14 levels, suggesting that culture physiology had not fully recovered from freezing by day 15, despite showing apparently strong growth before re-suspension. However, plating after day 15 revealed the continued presence of viable cells in all cultures, albeit at lower densities in some. This is particularly significant for vancomycin- and pgml-selected cultures, because it suggests that eventual extinction in the middle of week three was a genuine phenomenon caused by slow growth, consequent of an inability to evolve responses to their stressors (i.e. rather than an interactive effect of recovering from freezing and exposure to more concentrated stressor).

Figure 2.4.4 shows extinction of cultures by day under selection, determined by the absence of viable cells when they were scraped onto agar. These data demonstrate the greater difficulty faced by pgml-selected cultures than other cultures (notably pexiganan and melittin) in overcoming increased intensity of selection. The sudden demise of vancomycin-selected lines between days 16 and 18 also suggests an upper limit of the ability of cultures in this selection protocol to evolve resistance to this particular stressor. The persistence of some viable

cells in two pexiganan-selected lines was confirmed by plating, and these cells were cultured up to the end of week 4. However undetectably low densities, as determined photometrically, meant that they were considered functionally extinct in comparison to other cultures by the end of week 3. The remaining three pexiganan-selected cultures persisted at very low densities, but were not considered extinct because their ODs were consistently above those of sterile SGM.

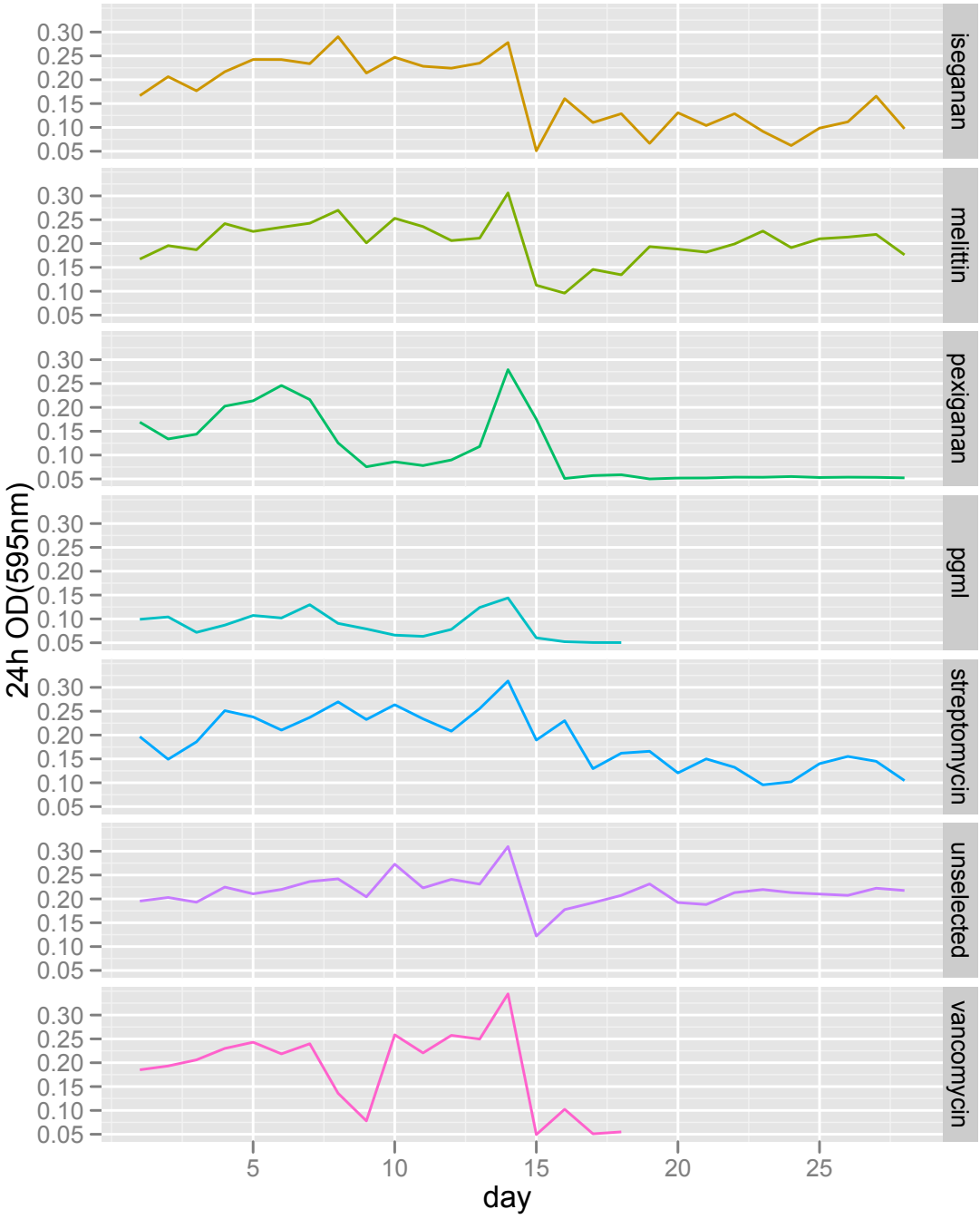


Figure 2.4.3. Daily 24h OD_{595} (means, threshold OD = 0.05) of selected cultures

Optical Density (595nm) of *S. aureus* cultures under standardised weekly-increasing selection from a range of antimicrobial stressors was measured daily when the culture was sub-cultured into the following day's media. There were five replicates per treatment, the ODs of which were averaged if they were >0.05. Cultures of ODs <0.05 are considered functionally extinct.

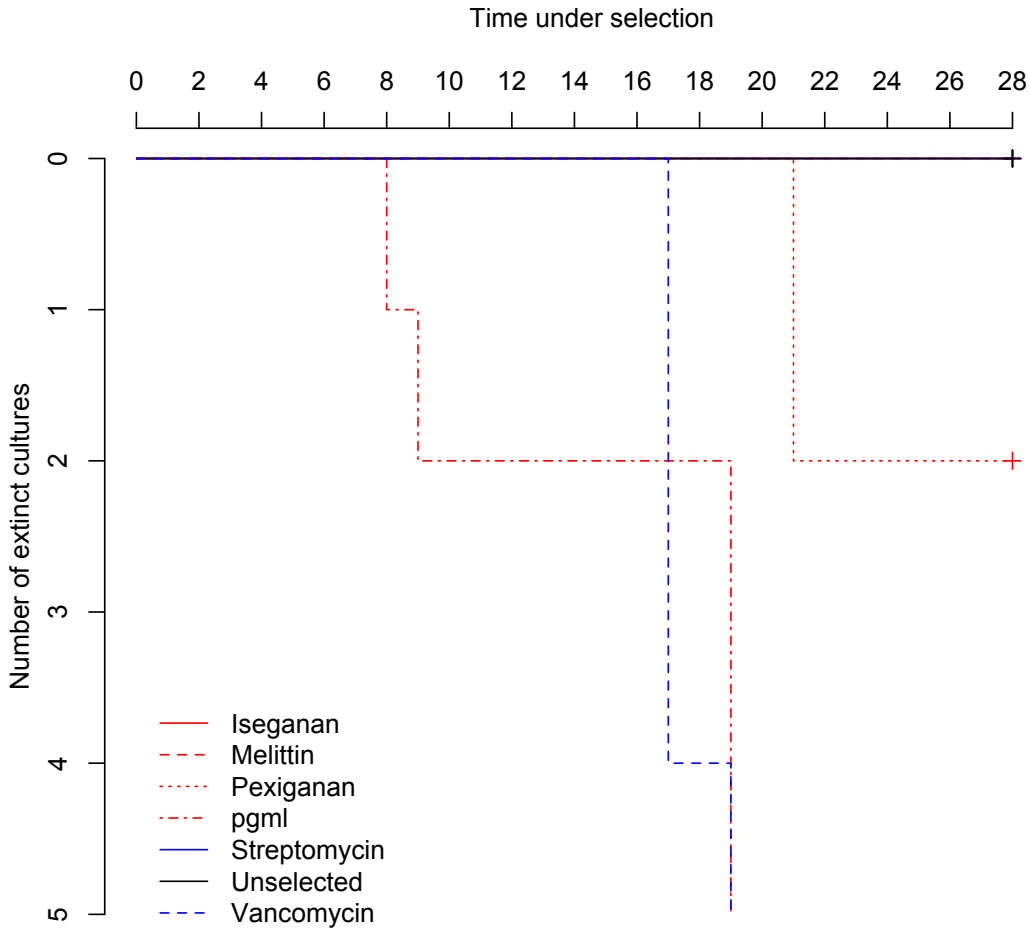


Figure 2.4.4. Functional Extinctions during the AMP/antibiotic selection protocol

S. aureus were serially cultured under standardised weekly-increasing selection from a range of antimicrobial stressors for up to four weeks. There were five replicates per treatment. Cultures selected by some stressors (Vancomycin and pgml) became totally extinct, confirmed by a lack of growth after plating on agar. Some persisted at extremely low densities in medium (measured photometrically) but displayed some growth after plating, which were considered functionally extinct. Surviving lines are compressed into the line at the top of the figure.

2.5 - Discussion

This experiment was designed to measure the costs of resistance to AMPs individually and in combination, by measuring performance in terms of growth and viability during selection, and comparing these metrics to cultures under selection from antibiotics. Taken together, these results are consistent with the theoretical predictions of how bacteria should perform under selection. Crucially, there were no categorical differences in how AMP-selected and antibiotic-selected *S. aureus* performed under selection, either in terms of fitness or their ability to evolve a response to a stressor. The responses to single AMPs are in the range set by vancomycin and streptomycin, showing that *S. aureus* adapted to grow in AMPs suffer no greater costs than those that must be borne by viable natural isolates. Therefore, this experiment shows that there is no fundamental difference between the costs of resistance to AMPs and antibiotics. The weaker response to a combination of AMPs indicates that additional costs of resistance to multiple stressors and interactions between component stressors constrain the emergence of AMP resistance in natural infections.

2.5.1 – Trade-offs in resistance evolution

A strong trade-off between general fitness and resistance to stressors should be manifested as a negative relationship between MIC and maxG over time. However, Table 2.4.2 suggests the absence of such a relationship in these data. This suggests that intrinsic trade-offs do not constrain resistance to individual AMPs in this experiment. However the data do not show the predicted trade-off for vancomycin- or streptomycin-selected bacteria either, so this conclusion should be treated cautiously.

There are two apparent trajectories for bacteria under selection in this protocol. Cultures in treatments that did not go extinct (iseganan, melittin, streptomycin) showed a slight depression of r0*max* (a proxy of

fitness) but survived the duration of the experiment, and even appear to show some compensatory evolution of r0*max* in the final week of the experiment. This suggestion corresponds with the ability of bacteria under selection from these stressors to evolve in terms of dose-response: Fig 2.4.1 suggests that, over the course of the whole experiment, dose-response evolved most in iseganan-, melittin- and streptomycin-selected cultures, although the changes were not necessarily synchronous. In evolutionary terms this means that these cultures show the greatest shift in their performance in varied environments, and can grow better at higher concentrations of their selective stressor at the cost of weaker growth at lower concentrations.

Interestingly, streptomycin-selected cultures showed both the quickest response to selection in terms of dose-response - which was almost flat by the end of week 1 – a concurrent depression of r0*max*, and a reasonably stable r0*max* thereafter. The ancestor uses a rapid efflux pump to resist tetracycline (A. Williams & S. Foster, Pers Comm; Hillen and Berens, 1994), which could possibly have simply been modified and used to export streptomycin. On the other hand, iseganan and melittin-selected cultures took longer to evolve resistance to their stressors, and a more gradual depression of r0*max* over the course of weeks 1 to 3. This is consistent with the theoretical prediction that responses to selection should be proportional to cost (Levin et al., 2000; Andersson, 2006).

The second trajectory witnessed in this experiment is an inability to adapt to the environment, hallmarked by a decline in r0*max* over time, decreased OD in selection cultures, and eventual extinction. This pattern is displayed by pexiganan-, pgml- and vancomycin-selected cultures. The decreasing r0*max* could be a consequence of two processes. First, the cultures may simply be highly stressed after their prolonged exposure to AMPs or antibiotics. A second possibility is that that the apparent decrease in r0*max* over time is semi-artefactual, and is in fact a consequence of variation in numbers of starting bacteria entering the

dose-response assay resulting in varied maximal rates of change in the assay. However, for reasons explained in Appendix 1, although this issue could compromise the absolute precision of the results, it does not completely invalidate the conclusion. The decreases in r0*max* are corroborated by their correspondence with falling OD and extinction in the selection cultures themselves. Although there is some (limited) potential for the dose-response and r0*max* values to show low fitness as an artefact of low population size in the selection cultures, low population size in the selection cultures is itself caused by low fitness.

Pgml- and vancomycin-selected cultures were the quickest to show compromised fitness and to go extinct. This is particularly remarkable for the pgml-selected lines, because it took another week for any pexiganan-selected cultures to go extinct, and melittin-selected cultures survived the duration of the experiment. This meets the theoretical prediction that the evolution of resistance is not favoured by this combination treatment (Aaron et al., 2000; Fischbach, 2011). This study is the first to show such a pattern using AMPs. It would be interesting to see what the nature of the interaction between pexiganan and melittin is through killing-curve and checkerboard assays, to test the theoretical notion that the pace of evolution of resistance to combinations of stressors should be associated with functional interactions (Hegreness et al., 2008; Michel et al., 2008; Yeh et al., 2009; Torella et al., 2010).

It is interesting that pexiganan-selected cultures went extinct in this protocol, but not in the recently published work of Habets & Brockhurst (Habets and Brockhurst, 2012). This suggests an upper limit of *S. aureus*' ability to evolve pexiganan resistance in this experimental protocol. The difference between this result and those of Habets & Brockhurst may be a function of differences in minimum population size (5µl and 40µl of 24h culture were transferred in each experiment, respectively) altering the probability of beneficial mutants arising and remaining in the population. Also, this experiment differs from Habets & Brockhurst's insofar as it was concerned with the dynamics of resistance

evolution rather than the potential for it, which manifested in differences in experimental protocols, and a less forgiving selection regime in the present study for any cultures that did not quickly evolve. Habets & Brockhurst selected *S. aureus* for pexiganan resistance to test for cross-resistance: this experiment was concerned with how the bacteria behave during selection.

2.5.2 – No difference between antibiotics and AMPs

The most critical trend in the data from this experiment is the lack of any apparent difference in the performance of *S. aureus* under selection from streptomycin, vancomycin, or the panel of AMPs. Streptomycin and vancomycin-selected bacteria performed as predicted: *S. aureus* struggled to grow in concentrated vancomycin, but rapidly evolved to grow in high concentrations of streptomycin. By incorporating antibiotics into the selection protocol, this study is the first to measure the costs of AMP resistance in a fully parameterised fitness framework. If streptomycin and vancomycin are accepted as extremes of the selection that *S. aureus* can be exposed to from antibiotics, then the AMPs used in this study select for the same range of responses as antibiotics. Critically, although *S. aureus*' resistance to pexiganan and vancomycin did not increase hugely in this experiment, they have been demonstrated previously in other studies or environmental isolates. Therefore, this study has shown no difference in the ability of bacteria to evolve resistance to AMPs or antibiotics *in vitro*.

These findings have two broad implications, concerning the evolution of immunity and the potential to use AMPs as drugs. First, it excludes the hypothesis that the rarity of AMP resistance in natural isolates is regulated by the intrinsic properties of AMP-microbe interactions (Hancock and Diamond, 2000; Hancock, 2001; Zasloff, 2002). On this basis, other properties of immune systems or AMP-microbe ecology must constrain microbial resistance. The lack of any factorial difference in the costs of AMP resistance in this study suggest that cost is not

necessarily a limiting factor, consistent with previous work (Pränting et al., 2008). Indeed, the comparison of pgml-selected *S. aureus* to pexiganan- and melittin-selected cultures suggests that the simultaneous action of numerous different immunological stressors constrains resistance evolution. This study is the first proof-of-principle experimental evidence of this process. Assuming that AMPs are bactericidal, analogous selective processes are likely *in vivo* when multiple host AMPs are synthesised after infection. In this case, plastic phenotypic responses to immunological stress in an infection are likely to be more facile than evolved resistance, and thereby dampen the effects of selection from AMPs on bacteria (Kahl et al., 1998; Lewis, 2001; Sadowska et al., 2002; Lewis, 2005; Moisan et al., 2006; Cai et al., 2009)

As well as the evolutionary ecology implications of these results, there is an important applied aspect. AMPs are currently under development as a new class of clinical antibiotics, based on the now discredited judgment that AMPs are somehow resistance-proof. If AMP resistance confers cross-resistance, then it might also be effective in helping infectious bacteria to evade the effects of AMPs when they are used in an immune response. Use of AMPs as therapeutic antibiotics might therefore select for more persistent and harmful pathogens. Experiments to test whether this is so are described in Chapter 3.

Chapter Three

The ecology of antimicrobial peptide-resistant Staphylococcus aureus *in* Tenebrio molitor

"There may be a danger…It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body.

…there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant. Here is a hypothetical illustration. Mr. X. has a sore throat. He buys some penicillin and gives himself, not enough to kill the streptococci but enough to educate them to resist penicillin. He then infects his wife. Mrs. X gets pneumonia and is treated with penicillin. As the streptococci are now resistant to penicillin the treatment fails. Mrs. X dies. Who is primarily responsible for Mrs. X's death? Why, Mr. X whose negligent use of penicillin changed the nature of the microbe. Moral: If you use penicillin, use enough"

Alexander Fleming,
Nobel Prize lecture.
December 11th, 1945

"The evolution of resistance to any antibiotic makes it less useful in treating disease…it also deprives any organism that produces it of part of its antibacterial armoury. This would not normally be a matter for concern; but in the case of antimicrobial peptides, we ourselves are the producers*. The evolution of resistance to human antimicrobial peptides, therefore, may have much more serious consequences than the evolution of resistance to conventional antibiotics, because our ability to resist infection might be permanently compromised."*

Graham Bell & Pierre-Henri Gouyon, 2003.
Arming the enemy: the evolution of resistance to self-proteins.
Microbiology 149, 1367-1375

3.1. Abstract

Antimicrobial peptides (AMPs) have been proposed as a solution to the increasing ineffectiveness of conventional antibiotics, because AMP resistance has rarely been observed in natural systems. However if using AMPs in a clinical setting selects for resistance and cross-resistance to other AMPs, then AMP therapy could coincidentally select for bacteria that are more resistant to AMPs deployed as part of immune responses. To test these ideas in an animal model, AMP-resistant, antibiotic-resistant and unselected *Staphylococcus aureus* generated in experiments described in Chapter 2 were inoculated into *Tenebrio molitor*. There were treatment-specific effects on beetle survival and long-term persistence of infection, but these effects were not necessarily related to AMP resistance of the cultures. However all but one of the AMP-resistant bacterial populations were significantly better at surviving the first 24 hours after inoculation into beetles than control strains. These results suggest that putative AMP therapies should be used with extreme caution, and confirm that AMPs are important effectors of innate immunity. Complementary to *in-vitro* data (Chapter 2), these data show that AMP-resistant *S. aureus* are not less fit in a host than their unselected ancestors, suggesting that intrinsic cost does not constrain natural AMP resistance.

3.2. Introduction

The evolutionary constraints on antimicrobial peptide (AMP) resistance have been extensively discussed in Chapter 2, focusing mainly on costs of this trait. This chapter addresses a number of subsequent issues, but incorporating an additional biomedical angle. The biomedical arguments for the potential of AMPs as a new class of antibiotic are laid out, followed by an alarming counter-argument.

3.2.1. The antibiotic resistance problem, and the potential of AMPs as novel therapeutic compounds

After being widely hailed as the greatest innovation of the twentieth century, the effectiveness of antibiotics is waning just seventy years after they first became widely available (Davies and Davies, 2010). The dangers of negligent use of antibiotics was recognised almost from the moment of their discovery, hallmarked by Alexander Fleming's warning of the dangers of negligent use of penicillin in his Nobel lecture. Bacteria resistant to penicillin were subsequently isolated from the environment just years after its commercial introduction (Chambers and DeLeo, 2009). Highly-resistant strains are now commonplace, and publicly prominent 'superbugs' that can happily survive in what were formerly antibiotics of last resort such as oxicilin or vancomycin (Lipsitch and Samore, 2002; Weigel, 2003; Fischbach and Walsh, 2009) are a particularly thorny problem for immune-compromised hospital patients. Many of these bacteria are resistant to panels of antibiotics, and resistance to certain antibiotics can infer cross-resistance to others e.g. β-lactam resistance can confer quinolone resistance (Sanders et al., 1984). There are almost innumerable reviews on the physiology and evolution of antibiotic resistance, and it is beyond the scope of this thesis to recount this information.

A proposed solution to the problem of antibiotic resistance has been to co-opt the antimicrobial peptides used by innate immune systems of, for example, frogs (Bechinger et al., 1993) and bees (Bulet et al., 2004), and use these therapeutically. This interest was initially motivated by the apparent 'resistance-proof' nature of these compounds (Hancock, 2000; 2001; Zasloff, 2002), and it was thought that AMP resistance simply could not evolve due to the mechanisms by which AMPs kill bacteria (see Chapter 2). However, as demonstrated in Chapter 2 and other experimental studies (for example Perron et al., 2006; Pränting et al., 2008; Habets and Brockhurst, 2012), this is simply not the case. Despite the mounting evidence that AMPs are far from resistance-proof, interest

continues in ‘AMP therapy’, seeking to use them as bactericidal or immunomodulatory drugs (Easton et al., 2009; Yeung et al., 2011). If bacteria were to evolve resistance to therapeutic AMPs and thereby become cross-resistant to AMPs in their immunological context, the consequences for hosts could be dire.

3.2.2. Cross-resistance to antibiotics: Cross-resistance to AMPs?

Cross-resistance is a common trait of antibiotic-resistant bacteria (Levin et al., 2000; Cafiso et al., 2010). Cross-resistant strains emerge either due to selection from diverse antibiotics in antibiotic-rich environments such as hospitals or farms, or because the physiological mechanisms conferring resistance to one antibiotic are effective in resisting another. The idea that AMPs and antibiotics exert different selective pressures is being gradually eroded (Perron et al., 2006; Pränting et al., 2008; Habets and Brockhurst, 2012), so the notion that AMP-cross-resistance could occur is as credible as for conventional antibiotics. The processes that could select for cross-resistance to AMPs are analogous to the same processes with antibiotics: resistant strains may emerge in AMP-rich environments (i.e. hosts), or the range of mechanisms that confer resistance to one AMP may exert broad effects (Bell and Gouyon, 2003; Peschel and Sahl, 2006). Cross-resistance to AMPs is plausible functionally, because the structures of AMPs are highly conserved (Zasloff, 2002) and the repertoire of known host resistance mechanisms is somewhat limited (Peschel and Sahl, 2006; Gruenheid and Le Moual, 2012). Since AMPs have been proposed as a novel class of antibiotics (Hancock, 2000; Zasloff, 2002; Reddy et al., 2004), it is important for public and environmental health to determine whether AMP resistance confers cross-resistance in a similar fashion to antibiotic cross-resistance, due to the risk of AMP therapy selecting strains that are highly resistant to AMPs in natural settings. The most alarming potential consequence of AMP therapy is selection of bacteria that are better able to survive an immune response (Buckling and Brockhurst, 2005).

3.2.3. Existing evidence of broad-spectrum AMP resistance

Despite close to a decade's interest in the notion of universal AMP resistance, there has been surprisingly little experimental work that has specifically focused on the issue. Older studies showed that cross-AMP-resistance tended to be limited to structurally similar or closely related molecules (Bell and Gouyon, 2003). A recent paper showed that *Staphylococcus aureus* selected to live in high concentrations of pexiganan – one of the magainin group of AMPs isolated from the frog *Xenopus laevis*, and the only AMP currently available commercially – can also show elevated resistance to the human defensin HNP-1, although this effect was not universal across replicate lines (Habets and Brockhurst, 2012). This was the first study to show that resistance is not necessarily confined to molecules within a given class, and is therefore circumstantial evidence that molecular mechanisms of specific AMP resistance can be co-opted for defence against AMPs to which the resistant bacteria is naïve.

The only study so far that has shown an advantage of AMP resistance in a living host model is Pränting et al. (2008), in which one of four *Salmonella enterica* mutants resistant to PR-39 (a porcine AMP) survived better than the wild-type strain with which it was co-injected into a mouse. This is an interesting result, but the limitations of working with vertebrate models mean that the experiment is replicated only once in one individual host, and it is possible that host-mediated effects led to the greater prevalence of resistant mutants in the mouse. Consequently there is ample scope for a broad-reaching study of the effects of AMP resistance on bacterial persistence in a host, and the pathology associated with infection by AMP-resistant bacteria.

3.2.4. Potential outcomes of infection by AMP-resistant bacteria

There are three basic outcomes in terms of *in vivo* persistence ("persistence outcomes") for bacteria with a history of exposure to AMPs

upon inoculation into a host: the infection can either be more susceptible to the immune system, less susceptible, or remain unchanged relative to ancestral populations (Figure 3.2.3.1). However these three outcomes are products of distinct ecological processes, depending on whether previous exposure to AMPs selected for resistance in the population or merely stressed it (Figure 3.2.3.1). It is important to define the differences between exposure to AMPs; stress, i.e. plastic physiological responses to exposure to AMPs leading to sub-optimal cellular function; and selection for resistance, i.e. exposure leading to heritable changes in traits that allow the bacteria to effectively cope with AMPs in the environment.

If bacteria do not become resistant to AMPs after exposure, then the persistence outcome should be the same as the ancestor's. It is also possible that bacteria could become more recalcitrant after infection without prior AMP resistance evolution if mutations emerge that are neutral during the selection process, but are serendipitously beneficial after inoculation into a host, such as increased growth rate or antigenic modification. This latter process is a backdrop to all the potential persistence outcomes that could arise after exposure to AMPs.

Once they are introduced to a host, bacteria that have evolved costly AMP resistance are much more interesting than those that were simply stressed by exposure during the selection protocol, because the costs and benefits of resistance will be amplified in the face of an immune response. Benefits will be amplified because *in vitro* AMP resistance may confer systemic immune resistance as predicted in the literature. Costs will be amplified because an antibacterial immune response is a stressful environment for bacteria, in which cells bearing the greatest net benefit after selection are likely to be the most competitive. If the benefits of *in vitro* AMP resistance are outweighed by their cost *in vivo* then an infection will be more susceptible to host immunity, and *vice-versa*. It is also possible that the costs and benefits of AMP resistance are balanced, or that the costs of *in vitro* resistance are mitigated by

compensatory mutations, in which case there will be no interaction between *in vitro* and *in vivo* resistance.

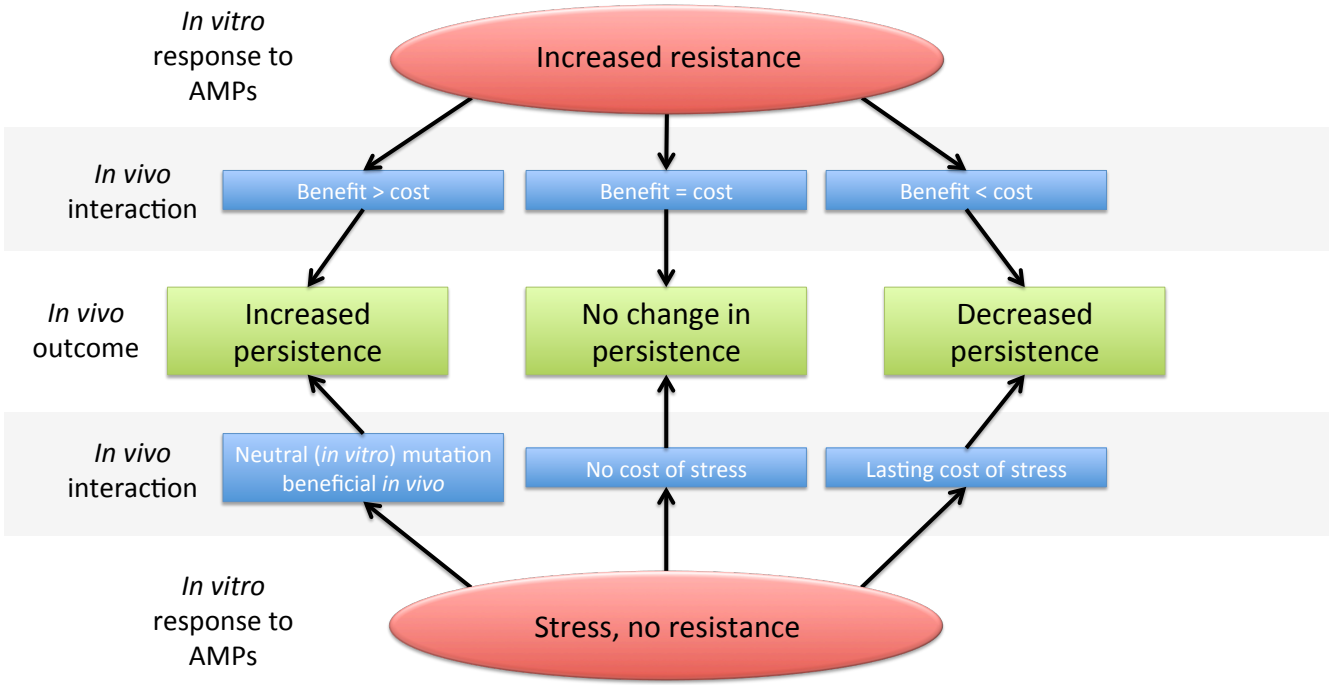


Figure 3.2.3.1. Potential interactions between responses to *in vitro* exposure to antimicrobial peptides and inoculation into hosts

Bacteria serially exposed to antimicrobial peptides (AMPs) *in vitro* can either evolve AMP resistance, or be stressed but evolve no resistance (red ovals). When confronted with AMPs as part of a hosts' immune response, the persistence of formerly AMP-exposed strains can be changed relative to that of their ancestor (green boxes). The direction of this change will be determined by the cost: benefit ratio of resistance for AMP-resistant strains, or the heritable and epigenetic costs and benefits of stress and random mutation for AMP-susceptible strains.

3.2.5. Goals of the present study

Experiments described in Chapter 2 generated AMP-resistant bacteria with controlled phylogeny and common ancestor. This material presents a unique opportunity to compare the effects of AMP resistance on both the fitness of the resistant bacteria in hosts, and the fitness of hosts infected by the resistant bacteria. The remainder of this chapter describes a series of experiments that compare the persistence and pathogenicity of AMP-selected, antibiotic-selected and unselected *S. aureus* derived from the selection experiment in Chapter 2. Complementary to the benefits of using parasites with a known and controlled evolutionary history, these strains are ideal for studies of direct clinical relevance since *S. aureus* is an opportunistic pathogen, and the antibiotic (tetracycline) resistance label universal to these cultures facilitates controlled recovery of the bacteria from a model host.

The mealworm beetle *Tenebrio molitor* is a convenient and powerful host organism for post-infection survival and bacterial persistence experiments (Haine et al., 2008a; Pursall and Rolff, 2011). The ease with which several hundred insects can be individually managed means that many individuals can be simultaneously treated, as required for meaningful survival studies. Additionally, insects gain a distinct advantage over vertebrates as models for survival experiments from their legal status as lab animals in the UK, which means that it is legal to keep infected individuals alive for indeterminate periods after infection. *T. molitor*'s large body size and architecturally simple haemocoel mean it is also fairly straightforward to quantitatively measure infection over time. In this context it has already been used as a vehicle to study persistence of *S. aureus*. It has been suggested that induced AMPs are a major component of *T. molitor*'s immune response to *S. aureus* (Haine et al., 2008a), making investigation of the interaction between AMP resistance, pathology and persistence even more attractive in this combination of host and parasite.

The benefits of long-term *in vivo* persistence for an opportunistic pathogen like *S. aureus* are obvious. The fitness costs of pathology are similarly obvious for a host, and of course host fitness equals zero when it is killed by a parasite. A case can also be made that induction of host morbidity is adaptive for *S. aureus*. Pathogenicity (often confusingly referred to as 'virulence' in the literature, discussed by Alizon et al. (2009)) is thought to be adaptive for a parasite if it aids transmission (Anderson and May, 1982; but see Ebert and Bull, 2003). *S. aureus* cells are not motile and do not sporulate, and so they can only be transmitted by breaching the host's dermal tissues, which can be achieved by killing and putrefying the host. The ability to kill a host can consequently be considered a fitness metric for sub-cutaneous *S. aureus* infection, as can – more obviously – the ability to survive a host's immune response. Host mortality can therefore be considered a measure of positive microbial fitness in this system, as well as of negative fitness in the host. Therefore, understanding the temporal dynamics of infection in relation to host mortality creates a clear picture of the fitness of both host and parasite, and the dynamic interplay between these two antagonists.

The potential for elevated immuno-resistance and pathogenicity of AMP-resistant strains are addressed by two experiments described in the remainder of in this chapter. The first measured persistence of AMP-resistant *S. aureus* (ARSA) in *T. molitor* up to one week after inoculation, as a measure of the ability of ARSA to survive host immunity. In the second experiment, survival of ARSA-infected *T. molitor* was observed, which is taken as a measure of the pathology caused by infection with these cultures. These experiments were controlled using beetles infected with antibiotic-selected or unselected control cultures from the previous selection experiment, and with the ancestor of all the selected bacteria. Therefore the phylogeny of the infection, the historical intensity of selection, and the potential for random evolution of the infectious bacteria are all controlled in these experiments.

3.3. Materials and methods

3.3.1. *T. molitor* culture

Final-instar *Tenebrio molitor* were purchased from a commercial supplier (www.livefoods.co.uk), and incubated in rat chow (Harlan Laboratories, UK) and apple in an insectary at 26^{o}C ±1 in a 12:12 light/dark cycle. Females were sexed as pupae and kept individually in rat chow. Eclosion was recorded daily. All beetles were virgins when treated.

3.3.2. *S. aureus* preparation, storage & culture

S. aureus were revived from glycerol stocks (preparation described in Chapter 2.3) by partially thawing one of the two aliquots until enough had thawed to pipette 100 µl of the glycerol stock. This volume was inoculated into 5ml LB (Sigma L2897-1KG) containing 5 µg ml^{-1} tetracycline (Sigma T7660-5G) and 5.6µg ml^{-1} amphotericin-B (Sigma A9528) and grown at 30^{o}C for 24 hours, shaking continuously at 100 rpm.

A 500µl aliquot of each 24-hour culture was pelleted by 5 minutes centrifugation at 4500g. Pellets of bacteria within each treatment were then pooled by communal resuspension in 1 ml Tryptic Soy Broth (Sigma T8907) with glycerol (80% water: 20% glycerol v/v), and frozen at -90^{o}C. Thus a frozen, pooled stock was prepared from each treatment in Chapter 2.

Vancomycin- and pgml-selected cultures went extinct at day 14 during selection (see Chapter 2.4), so unselected bacteria from day 14 were also pooled as an unselected control for these selection lines. Pexiganan-selected bacteria from day 14 were also pooled as a selected control. Descriptions of pooled bacteria are summarised in Table 3.3.1.

Table 3.3.1 – Bacterial populations pooled for *T. molitor* infection

Culture (treatment/timepoint)	Purpose
Ancestor, day 0	Pre-selection control
Iseganan, day 28	AMP-selected
Melittin, day 28	AMP-selected
Pexiganan, day 28	AMP-selected
Unselected, day 28	Unselected day 28 control
Streptomycin, day 28	Antibiotic-selected
pgml, day 14	AMP combination-selected
Vancomycin, day 14	Antibiotic-selected
Pexiganan, day 14	Selected day 14 control
Unselected, day 14	Unselected day 14 control

Prior to inoculation into *T. molitor*, pooled bacterial populations were revived from glycerol stocks by stabbing a sterile pipette tip approx. 2mm into the surface of the frozen stock, and dropping this tip into LB containing tetracycline and amphotericin-B (5 μg ml^{-1} and 5.6 μg ml^{-1} respectively). This approach was chosen in favour of streaking the glycerol stock onto agar and selecting one colony to preserve diversity within the culture, which can be important in resistance to bacterial stress (Lee et al., 2010). After inoculation, cultures were grown for 24 hours at 30 ^{o}C, shaking continuously at 100rpm. 24-hour cultures were pelleted by centrifugation at 4500g for 5 minutes, then washed and re-suspended in an equal volume of sterile PBS (NaCl 150mM, Na_2HPO_4 10mM, pH 6.5).

50μl of each inoculated culture (diluted x 10^{-5}) was plated on with 20 sterile glass beads on LB 1.5% agar containing tetracycline and amphotericin-B, to check for contamination and to quantify density of colony forming units (CFU) in the inocula. Plates were incubated for 24 hours at 37^{o}C, and photographed on a MicroPublisher 3.3 RTV camera (QImaging, Burnaby, BC, Canada). CFUs were automatically counted using OpenCFU (available at www.sourceforge.net, designed by Quentin Geissmann, University of Sheffield). The estimated CFU densities of the injected inocula are presented in Appendix 7.

3.3.3. *T. molitor* inoculation

Injections were performed by first swabbing the ventral surface of the abdomen with 80% EtOH, and loading a fine-pulled glass electrode (Narishige) with 5μl of the inoculum. A small hole was pierced between the third and forth abdominal sternites with a sterile pin. Inocula were then injected into the haemocoel by pushing the electrode through this hole (Figure 3.3.1) and pneumatically pressurising the sample in the electrode.

Figure 3.3.1. Inoculating *T. molitor* with a pulled glass electrode. Photo by Richard Naylor

T. molitor were injected with *S. aureus* or sterile PBS by loading a glass electrode with 5 µl of liquid and injecting it through a hole pierced between the third and fourth abdominal sternites.

3.3.4. Survival study - Monitoring mortality of infected *T. molitor*

Females were treated eight days after eclosion (n=50 per treatment), and mortality of each individual beetle was checked at least every two days. Sterile PBS was injected into controls as a procedural control. Mortality was recorded when beetles ceased to move and would not respond to mechanical stimulus. These checks were carried out up to 30 days post-infection.

An accelerated failure time (AFT) model was fitted to the data using a Weibull distribution (see Appendix 6 for model selection).

3.3.5. Persistence of selected cultures in *T. molitor*

3.3.5.1. Persistence study - Recovering inoculated *S. aureus* by perfusion bleeding

Beetles used in this experiment were between seven and ten days old when injected. Bacteria were recovered using a 'perfusion bleed' assay, refined from that of Haine et al. (2008a). See see Appendix 2 for details of the refinement of this technique..

Subject *T. molitor* were washed for 10 seconds in 80% EtOH. Genitalia were everted by gently squeezing the abdomen (Figure 3.3.2a), swabbed with 80% EtOH, and a small incision was made with a scalpel (Figure 3.3.2b). A 30 mm, 30-gauge needle was inserted through the plural membrane exposed between the abdomen and thorax on the side of the body laterally opposite to the genital incision, and pushed into the abdominal haemocoel (Figure 3.3.3a & b). 500µl sterile PBS was pushed through the needle, washing haemolymph out of the abdomen via the genital incision into a sterile collection tube (Figure 3.3.3c).

Thirty beetles were assigned to each bacterial treatment. Prior to inoculation, these beetles were randomly assigned to be bled thirty

minutes, twenty-four hours, or seven days after infection, to capture the full dynamic range of what is known to date about *T. molitor* antimicrobial immunity (Haine et al., 2008b). Beetles in some treatments died between infection and bleeding, so not all timepoints have a full complement of ten datapoints, particularly seven days after infection. Unfortunate external issues surrounding lab space meant that not all experiments could be completed. Consequently the dataset for beetles infected with iseganan-selected bacteria is not complete. As inoculations had to be completed on different days, fifteen beetles were additionally injected with sterile PBS on every day when bacteria were injected as a procedural sterility control (see Appendix 2). Five of these PBS-injected beetles were bled in parallel to infected beetles every day that bleeds were undertaken.

50µl of each perfused haemolymph sample was plated with 20 sterile glass beads onto a 950mm LB agar (1.5%) plate, containing tetracycline and amphotericin-B (as before). If beetles were bled 30 minutes after infection, perfused haemolymph samples were first diluted 10-fold and 100-fold, and each dilution was plated. The most conservative colony forming unit (CFU) estimate from plating these two dilutions was used in subsequent analyses. Plates were incubated and colonies counted as described in 3.3.2.

3.3.5.2. Persistence study – data analysis

The effect of bleed date on CFU was first analysed to test for any systemic contamination specific to a particular day, including data from beetles injected with PBS. These data were highly zero-inflated, so a zero-hurdle model using a binomial error distribution with a logit link was used.

Two subsequent analyses were conducted to assess the interaction of AMP resistance and persistence in *T. molitor*, after excluding the PBS-treated beetles. First, the reduction of CFU estimates over time by

treatment was modelled. Secondly, notable greater variation in CFU estimates 24 hours post-infection motivated specific analysis of these data.

Non-linear least-squares regression was used to fit a negative exponential function of the form

$$\log(\boldsymbol{CFU\ estimate}) \sim \boldsymbol{a} + \boldsymbol{b} * \log(\boldsymbol{time})$$

to CFU data over time as linear models on log-log scales. The decay rates of the fitted functions (***b***) are a measure of the ability of inocula to persist in the host.

24-hour CFU data were reanalysed separately from the rest of the data. A generalized linear model with a quasipoisson error distribution was fitted to the data, with treatment as a predictor variable. Initially inoculated CFU and date of treatment were non-significant factors of the maximal model and were excluded. To simplify the model, treatment groups showing significant differences from the intercept were grouped one-by one, with the end result that all ARSA strains (i.e. significantly different from the intercept) with the exception of pexiganan day 28 were grouped into one term of the model (ARSA). This process generated one group of equal statistical difference from the other inputs to the model.

Figure 3.3.2. Preparing *T. molitor* for perfusion bleeding. Photos by Richard Naylor.

Beetles were prepared for perfusion bleeding by everting the genitalia and make a small incision on its side

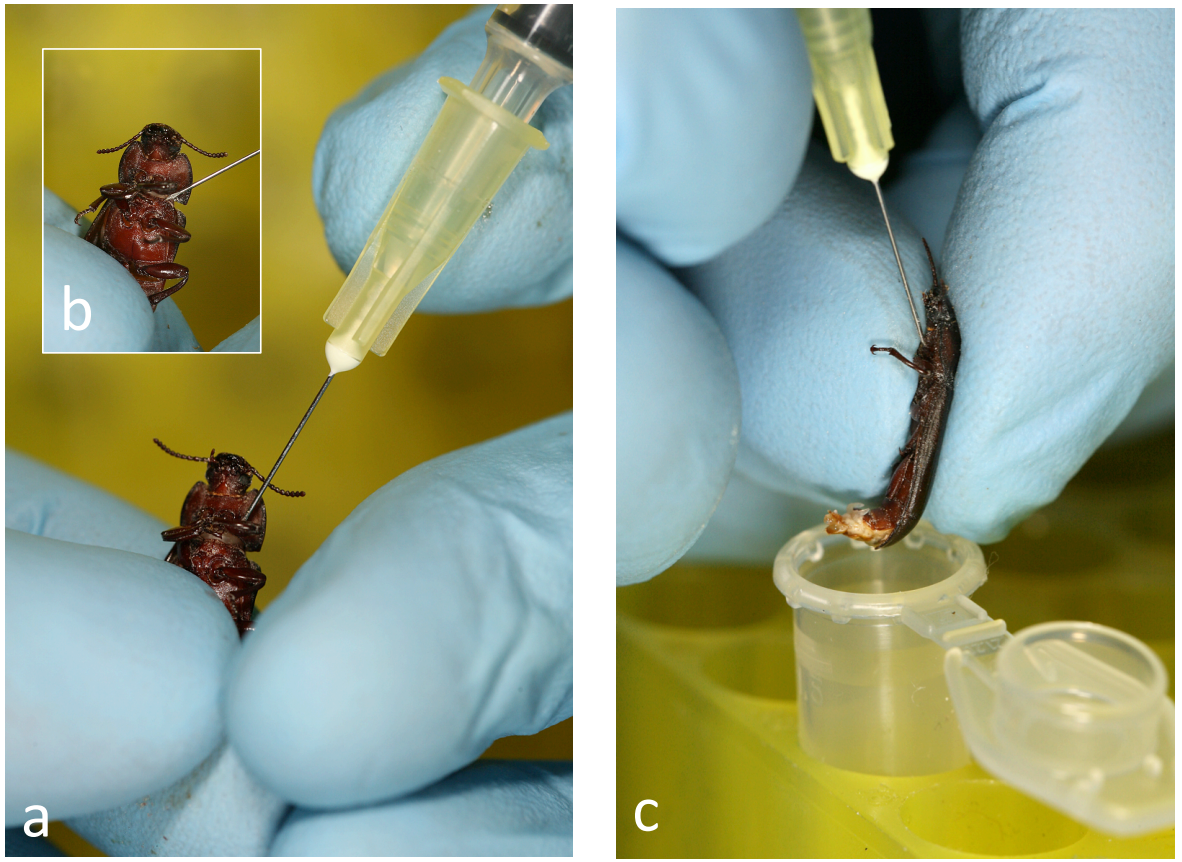


Figure 3.3.3. Perfusion bleeding *T. molitor.* Photos by Richard Naylor.

Needles were inserted into plural membrane exposed between the abdomen and thorax (a, b) and used to flush 500 µl sterile PBS through the haemocoel, removing infectious bacteria. Perfusions escaped via an incision on the beetles' posteriors and were collected in sterile centrifuge tubes, prior to plating on agar to quantify densities of viable bacteria in the haemocoel.

3.4 Results

3.4.1. Survival of *S. aureus*-infected *T. molitor*

There were strong effects of treatment group on beetle survival (Figure 3.4.1.1). Relative to the ancestral (day 0) control bacteria, pexiganan-selected (day 28), streptomycin-selected (day 28), and unselected (day 28) bacteria were significantly more pathogenic (Table 3.4.1.1.) Beetles inoculated with iseganan-selected (day 28), pexiganan-selected (day 28), and pgml-selected (day 14) *S. aureus* all died significantly slower than ancestor-infected control beetles. Survival of beetles inoculated with unselected (day 14), melittin-selected (day 14), vancomycin-selected (day 14) *S. aureus* or PBS did not significantly deviate from that of ancestor-treated beetles.

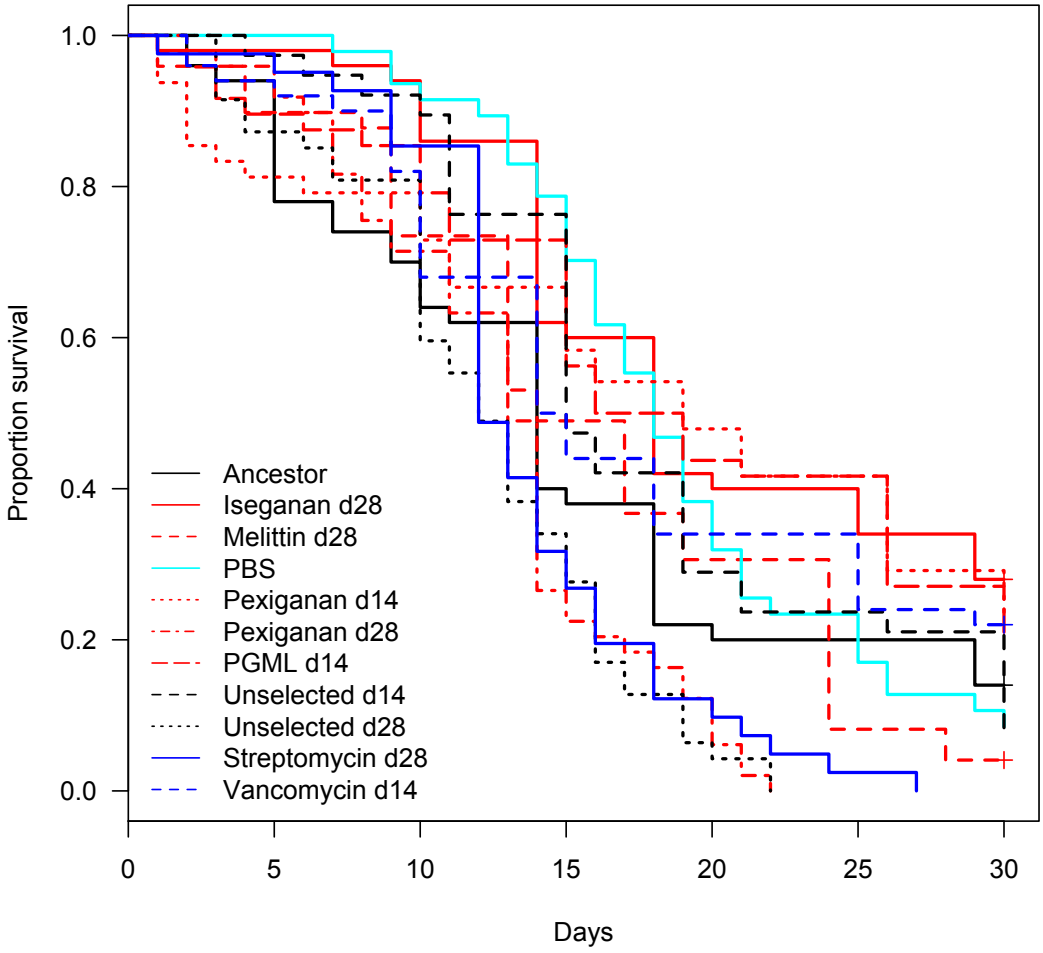


Figure 3.4.1.1. Post-infection survival of *T. molitor* infected with selected and unselected *S. aureus*

T. molitor were infected on day 0 with *S. aureus* with a history of exposure to a range of antimicrobial peptides and antibiotics, and the ancestor of these strains. Survival of beetles within each treatment group was subsequently monitored up to 30 days after infection.

Table 3.4.1.1. Survival of T. molitor infected with selected bacteria: Accelerated failure time model (Weibull distribution). Significant differences from ancestral bacteria highlighted in blue (better survival) and red (worse survival)

Treatment	*Coefficient*	*Standard error*	*Wald statistic*	*p*
Intercept (Ancestor)	2.9330	0.0754	38.91	<0.0001
Iseganan (d28)	0.3054	0.1115	2.74	0.0062
Pexiganan (d14)	0.2438	0.1105	2.21	0.0273
pgml (d14)	0.2328	0.1097	2.12	0.0339
Vancomycin (d14)	0.1634	0.1089	1.50	0.1337
Melittin (d28)	-0.0386	0.1039	-0.37	0.7102
PBS	0.0988	0.1063	0.93	0.3528
Unselected (d14)	0.0774	0.1121	0.69	0.4902
Pexiganan (d28)	-0.3203	0.1029	-3.11	0.0019
Unselected (d28)	-0.3565	0.1039	-3.43	0.0006
Streptomycin (d28)	-0.2591	0.1075	-2.41	0.0159

3.4.2. Persistence of selected bacteria in *T. molitor*

Bacterial treatment affected CFU recovered from infected beetles. Log-10 CFU data are presented in Figure 3.4.2.1.

There was some bacterial growth from samples derived from PBS-injected beetles. This was centred on perfusion bleeds conducted one particular day. However there was no significant effect of day on CFU in infected individuals (Zero hurdle model, binomial error distribution with logit link, $p>0.05$), so PBS data were discounted from subsequent analyses.

There is some variation between treatments in decay rates of CFU over time (summarised in Figure 3.4.2.2). However, in common with the results from the survival study, this does not map directly onto the evolutionary history of the inocula. CFU of streptomycin-selected *S. aureus* has the flattest response to time, and its confidence interval does not overlap with that of the ancestor, suggesting it has evolved to be a more persistent parasite than its ancestor. The difference between streptomycin-selected cultures and other cultures grown up to 28 days suggests that this is an effect of selection for streptomycin resistance rather than a function of time in serial culture during selection (Chapter 2). By contrast, persistence of vancomycin-selected *S. aureus* has apparently not evolved at all relative to the ancestor. CFU of both unselected and AMP-selected *S. aureus* (at both days 14 and 28) evolved moderately slower decay rates in the host than the ancestor, but this change was not significant. Decay rate of the vancomycin-selected culture was indistinguishable from the ancestor. The fitted models are plotted in Appendix 3.

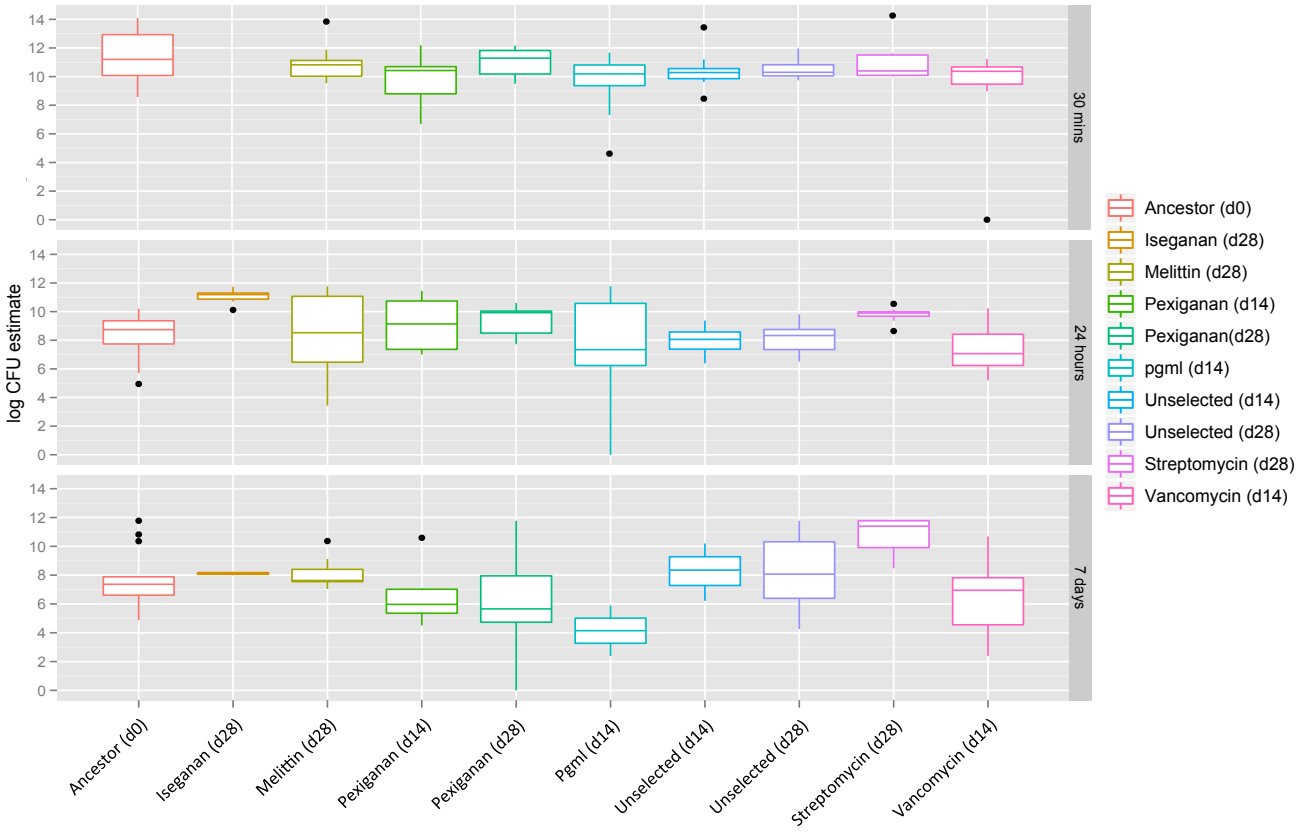
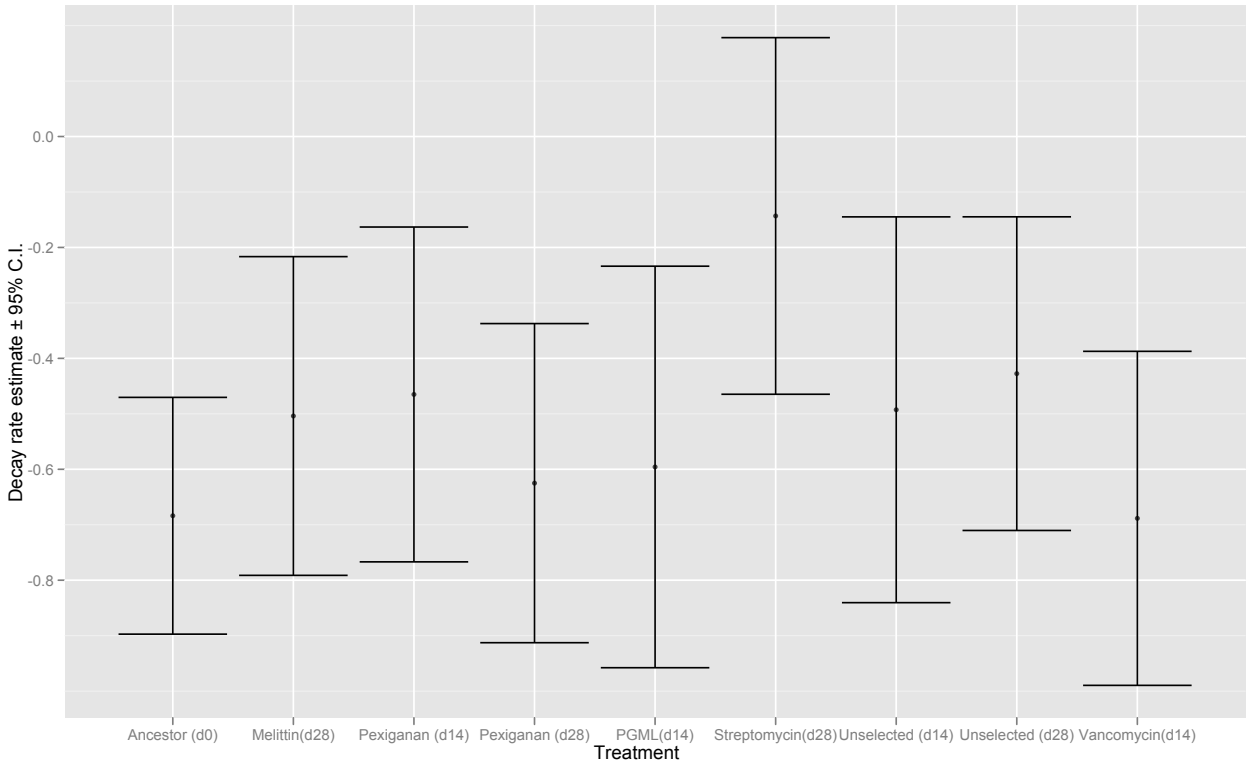


Figure 3.4.2.1. Persistence of selected *S. aureus* in *T. molitor* by treatment and time.

T. molitor were infected on day 0 with *S. aureus* with a history of exposure to a range of antimicrobial peptides and antibiotics, and the ancestor of these strains. These bacteria were recovered 30 minutes, 24 hours and 7 days post-infection and plated on agar to quantify the densities of viable cells (CFU) *in vivo*. Top and bottom error bars show the 95th and 5th percentile, respectively.



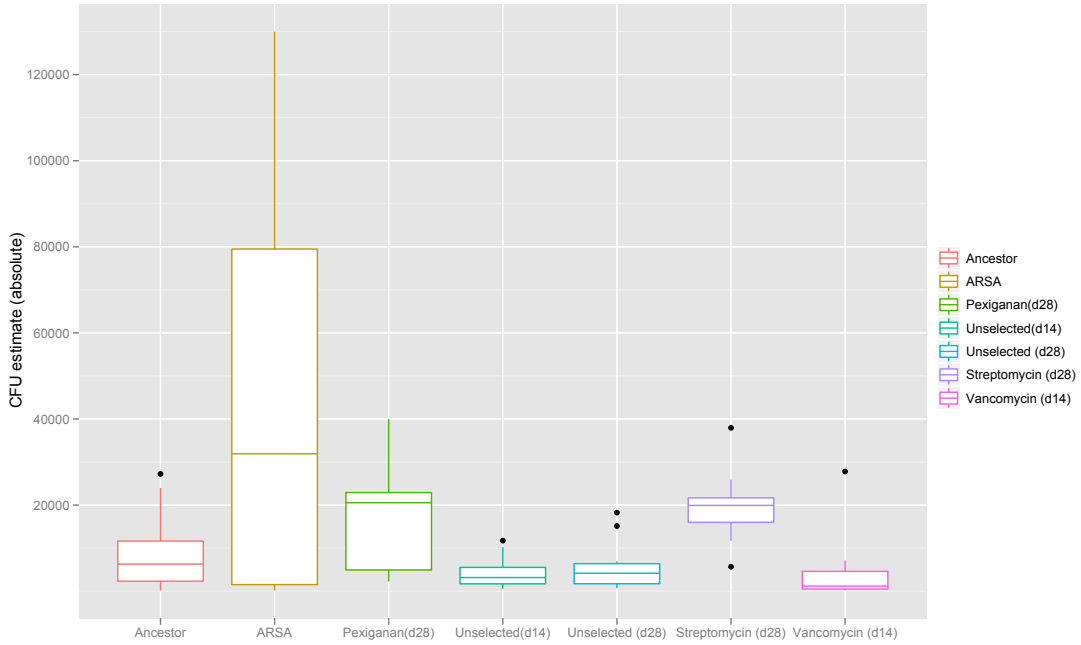
Figures 3.4.2.2. Decay rates of log CFU estimates (selected S. aureus) in *T. molitor*, ±95% confidence intervals

T. molitor were infected on day 0 with *S. aureus* with a history of exposure to a range of antimicrobial peptides and antibiotics, and the ancestor of these strains. These bacteria were recovered 30 minutes, 24 hours and 7 days post-infection and plated on agar to quantify the densities of viable cells (CFU) *in vivo*. Negative exponential functions were fitted to the data to estimate the rate of decline of each bacterial treatment in the host. These rates are plotted with 95% confidence intervals to show the variation in the ability of these strains to survive host immunity.

3.4.2.3. Comparison of 24h infection titers

Although persistence of infection over seven days was the main trait of interest, Figure 3.4.2.1 suggested that an interesting and somewhat distinct phenomenon was occurring 24 hours post-infection, and that AMP-selected bacteria were surviving at a greater density than antibiotic-selected or unselected cultures, contrary to the prediction that such differences would manifest most in the later phases of a persistent infection.

With the exception of pexiganan-selected (day 28) *S. aureus*, all AMP-selected cultures survived to a higher density 24-hours post infection than other cultures, independent of CFU 30 minutes or seven days after infection (Generalised Linear Model with quasipoisson family, $t=4.258$, $df=100$, $p<0.001$) There was also a marginal effect of streptomycin resistance ($p=0.08$). The data are presented in Figure 3.4.2.4 as they were entered into this simplified model.



Figures 3.4.2.4. Persistence of grouped AMP-resistant *S. aureus* (ARSA) and other selected cultures 24 hours post-infection

T. molitor were infected with *S. aureus* with a history of exposure to a range of antimicrobial peptides and antibiotics, and the ancestor of these strains. All antimicrobial peptide-resistant *S. aureus* (ARSA) - with the exception of those exposed to pexiganan for 28 days - showed significantly better survival in the host than other cultures 24 hours post-infection. ARSA data are grouped to reflect statistical model simplifications. Top and bottom error bars show the 95th and 5th percentile, respectively.

3.5. Discussion

This experiment was designed to test if AMP resistance is beneficial or deleterious in a host. Understanding this interaction is informative in either direction: either the rarity of AMP resistance (Chapter 2) can be explained by costs of evolving AMP resistance outweighing benefit in terms of survival in a host; or AMP-resistant bacteria persist better than non-resistant populations, and therefore using AMPs as drugs could select for more pathogenic bacteria. These data show that the relationships between *S. aureus* resistance to specific compounds, *in vivo* persistence and pathogenicity are complicated. This complexity is likely to be enhanced by the experimental evolution approach used to raise resistance in the inocula, which provides opportunity for random evolution of traits that would be neutral in the *in vitro* selection protocol, but advantageous in a host. There are, however, some striking trends in the data, despite the potential for confounding variation. I will first discuss the *in vivo* density of ARSA 24 hours post-infection, followed by the pathogenicity and long-term persistence of these strains in the host.

Barring cultures that were exposed to pexiganan for 28 days, all *S. aureus* that were exposed to AMPs in the selection protocol survived 24 hours in *T. molitor* much better than other cultures (Figure 3.4.2.4). This is the first evidence from a living host organism that providing scope for bacteria to evolve resistance to AMPs 'arms the enemy' with resistance to host immunity (Bell and Gouyon, 2003; Buckling and Brockhurst, 2005), and a stark and compelling warning of dangers associated with 'AMP therapy'. Furthermore, it directly complements recent work showing that pexiganan resistance can lead to *in vitro* cross-resistance (Habets and Brockhurst, 2012).

The implications of the interaction between a history of exposure to AMPs and 24-hour persistence are manifold, in terms of both our functional understanding of immunity and the biomedical implications of 'AMP therapy'. Regarding immunity first, the interaction between AMP

resistance and persistence corroborates previous evidence that AMPs are genuinely antimicrobial in immune responses (Andreu and Rivas, 1998; Bulet et al., 2004; Charroux and Royet, 2010), contrary to the proposition in the introduction to Chapter 2. This effect is only apparent 24 hours after infection, which is coincident with a temporal peak in expression of *T. molitor* AMPs (P. Johnston & J. Rolff, in prep). This is the one of the few organisms, if not the only organism, in which the evolutionary consequences of the temporal dynamics of immunity are being studied (Haine et al., 2008b). Understanding the timing of immunity in this organism could be an important first step in understanding how temporal dynamics of immunity regulate micro- and macro-evolution of bacterial parasites. The data presented in this chapter suggest that AMPs may well be very important in these processes (Haine et al., 2008a).

There are clear differences between the pathogenicity of the selected bacteria, although the trends in these data are less clear with respect to AMP resistance. The elevated pathogenicity (relative to the ancestral culture) of 28-day streptomycin-selected, pexiganan-selected and unselected bacteria suggests that pathogenicity in this experiment is to some extent a function of time in serial culture. However this effect was not mirrored in cultures that had been selected for melittin and iseganan resistance for 28 days, thus indicating that other processes are at work. Perhaps *in vitro* AMP resistance in these lines is not beneficial *in vivo*, or associated trade-offs negatively regulate pathogenicity.

The results of the beetle survival experiment are particularly surprising with respect to the pexiganan-selected (day 28) culture, because the parent cultures performed poorly during selection (Chapter 2), and showed no signs of persisting better than the other AMP-selected cultures either in terms of decay rate or absolute CFU: rather, this was the only AMP-selected culture to *not* persist significantly better than non-AMP-resistant cultures 24 hours after infection, although it showed more variation in seven-day CFU than any other treatment. It may be that the

level of *in vitro* resistance or tolerance these cultures evolved is optimal in the host beyond but not before seven days after infection. Perhaps these cultures evolved traits during the selection protocol that aid in long-term *in vivo* survival but not *in vitro.* For example, a low metabolic rate and an ability to evade the immune system in tissues would allow persistent cells to maintain a low level of latent infection until *in vivo* conditions became permissive for re-growth. The fact that mortality of *T. molitor* infected with these pexiganan-selected bacteria occurred mostly after day seven is consistent with such an infection dynamic. Unfortunately it is impossible to quantify such processes over a longer time frame because bacteria killing the host would lead to a self-selecting sample of hosts without a resurgent infection.

It is also remarkable that streptomycin-selected *S. aureus* were so much fitter by all metrics than cultures exposed to other treatments, and were more pathogenic than most treatments. This strain clearly persists better than any other over the first week after infection, significantly more so than the ancestor (Figure 3.4.2.3). This result defies all expectations from the start of the experiment, as streptomycin was included as a 'soft-selection' control in the selection protocol. Nevertheless, *S. aureus* exposed to this antibiotic showed the quickest evolution of dose-response in Chapter 2, and the most stable dose-response curve subsequently, i.e. evolutionary innovation was rapid, and there was ample time thereafter for refinement. As mentioned in Chapter 2, these cultures may have had a head-start in evolving resistance to their stressor: the ancestor uses a rapid efflux pump to resist tetracycline (A. Williams & S. Foster, Pers Comm; Hillen and Berens, 1994), which suggests that these cultures might have co-opted and developed their ancestral tetracycline resistance machinery for streptomycin resistance in a way that cultures exposed to other stressors could not. Likewise, it is eminently conceivable that these cultures' mechanisms of antibiotic resistance endow them with a degree of resistance to innate immunity, although a recent assay of these streptomycin-selected populations did

not detect any cross resistance to AMPs *in vitro* (J. Purves, Pers. Comm.).

Whatever the mechanisms underpinning the pathogenesis of streptomycin- and pexiganan-selected cultures may be, these results should be a stark warning to biomedical scientists with designs on developing components of innate immune responses as novel therapeutic antimicrobials (Hancock, 2000; Zasloff, 2002). Supporting earlier studies (Torella et al., 2010), I demonstrate that the evolutionary processes governing interactions between *in vitro* and *in vivo* microbial resistance are complex and unpredictable. My experiments show a marked increase in the pathogenicity of *S. aureus* that were selected for resistance to a supposedly benign antibiotic (streptomycin), allowed to drift in evolutionary space for around 2000 generations, or selected for resistance to a supposed wonder-drug (pexiganan); despite the low fitness of the latter *in vitro*. Moreover, the pathogenesis of lines grown in pexiganan for 28 days powerfully supports the hypothesis that using AMPs as drugs may 'arm the enemy' (Bell and Gouyon, 2003; Buckling and Brockhurst, 2005), and demonstrates that *in vitro* experiments should not be considered a reliable predictor of what will happen in dramatically different and variable *in vivo* conditions.

In contrast to the pathogenic and unpredictable pexiganan- and streptomycin-selected cultures, the pathogenicity of other selection lines scaled well with their performance during the preceding real-time evolution experiment (Chapter 2). Vancomycin-selected cultures showed little response to selection and, correspondingly, there were no differences between vancomycin-selected cultures and the ancestor in *T. molitor*. It is difficult to account for why there should be no difference between survival of beetles infected with melittin-selected bacteria and the ancestor, apart from to suggest that the benefits of melittin resistance outweigh its costs *in vitro*, but that the sum is closer to zero *in vivo*. Also, the results of the AFT model suggest that there was no difference between the sterile procedural control (PBS) and pathogenic

ancestral bacterial culture, which is cause for minor concern, but the data (Figure 3.4.1.1) suggest that this is likely an artefact of imperfections inherent in any statistical model: the proportion of beetles surviving after injection with PBS is consistently greater than that of infected individuals, and importantly it is around 0.2 greater than that of beetles inoculated with the ancestral bacteria, until around day 22 after treatment, when only 30% of the original PBS-treated group survive, followed by a reversal of the trend between the ancestor- and PBS-treated groups. Thus the AFT analysis is likely skewed by only 15 individuals, but unfortunately it is difficult to model these data more precisely.

S. aureus exposed to pexiganan for 14 days, iseganan for 28 days, or the 50:50 combination of pexiganan and melittin killed *T. molitor* significantly more slowly than their ancestor. This is an interesting result for several reasons. Crucially, it suggests that therapeutic use of AMPs as antibiotics does not always necessarily lead to the evolution of more pathogenic infection, although the reverse effect in the pexiganan-selected (day 28) cultures, discussed earlier, should strongly discourage any attempts to draw generalisations about AMP-mediated selection from these data. Once again, the disparity between *in vitro* and *in vivo* results may be explained by a different balance of costs and benefits of resistance in these environments. However, this does not appear to be the case for pgml-selected cultures. It seems that this combination treatment of bacteria in the selection protocol, which was intended to be a step closer to the natural environment, has blocked evolution of traits that would be favoured in both the *in vivo* or *in vitro* environment (with the caveat that there was some effect of treatment in terms of 24-hour survival in *T. molitor*, although this was the weakest significant effect), consistent with theoretical predictions (Chait and Craney, 2007; Fischbach, 2011). These cultures showed little response to exposure to a combination of AMPs measured by *in vitro* assays, nor did they report any benefit from this *in vitro* history when exposed to a natural panel of stressors *in vivo*. This further substantiates the conclusion reached in

Chapter 2, that the concurrent deployment of multiple AMPs during immune responses likely slows the evolution of infectious bacteria and constrains the evolution of AMP resistance. The environment of multiple AMPs, rather than selecting for generalist resistance with an additional associated cost of a slower response to selection, appears to have blocked selection for any traits that could be beneficial in either *in vitro* or *in vivo* environments, apart from, in these experiments, 24 hours after infection. Finally it is intriguing that cultures grown for 14 days in pexiganan should be less pathogenic than their 28-day counterparts, despite appearing to be somewhat more persistent in *T. molitor*. These disparities are strongly consistent with the suggestion that the *in vitro* strategy for cultures exposed to pexiganan in the selection protocol switched from resistance to tolerance around day 14, and that this has knock-on consequences for these strains *in vivo*, where they are opposite in every trait measured.

In conclusion, these data show that ARSA are not fundamentally weaker than antibiotic-selected or unselected *S. aureus* when injected into *T. molitor*. On the contrary, there is no systemic difference between AMP-selected and other cultures by survival and persistence metrics, which in conjunction with the observations of the evolution of these strains suggests that AMPs are not the 'wonder drugs' they were thought to be in an evolutionary theatre. The only systemic difference that could be detected between ARSA and other strains was that ARSA survived the first twenty-four hours after infection far better than other strains. Although this did not manifest in differences in persistence rates or host morbidity in this study, it demonstrate scope for these processes, since long-term persistence - and thereby pathology and morbidity – are functions of the early survival of an infection.

The biomedical implications of the interaction between historical exposure to AMPs and 24-hour persistence are fundamental if AMP therapy is to become a reality (Yeung et al., 2011). Microbial contributions to host pathology are likely to be determined by infectious

cells that survive initial immune responses and persist. Such persistence is a function of earlier bacterial densities, so there is more scope for damaging infection when the inoculum survives the initial few hours or days of the immune response. Although the experiments in the present study did not detect any differences between treatments in 7-day infection titers, late-infection persistence is a function of the early density of the infection. Therefore, the elevated ability of AMP-resistant bacteria to survive 24 hours in a host provides more scope for long-lasting, damaging infection, even if that was not detected in these experiments. Thus, these results run absolutely contrary to the notion that *"antimicrobial peptides have clear and obvious therapeutic potential"* (Hancock, 2000). On the contrary, *"large-scale use of chemotherapeutic AMPs may ultimately help pathogenic bacteria colonise…animals and plants that were previously off-limits to them"* (Buckling and Brockhurst, 2005), and medicinal use of AMPs could have important evolutionary effects on the evolution of pathogenic bacteria, with potentially dire consequences for patients.

Summary of Chapters 2 and 3

Chapters 2 & 3 demonstrate that there are evolutionary costs of being selected in vitro *for resistance to components of host immunity – specifically antimicrobial peptides (AMPs) – which manifest as weaker growth in pristine environments and specific adaptation to grow at high concentrations of these stressors. These costs are to some extent alleviated in a host animal, when exposure to the stressors against which resistance has been acquired facilitates better survival of the infection at certain times after inoculation. Furthermore, I have shown that an improved ability to survive in a host need not be related to specific selection from immune effectors, and that selection from other stressors can also improve persistence* in vivo. *Critically the data show that development of AMPs as clinical antibiotic drugs could select for more persistent pathogens.*

However, I have not shown any fundamental difference in the costs experienced by bacteria exposed to AMPs and those exposed to antibiotics. There must therefore be another reason that AMP resistance has not been commonly reported in environmental isolates of bacteria, and the data constitute the first experimental evidence that this phenomenon could be mediated by the multiplicity of AMPs that are simultaneously produced by infected hosts.

These first two Chapters show that resistance in an opportunistically pathogenic bacterium is regulated by benefits in some environments, which are offset by costs in others. In short, resistance may not always be the optimal strategy for a parasite.

Chapters 4 & 5 seek to develop the reciprocal of this framework. The core question motivating these Chapters is "why do hosts not always resist their parasites?" *Since the outcome of infection is determined by*

the balance of a parasite's evolutionary resistance to a host and the host's ecological resistance to a parasite, presumably there are endogenous factors that constrain a host's ability to clear parasitic infection. Chapter 4 seeks to measure the cost of an immunological resistance response to simulated microbial infection, by measuring a fitness cost of the immune challenge and demonstrating immunological self-harm to host tissue. Chapter 5 develops this framework by investigating the transcriptomic basis of immunological resistance, which shows that numerous mRNAs transcribed after an infection are predicted to have self-damaging secondary functions.

Chapter Four

Costs of immunological resistance: Could autopathology generate negative immunological feedbacks?

4.1. Abstract

Tolerance is quickly emerging as an important force in animal host and parasite evolution. Although tolerance has been recognised phenomenologically, very little is known about its causes or mechanisms. A case can be made that the optimal immunological strategy for an infected host shifts from resistance to tolerance over time after infection, because constitutive immunity is predicted to be self-harming (immunopathology), and cumulative costs of immunopathology will eventually outweigh the benefits of completely killing a dwindling infection. *Tenebrio molitor* infected with live or dead *Staphylococcus aureus* show no difference in survival, suggesting that endogenous pathology associated with the response to microbial immune elicitors is a major component of total pathology. Inoculating *T. molitor* with dead *S. aureus* also led to wanton haemocoelic melanin deposition, suggesting a link between this constitutive immune effector and self-damage. Since a host cannot continuously invest in traits that will eventually kill it, a link can be tentatively made between immunopathological constitutive defences and eventual tolerance.

4.2. Introduction

4.2.1. Pathology and persistent infection are the produce of microbial and host traits.

From the moment of association, the fates of an infected host and its infecting parasites are locked together (Little et al., 2010), and the fitness of each protagonist is dependent upon phenotypes expressed by both (Long and Graham, 2011). A host's "decision" to express a given immune effector of parasite resistance is determined by the benefit of expression relative to the endogenous costs, and the same logic holds for a parasite's expression of any costly "virulence factor" that aids survival in the host. Therefore, activity of individual immune effectors should be modulated as the marginal benefit of its use diminishes, for

example as parasite densities decrease. Consequently, the costs to a host of expressing a certain immune phenotype will have knock-on effects on parasites, because endogenous host costs form a negative feedback on the host's ability to invest in that trait. This cost: benefit of resistance framework forms the basis of classical ecological immunology theory (Sheldon and Verhulst, 1996; Rolff and Siva-Jothy, 2003). In the light of the recent recognition of parasite tolerance in animals as an alternative immunological strategy to resistance, I propose that the cost-benefit framework could generate negative feedbacks that will lead to host-parasite coexistence. These interdependencies generate a vast and varied array of potential feedbacks and outcomes, the exciting potential for which has only recently been recognised.

The causes and consequences of pathogen-driven persistence are well characterised (see Chapter 1). However it has recently been recognised that an infection not dying in animal hosts can be host-driven, i.e. the host tolerates the infection (Raberg et al., 2007). Theory suggests that tolerance is an important outcome, both in terms of short-term selection on individual hosts and parasites, and long-term host-parasite coevolution (Råberg et al., 2008; Little et al., 2010). However, to date it has been studied mainly on a phenomenological and theoretical level (e.g. Ayres and Schneider, 2008; Schneider and Ayres, 2008; Schneider and Chambers, 2008, Best et al., 2010; Long and Boots, 2011). Consequently, there is a pressing need to study the causes and mechanisms of tolerance. This chapter is concerned with understanding why parasite tolerance is induced.

There is an explicit but apparently unrecognised conceptual connection between tolerance and auto-reactivity caused by a host's immune system: "virulence" (total damage to a host) is defined as the sum of host self-damage and parasite-mediated damage (Long and Graham, 2011). Tolerance is defined as the inverse of virulence (Baucom and de Roode, 2010; Schneider, 2011), i.e. hosts remain healthy independent of parasite burden, and are isolated from damage associated with infection.

In other words, tolerance is coincident with low immunopathology. Individual hosts cannot modulate the costs of evolving an immune response because they are fixed, but they can modulate investment into resisting infection. By doing so they modulate the inducible costs of resistance, the negative effects of which are life-history trade-offs and immunopathology (Graham et al., 2010; Schmid-Hempel, 2011). There is therefore a strong conceptual connection between hosts avoiding the immunopathological costs of resistance, and the induction of tolerance.

Theory and empirical studies show that, when considered as life-long strategies, resistance and tolerance are negatively correlated (Raberg et al., 2007). Tolerance, resistance and virulence are not lasting states, and expression of immunity is modulated in response to infection. Consequently, rather than considering them as stable strategies throughout a host's lifetime, one must consider their dynamic relationship. Could the induction of resistance and tolerance also be negatively correlated over the lifespan of an individual host? I propose that tolerance should be dynamically induced after immunopathological resistance, because this process leads to increasing net cost and decreasing gain, particularly as parasite densities decrease. The mechanistic basis of this argument is that the collateral physiological and structural damage caused by resistance mechanisms negatively feeds back on a host’s ability to invest in resistance, and so less autotoxic immune effectors are deployed. In short, there is an upper limit on how long and how intensively a host can continue investing in a process that damages it. A ‘black-box’ evolutionary ecology argument - in terms of temporal patterns of investment and negative feedbacks - and a mechanistic biochemical argument of physiological damage and biochemical interactions are outlined separately below.

4.2.2. The ‘black box’ evolutionary ecology argument for immunopathology driving tolerance

Immunopathology should only be maintained when its benefits (fitness retained by parasite killing) outweigh its costs (collateral self-damage). One can therefore assume that autopathological immune responses have been tuned by natural selection to minimise the ratio of autopathology to parasite killing, i.e. to minimise the secondary cost of parasite resistance, and make the trade-off between these two traits as favourable as possible. One can consequently assume that immunopathology is proportional to parasite killing. The rate of investment into immunopathological resistance traits therefore ought to decrease as net immunopathology increases over time. At this point a host must employ less autoreactive immune effectors to prevent further growth of the infection. If the antiparasitic effect of an immune effector is positively related to its autoreactivity, then an initial rapid initial burst of autopathology should diminish over time, associated with an observed outcome eventually consistent with tolerance (Figure 4.2.1). Fitness loss is therefore minimised by trading future unpredictable pathogenic damage for current predictable immunopathological damage.

It might be argued that, rather than optimizing tolerance, immunopathology should promote more specific resistance responses. The counterargument is that, unfortunately for hosts, the most potent resistance responses are probably also autotoxic, because these attack the most fundamental features of parasites. In this case, immunopathology is a secondary effect of the requirement for potent resistance responses. It is therefore possible that resistance responses are universally autotoxic to some extent.

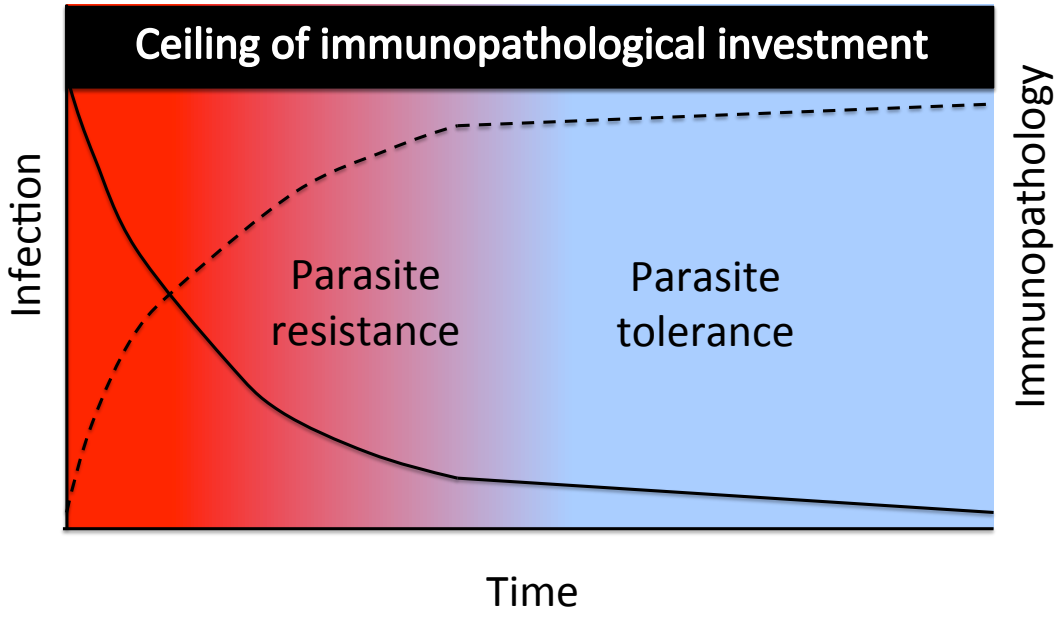


Fig 4.2.1. Shifting optimality of resistance and tolerance in response to immunopathology.

If activation of constitutive resistance responses causes proportional damage to host and parasite, then the host's ability to invest in resistance has an upper limit. Accumulation of immunopathological damage constrains rate of investment in immunopathological processes. Hosts approaching this limit must reduce investment in autotoxic responses, with a subsequent decline in rates of parasite killing. This leads to a continuous switch from parasite resistance to tolerance.

4.2.3. The biochemical argument for immunopathology as a cause of tolerance

The proposal that tolerance is a concession to avoiding immunopathology associated with resistance is predicated on the assumption that resistance should be more autopathological than tolerance. I propose that constitutive immune defences are by definition resistance responses, and that constitutive defences are selected for an ability to attack fundamental and conserved traits, with the secondary drawback of immunopathology.

As discussed in Chapter 1, constitutive defences are activated immediately upon detection of parasites. Such defences are therefore under strong selection for generalism in their efficacy, capable of killing or neutralising the phylogenetically diverse array of parasites that a host may encounter. As such, they are also under selection to be largely non-specific. Their effectiveness must also be stable over evolutionary time. Finally, constitutive defences must be rapid and potent in order to minimise colonization of the host by the parasite. I predict that selection for these functions favours constitutive immune effectors that attack highly-conserved physiological, cytological or histological traits. The downside of the generalist power of such effectors is that the conserved traits they attack are likely to be shared by both the parasites they attack and the hosts they protect. Consequently, there is an evolutionary-biochemical argument that immunopathology should be associated with constitutive immune defences when these defences are involved in resisting a parasite.

4.2.4 The putative biochemistry of tolerance

Our understanding of the mechanistic bases of tolerance is thus far weak (Baucom and de Roode, 2010). Arguably, the dual phenomena of a long-lasting induced humoral immune response and coincident persistent infection in *Tenebrio molitor* (Haine et al., 2008a) is the only

study which makes a link between a particular functional group of immune effectors (i.e. unidentified soluble antimicrobial compounds, possibly AMPs) and a persistent sepsis: these phenomena are also consistent with tolerance. A complementary result from a recent study is that an insect AMP is required for control and maintenance of a symbiotic bacterium (Haag et al., 2011; Login et al., 2011). These results are significant for the current discussion because AMPs should not be toxic to metazoa (see thesis introduction), and so their putative role in tolerance responses is consistent with induced tolerance as a less costly alternative to resistance. Interestingly, there is biochemical evidence from the moth *Manduca sexta* that an inducible defence (lysozyme) inhibits autotoxic catalysis of prophenoloxidase, suggesting selection for induced defences to dampen the action of constitutive defences (Rao et al., 2010).

4.2.5. Melanisation and immunopathology.

It can be argued that any organism with an un-compartmentalised body cavity is selected for particularly potent constitutive defences. This is because any infection has the potential to become rapidly systemic, rather than being spatially confined by physical structures. This *Bauplan* also means that the autopathological effects of immune responses should manifest at an organismal level as toxins and reactive molecules are circulated around all tissues. As discussed in Chapter 1, melanin and the biochemical cascades involved in its production have been frequently used as metrics of invertebrate immune function (Rolff and Siva-Jothy, 2003; Eleftherianos et al., 2007; 2007; Pauwels et al., 2011; Laughton et al., 2011b) but also see (Cerenius et al., 2008). The antimicrobial killing power of melanisation is thought to be mediated by the production of highly toxic intermediates such as oxidized phenols, quinones and reactive oxygen species, anoxia induced by melanin deposition on cell surfaces (Nappi and Ottaviani, 2000; Zhao et al., 2007), and externalising phagocytosed microbes by coating aggregated nodules of phagocytes in sclerotized melanic tissue. These cascades

are strong candidates as immune effectors with autopathological side-effects, because these processes do not discriminate between prokaryotic and eukaryotic cells and are undertaken in the presence of both within the haemocoel (Figure 4.2.2). Furthermore, it ought to produce an easily measurable phenotype in the form of indiscriminate blackening of host tissues.

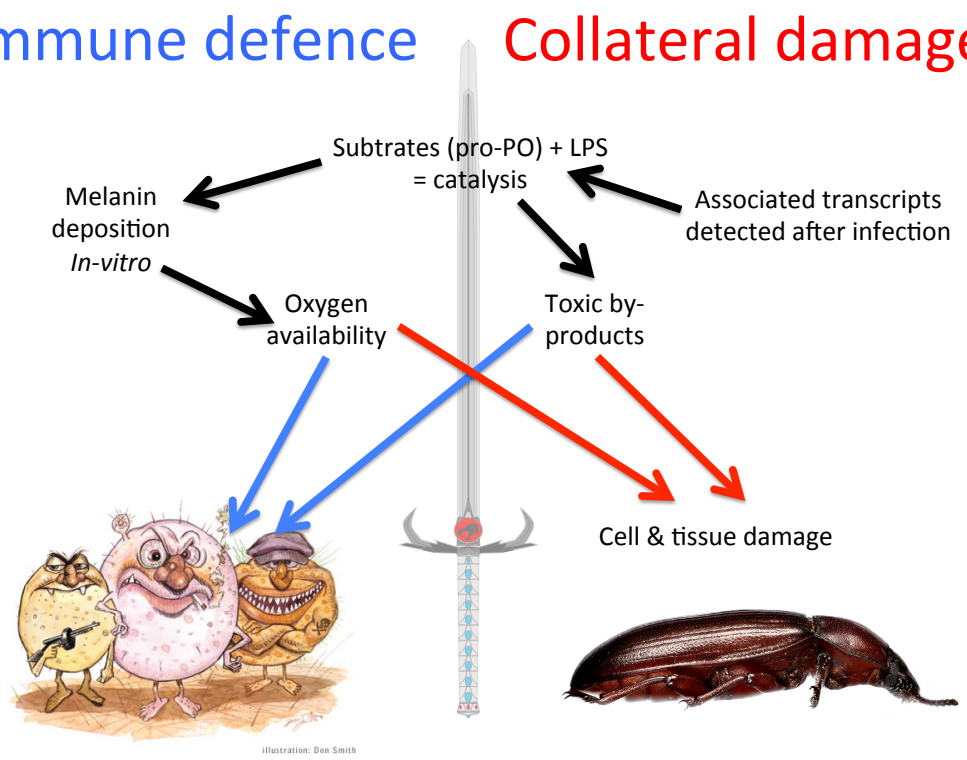


Figure 4.2.2. The potential of melanisation and melanin-producing cascades to act as an immunological "double-edged sword"

Activation of melanin producing-pathways in response to infection leads to wanton melanin deposition and release of toxic by-products. These combat infection but can circulate freely in the open haemocoel, secondarily damaging to host tissue.

With specific reference to the system in hand, there is some circumstantial evidence that melanisation is associated with self-harm in *Tenebrio*, although it has rarely been framed as such and explicit experiments are scarce. The most direct example (Sadd and Siva-Jothy, 2006) showed an association between melanisation of a nylon implant and decreased malpighian tubule function. Notably, this damage was not systemic, but that may have been because the nylon filament implant used to stimulate melanisation was spatially discrete. More recently, a strain-specific positive relationship has been demonstrated between *Tenebrio* mortality, PO activity and melanisation after infection with *Aeromonas hydrophilia* (Noonin et al., 2010), although it is impossible to form conclusions about causality in this case since there was no direct manipulation of PO. A more direct link between PO and fitness is given by a recent study showing an association between elevated larval PO activity stimulated by non-infectious material and reduced adult lifespan (Pursall, 2010; Pursall and Rolff, 2011), although these results are also correlative. The only experimental manipulation of *Tenebrio* melanisation activity to date showed that RNAi knockdown of a protein with a role in the negative regulation of melanisation was associated with rapid tissue melanisation and mortality (Zhao et al., 2005), but unfortunately this study did not address the interaction with pathogen resistance or tolerance. Armitage & Siva-Jothy (2005) showed that *T. molitor* selected for more melanic cuticle were both more immunocompetent and were better able to retain fitness after activation of melanin-producing pathways, suggesting variation in ability to cope with coincident self-damage after melanisation. Perhaps the most compelling evidence that melanisation plays a role in determining resistance outcomes comes from an organism in which PO and melanin-associated immunity is understudied: a single mutation in a signaling protease involved in *Drosophila*'s melanisation response had dramatic and microbe-specific effects on resistance and tolerance (Ayres and Schneider, 2008).

This chapter details a series of studies to test the hypothesis that immunopathology is a major cost of resistance responses. This is

intended to pave the way to understanding if autopathological immune processes drive the induction of tolerance. This is achieved by two experiments. The first aimed to measure what proportion of post-infection fitness loss (virulence) is driven by immunopathology, by monitoring survival of beetles inoculated with a range of dead or live bacterial (*S. aureus*) challenges. Doses were varied because immunopathology is predicted to be proportional to dose, so variation in virulence after infection with dead cellular material can be attributed to self-damage. Insects are ideal models to address these questions because their open haemocoel means each individual is effectively a model abscess, and so infection and immunity are manifested at an organismal level with measurable fitness consequences. The second experiment consisted of dissecting beetles that had been inoculated with dead *S. aureus* and looking for morphological evidence of self-harm, specifically melanisation.

4.3. Materials and methods

4.3.1. Quantifying the contribution of immunopathology to host mortality

A beetle survival study was performed to assess how much post-infection fitness can be attributed to *T. molitor*'s reaction to dead bacterial cellular material (i.e. endogenous damage only), and how much to live bacteria at the same dose. Beetles were prepared for these studies exactly as in Chapter 3, with the following differences:

1. Beetles were injected with bacteria grown from a single colony of *S. aureus* JLA513 (SH1000 background), grown in LB media with tetracycline at 5 μg ml^{-1} and amphotericin-B at 5.6 μg ml^{-1}. In the preliminary study, *S. aureus* were cultured for 48h to allow time for them to enter stationary phase and deplete their media of nutrients. Since any immunopathological effects on post-infection fitness are predicted to be dose-dependent, three concentrations were used. Concentrations were

varied by aliquoting and centrifuging (5 minutes at 4500 rpm) the starting culture, then resuspending the pellets in 75%, 100% or 125% of the starting volume of supernatant. An aliquot of the supernatant was also taken. The resuspended cultures were split again, and one aliquot of each was killed by heat shock (12 minutes at 65c in a water bath). This created six aliquots of bacteria (Table 4.3.1.): live-high dose (LH), live-medium dose (LM), live-low dose (LL), dead-high dose (DH), dead-medium dose (DM) and dead-low dose (DL). In addition beetles were treated with supernatant (SN) to control for the effect of toxins released into the growth medium by *S. aureus* during growth. It was thought necessary to suspend the pelleted bacteria in this growth medium because live *S. aureus* will produce extracellular toxins in the host but dead bacteria will not. Suspending bacteria in toxic supernatant makes this problem systemic, and any difference between the survival of bacteria injected with the same dose of live or dead bacteria can therefore be attributed to variation in toxins. The difference between beetles treated with bacterial material and beetles treated with supernatant can be attributed to the presence or absence of cellular material. The treatments for this first survival study are listed in table 4.3.1.

Table 4.3.1. *S. aureus* preparations inoculated into *T. molitor* before monitoring beetle survival

Abbreviation	*Description*	*CFU x 10^{-5}*
DH	Dead bacteria, high dose	550 (dead)
DM	Dead bacteria, medium dose	263 (dead)
DL	Dead bacteria, low dose	154 (dead)
LH	Live bacteria, high dose	550
LM	Live bacteria, medium dose	263
LL	Live bacteria, low dose	154
SN	Supernatant: *S. aureus* toxins	0
PBS	Sterile PBS: wounding control	n/a
C	Untreated controls	n/a

2. All beetles used in these experiments were sexed and individually isolated as pupae from long-term stocks at the University of Sheffield. All beetles were treated seven days after imaginal eclosion, and 25 individuals were used per treatment. Shortly after the experiment was completed it became apparent that this stock of beetles was infected with microsporidia, and it is possible that this infection was present in the beetles used in the survival study.

4.3.2. Assessment of self-melanisation after live and dead bacterial challenges

To assess beetle-driven blackening of self-tissues after inoculation with bacteria, 10 adult virgin *T. molitor* were injected with 5µl dead (heat-shocked by 12 minutes at 65c in a water bath) 24h culture of *S. aureus* JLA 513 (c. 10^7 cells) as described above and in Chapter 3. These cells were pelleted from washed and suspended in a volume of sterile PBS equal to the volume of growth medium the culture was grown in. The inviability of heat-killed bacteria was confirmed by spreading a suspension of these bacteria on LB agar (1.5%). 10 controls were injected with 5µl of sterile PBS.

Observations of melanisation were conducted by dissecting *Tenebrio* elytra and wings and entering the abdomen through the exposed plural membrane. These dissections were conducted 36-48h after infection. Beetles were preserved for up to a week in 100% absolute EtOH before dissection. Images were taken on a MicroPublisher 3.3 RTV camera (QImaging, Burnaby, BC, Canada) attached to either a Leica dissecting stereoscope or Leitz Diaplan compound microscope (Wetzlar, Hessen, Germany).

4.4. Results

4.4.1. Comparing survival of *T. molitor* inoculated with dead or living *S. aureus*

Survival curves of treated beetles are presented in Figure 5.4.1.1a. Beetles treated with live or dead *S. aureus* or *S. aureus* culture supernatant died quicker than untreated or PBS-treated controls over the first seven days after treatment (figure 5.4.1.1b). Over these first few days all beetles treated with *S. aureus* cellular material died at a greater rate than those treated with supernatant toxins, PBS, or untreated controls; apart from those given a low dose of dead cells. However, supernatant also negatively affected beetle survival.

A Cox's proportional hazards model was fitted to these data, which suggested an increase in mortality hazard (relative to untreated controls) of beetles treated with live or dead *S. aureus* cellular material (excluding a low dose of live cells), which is marginally not but significantly greater in magnitude than the risk of mortality of supernatant-treated beetles (Table 5.4.1, Figures 4.4.1.2. & 4.4.1.3). Hazard ratios from the model are presented in Figure 4.3.1.4.

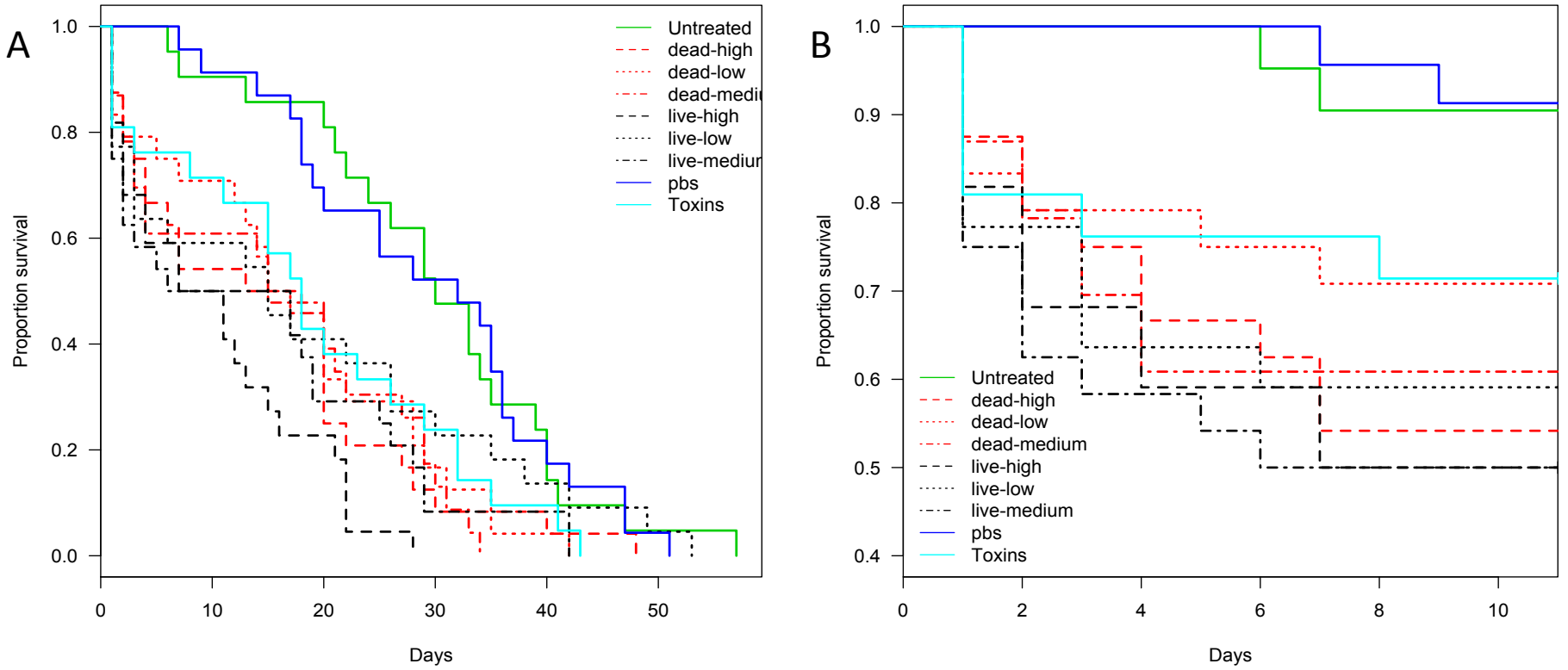


Figure 4.4.1.1. Survival of *T. molitor* infected with varied doses of dead and live *S. aureus*, showing (A) all data to day 60 and (B) the initial ten days after inoculation

T. molitor were injected with varied doses of dead and live *S. aureus* and their survival was monitored daily, to assess the relative contributions of microbe and host to virulence.

Table 4.4.1. Survival of *T. molitor* after inoculation with dead and live *S. aureus*: Cox's proportional hazards model output

Treatment	*Coefficient (Intercept = untreated controls)*	*Wald statistic*	*Z-statistic*	*Pr(>|z|)*
DH	0.8835	0.3061	2.89	0.0039
DL	0.8350	0.3065	2.72	0.0064
DM	0.9567	0.3117	3.07	0.0021
LH	1.5004	0.3215	4.67	<0.0001
LL	0.4617	0.3108	1.49	0.1375
LM	0.9749	0.3064	3.18	0.0015
PBS	0.0552	0.3067	0.18	0.8572
SN	0.6876	0.3148	2.18	0.0290

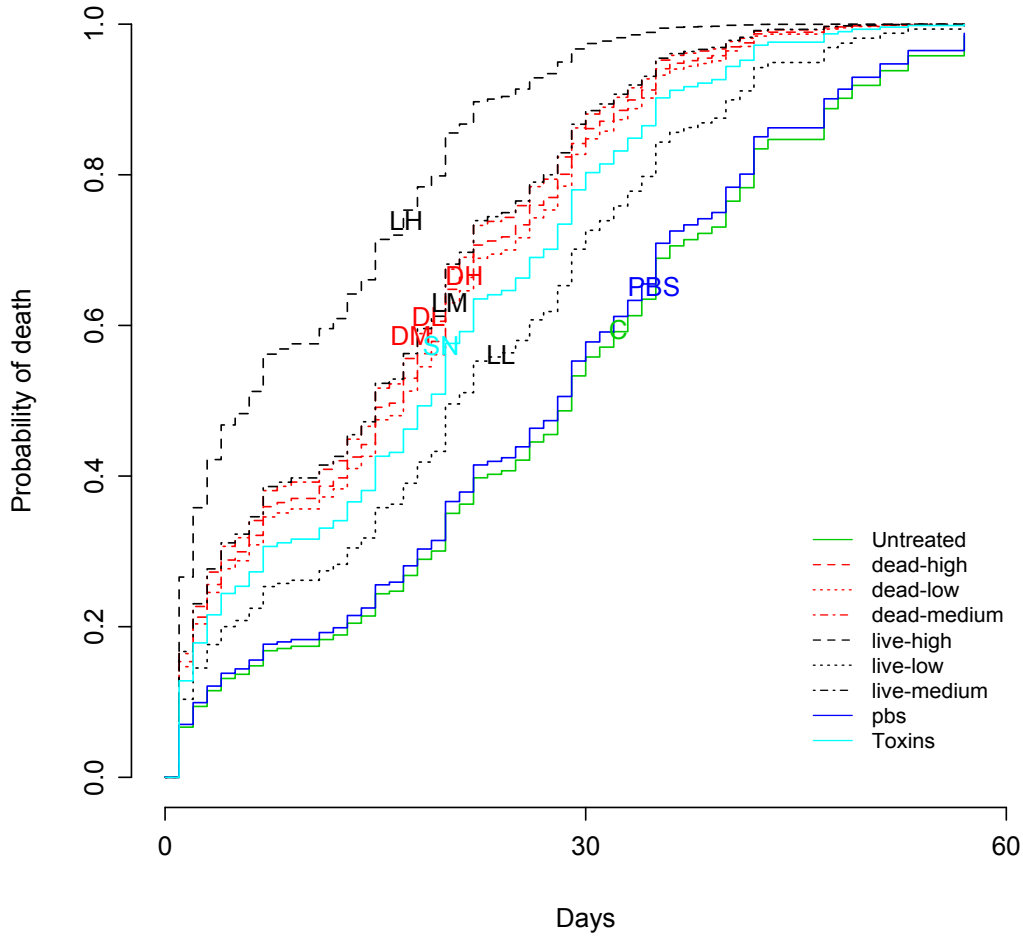


Figure 4.4.1.2. Survival study - mortality hazard functions (Cox's proportional hazards model)

T. molitor were inoculated with varied doses of dead (red) and live (black) *S. aureus*, to assess the relative contributions of host and microbial pathology to host survival. There was a greater but non-significant hazard of mortality for beetles injected with dead bacteria than those injected with the solvent in which the dead bacteria were suspended, suggesting that endogenous pathology negatively impacts on host survival. See Table 4.3.1 for treatment key.

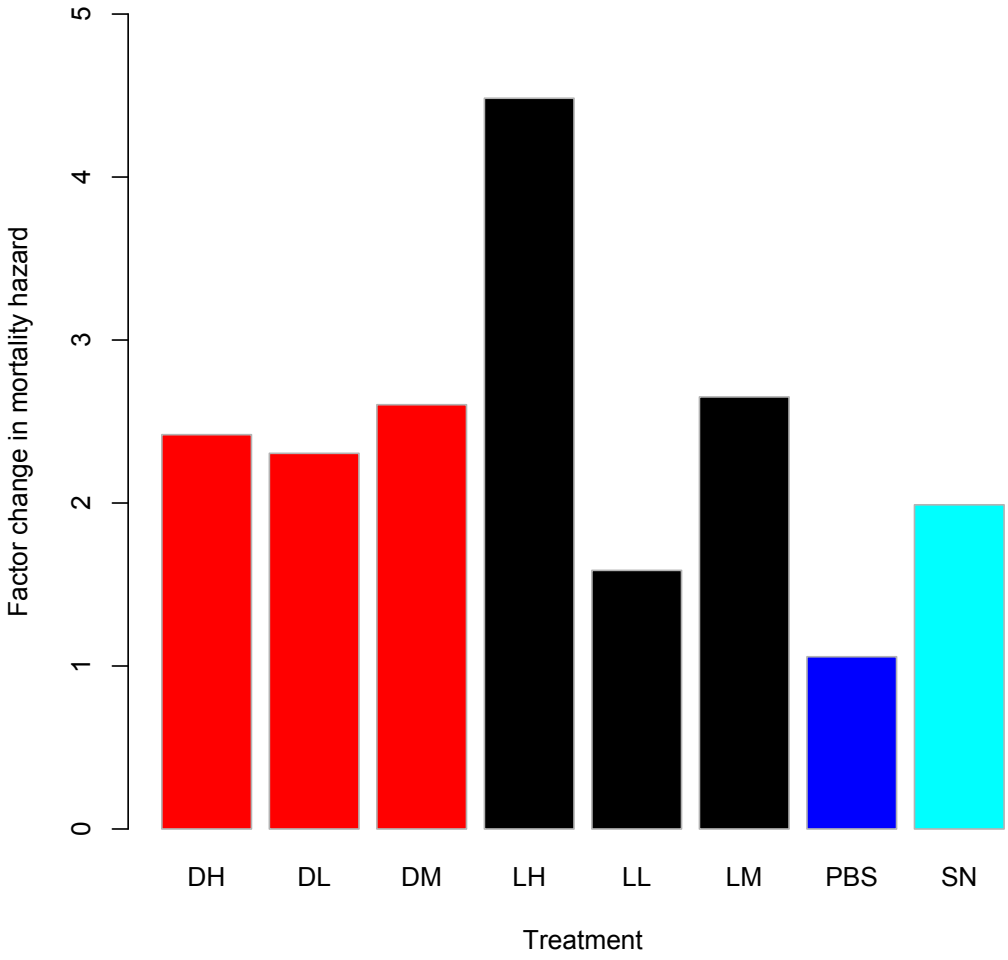


Figure 4.4.1.3. Factoral change in risk of mortality of T. molitor injected with dead (red) and live (black) bacterial challenges, and treatment controls (PBS and *S. aureus* toxins; dark and light blue respectively)

T. molitor were inoculated with varied doses of dead and live *S. aureus* and a Cox's proportional hazards model was fitted to the data. Exponentiating the coefficients of the model give the change in risk of mortality relative to the intercept (untreated controls). See Table 4.3.1 for treatment key.

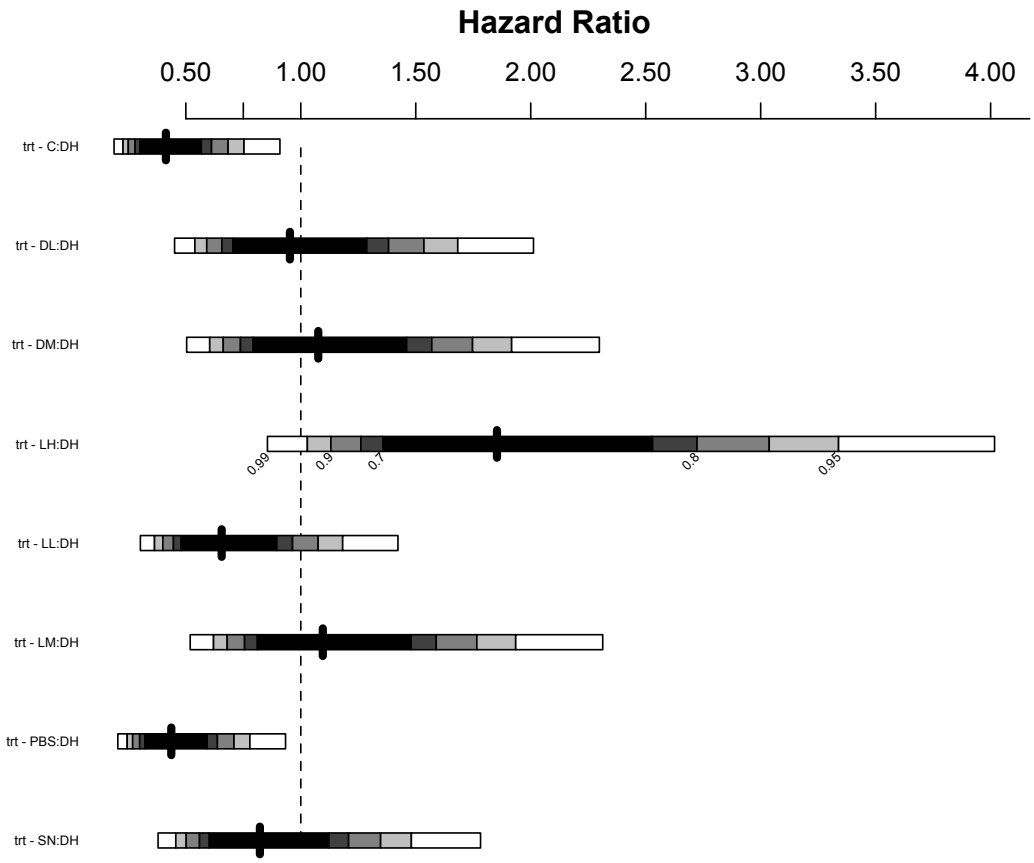


Figure 4.3.1.4. Hazard ratios of *T. molitor* infected with dead and live *S. aureus*.

T. molitor were injected with varied doses of dead and live *S. aureus* and a Cox's proportional hazards model was fit to the survival data. This figure shows the hazard coefficients and confidence intervals from the model. The dashed line represents the survival coefficient of dead/high (DH) dose-treated beetles. See Table 4.3.1 for treatment key.

4.4.2. Observations of self-melanisation after inoculation with dead and live bacteria

PBS-injected beetles had clear, white fat body tissue that showed no apparent discolouration (examples in Figure 4.4.2.1a) In contrast, beetles injected with cellular suspensions developed melanised nodules throughout the abdominal haemocoel (Figure 4.4.2.1b). There were no qualitative differences between beetles treated with either live or dead bacteria. Blackening of the dorsoventral area of the pleural membrane in the downstream region of the dorsal vessel was commonly observed. These observations demonstrate systemic self-melanisation after a simulated bacterial challenge.

Interestingly, fine dissection of tissue nodules and blackened membranes revealed that microsporidia-like cells were commonly associated with melanic deposits (Figure 4.4.2.4). These cells were not associated with melanic deposits in PBS-injected beetles (Figure 4.4.2.3).

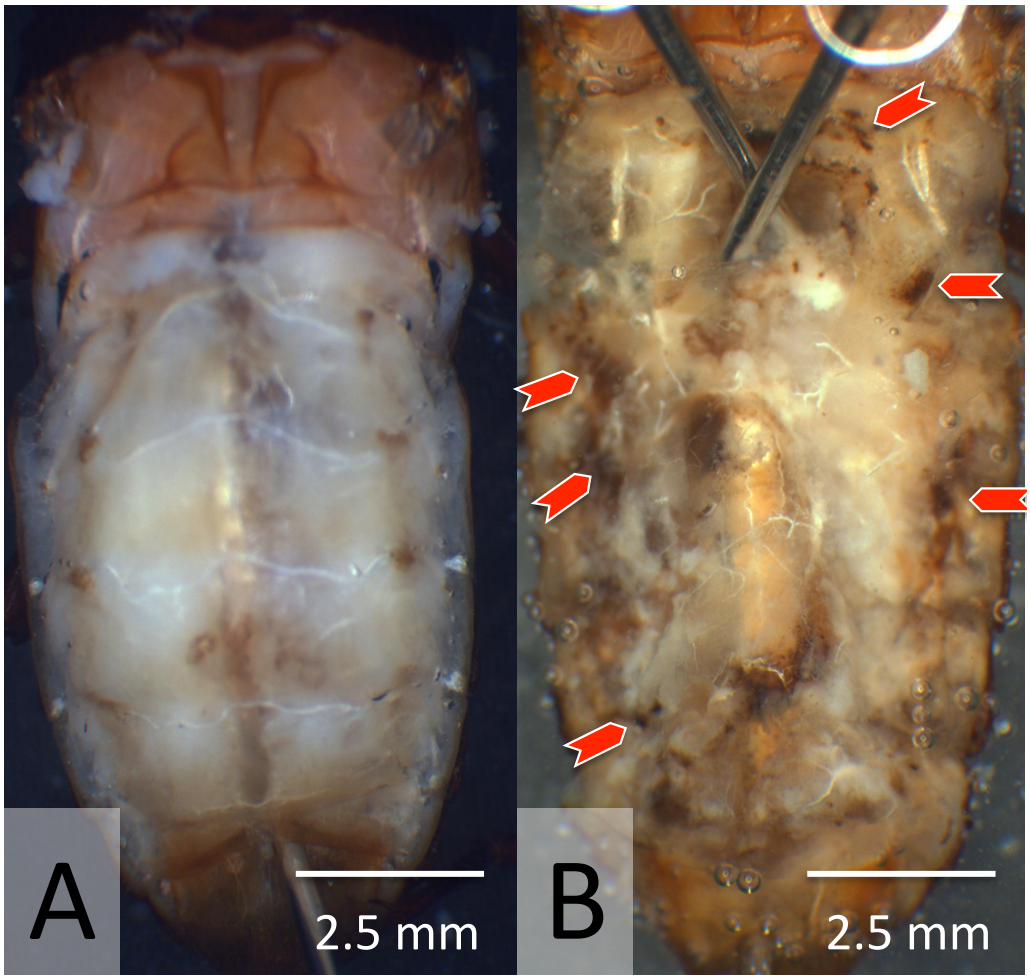


Figure 4.4.2.1. Exemplar dorsal views of *T. molitor* abdomens 72 hour after injection with sterile PBS (A) or dead *S. aureus* (B).

T. molitor were injected with dead bacteria to stimulate an immune response, and the abdomens of inoculated animals was compared to those of healthy individuals injected with sterile saline, after elytra and wings were removed. Injection of dead bacterial matter stimulates and immune response in the absence of prolonged pathology from the inoculum. The profound blackening and wasting of tissues shown in panel B can therefore be attributed to endogenous host processes.

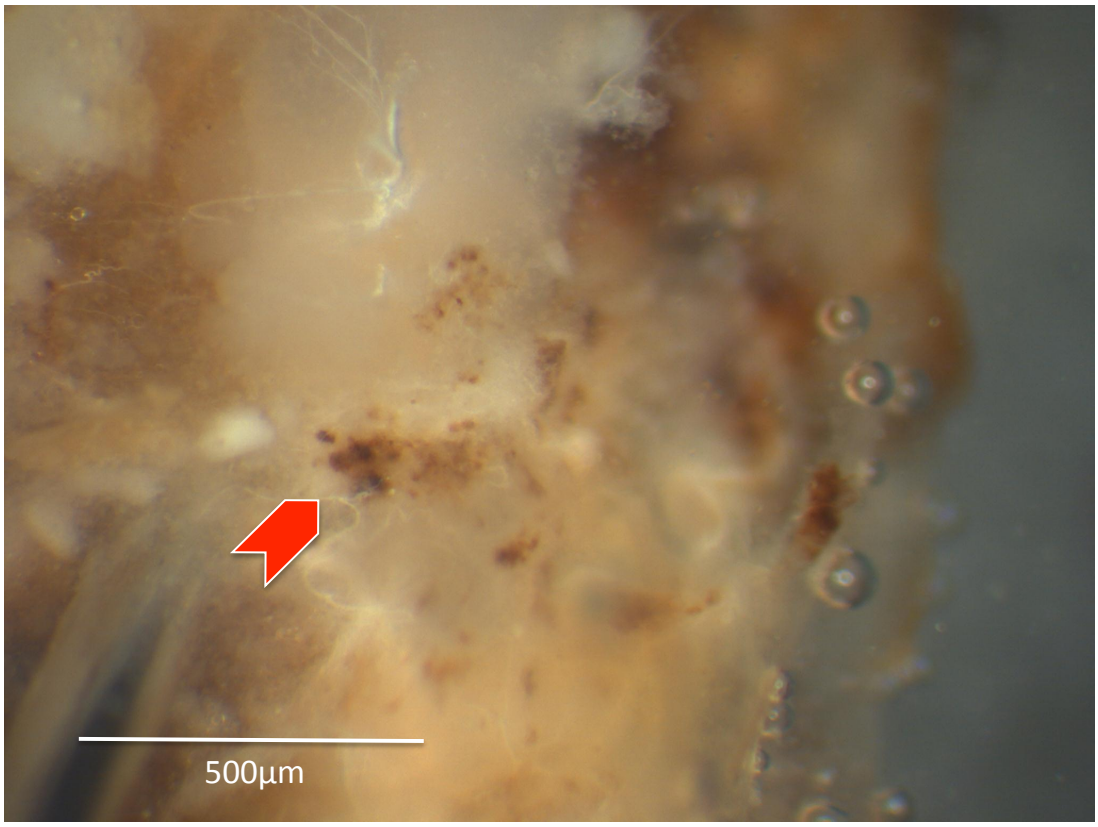


Figure 4.4.2.2. Dissected *T. molitor* fat body, 72 hours after infection with dead *S. aureus*.

T. molitor were injected with dead bacteria to stimulate an immune response in the absence of prolonged pathology from the inoculum. This micrograph shows dark melanised deposits on dissected fat body tissue, deposited by the host in response to injection of dead bacteria. Such deposits were found throughout the haemocoels of immune-challenged beetles.

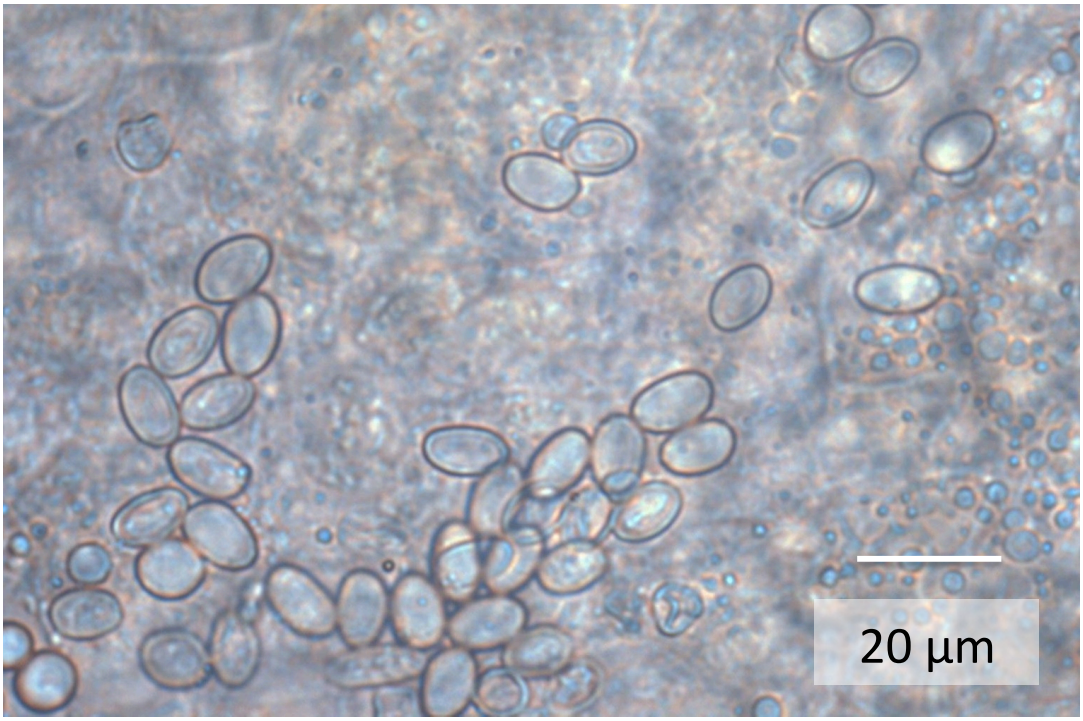


Figure 4.4.2.3. Phase-contrast micrograph, showing dissected microsporidia-like cells attached the inner surface of the pleural membrane of PBS-injected *T. molitor*

When microsporidia-like cells were found in *T. molitor* whose immune systems had not been activated by injection with dead bacterial matter, they were not associated with melanic deposits.

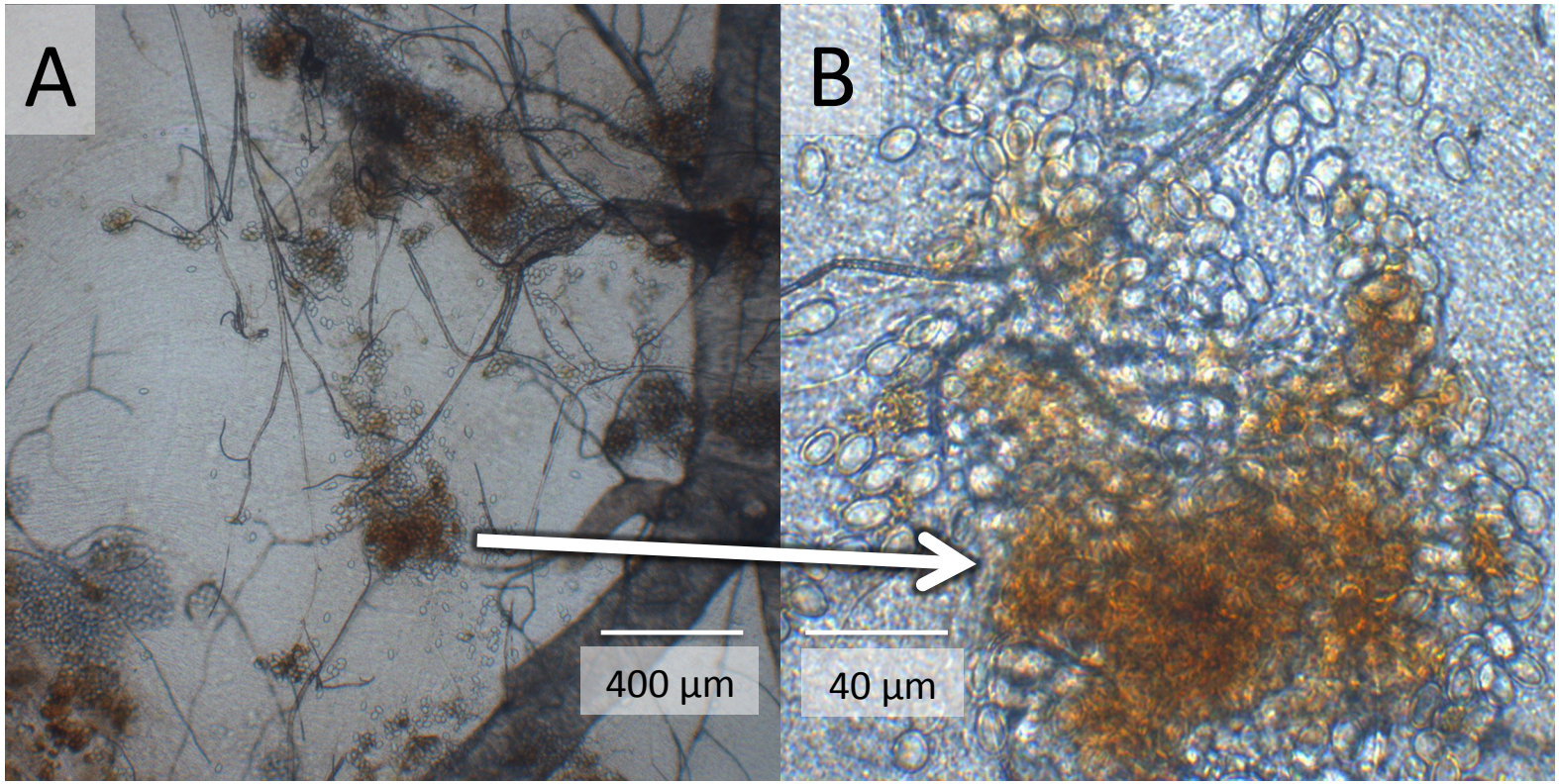


Figure 4.4.2.4. Melanic deposits on microsporidia-like cells from *T. molitor* infected with dead *S. aureus*, dissected from the inner surface of the abdominal plural membrane.

Melanin is associated with microsporidia-like cells in T. molitor only after the beetle is injected with dead bacterial matter: (A) Plan view of dissected melanic spots. (B) Phase-contrast micrograph, showing a close magnification of melanised microsporidia-like cells.

4.5. Discussion

The two experiments described were designed with the goal of assessing what proportion of virulence in *T. molitor* can be attributed to immunopathology, and whether there is morphological evidence of attacks on self-tissue after infection. Some inference can be drawn from the results, particularly in light of published literature about the functions of PO, melanin and immunopathology in a wider sense. However results from the survival study should be treated with care due to the potentially confounding effect of suspending bacterial preparations in culture supernatant. There are clear negative effects of being injected with supernatant, which may mask the fitness effects of inoculation with bacterial material.

Survival of beetles treated with dead or live *S. aureus* (Figure 4.4.1.1) ranks as predicted, with a consistently greater risk of mortality in beetles treated with bacterial cellular material (apart from treatment LL) than in beetles treated with toxins (Figure 4.4.1.3 & Table 4.4.1). Notably, Figure 4.4.1.1b suggests that the differences between groups arose mostly over the first week after treatment, consistent with the proposition that the initial phases of immune responses are responsible for fitness loss. However the differences between the groups treated with supernatant and cellular material were not significant, so the toxic effects of the supernatant solvent cannot be separated from effects caused by immunopathology. The lack of significance in these data is likely accentuated by the relatively small sample sizes (25 per group).

These observations suggest that a further study with a larger sample size and bacterial preparations suspended in a non-toxic solvent might yield stronger positive results, particularly if a different analytical approach were used to compartmentalize mortality over discrete periods of the survival curve. There would be particular value in focusing on the first ten days after inoculation, because this appears is when the majority of fitness is lost in beetles treated with bacterial cellular material,

because this is when constitutive defences are most active, and because this period is most relevant for fitness in the wild. Although I attempted such a study, uncontrollable factors led to the failure of that particular experiment (see Appendix 4), and it could not be repeated due to time constraints. However it is of course also possible that survival is not a suitable proxy for immunopathological damage – indeed the idea that investment in immunopathological effectors is condition-dependent to some extent predicts this – or that the hypothesis is simply void. The numerous autocorrelations between harmful processes during the initial days after inoculation (wounding, pathogen-derived toxicity, immunopathology, and potentially tolerance) mean that a successful iteration of this experiment should ideally incorporate mechanistic manipulations, e.g. RNAi knockdown of proPO or melanin-inhibitory protein, which should respectively restore or further compromise survival after inoculation with dead bacteria if immunopathology does indeed compromise survival.

The observation of systemic melanisation of the haemocoel tissues in response to inoculation with dead bacteria is important for two reasons. First, it provides a new piece of circumstantial evidence that melanin and sclerotisation are important antibacterial immune effectors in insects. This accords with a growing body of more quantitative experimental data demonstrating an important role in resistance responses (Zhao et al., 2007; Cerenius et al., 2008; Pauwels et al., 2011). If this is taken as granted, then the second implication of these results is that the observations of self-melanisation and blackened microsporidia suggest that constitutive defences have been selected for nondiscriminatory attacks on biological material: providing a link between a modulated and easily quantified defence effector, the benefits of antimicrobial defence, and the costs of immunopathological collateral damage (Sadd and Siva-Jothy, 2006). A worthwhile sequel to these qualitative observations would be to quantify accrual of melanised tissue over a week after inoculation with dead and live bacteria, and measuring the temporal dynamics of PO and proPO over the week after inoculation would be

informative. If auto-melanisation is self-damaging and necessitates investment in alternative routes to infection management, and then PO activity should be coincident with intensive infection, and should negatively correlate induced antimicrobial activity. proPO should be depleted over time, in order to cut off the supply of immunological tools with which the host could further damage itself.

Should the principles outlined in this chapter withstand further testing, then the dominant paradigm of invertebrate immunology would be refined. The recent recognition of tolerance in animal systems has expanded the conception of what animal immune systems can actually do, showing that they are subtler than just blunt instruments for the elimination of parasites. Clearly there are situations when it is more advantageous for a host to allow parasites to live in its tissues than to remove them, and I suggest that avoiding immunopathology is intrinsically linked to these situations. Parasites are by definition not cost-neutral, suggesting that they should only be tolerated if the costs of their removal are greater than those of their tolerance. Since many of the costs of immunity are fixed overheads (Schmid-Hempel, 2011), autopathological immune effectors are strong candidates for tipping the scales of cost and benefit sufficiently to alter the optimum immunological strategy from resistance to tolerance.

To summarise, hosts' constitutive defences will be selected for functions that will make them secondarily autotoxic. If immune responses and therefore their constituent effectors are graded on a continuum between resistance and tolerance responses, then we predict that resistance responses will be immunopathological, because resistance should be the optimal strategy when an infection is established, and constitutive defences are used to control the first phase of an infection. However the optimality of resistance will change as the cumulative costs of resistance reach a threshold value and parasite densities are diminished. At this point less autotoxic immune effectors should be transcribed, with the tradeoff that they are less potently antimicrobial. There are no published

studies on how immunological transcriptomes change over time after bacterial infection, but a shifting assemblage of immune effectors from autoreactive and nonspecifically toxic molecules to specific disruptors of microbial physiology would be consistent with a switch from resistance to tolerance.

Chapter Five

Identification of immuno-modulated expressed sequence tags in Tenebrio molitor *by suppressive subtractive hybridization*

5.1. Abstract

Tenebrio molitor is constrained as a study system by a lack of molecular sequence data to complement physiological studies of immunity. A deeper understanding of the mechanistic bases of *T. molitor* responses to *S. aureus* infection could support the principle that immunopathology is important in determining the dynamics of infection and host mortality in this organism. It would also be a boon for future researchers using this beetle as a study system, for example in studies of its long-lasting immunity. Consequently *T. molitor* were infected with *S. aureus*, and immunomodulated expressed sequence tags (ESTs) were cloned and sequenced by Suppressive Subtractive Hybridisation. The output sequence data were assigned Gene Ontologies using Blast2GO, to predict functions. The data suggest that infected *T. molitor* produce enzymes with potential to harm both microbial parasites and the host, and therefore that immunopathology could be a strong determinant of immunity in this system.

5.2. Introduction

5.2.1. Why sequence *T. molitor*?

Tenebrio molitor is an emerging model study system. The primary scientific reasons for its use are that its large body permits extraction of relatively large volumes of haemolymph (up to around 10µl can be easily extracted) giving it a distinct edge over *Drosophila*. These volumes facilitate physiological assays (e.g. Barnes and Siva-Jothy, 2000) or biochemical studies (e.g. Zhao et al., 2005). In combination with *Staphylococcus aureus* it provides an ideal system to study persistent infection in insects (Haine et al., 2008a). *T. molitor's* main drawback as an immunological study system is the paucity of genomic and transcriptomic sequence data to describe its immune response, and functional manipulations are not possible on the same scale as in other insects, e.g. *Tribolium castaneum* (Bucher et al., 2002; Knorr et al.,

2009), *Drosophila* (Ayres and Schneider, 2008). Our knowledge of the mechanisms of *T. molitor* immunity is based on biochemical studies (Moon et al., 1994a; Zhao et al., 2005; Fabrick et al., 2009; Jiang et al., 2011), or circumstantial inference (Haine et al., 2008a), which would be well-complemented by molecular sequence data.

It is also useful to begin to develop an understanding of the transcriptional changes in *T. molitor* specifically in response to *Staphylococcus aureus*, to begin to fill in the mechanistic blanks in on-going projects using this beetle as a model organism. In terms of work included so far in this thesis, it would be informative to know whether mRNAs for enzymes with potential to cause autopathology are abundantly transcribed shortly after detection of *S. aureus*. Such a finding would be consistent with the hypothesis that self-damaging immune effectors have not been selected against because generalist toxicity is a fundamental requirement of resistance to microbial infection. With transcriptomic information, future research could test this idea through loss-of-function studies. Transcriptomic data could also be used to test the proposal that long-lasting induced humoral immunity has evolved in *T. molitor* as a mechanism to control persistent infection (Haine et al., 2008a). This goal could be achieved by monitoring transcription of specific compounds along the time course of the long-lasting immune response, followed by specific knockdown of constituent effectors of long-lasting immunity. Such investigations of long-lasting *T. molitor* immunity would be a step towards pinning functions in parasite tolerance to specific immune effectors.

5.2.2. Suppressive subtractive hybridisation: an overview, and application in this study

Modern high-throughput ‘next-gen’ molecular sequencing technology is rapidly producing a massive edifice of data, by capturing the full diversity of RNA or DNA of the organisms it is used to sequence. These techniques permit thorough and rigorous analysis of the full diversity of

nucleic acids in a sample, which hugely enriches our insight into how organisms and evolution work. Additionally, full transcriptome analysis, for example using RNA-seq technology, allows creation of contigs that can be paired with proteomic data. These approaches are hugely valuable, but are not always the best fit for purpose. If, for example, a researcher is interested in responses to a specific treatment, next-gen technology will provide a thorough answer, but it will often be buried in volumes of extraneous data that can only be removed with time-consuming bioinformatic analysis. The present study requires a qualitative assessment of a targeted subset of *T. molitor's* transcriptome in response to *S. aureus* infection, for which next-gen sequencing would be overkill.

Suppressive subtractive hybridisation (SSH) is a means to a handle on the transcriptome of *S. aureus*-infected *T. molitor*, although it has now largely been superseded by next-gen sequencing technology. It is a selective PCR-based transcriptomic method used to amplify and clone cDNAs that are differentially expressed in two samples. It can be used to detect transcriptional differences between "driver" cDNA from treated experimental subjects and controls, by hybridising and removing equimolar cDNAs, leaving only cDNAs over-expressed in the driver population. Also, because SSH randomly samples mRNAs from the full diversity of the transcriptome, the probability of transcripts associated with a certain gene being returned in the final analysis is related to the abundance of transcripts of that gene. It is therefore a semi-quantitative approach to transcriptomic sequencing. It lacks the depth and breadth of next-gen technologies like 454 and Illumina, but it is a cheaper, less labour-intensive approach to revealing qualitative differences in expression.

The output of SSH is a collection of expressed sequences tags (ESTs) – mRNA sequences between around 0.1 – 1 mb long that can then be used in subsequent expression studies. Here, SSH is used to characterise expressed sequence tags (ESTs) from *T. molitor* infected

with *S. aureus*. Output ESTs were mapped with Gene Ontologies (GOs) (Conesa et al., 2005). GOs are useful new tools for molecular biologists, facilitating classification of genes using a standardised syntax. They allow classification of genes based on the cellular component in which they occur, the molecular function of the product, and the functional biological significance of the product. Once sequences had been mapped to GOs, upregulation of output from the SSH was validated by quantitative real-time PCR.

5.3. Materials and methods

All experimental work was conducted at the Institute of Phytopathology and Applied Zoology, Justus-Liebig-Universität Gießen, Germany. Sequencing was outsourced to GATC (Konstanz, Germany). Further confirmation of immune responses and sequence data by qPCR was conducted at the University of Sheffield.

5.3.1. Larval and bacterial culture

T. molitor larvae used to generate cDNA for SSH were purchased from a commercial supplier (Fressnapf, Germany) and maintained in ground meal with apple at 28^{o}C in the dark. For qPCR analyses, larvae from long-term cultures maintained at the University of Sheffield were raised on ground rat chow (Harlan Laboratories, UK) and apple in an insectary at 26^{o}C ±1 in a 12:12 light/dark cycle. Bacteria were grown by inoculating a single colony of *S. aureus* (SH1000 tetracycline-resistant strain JLA513, kindly supplied by Simon Foster, University of Sheffield) into Luria-Bertani (LB) medium supplemented with 5 µg ml^{-1} tetracycline and 5.6 µg ml^{-1} amphotericin-B. This preparation was grown for 48 hours at 30^{o}C, allowing the culture to reach stationary phase in accordance with previous *Tenebrio* infection studies (Haine et al., 2008a).

5.3.2. Infection and RNA extraction

For creation of a cDNA library larval *Tenebrio* were used rather than imagoes for their large fat body and subsequently higher mRNA yields, and to circumvent diluting the source pool of mRNA with heavily transcribed sex-related genes in adults.

Fourteen final-instar larvae were chilled on ice for 15 minutes, and then a small patch of the dorso-posterior surface of the animals was swabbed with 80% EtOH. A sterile pin was used to pierce between the third and fourth body segments, through which 6.3 x 10^4 CFU of *S. aureus* culture suspended in 5 µl LB was injected via a sterilised glass needle. Fourteen controls were injected with sterile PBS.

RNA was extracted eight hours post-infection, since previous studies have shown strong transcription at this interval post-infection in insects (Altincicek et al., 2008b; Vogel et al., 2011b). Larvae were homogenized in liquid nitrogen, and stored at -80^{o}C.

RNA was isolated from homogenate by phenol-chloroform extraction. Around 10 mg of frozen homogenate was suspended in 1 ml Triazol (Molecular Research Center, TR-118) and vortexed, before adding 150 µl 1-Bromo-3-chloropropane (Sigma B9673). This preparation was vortexed and allowed to stand two minutes at room temperature, then five minutes on ice. It was centrifuged (10 minutes at 4^{o}C, 16000 g), before adding the transparent phase to 550 µl isopropanol and incubating this mixture at 4^{o}C for 24 hours. The incubated preparations were centrifuged (30 minutes, 4^{o}C, 16000 g), and the supernatants removed. 80% ethanol was added to the pellets, which were then spun again (10 minutes, 4^{o}C, 16000 g) to precipitate RNA. The supernatants were removed and the pellets dried for 10 minutes on ice. Pellets were then dissolved in RNA storage solution (Ambion, AM7000) and stored at -20^{o}C.

5.3.3. Constructing a subtracted cDNA library

cDNA was constructed from extracted RNA using a SMART PCR cDNA Synthesis Kit (Clontech, 634902). Sequences over-expressed in “driver” cDNA (derived from experimental animals) relative to controls were isolated using the PCR-select cDNA subtraction kit (Clontech, 120911). Both kits were used according the protocols described by Altincicek & Vilcinskas (2007).

5.3.4. Cloning and sequencing

cDNA probes were transformed into competent *E. coli* supplied with a pGEM-t-Easy kit (Promega A1360). These cells were plated on LB 1% agar, and 288 clones were randomly selected and inoculated into soft agar on three 96-well plates. These plates were incubated overnight at 37^{o}C, and then sent to a commercial sequencing provider (GATC, Konstanz, Germany).

5.3.5. Bioinformatic analyses

Vector sequences were removed using seqtrim (*http://www.scbi.uma.es/cgi-bin/seqtrim/seqtrim_login.cgi*, (Falgueras et al., 2010) and trimmed sequences were searched against the NCBI non-redundant protein database using BLAST2GO (*http://www.blast2go.org,* (Conesa et al., 2005). Trimmed sequences were assembled using cap3 (95% sequence identity, 30 bp overlap). Sequences retrieving a BLASTx hit with an e-value of $<10^{-3}$ were mapped for gene ontologies (GOs) and annotated. 82 of the 189 BLAST-searched sequences were annotated with GOs. Further analyses were only performed on annotated sequences.

5.3.6. Quantitative PCR

q-PCR was performed on an Applied Biosystems StepOnePlus platform, using iTaq SYBR Green Supermix With ROX (BioRad 172-5851) with 5 ng of cDNA per 15 µl reaction, according to the instructions of the manufacturer.

Primers were designed for sequences aligned with known immune effectors (Table 5.1) using Primer3 (*http://frodo.wi.mit.edu/primer3*) according to the parameters described in Appendix 8. Primers that annealed to secondary target sequence structures predicted by the mfold web app (*http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form*; (Zuker, 2003), or that exhibited self-complementarity (predicted by OligoCalc: *http://www.basic.northwestern.edu/biotools/oligocalc.html*; (Kibbe, 2007) were excluded. Since qPCR was performed to validate the SSH (i.e. to ensure that *S. aureus* infection of *Tenebrio* induces an immune response) expression of the previously sequenced *Tenebrio* immunological genes Tenecin-1 (Moon et al., 1994a) and pro-phenoloxidase (NCBI accession number AB020738.1) were also included in the qPCR analysis. Confirming the up-regulation of these sequences would constitute a confirmed panel of immune effector sequences available for future studies. I used a ribosomal protein (rpl27a, NCBI accession number X99204.1) as an internal control because this gene should be constitutively expressed. Relative expression was calculated as the exponent of the CT value of the control gene minus that of the target genes.

cDNA from the original SSH experiment was unavailable for qPCR confirmation. Therefore qPCR of these sequences was performed on pooled cDNA from *Tenebrio* larvae drawn from long-term stocks at the University of Sheffield that were treated exactly as the animals used to generate cDNA for the initial SSH (13 individuals injected with *S. aureus*, 13 with PBS). This opens these experiments to the possibility of confounding inter-population differences in the specifics of immunity, but

a broad pattern of up-regulation of immunoresponsive genes suggests effective treatment. RNA extraction was replicated twice and both samples were run used in the assay, replicated twice for each gene of interest, generating four CT values per gene per treatment or control larvae. The most conservative CT value was used.

Table 5.3.1. Primers used for qPCR

Alignment	*NCBI accession number*	*Left primer 5'-3'*	*Right primer 5'-3'*
Multicopper oxidase	n/a	GTGACATCTCGTGGTCCTCTC	CAGGCTACTGGTTGTTCCACT
Tyrosine hydroxylase	n/a	TTCACGACTCCGCTTTCTTT	AATTGGACTTGCTTCACTTGG
Ferritin	n/a	GGGTCGGTCTTGATTTCTTGT	AAGGCAATAGTCGCATCCA
Attacin 2	n/a	TCCACCTTCCATTTCGTTTC	ATTCACCTCTTTGGCGTTTG
Tenecin 1	D17670.1	GGAAGCGGCAACAGCTGAAGAAAT	AACGCAGACCCTCTTTCCGTTACA
ProPO	AB020738.1	GCACGAGCTGGAATTGTGT	GGTCGAACAACAGGAGGATG
Ribosomal protein L27a	X99204.1	GCATGGCAAACACAGAAAGCATC	ATGACAGGTTGGTTAGGCAGGC

5.4. Results & discussion

The SSH analysis produced a clutch of positive reads from sequencing of pooled subtracted mRNA from *T. molitor* larvae eight hours post-infection. Many of these sequences aligned strongly with sequences of known immunological relevance from other species, including a number of proteins with known auto-reactive functions. Sequences with promising immunological alignments are discussed below, categorised by functional group.

5.4.1. Subtracted cDNA library sequencing & predicted functions

Blast2GO revealed that 82 of the 288 clones produced by the SSH yielded ESTs showing ontologies with known genes (Appendix 5). These were primarily with other insect species and 55 further unknown sequences, including 5 contigs.

Figures 5.3.1 and 5.3.2 respectively show the breakdown of GOs in molecular and biological function at GO level 3. ESTs with known ontologies are discussed in detail below.

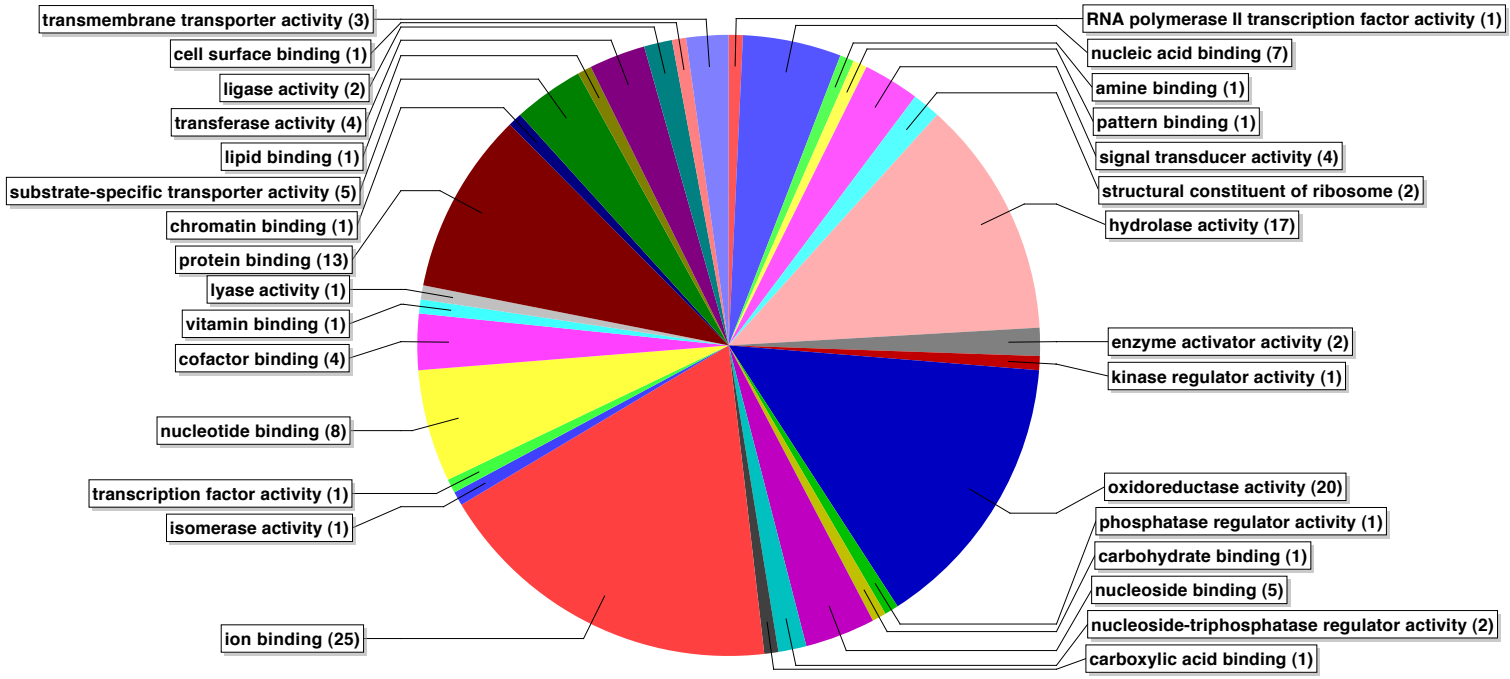


Figure 5.3.1. Annotated SSH results: Molecular function GOs (level 3)

Sequences produced by SSH analysis were annotated with gene ontologies (GOs) to putatively assign them functions based on those of homologous sequences. The detail of these proposed functions is based on the "level" of ontologies, which determines how many connections are made between each sequence and functions of homologs. This figure shows GOs for molecular function at level 3.

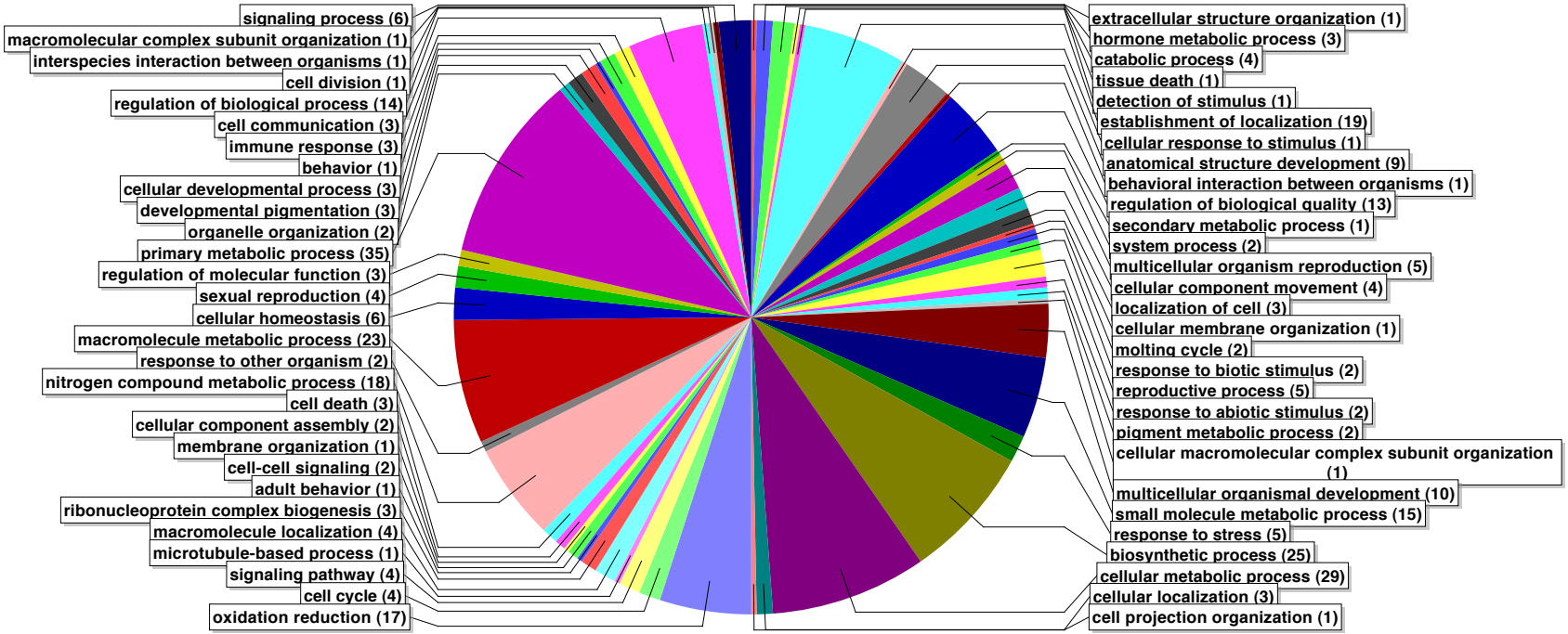


Figure 5.3.2. Annotated SSH results: Biological processes GOs (level 3)

Sequences produced by SSH analysis were annotated with gene ontologies (GOs) to putatively assign them functions based on those of homologous sequences. The detail of these proposed functions is based on the "level" of ontologies, which determines how many connections are made between each sequence and functions of homologs. This figure shows GOs for biological processes at level 3.

5.4.1.1a. Immune defence - signalling

Once an infection has breached an insect's dermal tissues, the first step of an immune response is the detection of invading microbes. Molecules for this purpose can be crudely categorised as receptors known as pattern recognition-receptors (PRRs) that specifically bind microbe-associated molecular patterns, or pro-enzymes that are activated upon incubation with microbes. The SSH returned an mRNA sequence hit aligning with a beta-glucan recognition protein. Beta-glucans are fundamental carbohydrate components of bacterial cell walls, making them suitable targets for PRRs such as the beta-glucan recognition protein returned by the SSH (Jiang et al., 2004).

5.4.1.1b. Immune defence – Enzymatic defences

The SSH revealed numerous oxidative and reductive enzymes, hydrolases, and proteases (Figures 5.3.1 & 5.3.2). Such enzymes are implicated in enzymatic cascades associated with melanin production (Zhao et al., 2007; Dittmer and Kanost, 2010; Prasain et al., 2012), in agreement with previous work showing that melanin production is important in fighting bacterial infection. These include a laccase, tyrosine hydroxylase, multicopper oxidase, dopa decarboxylase, and quinone oxidoreductase. Interestingly I did not detect differential expression of any prophenoloxidases (proPOs), suggesting that the frequent measurement of proPOs as a metric of investment in immunity may be misleading (consistent with Christensen et al., 2005), and that the roles of other enzymes in melanin production and invertebrate immunity ought to be considered more thoroughly and frequently.

22 ESTs associated with such oxidative enzymes were present in the pool (Appendix 5). Since SSH is a semi-quantitative process, the prevalence of these transcripts in the SSH output suggests that these enzymes are important mediators of immunity. Finding mRNAs for such oxidative enzymes is particularly important in light of the ideas proposed

in Chapter 4. Such oxidative enzymes produce toxic secondary products that can damage both host and parasite (reviewed in Gillespie et al., 1997; and chapters in Rolff and Reynolds, 2009).

5.4.1.1c. Immune defence – Iron scavengers

The SSH output contained 25 molecular function GOs for ion binding (Figure 1), largely comprising transferrins and ferritins. These enzymes scavenge iron (Beasley et al., 2011), thereby impairing microbial metabolism and increasing stress (Hammer and Skaar, 2011). For *Staphylococci* in particular, iron is a an important nutrient: iron-starved *S. aureus* struggle to grow in its absence, and iron paucity promotes biofilm formation as a stress response *in vitro* (Johnson et al., 2005). Bacteria in biofilms switch to persistence phenotypes and grow more slowly than in ideal culture conditions (Lewis, 2001; Balaban, 2004; Lewis, 2005). It is conceivable that reduced host tissue iron content could slow growth of infection as a tolerance strategy, by stimulating infectious bacteria to activate pathways normally associated with stressed phenotypes, consistent with predictions about the ultimate function of long-lasting immune responses (Schneider and Chambers, 2008). The ability to acquire iron has been shown to be crucial for virulence of the entomopathogenic bacterium *Photorhabdus luminescens* (Watson et al., 2010), substantiating the idea of selection for hosts to deprive infectious bacteria of iron. However, by upsetting iron homeostasis these defences also likely incur physiological costs of use.

5.4.1.1.d. Immune defence – Antimicrobial peptides

The SSH yielded ESTs that aligned with two antimicrobial peptides. BLAST2GO aligned one EST with an antimicrobial peptide from *Tribolium castaneum* (Coleoptericin). A second aligned with a predicted AMP (cp1) from the *T. castaneum* genome (NCBI reference sequence: XM_001809585.1), which is thought to be an attacin and has recently been discovered by a parallel SSH analysis in the burying beetle

Nicrophorus vespiloides (Vogel et al., 2011b). Interestingly, attacins are active only against growing gram-negative bacteria (Carlsson et al., 1991; 1998). The expression of this AMP in response to stationary-phase gram-positive bacteria suggests that the transcription of a range of AMPs is conjoined regardless of whether the products are effective against the infection, substantiating the suggestion that AMPs may interact in yet-unknown ways *in vivo* (Chapter 2).

5.4.1.2. Stress-associated proteins

Immune responses are often associated with the transcription of stress-associated genes (Altincicek et al., 2008b; Vogel et al., 2011a). *T. molitor* expressed cytochrome p450, known for a role in detoxification post-infection (Chahine and O'Donnell, 2011; Chung et al., 2011). One sequence in particular, aligned with stress-induced phosphoprotein 1, showed ontologies with various aspects of stress management, development and immune function.

5.4.1.3 Other GO-annotated sequences

In addition to detecting ESTs directly involved in the immune response, it is likely that the SSH process has detected ESTs associated with second order effects of immune challenge, which are involved in other metabolic, physiological and transcriptional processes. Based on the scheme of Altincicek et al (2008b) these transcripts are classified by involvement in metabolism, protein biogenesis and transcription, and development. There were also a number of sequences for which it was impossible to propose a classification. This study is concerned with finding effectors of immunity for future functional studies. Therefore, although transcription of these ESTs with functions other than parasite management must be modulated by infection in order for the SSH to detect them, they are not directly relevant to the present study, and so are listed separately in Appendix 5.

5.4.2. qPCR confirmation of immune responses

qPCR was used to validate the SSH technique by confirming that infecting larvae with *S. aureus* had indeed caused up-regulation of the ESTs returned by the SSH and caused an immune response. Attacin/cp1, Tenecin 1, and tyrosine hydroxylase showed strongly up-regulated expression after immune challenge, and multicopper oxidase and pro-phenoloxidase showed marginally up-regulated expression (Figure 5.4.1). This confirms both that the outputs from the SSH are up-regulated after infecting *T. molitor* larvae with *S. aureus*, and that this treatment causes an immune response.

Although qPCR showed a broad pattern of up-regulation of the target genes, infection was not associated with stronger expression of ferritin. This result was surprising, since the high frequency of ferritin reads in the pool of 288 sequences suggests that this mRNA was abundant. The strong expression of ferritin in the qPCR control group suggested that it is constitutively highly expressed regardless of treatment, and that redundant cDNAs have caused a false positive in the SSH. However this is a potential issue with any transcriptomic experiment. It is also possible that this discrepancy is due to the use of different larval populations to generate cDNA for the SSH and qPCR analyses.

With the exception of ferritin, the broad up-regulation of the target immune effectors in infected larvae suggests that the sequence data produced by the SSH are reliable, and up-regulated in response to *S. aureus* infection. This affirms the validity of the SSH methodology.

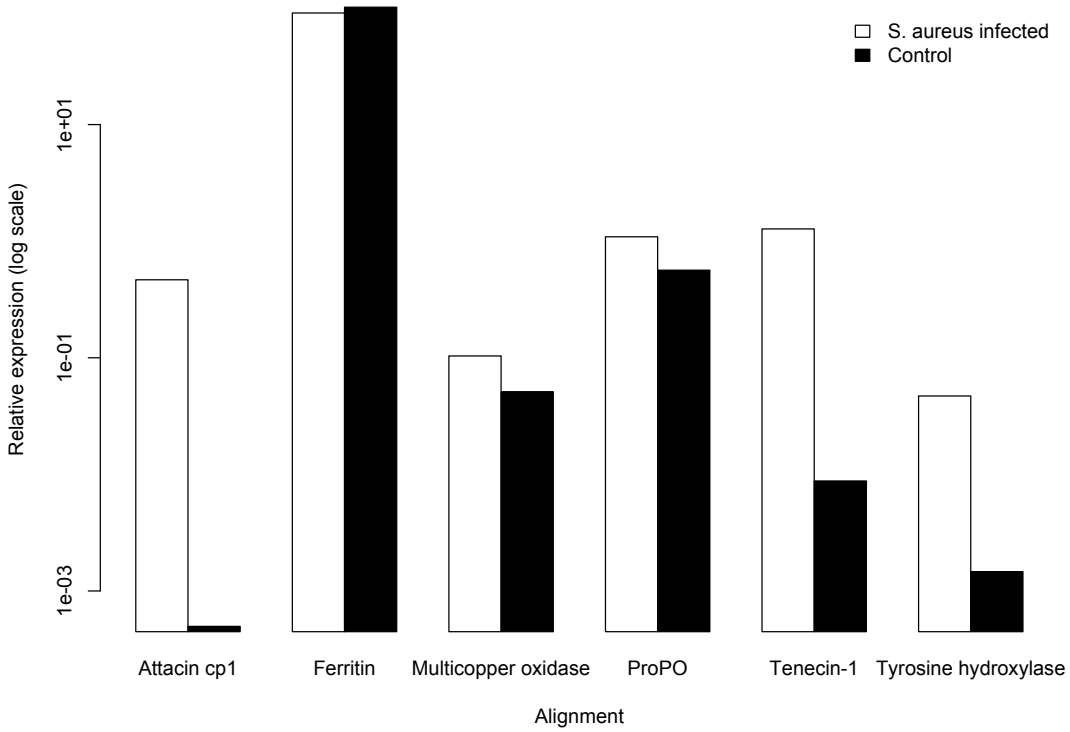


Figure 5.4.1. Expression of immunomodulated sequences identified by SSH, relative to ribosomal control gene rpl27a.

Expression of genes of interest was measured by qPCR to affirm up-regulation of immunomodulated *T. molitor* genes after *S. aureus* infection. Expression of each gene of interest was calculated relative to a ribosomal control gene by exponentiating the difference between CT values produced by target genes and control genes.

5.5. Summary and perspective

This experiment has revealed that a great diversity of mRNAs are detectable in *T. molitor* after infection with *S. aureus*. Many of these mRNAs align with others from databases that code for proteins with known immunological functions. A subset of these effectors - such as laccases, tyrosine hydroxylases, multicopper oxidases and quinone oxidoreductase – are involved in production of toxins that would be toxic to both the beetle and S. aureus infection when they are circulated around the haemocoel. These data are not proof of the essential role of generalist toxicity in resistance responses suggested in Chapter 4, but they are strongly consistent with this hypothesis. It would be informative to quantify expression of immune effectors with predicted autotoxic side-effects over the time-course of *T. molitor*'s immune response, and to see if their knockdown reduces self-melanisation and post-infection mortality. Similar approaches could also be used to probe ultimate function of long-lasting *T. molitor* immunity.

This study has contributed sequence data that can now be used in answering a range of questions regarding the evolution of immunity in *T. molitor* in conjunction with the expanding wealth of biochemical, physiological and ecological methods that have previously been used in this organism. These mRNA sequence data will help to understand the biology of this emerging study system, and through doing so can be used in answering key questions that have begun to be addressed in *T. molitor*.

5.6. Acknowledgements

This chapter would have been impossible without the support and assistance of numerous people and organisations, who deserve acknowledgment separate from the general acknowledgments at the beginning of this thesis.

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Andreas Vilcinskas showed tremendous generosity in hosting and funding my work in Gießen. I also wish to gratefully thank Alissa Singer and the Bellin-Sesay family for accommodation, entertainment and tolerance. I am enormously indebted to all members of AG Vilcinskas, particularly the limitlessly patient Meike Fischer, Dalial Freitak, and Eileen Knorr.

In Sheffield, Jennie Garbutt provided invaluable assistance and expertise in qPCR techniques, and designed primers for Tenecin-1 and proPO. Paul Johnston ran Blast2GO analyses of sequence data.

Chapter Six

Synthesis & general discussion

6.1. Summary of findings

The results presented in the preceding four Chapters complement existing data on the costs and benefits to bacteria of evolving resistance to host immunity; and the costs and mechanisms of inducing a host resistance response to infection. These findings are recapitulated in the table on the following page, before a discussion of the broader implications of a synthesis of these results.

Table 6.1. Key findings presented in this thesis

Chapter 2	• For the major human pathogen *Staphylococcus aureus,* the costs of genetic trade-offs caused by the evolution of resistance to antimicrobial peptides are not greater than those of evolving resistance to stressful antibiotics. • The evolutionary response to selection from a combination of antimicrobial peptides is weaker than the response to the constituents of the combination.
Chapter 3	• Populations of antimicrobial peptide-resistant *S. aureus* survive the initial twenty-four hours after inoculation into the mealworm beetle *Tenebrio molitor* significantly better than antibiotic-selected populations, unselected populations, and the ancestor of all these populations. • When *T. molitor* are infected with *S. aureus*, there is no effect of antimicrobial peptide resistance in the inocula on beetle survival.
Chapter 4	• Simulating an antibacterial immune response in *T. molitor* causes damage to self-tissue that has previously been shown to be associated with a decline in physiological function. • *T. molitor*'s response to simulated bacterial infection causes secondary damage to another microbe in the haemocoel.
Chapter 5	• *T. molitor*'s antibacterial immune response is mediated by a diversity of molecular mechanisms. A number of these are strongly reactive and could cause pathological self-harm when released into the haemocoel.

6.2. Costs and benefits of the evolution of antimicrobial peptide resistance

According to the experiment presented in Chapter 2, the costs of antimicrobial peptide (AMP) resistance are in the same range as costs of resistance to conventional antibiotics. As expected, *Staphylococcus aureus* exposed to the antibiotic streptomycin rapidly evolved resistance to this stressor, and cultures exposed to vancomycin showed very little response to selection. Cultures exposed to AMPs showed a response slower than that to streptomycin, but in contrast to vancomycin there was still a response. Vancomycin exerts its bactericidal action on the cell wall, and AMPs damage the cell membrane, so resistance to both is likely to involve costly change in fundamental structural traits. Vancomycin resistance has previously evolved in environmental *S. aureus* (Weigel, 2003; Wright, 2007) and was more costly than AMP resistance in my experimental protocol, therefore I have falsified the idea that AMP resistance is constrained by its inherent cost. Nevertheless there clearly are costs of AMP resistance, expressed as a depression of r0 in low concentrations of AMP and un-supplemented media, but mitigation of these costs was also observed.

The costs of AMP resistance appear, however, to be somewhat mitigated when the resistant bacteria are inoculated into *T. molitor*. Recent unpublished transcriptomic data (P. Johnston & J. Rolff, Pers. Comm.) show that AMPs are most strongly expressed by *T. molitor* over the first twenty-four hours after bacterial infection, and accordingly significantly more AMP-resistant *S. aureus* were recovered from the beetle twenty-four hours after infection. This shows that fitness of strains that have endured selection for resistance is environmentally determined, and that the outcome of infection is in part determined by the genetic costs and benefits of parasite traits. AMP-resistant strains can better survive immunity, and so have a greater chance of persisting, growing to escape the haemocoel and being transmitted, and are therefore fitter in a host. Importantly, this is a significant biomedical

finding, as it suggests that using AMPs as therapeutic antimicrobials could be a profoundly bad idea (Bell and Gouyon, 2003; Habets and Brockhurst, 2012). If "AMP therapy" becomes a medical reality, my data show that the resistant strains that will probably consequently evolve will cause more persistent and more pathogenic infection.

6.3. Evolutionary conservation of self-harming immunity

My work in this thesis was also concerned with the costs of host resistance to infection, and whether these costs can be implicated as causes of tolerance. I presented data in Chapter 4 that are consistent with a connection between self-melanisation and resistance to bacterial infection in *T. molitor* (Zhao et al., 2007). Although I unfortunately could not measure a fitness cost coincident with this self-melanisation, previous work (Sadd and Siva-Jothy, 2006) showed that this phenomenon compromises fundamental physiological function. Chapter 5 shows that *T. molitor*'s immune response to *S. aureus* infection is mediated in part by reactive enzymes that produce by-products toxic to fundamental conserved cellular traits (Zhao et al., 2007). When these products are released into the open haemocoel they will harm infectious bacteria, with the secondary effect of systemic self-damage. Although melanisation may not necessarily be a downstream effect of these processes (Leclerc et al., 2006), it can be considered an indicator of them. I suggest that the release of such nonspecific toxins has been conserved as part of the immune system precisely because they will damage a wide range of living cells, and they are therefore potent and versatile components of constitutive immune defence. In short, I propose that intrinsically costly immunopathology has been conserved because broad-spectrum toxicity is a fundamental requirement of immune systems, which must act quickly and decisively against potential infection by organisms from any taxonomic kingdom.

6.4. Reciprocal costs of resistance define host-parasite associations

I suggest that applying the same intellectual framework to hosts and their infections could be extremely constructive in building our understanding of how these protagonists coevolve. The concept of costs of immunity is well-established, and the evolutionary immunology framework has been used to study the determinants of parasite resistance for nearly twenty years (Sheldon and Verhulst, 1996). The concept of cost is not quite so pervasive in microbiology and pathogen literature, largely because researchers in these fields are understandably more concerned with how microbes harm hosts than why. Nevertheless there are clear genetic costs associated with evolving resistance to chemical stressors, and costly trade-offs to expressing the phenotypes used to survive host immunity. I propose that there is scope to expand the framework developed in evolutionary immunology beyond just the host to include parasites as well, because the costs to parasites of resisting immunity will determine the outcome of infection just as strongly as the costs to the host of resisting the parasite.

Ultimately, hosts and the organisms that inhabit them are each simply components of the other's environment that can facultatively cause stress, and from this perspective both are governed by the same evolutionary rules when it comes to resisting these stresses. In their most reduced form, the interactions between hosts and infections are reciprocal responses of each party to chemicals associated with the other, and these responses are frequently resistant. Both are subject to evolutionary and deployment costs of resistance, benefits of resistance, endogenous negative feedbacks on resistance, and usually antagonism from the other in expressing resistance. Applying this framework simultaneously to both host and infection is likely to yield a much more complete picture of how hosts and parasites coevolve. An additional benefit of such a conceptual framework is that it will aid integration between host-parasite and mutualism research, by building a single

system by which the costs and benefits of any symbiotic association can be assessed. Since mutualistic associations have parasitic origins, the proposed integrative conceptual framework could well allow us to better understand what determines the positions of microbes on the continuum between pathogen and mutualist (Douglas, 2010; Lazzaro and Rolff, 2011). I view my work in this thesis, particularly Chapter 3, as a case study in this approach.

6.5. Is tolerance a low-cost alternative to resistance?

Although I have presented no work explicitly studying tolerance, there are numerous theoretical connections between the costs of resistance and parasite tolerance. Considering the recent surge of academic interest in this formerly under-appreciated aspect of immunity (Schneider and Ayres, 2008), it is worthwhile discussing how the results presented herein and their more general context could be related to the evolution and activation of tolerance.

Theory predicts that resistance and tolerance are negatively correlated (Roy and Kirchner, 2000), which has been demonstrated empirically both in animals (Raberg et al., 2007) and plants (Koskela et al., 2002; Rodenburg et al., 2006; Rowntree et al., 2011a). Also, it is generally accepted that resistance and tolerance are not discrete phenotypes, and that host responses range continuously between the two (Graham et al., 2010; Rowntree et al., 2011a). These observations suggest that the optimality of each strategy is determined by a continuous variable. Prevalence of tolerance can be defined as the inverse of the prevalence of pathology (Rowntree et al., 2011a). Pathology is an expression of the cost of damage caused by the infection and the cost of immunity i.e. resistance. If tolerance and resistance are successful, then the host is isolated from the cost of damage caused by infection and the benefits of each strategy are equal, so these variables can be removed. On this basis, the prevalence of tolerance is inversely related to the costs of

resistance. Synthesising these principles suggests that tolerance is less endogenously costly than resistance.

If tolerance is less inherently costly than resistance, why should resistance ever evolve? There are two likely reasons. First, that the machinery of tolerance, whatever it might be, will be governed by tradeoffs and therefore be imperfect, just like resistance. This being so, a tolerant host will still suffer some diminished costs of parasitism. Secondly, as long as the parasite lives in the host there is a risk that it will evolve means to subvert the host's expression of tolerance. Since a parasite should only harm its host if it aids transmission (Anderson and May, 1982) and tolerance by definition prevents this, one can predict that selection to subvert tolerance is strong.

There is some empirical evidence that is supportive of these ideas. Recent work has shown that protein nutrition affects antimicrobial peptide expression (i.e. resistance genes) in *Drosophila melanogaster* (Fellous and Lazzaro, 2010), and the ability to resist viral infection in the Indian mealmoth *Plodia interpunctella* (Boots, 2011). Resource availability is a strong determinant of condition, so resistance and condition are positively correlated. Combined with the aforementioned idea that tolerance is less costly than resistance, I suggest that resistance is optimal when the host is in sufficiently good condition to mount a resistance response. If the cost of resistance is independent of condition, then the marginal gains from resistance are diminished as condition declines. However marginal gains can be rescued by increased investment in tolerance if the associated costs are less than costs of resistance, but at the risk of future parasitism and damage. The optimality of each strategy is therefore determined by cost, and can be written as:

$$Optimality\ of\ strategy$$

$$=$$

$$(Condition - cost\ of\ strategy) * risk\ of\ future\ damage\ by\ the\ parasite$$

If an infected organism can "calculate" this for tolerance and resistance, then the ratio of the optimalities of each separate strategy should determine the ratio of investment into each process:

$$Optimality\ of\ resistance: optimality\ of\ tolerance$$
$$=$$
$$investment\ in\ resistance: investment\ in\ tolerance$$

Thus I suggest that costs of resistance play an important role in the evolution and induction of tolerance. In this context it is significant that tolerance and resistance to microbial infection in *D. melanogaster* can be altered by mutation in a single gene critical for melanisation, a mediator of costly antimicrobial resistance (Ayres and Schneider, 2008). If it is accepted that the conceptual framework governing resistance in host-parasite systems can work reciprocally, then the reasoning applied to host tolerance of parasites should also determine whether infection is host-resistant and pathological, or host-tolerant and benign.

6.6. Synthesis and future directions

There is a diverse range of experiments that could follow those described in Chapters 2 - 5, with implications for the specific topics covered in each individual Chapter and for the broader proposed framework.

6.6.1. Further assessments of the costs of AMP resistance

Although a genetic cost of evolving AMP resistance has been shown, it would be ideal to quantify competitiveness of the selected strains against one another, particularly for those exposed to the combination of pexiganan and melittin. Ideally this experiment would be fully reciprocal and involve all the selected cultures. This would constitute powerful

evidence to show whether or not the apparent rarity of natural AMP resistance is driven by reduced competitiveness of resistant mutants when they arise *in vivo*. To complete such an experiment with the strains evolved in Chapter 2 would require transformation of the cultures with distinctive markers.

6.6.2. Mechanisms of AMP resistance

Functional studies would allow a deeper understanding of how AMP resistance is mediated. Although mechanisms of AMP resistance are already known (Peschel and Sahl, 2006; Gruenheid and Le Moual, 2012), this would be the first time they have been shown in experimentally evolved cultures. The known mechanisms of AMP resistance could be elucidated using an array of phenotypic assays, for example by measuring extracellular protease activity. Relating the fitness of each strain to specific mechanisms, and particularly to the induction of specific mechanisms, would build understanding of the costs of activating these resistance mechanisms, thereby applying the evolutionary immunology cost framework to bacteria. Understanding the costs that bacteria stand by activating such mechanisms is also likely to aid our understanding of how host control of the evolution of infection is mediated via bacterial physiology.

6.6.3. Phylogeny of AMP-resistant cultures

Implicitly, the genomes of populations that have to overcome greater evolutionary hurdles must change more than those of populations that have easily overcome stresses. On this basis, sequencing and phylogeny of the cultures that evolved in Chapter 2 show how parsimonious evolution under selection from each stressor was. This would test the prediction that the cultures that went extinct under selection are reasonably close to the ancestor, whereas those that showed a more pronounced response to selection will have moved further through evolutionary space.

6.6.4. Inhibiting autopathological immunity

Inhibiting mechanisms of autopathological immunity will help to clarify whether the costs of resistance drive the induction of tolerance. However inference from such functional studies must made carefully. As discussed in Chapters 1 & 4, it is possible that the antimicrobial and self-damaging effects coincident with melanin production are mediated by upstream compounds, and so inhibition of prophenoloxidase, for instance, may not mediate any effect on either antimicrobial activity or self-damage. Furthermore, the results of Chapter 5 show that a diverse range of reactive enzymes and therefore toxins are produced after infection, so selective knock-down or mutation of select mechanisms may not cause any measurable fitness effect. These problems notwithstanding, if a thorough buffer can be imposed on autopathological immunity, it could test if this phenomenon has been maintained because of the necessity for constitutive defence to be effective against a diversity of potential infections.

6.6.5. Resource manipulation of infected *T. molitor*

If host investment into resistance and tolerance is affected by condition as proposed in Section 6.5, then manipulating nutritional quality should generate predictable outcomes in infection titers: reduced nutritional quality should yield elevated tolerance of infection. Such experiments could be particularly informative about the dynamic balance between resistance and tolerance if they were 2-factoral, and incorporated different parasites that varied in their ability to resist host immunity, such as the AMP-resistant lines evolved the experiment described in Chapter 2. This would constitute an experiment that fully embraces the idea that hosts and infections are governed by the same processes, with the same evolutionary and ecological outcomes.

6.6.6. Outlook

Resistance is costly, and this principle extends to diverse and disparate organisms. Applying it to associated organisms is useful in developing our understanding of the benefits, costs and feedbacks that govern their coevolution, and creating generalisations that can be extended to all organisms.

I believe that future experiments should better consider the interconnectedness of the costs experienced by hosts and parasites as they each try to resist the other, because these will lead to feedbacks that will strongly determine the outcome of infection. More fundamentally the parallels in the evolutionary ecology of both parties are manifold and profound, and the same framework can be applied to them. An integrative framework that applies the same logic to both partners in interspecific associations could be useful in understanding the full spectrum of interactions from pathogen to mutualist. As emphasised by Graham et al. (2010), we must consider the fitness of host and parasite as completely interconnected, and measuring the fitness of both and the strength of the interaction is vital in understanding coevolution. This thesis has discussed the dual notions of costs of bacterial resistance to immunity, and costs of host resistance to parasites; which is a case study in trying to think about hosts and parasites in the same way. As technological advances and novel approaches like deep sequencing, meta-genomics and new analytical tools allow us to fathom greater depths of the complexity of life at all levels of organisation, it will become increasingly possible to use unified conceptual approaches to understand infection, immunity, and ultimately probe what drives coevolution.

Aaron, S.D., Ferris, W., Henry, D.A., Speert, D.P., and Macdonald, N.E. (2000). Multiple combination bactericidal antibiotic testing for patients with cystic fibrosis infected with Burkholderia cepacia. Am. J. Respir. Crit. Care Med. *161*, 1206–1212.

Alizon, S., Hurford, A., Mideo, N., and van Baalen, M. (2009). Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. Journal of Evolutionary Biology *22*, 245–259.

Altincicek, B., and Vilcinskas, A. (2007). Identification of immune-related genes from an apterygote insect, the firebrat Thermobia domestica. Insect Biochem. Mol. Biol. *37*, 726–731.

Altincicek, B., Knorr, E., and Vilcinskas, A. (2008a). Beetle immunity: Identification of immune-inducible genes from the model insect Tribolium castaneum. Developmental & Comparative Immunology *32*, 585–595.

Altincicek, B., Knorr, E., and Vilcinskas, A. (2008b). Beetle immunity: Identification of immune-inducible genes from the model insect Tribolium castaneum. Developmental & Comparative Immunology *32*, 585–595.

Amatngalim, G.D., Nijnik, A., Hiemstra, P.S., and Hancock, R.E.W. (2010). Cathelicidin Peptide LL-37 Modulates TREM-1 Expression and Inflammatory Responses to Microbial Compounds. Inflammation *34*, 412–425.

An, C., and Kanost, M.R. (2010). Manduca sexta serpin-5 regulates prophenoloxidase activation and the Toll signaling pathway by inhibiting hemolymph proteinase HP6. Insect Biochem. Mol. Biol. *40*, 683–689.

Anderson, R., and May, R. (1982). Coevolution of hosts and parasites. Parasitology *85*, 411–426.

Andersson, D.I. (2006). The biological cost of mutational antibiotic

resistance: any practical conclusions? Curr. Opin. Microbiol. *9*, 461–465.

Andreu, D., and Rivas, L. (1998). Animal antimicrobial peptides: an overview. Biopolymers *47*, 415–433.

Armitage, S.A.O., and Siva-Jothy, M.T. (2005). Immune function responds to selection for cuticular colour in Tenebrio molitor. Heredity (Edinb) *94*, 650–656.

Ayres, J.S., and Schneider, D.S. (2008). A Signaling Protease Required for Melanization in Drosophila Affects Resistance and Tolerance of Infections. Plos Biol *6*, e305.

Balaban, N.Q. (2004). Bacterial Persistence as a Phenotypic Switch. Science *305*, 1622–1625.

Barnes, A.I., and Siva-Jothy, M.T. (2000). Density-dependent prophylaxis in the mealworm beetle Tenebrio molitor L. (Coleoptera: Tenebrionidae): cuticular melanization is an indicator of investment in immunity. Proc. Biol. Sci. *267*, 177–182.

Baucom, R.S., and de Roode, J.C. (2010). Ecological immunology and tolerance in plants and animals. Funct Ecology *25*, 18–28.

Beasley, F.C., Marolda, C.L., Cheung, J., Buac, S., and Heinrichs, D.E. (2011). Staphylococcus aureus transporters Hts, Sir, and Sst capture iron liberated from human transferrin by Staphyloferrin A, Staphyloferrin B, and catecholamine stress hormones, respectively, and contribute to virulence. Infect. Immun. *79*, 2345–2355.

Bechinger, B., Zasloff, M., and Opella, S.J. (1993). Structure and orientation of the antibiotic peptide magainin in membranes by solid-state nuclear magnetic resonance spectroscopy. Protein Sci. *2*, 2077–2084.

Bell, G., and Gouyon, P.-H. (2003). Arming the enemy: the evolution of resistance to self-proteins. Microbiology *149*, 1367–1375.

Besier, S., Smaczny, C., Mallinckrodt, von, C., Krahl, A., Ackermann, H., Brade, V., and Wichelhaus, T.A. (2007). Prevalence and clinical significance of Staphylococcus aureus small-colony variants in cystic fibrosis lung disease. J. Clin. Microbiol. *45*, 168–172.

Best, A., White, A., and Boots, M. (2010). Resistance is futile but tolerance can explain why parasites do not always castrate their hosts. Evolution *64*, 348–357.

Boman, H.G. (1995). Peptide antibiotics and their role in innate immunity. Annual Review of Immunology *13*, 61–92.

Boman, H.G., and Hultmark, D. (1987). Cell-Free Immunity in Insects. Annu. Rev. Microbiol. *41*, 103–126.

Boots, M. (2008). Fight or learn to live with the consequences? Trends in Ecology & Evolution *23*, 248–250.

Boots, M. (2011). The Evolution of Resistance to a Parasite Is Determined by Resources. Am Nat *178*, 214–220.

Brennan, C.A., Delaney, J.R., Schneider, D.S., and Anderson, K.V. (2007). Psidin Is Required in Drosophila Blood Cells for Both Phagocytic Degradation and Immune Activation of the Fat Body. Current Biology *17*, 67–72.

Brockhurst, M.A. (2011). Evolution. Sex, death, and the Red Queen. Science *333*, 166–167.

Brogden, K.A. (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. *3*, 238–250.

Bucher, G., Scholten, J., and Klingler, M. (2002). Parental RNAi in Tribolium (Coleoptera). Curr. Biol. *12*, R85–R86.

Buckling, A., and Brockhurst, M. (2005). Microbiology: RAMP resistance. Nature *438*, 170–171.

Bulet, P., Hetru, C., Dimarcq, J.L., and Hoffmann, D. (1999). Antimicrobial peptides in insects; structure and function. Developmental & Comparative Immunology *23*, 329–344.

Bulet, P., Stöcklin, R., and Menin, L. (2004). Anti‐microbial peptides: from invertebrates to vertebrates. Immunological Reviews *198*, 169–184.

Cafiso, V., Bertuccio, T., Spina, D., Campanile, F., Bongiorno, D., Santagati, M., Sciacca, A., Sciuto, C., and Stefani, S. (2010). Methicillin resistance and vancomycin heteroresistance in Staphylococcus aureus in cystic fibrosis patients. Eur J Clin Microbiol Infect Dis *29*, 1277–1285.

Cai, Y., Yu, X.-H., Wang, R., An, M.-M., and Liang, B.-B. (2009). Effects of iron depletion on antimicrobial activities against planktonic and biofilm Pseudomonas aeruginosa. J. Pharm. Pharmacol. *61*, 1257–1262.

Cameron, D.D., Coats, A.M., and Seel, W.E. (2006). Differential Resistance among Host and Non-host Species Underlies the Variable Success of the Hemi-parasitic Plant Rhinanthus minor. Annals of Botany *98*, 1289–1299.

Carlsson, A., Engström, P., Palva, E.T., and Bennich, H. (1991). Attacin, an antibacterial protein from Hyalophora cecropia, inhibits synthesis of outer membrane proteins in Escherichia coli by interfering with omp gene transcription. Infect. Immun. *59*, 3040–3045.

Carlsson, A., Nyström, T., de Cock, H., and Bennich, H. (1998). Attacin--an insect immune protein--binds LPS and triggers the specific inhibition of bacterial outer-membrane protein synthesis. Microbiology (Reading, Engl.) *144 (Pt 8)*, 2179–2188.

Cerenius, L., Kawabata, S.-I., Lee, B.L., Nonaka, M., and Söderhäll, K. (2010). Proteolytic cascades and their involvement in invertebrate immunity. Trends in Biochemical Sciences *35*, 575–583.

Cerenius, L., Lee, B.L., and Söderhäll, K. (2008). The proPO-system: pros and cons for its role in invertebrate immunity. Trends in

Immunology *29*, 263–271.

Chahine, S., and O'Donnell, M.J. (2011). Interactions between detoxification mechanisms and excretion in Malpighian tubules of Drosophila melanogaster. J. Exp. Biol. *214*, 462–468.

Chait, R., and Craney, A. (2007). Antibiotic interactions that select against resistance. Nature.

Chambers, H.F., and DeLeo, F.R. (2009). Waves of resistance: Staphylococcus aureus in the antibiotic era. Nat. Rev. Microbiol. *7*, 629–641.

Charroux, B., and Royet, J. (2010). Drosophila immune response: From systemic antimicrobial peptide production in fat body cells to local defense in the intestinal tract. Fly (Austin) *4*, 40–47.

Christensen, B.M., Li, J., Chen, C.-C., and Nappi, A.J. (2005). Melanization immune responses in mosquito vectors. Trends in Parasitology *21*, 192–199.

Chung, H., Boey, A., Lumb, C., Willoughby, L., Batterham, P., and Daborn, P.J. (2011). Induction of a detoxification gene in Drosophila melanogaster requires an interaction between tissue specific enhancers and a novel cis-regulatory element. Insect Biochem. Mol. Biol. *41*, 863–871.

Collins, J.F., Wessling-Resnick, M., and Knutson, M.D. (2008). Hepcidin regulation of iron transport. pp. 2284–2288.

Conesa, A., Götz, S., García-Gómez, J.M., Terol, J., Talón, M., and Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics *21*, 3674–3676.

Costerton, W., Veeh, R., Shirtliff, M., Pasmore, M., Post, C., and Ehrlich, G. (2003). The application of biofilm science to the study and control of

chronic bacterial infections. Journal of Clinical Investigation *112*, 1466–1477.

D'Arcy Hart, P. (1999). A change in scientific approach: from alternation to randomised allocation in clinical trials in the 1940s. BMJ *319*, 572–573.

Davies, J., and Davies, D. (2010). Origins and Evolution of Antibiotic Resistance. Microbiology and Molecular Biology Reviews *74*, 417–433.

Dean, I., and Siva-Jothy, M.T. (2011). Human fine body hair enhances ectoparasite detection. Biology Letters.

Dean, P., Potter, U., Richards, E., Edwards, J., Charnley, A., and Reynolds, S. (2004). Hyperphagocytic haemocytes in Manduca sexta. Journal of Insect Physiology *50*, 1027–1036.

Defaye, A., Evans, I., Crozatier, M., Wood, W., Lemaitre, B., and Leulier, F. (2009). Genetic ablation of Drosophila phagocytes reveals their contribution to both development and resistance to bacterial infection. Journal of Innate Immunity *1*, 322–334.

Dittmer, N.T., and Kanost, M.R. (2010). Insect multicopper oxidases: diversity, properties, and physiological roles. Insect Biochem. Mol. Biol. *40*, 179–188.

Douglas, A.E. (2010). The symbiotic habit (Princeton Univ Pr).

D'Costa, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W.L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., et al. (2011). Antibiotic resistance is ancient. Nature *477*, 457–461.

Easton, D.M., Nijnik, A., Mayer, M.L., and Hancock, R.E.W. (2009). Potential of immunomodulatory host defense peptides as novel anti-infectives. Trends Biotechnol. *27*, 582–590.

Ebert, D., and Bull, J.J. (2003). Challenging the trade-off model for the evolution of virulence: is virulence management feasible? Trends in

Microbiology *11*, 15–20.

Ebert, D., and Bull, J.J. (2008). The evolution and expression of virulence. In Evolution in Health and Disease, S.C. Stearns, and J. Koella, eds. (Evolution in health and disease).

Eleftherianos, I., Boundy, S., Joyce, S.A., Aslam, S., Marshall, J.W., Cox, R.J., Simpson, T.J., Clarke, D.J., and Reynolds, S.E. (2007). An antibiotic produced by an insect-pathogenic bacterium suppresses host defenses through phenoloxidase inhibition. Proceedings of the National Academy of Sciences *104*, 2419.

Enright, M.C., Robinson, D.A., Randle, G., Feil, E.J., Grundmann, H., and Spratt, B.G. (2002). The evolutionary history of methicillin-resistant Staphylococcus aureus (MRSA). Proc. Natl. Acad. Sci. U.S.a. *99*, 7687–7692.

Fabrick, J., Oppert, C., Lorenzen, M.D., Morris, K., Oppert, B., and Jurat-Fuentes, J.L. (2009). A Novel Tenebrio molitor Cadherin Is a Functional Receptor for Bacillus thuringiensis Cry3Aa Toxin. Journal of Biological Chemistry *284*, 18401–18410.

Falgueras, J., Lara, A.J., Fernández-Pozo, N., Cantón, F.R., Pérez-Trabado, G., and Claros, M.G. (2010). SeqTrim: a high-throughput pipeline for pre-processing any type of sequence read. BMC Bioinformatics *11*, 38.

Fellous, S., and Lazzaro, B.P. (2010). Larval food quality affects adult (but not larval) immune gene expression independent of effects on general condition. Molecular Ecology *19*, 1462–1468.

Fischbach, M.A. (2011). Combination therapies for combating antimicrobial resistance. Curr. Opin. Microbiol.

Fischbach, M.A., and Walsh, C.T. (2009). Antibiotics for Emerging Pathogens. Science *325*, 1089–1093.

Folstad, I., and Karter, A.J. (1992). Parasites, bright males, and the immunocompetence handicap. American Naturalist 603–622.

Foschiatti, M., Cescutti, P., Tossi, A., and Rizzo, R. (2009). Inhibition of cathelicidin activity by bacterial exopolysaccharides. Mol. Microbiol. *72*, 1137–1146.

Frank, S.A. (2002). Immunology and evolution of infectious disease (Princeton Univ Pr).

Fricke, W.F., Wright, M.S., Lindell, A.H., Harkins, D.M., Baker-Austin, C., Ravel, J., and Stepanauskas, R. (2008). Insights into the Environmental Resistance Gene Pool from the Genome Sequence of the Multidrug-Resistant Environmental Isolate Escherichia coli SMS-3-5. Journal of Bacteriology *190*, 6779–6794.

Ganz, T. (2003). Defensins: antimicrobial peptides of innate immunity. Nat. Rev. Immunol. *3*, 710–720.

Gillespie and, J.P., Kanost, M.R., and Trenczek, T. (1997). Biological mediators of insect immunity. Annu. Rev. Entomol. *42*, 611–643.

Graham, A.L., Shuker, D.M., Pollitt, L.C., Auld, S.K.J.R., Wilson, A.J., and Little, T.J. (2010). Fitness consequences of immune responses: strengthening the empirical framework for ecoimmunology. Funct Ecology *25*, 5–17.

Gross, J., Schumacher, K., Schmidtberg, H., and Vilcinskas, A. (2008). Protected by fumigants: beetle perfumes in antimicrobial defense. J. Chem. Ecol. *34*, 179–188.

Gruenheid, S., and Le Moual, H. (2012). Resistance to antimicrobial peptides in Gram-negative bacteria. FEMS Microbiology Letters *330*, 81–89.

Haag, A.F., Baloban, M., Sani, M., Kerscher, B., Pierre, O., Farkas, A., Longhi, R., Boncompagni, E., Hérouart, D., Dall'Angelo, S., et al. (2011).

Protection of Sinorhizobium against Host Cysteine-Rich Antimicrobial Peptides Is Critical for Symbiosis. Plos Biol *9*, e1001169.

Habets, M.G.J.L., and Brockhurst, M.A. (2012). Therapeutic antimicrobial peptides may compromise natural immunity. Biology Letters.

Haine, E.R., Moret, Y., Siva-Jothy, M.T., and Rolff, J. (2008a). Antimicrobial defense and persistent infection in insects. Science *322*, 1257–1259.

Haine, E.R., Pollitt, L.C., Moret, Y., Siva-Jothy, M.T., and Rolff, J. (2008b). Temporal patterns in immune responses to a range of microbial insults (Tenebrio molitor). Journal of Insect Physiology *54*, 1090–1097.

Haine, E.R., Rolff, J., and Siva-Jothy, M.T. (2007). Functional consequences of blood clotting in insects. Developmental & Comparative Immunology *31*, 456–464.

Hall, B.G., and Barlow, M. (2004). Evolution of the serine beta-lactamases: past, present and future. Drug Resist. Updat. *7*, 111–123.

Hammer, N.D., and Skaar, E.P. (2011). Molecular Mechanisms of Staphylococcus aureus Iron Acquisition. Annu. Rev. Microbiol. *65*, 129–147.

Hancock, R.E. (2000). Cationic antimicrobial peptides: towards clinical applications. Expert Opin Investig Drugs *9*, 1723–1729.

Hancock, R.E. (2001). Cationic peptides: effectors in innate immunity and novel antimicrobials. The Lancet Infectious Diseases *1*, 156–164.

Hancock, R.E.W., and Diamond, G. (2000). The role of cationic antimicrobial peptides in innate host defences. Trends in Microbiology *8*, 402–410.

Hegreness, M., Shoresh, N., Damian, D., Hartl, D., and Kishony, R. (2008). Accelerated evolution of resistance in multidrug environments.

Proc. Natl. Acad. Sci. U.S.a. *105*, 13977–13981.

Hillen, W., and Berens, C. (1994). Mechanisms underlying expression of Tn10 encoded tetracycline resistance. Annual Reviews in Microbiology *48*, 345–369.

Holden, M.T.G., Heather, Z., Paillot, R., Steward, K.F., Webb, K., Ainslie, F., Jourdan, T., Bason, N.C., Holroyd, N.E., and Mungall, K. (2009). Genomic evidence for the evolution of Streptococcus equi: host restriction, increased virulence, and genetic exchange with human pathogens. PLoS Pathog *5*, e1000346.

Huang, H.W. (2000). Action of Antimicrobial Peptides: Two-State Model †. Biochemistry *39*, 8347–8352.

Hurst, G.D.D., Anbutsu, H., Kutsukake, M., and Fukatsu, T. (2003). Hidden from the host: Spiroplasma bacteria infecting Drosophila do not cause an immune response, but are suppressed by ectopic immune activation. Insect Molecular Biology *12*, 93–97.

Iwanaga, S., and Lee, B.L. (2005). Recent advances in the innate immunity of invertebrate animals. J. Biochem. Mol. Biol. *38*, 128–150.

Jarosz, L.M., Ovchinnikova, E.S., Meijler, M.M., and Krom, B.P. (2011). Microbial Spy Games and Host Response: Roles of a

Pseudomonas aeruginosa Small Molecule in

Communication with Other Species. PLoS Pathog *7*, e1002312.

Jarraud, S., Mougel, C., Thioulouse, J., Lina, G., Meugnier, H., Forey, F., Nesme, X., Etienne, J., and Vandenesch, F. (2002). Relationships between Staphylococcus aureus genetic background, virulence factors, agr groups (alleles), and human disease. Infect. Immun. *70*, 631–641.

Jiang, H. (2008). The biochemical basis of antimicrobial responses in Manduca sexta. Insect Science *15*, 53–66.

Jiang, H., Ma, C., Lu, Z.-Q., and Kanost, M.R. (2004). Beta-1,3-glucan recognition protein-2 (betaGRP-2)from Manduca sexta; an acute-phase protein that binds beta-1,3-glucan and lipoteichoic acid to aggregate fungi and bacteria and stimulate prophenoloxidase activation. Insect Biochem. Mol. Biol. *34*, 89–100.

Jiang, R., Zhang, B., Kurokawa, K., So, Y.I., Kim, E.H., Hwang, H.O., Lee, J.H., Shiratsuchi, A., Zhang, J., Nakanishi, Y., et al. (2011). 93-kDa Twin-domain Serine Protease Inhibitor (Serpin) Has a Regulatory Function on the Beetle Toll Proteolytic Signaling Cascade. Journal of Biological Chemistry *286*, 35087–35095.

Johnson, M., Cockayne, A., Williams, P.H., and Morrissey, J.A. (2005). Iron-responsive regulation of biofilm formation in staphylococcus aureus involves fur-dependent and fur-independent mechanisms. Journal of Bacteriology *187*, 8211–8215.

Kadioglu, A., Weiser, J.N., Paton, J.C., and Andrew, P.W. (2008). The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease. Nat. Rev. Microbiol. *6*, 288–301.

Kahl, B., Herrmann, M., Everding, A.S., Koch, H.G., Becker, K., Harms, E., Proctor, R.A., and Peters, G. (1998). Persistent infection with small colony variant strains of Staphylococcus aureus in patients with cystic fibrosis. J. Infect. Dis. *177*, 1023–1029.

Kahm, M., Hasenbrink, G., Lichtenberg-Fraté, H., Ludwig, J., and Kschischo, M. (2010). grofit: fitting biological growth curves with R. Journal of Statistical Software *33*, 1–21.

Keren, I., Kaldalu, N., Spoering, A., Wang, Y., and Lewis, K. (2004). Persister cells and tolerance to antimicrobials. FEMS Microbiology Letters *230*, 13–18.

Kibbe, W.A. (2007). OligoCalc: an online oligonucleotide properties calculator. Nucleic Acids Res. *35*, W43–W46.

Kirkness, E.F., Haas, B.J., Sun, W., Braig, H.R., Perotti, M.A., Clark, J.M., Lee, S.H., Robertson, H.M., Kennedy, R.C., Elhaik, E., et al. (2010). Genome sequences of the human body louse and its primary endosymbiont provide insights into the permanent parasitic lifestyle. Proc. Natl. Acad. Sci. U.S.a. *107*, 12168–12173.

Knorr, E., Schmidtberg, H., Vilcinskas, A., and Altincicek, B. (2009). MMPs regulate both development and immunity in the tribolium model insect. PLoS ONE *4*, e4751.

Koskela, T., Puustinen, S., Salonen, V., and Mutikainen, P. (2002). Resistance and tolerance in a host plant-holoparasitic plant interaction: genetic variation and costs. Evolution *56*, 899–908.

Kruse, H., and Sørum, H. (1994). Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. Appl. Environ. Microbiol. *60*, 4015–4021.

Kulkarni, M.M., Barbi, J., McMaster, W.R., Gallo, R.L., Satoskar, A.R., and McGwire, B.S. (2011). Mammalian antimicrobial peptide influences control of cutaneous Leishmania infection. Cellular Microbiology *13*, 913–923.

Laughton, A.M., Boots, M., and Siva-Jothy, M.T. (2011a). The ontogeny of immunity in the honey bee, Apis mellifera L. following an immune challenge. Journal of Insect Physiology *57*, 1023–1032.

Laughton, A.M., Garcia, J.R., Altincicek, B., Strand, M.R., and Gerardo, N.M. (2011b). Characterisation of immune responses in the pea aphid, Acyrthosiphon pisum. Journal of Insect Physiology *57*, 830–839.

Lavine, M.D., and Strand, M.R. (2002). Insect hemocytes and their role in immunity. Insect Biochem. Mol. Biol. *32*, 1295–1309.

Lavine, M.D., Chen, G., and Strand, M.R. (2005). Immune challenge differentially affects transcript abundance of three antimicrobial peptides in hemocytes from the moth Pseudoplusia includens. Insect Biochem.

Mol. Biol. *35*, 1335–1346.

Lazzaro, B.P., and Rolff, J. (2011). Immunology. Danger, microbes, and homeostasis. Science *332*, 43–44.

Leclerc, V., Pelte, N., Chamy, L.E., Martinelli, C., Ligoxygakis, P., Hoffmann, J.A., and Reichhart, J.-M. (2006). Prophenoloxidase activation is not required for survival to microbial infections in Drosophila. EMBO Rep *7*, 231–235.

Lee, H.H., Molla, M.N., Cantor, C.R., and Collins, J.J. (2010). Bacterial charity work leads to population-wide resistance. Nature *467*, 82–85.

Lee, K.P., Simpson, S.J., and Wilson, K. (2008). Dietary protein-quality influences melanization and immune function in an insect. Funct Ecology *22*, 1052–1061.

Lee, S., Moon, H., Kawabata, S.I., Kurata, S., Natori, S., and Lee, B. (1995). A sapecin homologue of Holotrichia diomphalia: purification, sequencing and determination of disulfide pairs. Biological & Pharmaceutical Bulletin *18*, 457–459.

Lehrer, R.I., and Ganz, T. (1999). Antimicrobial peptides in mammalian and insect host defence. Current Opinion in Immunology *11*, 23–27.

Levin, B.R., and Rozen, D.E. (2006). Non-inherited antibiotic resistance. Nat. Rev. Microbiol. *4*, 556–562.

Levin, B.R., Perrot, V., and Walker, N. (2000). Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. Genetics *154*, 985–997.

Lewis, K. (2001). Riddle of biofilm resistance. Antimicrob. Agents Chemother. *45*, 999–1007.

Lewis, K. (2005). Persister cells and the riddle of biofilm survival. Biochemistry Mosc. *70*, 267–274.

Li, M., Lai, Y., Villaruz, A.E., Cha, D.J., Sturdevant, D.E., and Otto, M. (2007). Gram-positive three-component antimicrobial peptide-sensing system. Proc. Natl. Acad. Sci. U.S.a. *104*, 9469–9474.

Lipsitch, M., and Samore, M.H. (2002). Antimicrobial use and antimicrobial resistance: a population perspective. Emerging Infectious Diseases *8*, 347.

Little, T.J., Shuker, D.M., Colegrave, N., Day, T., and Graham, A.L. (2010). The Coevolution of Virulence: Tolerance in Perspective. PLoS Pathog *6*, e1001006.

Login, F.H., Balmand, S., Vallier, A., Vincent-Monegat, C., Vigneron, A., Weiss-Gayet, M., Rochat, D., and Heddi, A. (2011). Antimicrobial Peptides Keep Insect Endosymbionts Under Control. Science *334*, 362–365.

Long, G.H., and Boots, M. (2011). How can immunopathology shape the evolution of parasite virulence? Trends in Parasitology *27*, 300–305.

Long, G.H., and Graham, A.L. (2011). Consequences of immunopathology for pathogen virulence evolution and public health: malaria as a case study. Evolutionary Applications *4*, 278–291.

Long, G.H., Chan, B.H., Allen, J.E., Read, A.F., and Graham, A.L. (2008). Experimental manipulation of immune-mediated disease and its fitness costs for rodent malaria parasites. BMC Evolutionary Biology *8*, 128.

Loose, C., Jensen, K., Rigoutsos, I., and Stephanopoulos, G. (2006). A linguistic model for the rational design of antimicrobial peptides. Nature *443*, 867–869.

Maisnier-Patin, S., and Andersson, D.I. (2004). Adaptation to the deleterious effects of antimicrobial drug resistance mutations by compensatory evolution. Res. Microbiol. *155*, 360–369.

Maisnier-Patin, S., Berg, O.G., Liljas, L., and Andersson, D.I. (2002). Compensatory adaptation to the deleterious effect of antibiotic resistance in Salmonella typhimurium. Mol. Microbiol. *46*, 355–366.

Maisnier-Patin, S., Roth, J.R., Fredriksson, Å., Nyström, T., Berg, O.G., and Andersson, D.I. (2005). Genomic buffering mitigates the effects of deleterious mutations in bacteria. Nat Genet *37*, 1376–1379.

Margolis, E., and Levin, B.R. (2008). The evolution of bacteria-host interactions: virulence and the immune over-response. Evolutionary Biology of Bacterial and Fungal Pathogens 3–12.

Martinez, J.L. (2008). Antibiotics and Antibiotic Resistance Genes in Natural Environments. Science *321*, 365–367.

McKean, K.A., and Nunney, L. (2008). Sexual selection and immune function in Drosophila melanogaster. Evolution *62*, 386–400.

McKean, K.A., Yourth, C.P., Lazzaro, B.P., and Clark, A.G. (2008). The evolutionary costs of immunological maintenance and deployment. BMC Evolutionary Biology *8*, 76.

Meister, M., Lemaitre, B., and Hoffmann, J.A. (1997). Antimicrobial peptide defense in Drosophila. Bioessays *19*, 1019–1026.

Michel, J.-B., Yeh, P.J., Chait, R., Moellering, R.C., and Kishony, R. (2008). Drug interactions modulate the potential for evolution of resistance. Proc. Natl. Acad. Sci. U.S.a. *105*, 14918–14923.

Miteva, V.I., Sheridan, P.P., and Brenchley, J.E. (2004). Phylogenetic and physiological diversity of microorganisms isolated from a deep greenland glacier ice core. Appl. Environ. Microbiol. *70*, 202–213.

Moisan, H., Brouillette, E., Jacob, C.L., Langlois-Bégin, P., Michaud, S., and Malouin, F. (2006). Transcription of virulence factors in Staphylococcus aureus small-colony variants isolated from cystic fibrosis patients is influenced by SigB. Journal of Bacteriology *188*, 64–76.

Moon, H.J., Lee, S.Y., Kurata, S., Natori, S., and Lee, B.L. (1994). Purification and molecular cloning of cDNA for an inducible antibacterial protein from larvae of the coleopteran, Tenebrio molitor. J. Biochem. *116*, 53–58.

Moret, Y., and Schmid-Hempel, P. (2000). Survival for Immunity: The Price of Immune System Activation for Bumblebee Workers. Science *290*, 1166–1168.

Mucklow, P.T., and Ebert, D. (2003). Physiology of immunity in the water flea Daphnia magna: environmental and genetic aspects of phenoloxidase activity. Physiol. Biochem. Zool. *76*, 836–842.

Nappi, A.J., and Ottaviani, E. (2000). Cytotoxicity and cytotoxic molecules in invertebrates. Bioessays *22*, 469–480.

Nehme, N.T., Quintin, J., Cho, J.H., Lee, J., Lafarge, M.-C., Kocks, C., and Ferrandon, D. (2011). Relative roles of the cellular and humoral responses in the Drosophila host defense against three gram-positive bacterial infections. PLoS ONE *6*, e14743.

Nigam, Y., Maudlin, I., Welburn, S., and Ratcliffe, N.A. (1997). Detection of phenoloxidase activity in the hemolymph of tsetse flies, refractory and susceptible to infection with Trypanosoma brucei rhodesiense. J. Invertebr. Pathol. *69*, 279–281.

Noonin, C., Jiravanichpaisal, P., Söderhall, I., Merino, S., Tomás, J.M., and Söderhäll, K. (2010). Melanization and Pathogenicity in the Insect, Tenebrio molitor, and the Crustacean, Pacifastacus leniusculus, by Aeromonas hydrophila AH-3. PLoS ONE *5*, e15728.

Otti, O., Gantenbein-Ritter, I., Jacot, A., and Brinkhof, M.W.G. (2012). Immune response increases predation risk. Evolution *66*, 732–739.

Otto, M. (2008). Staphylococcal biofilms. Curr. Top. Microbiol. Immunol. *322*, 207–228.

Pauwels, K., De Meester, L., Decaestecker, E., and Stoks, R. (2011). Phenoloxidase but not lytic activity reflects resistance against Pasteuria ramosa in Daphnia magna. Biology Letters *7*, 156–159.

Perron, G.G., Zasloff, M., and Bell, G. (2006). Experimental evolution of resistance to an antimicrobial peptide. Proceedings of the Royal Society B: Biological Sciences *273*, 251–256.

Peschel, A., and Sahl, H.-G. (2006). The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nat. Rev. Microbiol. *4*, 529–536.

Peterson, L.R., and Dalhoff, A. (2004). Towards targeted prescribing: will the cure for antimicrobial resistance be specific, directed therapy through improved diagnostic testing? J. Antimicrob. Chemother. *53*, 902–905.

Pham, L.N., Dionne, M.S., Shirasu-Hiza, M., and Schneider, D.S. (2007). A Specific Primed Immune Response in Drosophila Is Dependent on Phagocytes. PLoS Pathog *3*, e26.

Prasain, K., Nguyen, T.D.T., Gorman, M.J., Barrigan, L.M., Peng, Z., Kanost, M.R., Syed, L.U., Li, J., Zhu, K.Y., and Hua, D.H. (2012). Redox potentials, laccase oxidation, and antilarval activities of substituted phenols. Bioorg. Med. Chem. *20*, 1679–1689.

Pränting, M., and Andersson, D.I. (2010). Mechanisms and physiological effects of protamine resistance in Salmonella enterica serovar Typhimurium LT2. Journal of Antimicrobial Chemotherapy *65*, 876–887.

Pränting, M., Negrea, A., Rhen, M., and Andersson, D.I. (2008). Mechanism and fitness costs of PR-39 resistance in Salmonella enterica serovar Typhimurium LT2. Antimicrob. Agents Chemother. *52*, 2734–2741.

Pursall, E.R. (2010). Long-term costs of early-life infection in the mealworm beetle, Tenebrio molitor.

Pursall, E.R., and Rolff, J. (2011). Immune responses accelerate ageing: proof-of-principle in an insect model. PLoS ONE *6*, e19972.

Raberg, L., Sim, D., and Read, A.F. (2007). Disentangling Genetic Variation for Resistance and Tolerance to Infectious Diseases in Animals. Science *318*, 812–814.

Rao, X.-J., Ling, E., and Yu, X.-Q. (2010). The role of lysozyme in the prophenoloxidase activation system of Manduca sexta: An in vitro approach. Developmental & Comparative Immunology *34*, 264–271.

Råberg, L., Graham, A.L., and Read, A.F. (2008). Review. Decomposing health: tolerance and resistance to parasites in animals. Philos. Trans. R. Soc. Lond., B, Biol. Sci. *364*, 37–49.

Reddy, K., Yedery, R., and Aranha, C. (2004). Antimicrobial peptides: premises and promises. International Journal of Antimicrobial Agents *24*, 536–547.

Rodenburg, J., Bastiaans, L., and Kropff, M.J. (2006). Characterization of host tolerance to Striga hermonthica. Euphytica *147*, 353–365.

Rolff, J., and Reynolds, S. (2009). Insect infection and immunity (Oxford University Press, USA).

Rolff, J., and Siva-Jothy, M.T. (2002). Copulation corrupts immunity: a mechanism for a cost of mating in insects. Proc. Natl. Acad. Sci. U.S.a. *99*, 9916–9918.

Rolff, J., and Siva-Jothy, M.T. (2003). Invertebrate ecological immunology. Science *301*, 472–475.

Rosales, C. (2011). Phagocytosis, a cellular immune response in insects. Isj *8*, 109–131.

Rowntree, J.K., Cameron, D.D., and Preziosi, R.F. (2011a). Genetic variation changes the interactions between the parasitic plant-ecosystem engineer Rhinanthus and its hosts. Philos. Trans. R. Soc. Lond., B, Biol.

Sci. *366*, 1380–1388.

Rowntree, J.K., Shuker, D.M., and Preziosi, R.F. (2011b). Forward from the crossroads of ecology and evolution. Philos. Trans. R. Soc. Lond., B, Biol. Sci. *366*, 1322–1328.

Roy, B.A., and Kirchner, J.W. (2000). Evolutionary dynamics of pathogen resistance and tolerance. Evolution *54*, 51–63.

Sadd, B.M., and Siva-Jothy, M.T. (2006). Self-harm caused by an insect's innate immunity. Proc. Biol. Sci. *273*, 2571–2574.

Sadowska, B., Bonar, A., Eiff, C., Proctor, R.A., Chmiela, M., Rudnicka, W., and Różalska, B. (2002). Characteristics of Staphylococcus aureus, isolated from airways of cystic fibrosis patients, and their small colony variants. FEMS Immunology & Medical Microbiology *32*, 191–197.

Sanders, C.C., Sanders, W.E., Goering, R.V., and Werner, V. (1984). Selection of multiple antibiotic resistance by quinolones, beta-lactams, and aminoglycosides with special reference to cross-resistance between unrelated drug classes. Antimicrob. Agents Chemother. *26*, 797–801.

Schmid-Hempel, P. (2011). Evolutionary Parasitology (Oxford Univ Pr).

Schneider, D.S. (2011). Tracing Personalized Health Curves during Infections. Plos Biol *9*, e1001158.

Schneider, D.S., and Ayres, J.S. (2008). Two ways to survive infection: what resistance and tolerance can teach us about treating infectious diseases. Nat. Rev. Immunol. *8*, 889–895.

Schneider, D.S., and Chambers, M.C. (2008). MICROBIOLOGY: Rogue Insect Immunity. Science *322*, 1199–1200.

Shaw, L.N., Aish, J., Davenport, J.E., Brown, M.C., Lithgow, J.K., Simmonite, K., Crossley, H., Travis, J., Potempa, J., and Foster, S.J. (2006). Investigations into sigmaB-modulated regulatory pathways governing extracellular virulence determinant production in

Staphylococcus aureus. Journal of Bacteriology *188*, 6070–6080.

Sheldon, B.C., and Verhulst, S. (1996). Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. Trends in Ecology & Evolution *11*, 317–321.

Siva-Jothy, M.T. (2000). A mechanistic link between parasite resistance and expression of a sexually selected trait in a damselfly. Proceedings of the Royal Society B: Biological Sciences *267*, 2523–2527.

Siva-Jothy, M.T., Moret, Y., and Rolff, J. (2005). Advances in Insect Physiology. *32*, 1–48.

Siva-Jothy, M.T., Tsubaki, Y., and Hooper, R.E. (1998). Decreased immune response as a proximate cost of copulation and oviposition in a damselfly. Physiological Entomology *23*, 274–277.

Stjernman, M., Råberg, L., and Nilsson, J.-Å. (2008). Maximum Host Survival at Intermediate Parasite Infection Intensities. PLoS ONE *3*, e2463.

Stowe, K.A., Marquis, R.J., Hochwender, C.G., and Simms, E.L. (2000). The evolutionary ecology of tolerance to consumer damage. Annual Review of Ecology and Systematics 565–595.

Strand, M.R. (2008). The insect cellular immune response. Insect Science *15*, 1–14.

Sugumaran, M. (2002). Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects. Pigment Cell Res. *15*, 2–9.

Sun, S., Negrea, A., Rhen, M., and Andersson, D.I. (2009). Genetic analysis of colistin resistance in Salmonella enterica serovar Typhimurium. Antimicrob. Agents Chemother. *53*, 2298–2305.

Tong, S.Y.C., Chen, L.F., and Fowler, V.G. (2012). Colonization, pathogenicity, host susceptibility, and therapeutics for Staphylococcus

aureus: what is the clinical relevance? Semin Immunopathol *34*, 185–200.

Torella, J.P., Chait, R., and Kishony, R. (2010). Optimal Drug Synergy in Antimicrobial Treatments. PLoS Comput Biol *6*, e1000796.

Tzou, P., Reichhart, J.-M., and Lemaitre, B. (2002). Constitutive expression of a single antimicrobial peptide can restore wild-type resistance to infection in immunodeficient Drosophila mutants. Proc. Natl. Acad. Sci. U.S.a. *99*, 2152–2157.

Vale, P.F., Wilson, A.J., Best, A., Boots, M., and Little, T.J. (2011). Epidemiological, evolutionary, and coevolutionary implications of context-dependent parasitism. Am Nat *177*, 510–521.

Vogel, H., Altincicek, B., Glöckner, G., and Vilcinskas, A. (2011a). A comprehensive transcriptome and immune-gene repertoire of the lepidopteran model host Galleria mellonella. BMC Genomics *12*, 308.

Vogel, H., Badapanda, C., and Vilcinskas, A. (2011b). Identification of immunity-related genes in the burying beetle Nicrophorus vespilloides by suppression subtractive hybridization. Insect Molecular Biology *20*, 787–800.

Wang, R., Braughton, K.R., Kretschmer, D., Bach, T.-H.L., Queck, S.Y., Li, M., Kennedy, A.D., Dorward, D.W., Klebanoff, S.J., Peschel, A., et al. (2007). Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat. Med. *13*, 1510–1514.

Wang, R., Khan, B.A., Cheung, G.Y.C., Bach, T.-H.L., Jameson-Lee, M., Kong, K.-F., Queck, S.Y., and Otto, M. (2011). Staphylococcus epidermidis surfactant peptides promote biofilm maturation and dissemination of biofilm-associated infection in mice. J. Clin. Invest. *121*, 238–248.

Ward, H., Perron, G.G., and Maclean, R.C. (2009). The cost of multiple

drug resistance in Pseudomonas aeruginosa. Journal of Evolutionary Biology *22*, 997–1003.

Watson, R.J., Millichap, P., Joyce, S.A., Reynolds, S., and Clarke, D.J. (2010). The role of iron uptake in pathogenicity and symbiosis in Photorhabdus luminescens TT01. BMC Microbiol *10*, 177.

Webster, J., and Woolhouse, M. (1999). Cost of resistance: relationship between reduced fertility and increased resistance in a snail—schistosome host—parasite system. Proc. Biol. Sci. *266*, 391–396.

Weigel, L.M. (2003). Genetic Analysis of a High-Level Vancomycin-Resistant Isolate of Staphylococcus aureus. Science *302*, 1569–1571.

Wickham, H. (2009). ggplot2. Wiley Interdisciplinary Reviews: Computational Statistics.

Wright, G.D. (2007). The antibiotic resistome: the nexus of chemical and genetic diversity. Nat. Rev. Microbiol. *5*, 175–186.

Wuerth, K., and Hancock, R.E.W. (2011). New insights into cathelicidin modulation of adaptive immunity. Eur. J. Immunol. *41*, 2817–2819.

Yeaman, M.R., and Yount, N.Y. (2003). Mechanisms of antimicrobial peptide action and resistance. Pharmacol. Rev. *55*, 27–55.

Yeh, P., Tschumi, A.I., and Kishony, R. (2006). Functional classification of drugs by properties of their pairwise interactions. Nat Genet *38*, 489–494.

Yeh, P.J., Hegreness, M.J., Aiden, A.P., and Kishony, R. (2009). Drug interactions and the evolution of antibiotic resistance. Nat. Rev. Microbiol. *7*, 460–466.

Yeung, A.T.Y., Gellatly, S.L., and Hancock, R.E.W. (2011). Multifunctional cationic host defence peptides and their clinical applications. Cell. Mol. Life Sci. *68*, 2161–2176.

Zanetti, M. (2005). The role of cathelicidins in the innate host defenses of mammals. Curr Issues Mol Biol *7*, 179–196.

Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. Nature *415*, 389–395.

Zasloff, M. (2009). Mysteries that still remain. Biochim. Biophys. Acta *1788*, 1693–1694.

Zhao, M., Söderhall, I., Park, J.-W., Ma, Y.G., Osaki, T., Ha, N.-C., Wu, C.F., Söderhäll, K., and Lee, B.L. (2005). A novel 43-kDa protein as a negative regulatory component of phenoloxidase-induced melanin synthesis. J. Biol. Chem. *280*, 24744–24751.

Zhao, P., Li, J., Wang, Y., and Jiang, H. (2007). Broad-spectrum antimicrobial activity of the reactive compounds generated in vitro by Manduca sexta phenoloxidase. Insect Biochem. Mol. Biol. *37*, 952–959.

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. *31*, 3406–3415.

Appendix 1. Issues with dose-response and r0*max* estimates

Practical constraints meant it was impossible to control the number of cells entering the dose-response assays. This may have knock-on effects on the apparent growth of cultures in the dose-response assay for three reasons:

1. Bacterial cultures grow roughly exponentially. Variation in the number of cells which is exponentiated will correspond with variation in the consequent slopes.
2. Bacteria in different phases of growth will also be in different physiological states, which may alter susceptibility to a stressor.
3. The molarity of a stressor per individual bacterial cell will depend on the number of cells entering the assay if the concentration of the stressor required for its effectiveness is not exceeded, since (y/x) = 2 (y/2x).

The assays and subsequent analyses were designed to buffer against this possibility:

1. 18h growth in unsupplemented media between leaving the selection protocol and entering the dose-response allowed time for exponential growth in stress-free conditions across all treatments
2. Six hours (the duration of the dose-response assay) is a sufficient window of opportunity for S. aureus to reach its maximal growth rate, and only the maximal possible r0 value is used in the analyses.
3. The calculation of a rate (r0) in place of a raw dN/dT figure ought to control confounding effects of starting OD on metrics of growth.

Appendix 2. Controlling contamination in perfusion bleed techniques

Chapter 3 makes use of "perfusion bleed" techniques originally developed by (Haine et al., 2008a) to hydraulically flush infectious bacteria from *T. molitor*'s haemocoel. Before embarking on the experiments described in Chapter 3 it became evident that there was room to improve the original protocol.

The essence of the perfusion bleed technique (Chapter 3, Figures 3.3.2 & 3.3.3) is to inject tetracycline-resistant *S. aureus* into *T. molitor's* abdomen and then flush the abdomen with a relatively large volume of PBS. A sub-sample of this solution is then spread with beads on an agar plate. This plate contains tetracycline and amphotericin-B, on the assumption that these antimicrobials will control for microbial contamination. The advantages of this assay over plating a diluted sample of neat haemolymph are that it reduces the potential for stochastic effects on the final number of CFU detected on the agar plate - for example caused by small localized abscesses acting as reservoirs of infection, that may not be detected by without forcing bacteria from the tissue with a pressurised solvent like PBS. Reflection on extensive experience of the assay (including generating part of the original (Haine et al., 2008a) dataset) revealed potential flaws that had to be addressed before the assay or the data used by (Haine et al., 2008a) could be regarded confidently. These flaws are described below:

1. **Experimental control**.

(Haine et al., 2008a) present no evidence of procedural control (i.e. perfusion bleeds of beetles injected with sterile liquid), negative control (i.e. to check for contamination of PBS or agar), or untreated control (i.e. perfusion bleeds of un-injected beetles). These flaws are fundamental and could profoundly alter the apparent dynamics of persistent infection in the *T. molitor / S. aureus* system if any contamination is present.

2. **Sensitivity to contamination.**

The perfusion bleed technique relies on the assumption that *T. molitor*'s resident bacteria are filtered out of the final assay by tetracycline dissolved in agar. However environmental microbes are commonly antibiotic resistant or indifferent (see Chapter 1 and Chapter 3). Thus there is potential for contamination of *T. molitor* with tetracycline-resistant environmental bacteria.

3. **Potential for contamination.**

There are three potential routes for any tetracycline-resistant bacteria that may be present in experimental beetles to enter the experimental sample. Such bacteria could be a) washed off the cuticle of experimental animals by PBS that leaks from where the hypodermic needle is inserted into the beetle and fall into the collection tube; b) endemic to haemolymph; or c) released from the gut into the haemocoel if the hypodermic needle pierces into the gut lumen.

I investigated the potential for these issues to confound the results of (Haine et al., 2008a), and thereby develop adequate control protocols for my own subsequent experiments in Chapter 3. I checked for potential contamination when negative controls were employed, refined the protocol, repeated part of (Haine et al., 2008a)'s study into the dynamics of persistent infection with a negative control and refined protocol, and checked the presence of *S. aureus* from frozen stocks of the samples generated by (Haine et al., 2008a).

For to test for potential contamination, I infected 10 beetles from long-term stocks at the University of Sheffield with *S. aureus* JLA 513 (a tetracycline-resistant clone that was integral in all subsequent experiments in Chapters 2-5, and the clone used by (Haine et al., 2008a)). Beetles were infected as described in Chapter 3, except that bacteria were unwashed and suspended in their growth medium. One colony of *S. aureus* was inoculated into 5 ml LB containing 5 μg ml^{-1} tetracycline and 5.6 μg ml^{-1} amphotericin B. Bacteria were grown for 48h

at 30°C, shaking at 100 rpm. I injected a further 10 beetles with un-inoculated growth medium, and another 10 were untreated. These beetles were perfusion bled with sterile PBS 5h after treatment as described in Chapter 3, and 5µl of the bleed was plated onto LB agar using sterile glass beads. The plates were incubated overnight at 30°C. CFU were counted manually. The resultant CFU data are plotted in Figure A2.1.

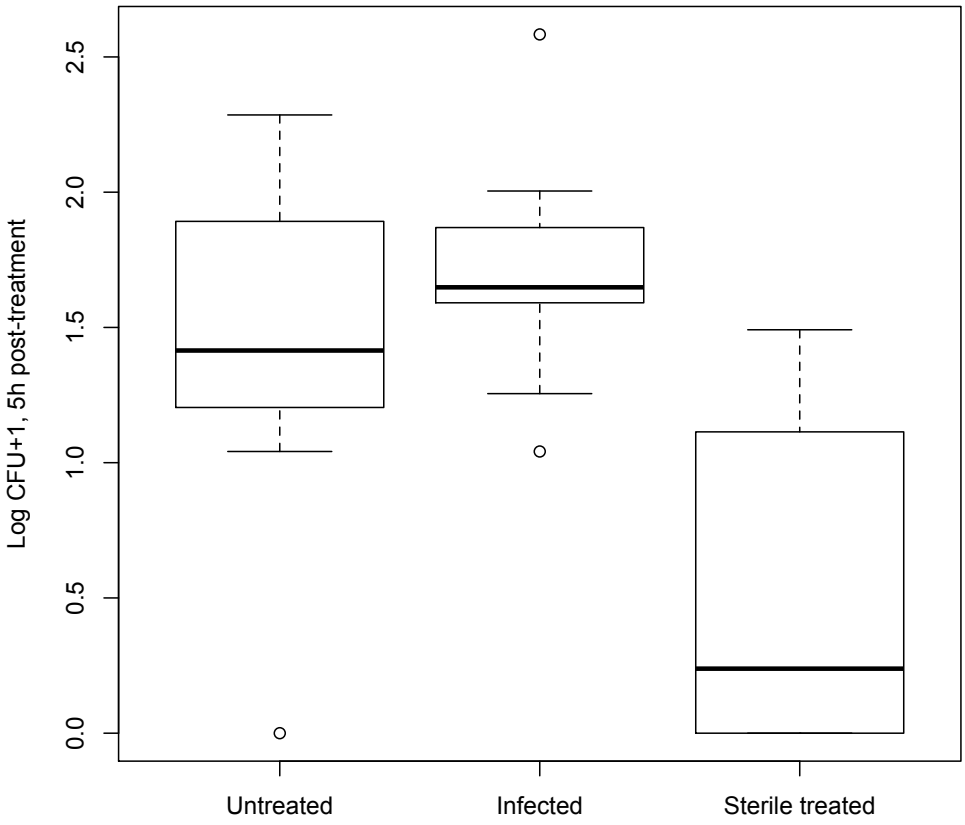


Figure A2.1. Quantifying contamination from perfusion bleeding *T. molitor*

Bacterial CFU from perfusion bleeds of *T. molitor* that were not injected with anything, injected with 5µl of an overnight culture of *S. aureus* JLA 513 (tetracycline resistant), or injected with sterile bacterial growth medium. 5µl of the perfusions were plated on LB agar containing 5 µg ml^{-1} tetracycline and 5.6 µg ml^{-1} amphotericin-B. These data show that the perfusion bleed assay used by (Haine et al., 2008a) is sensitive to contamination and would benefit from refinement.

The data in Figure A.2.1. demonstrated that the assay required further development to ensure sterility, and that bacteria capable of growing on tetracycline and amphotericin-B were present in *T. molitor*.

To localize the source of the contamination, I sampled the cuticle and haemolymph of beetles (ten per group). Pure haemolymph was extracted by piercing a hole in the beetle's plural membrane between the abdomen and thorax with a sterile pin, and pipetting the exuded haemolymph. This was mixed with sterile PBS in a 1:10 ratio, approximating the mixture of haemolymph and PBS in a perfusion bleed. To sample the cuticle, beetles were washed in sterile PBS by dunking each individual beetle in PBS in a centrifuge tube and vortexing briefly. I also perfusion bled ten uninfected beetles as before, but first washed them for ten seconds in 80% ethanol. 5 µl of the resulting samples were plated as before. To test whether the contamination shown in A2.1 was introduced during the plating process, beads were spread over plates with no beetle sample (n=3). PBS was drawn from the same tube when refilling the syringe between bleeding individual beetles, so to test if this could be contaminated during the bleeding procedure, a 50µl sample of the PBS was plated before and after bleeding (n=3). To test whether the contamination could be airborne, plates were also left open in the laminair flow cabinet in which the samples were plated (n=3). Plates were incubated and CFU were quantified as previously. The percentage of plates showing growth are plotted in Figure A.2.2. The CFU counts from samples of beetles that were washed in EtOH are presented in Figure A.2.3, compared to the data from untreated beetles (using the same data as A.2.1).

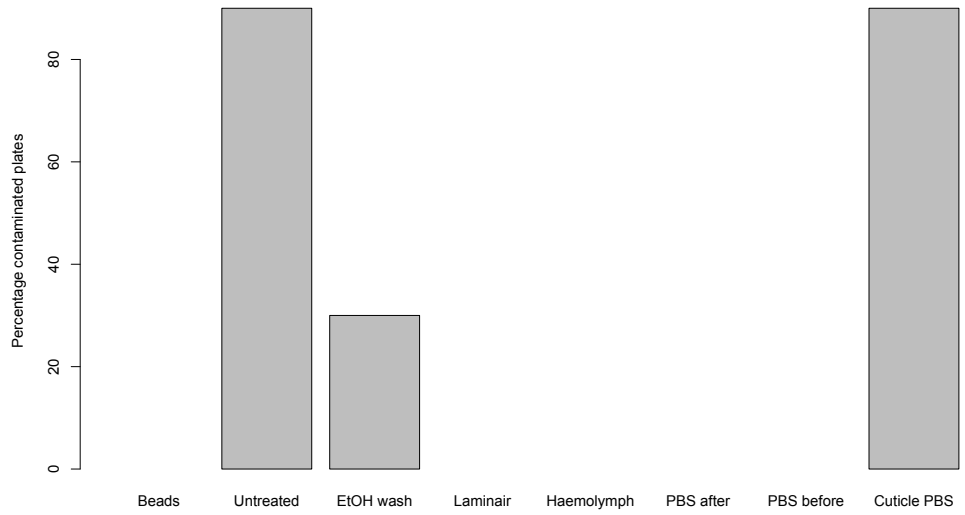


Figure A.2.2. Localising contamination in the perfusion bleed technique: percentage of plates showing positive growth.

Pure samples of beetle haemolymph, perfusion bled haemolymph (Untreated), PBS exposed to the cuticle (Cuticle PBS), and perfusion-bled haemolymph from beetles that were first washed in 80% EtOH (EtOH wash) were plated on LB containing tetracycline (5 μg ml^{-1}) and amphotericin-B (5.6 μg ml^{-1}). No colonies grew from pure haemolymph. Plating samples from perfusion-bled beetles or cuticular washes led to positive growth from 90% of samples in each treatment, suggesting that the contamination was cuticular. Washing the beetle's cuticle with EtOH before perfusion bleeding reduced the number of plates showing positive growth to 30%.

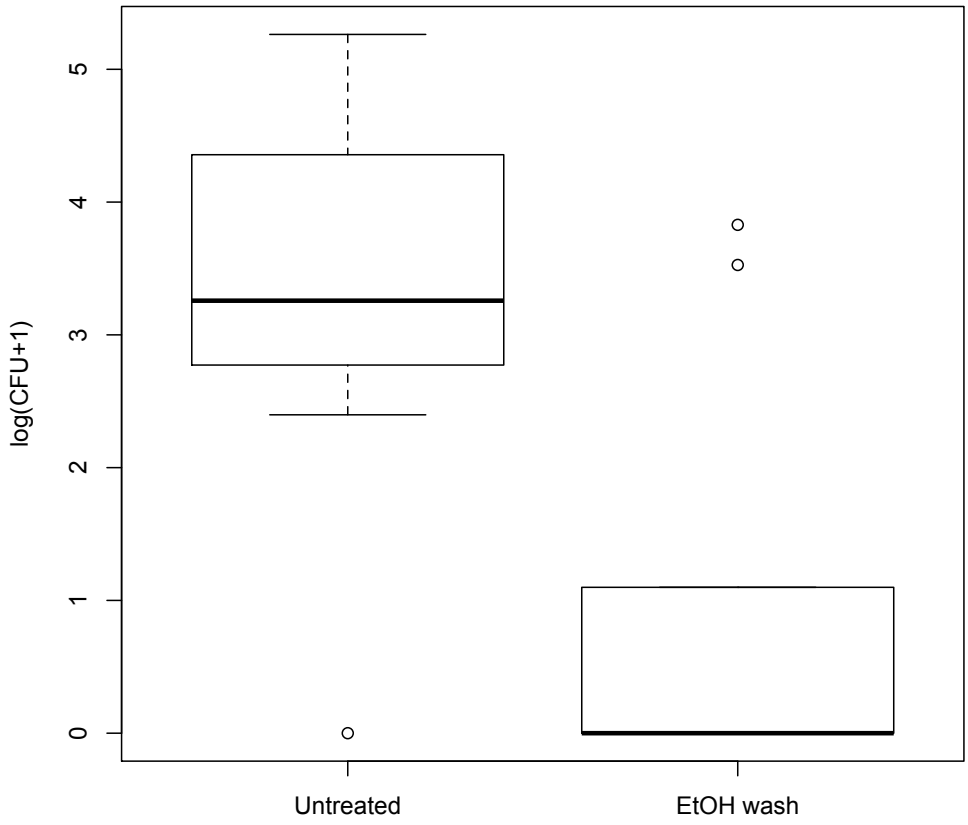


Figure A.2.3. CFU from perfusion bled beetles with and without EtOH washing.

Uninfected beetles were perfusion bled as by (Haine et al., 2008a), or after washing the beetle cuticle in ethanol for 10 seconds before bleeding. Washing with ethanol stopped growth in 70% of cases, and reduced it below that of the uninfected beetles in a further 10% (n=10 per treatment).

These data show that it is sensible to briefly wash all beetles in an experiment before perfusion bleeding. It also shows that an uninfected control is essential in studies using this procedure. Although the assay is not perfect, using this slightly refined protocol and including controls ensures that any contamination is systemic. If CFU counts from uninfected controls are significantly less than those from infected beetles, then the procedure is reasonably sound. If, however, CFU counts from uninfected controls are not significantly different from those of infected beetles, then it is possible if not likely that the measurement of infection in the infected beetles is not the injected bacteria, but rather background contamination. Comparisons should be made between uninfected beetles and infected beetles based on the day that the beetles were bled, since this is when cuticular contamination is controlled for with EtOH washing. Although plating neat haemolymph would from some perspectives be preferable because there was no evidence of contamination when beetles were bled this way (Figure A. 2.2), it seems likely that this method is open to stochastic because *S. aureus* infection in the beetle is likely to be localised in abscesses, and cells from such abscesses would not be washed from beetle tissues without the hydraulic pressure applied by the perfusion bleed technique.

The experiment in Chapter 3 detailing persistence of *S. aureus* in *T. molitor* incorporated all the above procedures into the experiment and analysis. Every day that beetles were infected for these experiments, a subset (15-30 individuals) was injected with sterile PBS in parallel to those injected with bacteria. Some (5-10) of these sterility controls were bled in parallel to their infected counterparts. This revealed that there was some background contamination on some specific bleed days, but the numbers of bacteria recovered from the uninfected controls were always significantly less than the infected beetles, showing that the CFU data reported were true positives.

I was concerned that a high-profile study (Haine et al., 2008a) may have reported an erroneous result. Therefore I tested whether the results

reported could have been false positives. Fortunately, Haine et al left frozen stocks of the pooled *S. aureus* they recovered from *T. molitor* at the University of Sheffield, which I had access to. Therefore I plated these samples on Mannitol salt phenol red agar (Sigma, 63567-500G-F). This is a selective high-salt medium, commonly used for diagnostic tests of *Staphylococci*. The contaminating cells recovered by perfusion bleeding (above) appeared Staphylococcal, likely *S. epidermis*. Mannitol salt phenol red agar is a commonly used diagnostic test that distinguishes between *S. aureus* and other Staphylococci by testing for the ability to ferment mannitol, which is indicated by a clear yellow halo around mannitol-fermenting cells. Bacteria left by (Haine et al., 2008a) fermented mannitol (Figure A.2.4), shows that *S. aureus* were present in some of these samples, showing that (Haine et al., 2008a) did manage to establish persistent *S. aureus* infection, but the accuracy of their assay is a little dubious. However I could not recover any living cells at all from the "28 days" timepoint reported previously. It may be that any Staphylococcal cells that were present did not survive freezing, or that those reported were false positives. It has also since been confirmed that, in agreement with (Haine et al., 2008a; 2008b), *T. molitor* induce long-lasting antimicrobial haemolymph activity in response to S. aureus infection (P. Johnston, Pers. comm.).

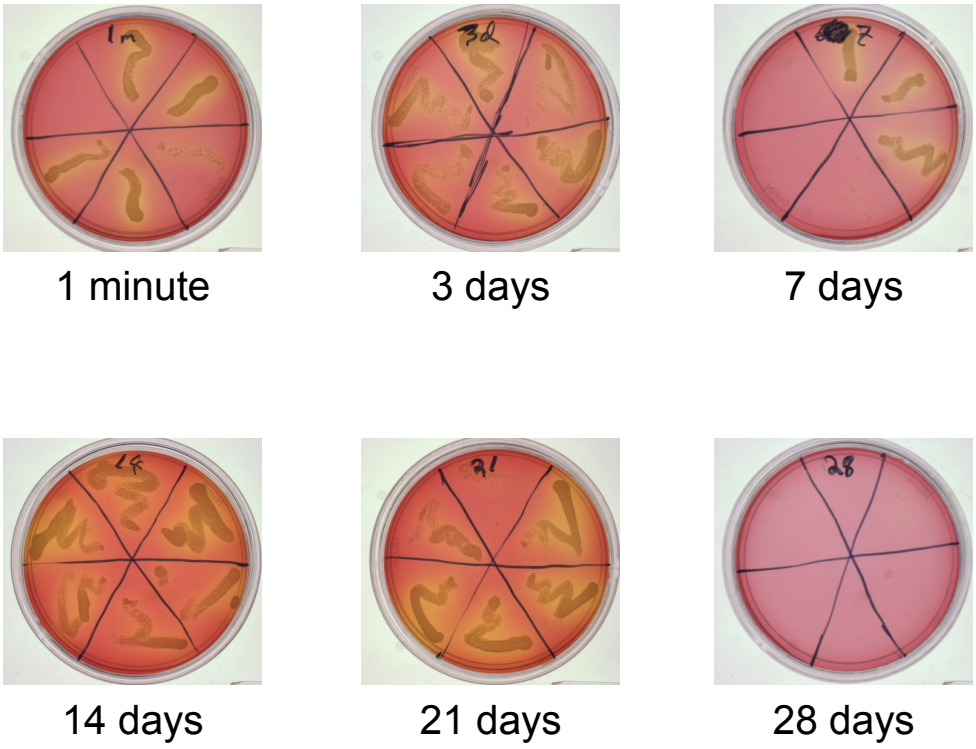


Figure A.2.4. Assaying presence of *S. aureus* in frozen samples from (Haine et al., 2008a)

(Haine et al., 2008a) claim to have infected T. molitor with S. aureus and recovered cells up to 28 days after infection, and that samples from replicate beetles at each time point were pooled and frozen at -90^{o}C. Subsequent concerns about methodology motivated re-plating these samples to test for the presence of *S. aureus*. Plating frozen samples of bacteria extracted from *T. molitor* up to 28 days after infection on Mannitol salt phenol red agar revealed that persistent *S. aureus* infection was established. Each scrape on each plate represents a different replicate frozen stock labeled as derived from that respective time-point. 6 replicate stocks were available for each 3 days, 14 days and 21 days. 5 were available for 1 minute, and 3 for 28 days. Golden halos around bacterial scrapes indicates the presence of *S. aureus*. No bacteria could be recovered from the sample of bacteria labeled to indicate that it contained bacteria extracted 28 days after *T. molitor* infection, which may indicate that either no S. aureus were present at this point, or that the cells did not survive freezing.

To further test (Haine et al., 2008a) and the applicability of perfusion bleeding *T. molitor* to assay for *S. aureus* persistence, part of their study was repeated, to check the validity of the previous results and the assay. Specifically, 70 beetles were injected with sterile PBS as a contamination control, and a further 70 were injected with washed S. aureus JLA 513, as described in Chapter 3. Beetles were perfusion bled and samples were plated as described in Chapter 3. Beetles were bled 1 minute after injection (samples diluted x 10^{-3}), 30 minutes after (samples diluted x 10^{-2}), 6 hours after, 3 days after, 7 days after, 14 days after, and 28 days after. Some beetles that were designated to be bled at later time-points died before they could be bled. The results (Figure A.2.5) show that this protocol established repeatable S. aureus infection. There was some contamination in some PBS-injected beetles, but most samples from sterility controls were clean. This shows that the approach used by (Haine et al., 2008a) was not perfect, but that modification by including a proper control and surface-sterilising beetles by washing them in ethanol before perfusion bleeding allows quantitative measure of infection and exclusion of most contamination.

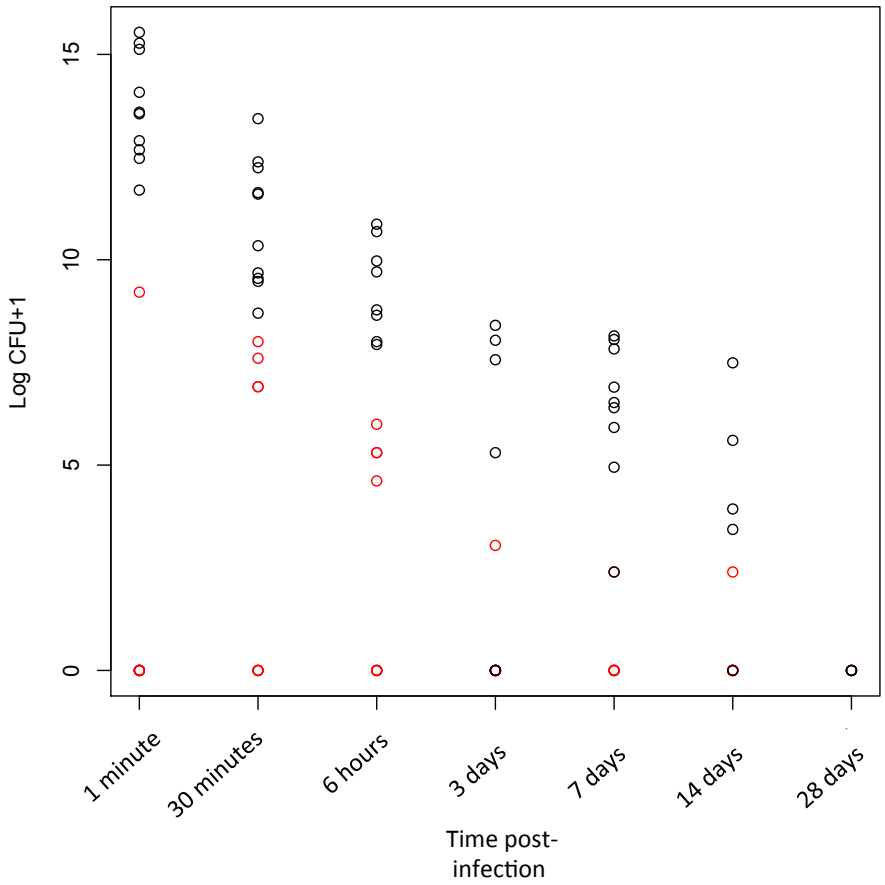


Figure A.2.5. Testing the application of perfusion bleeding techniques to persistent *S. aureus* infection of *T. molitor*.

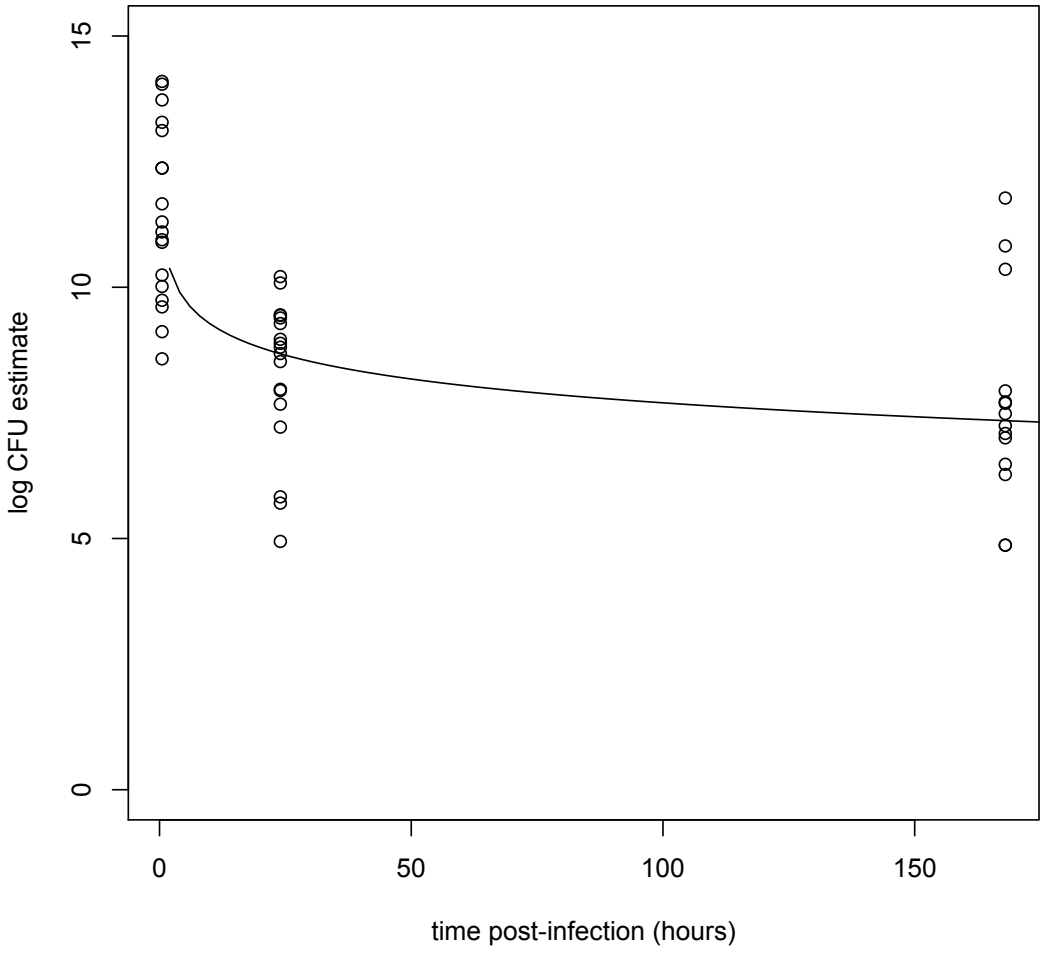
Beetles were infected and perfusion bled as previously (Haine et al., 2008a), with the modification of a sterile procedural control (red), and beetles were washed in ethanol before perfusion bleeding. This approach removes most systemic contamination across the full range of time points, as shown by the consistently greater CFU counts from *S. aureus*-infected beetles (black).

Appendix 3. Nonlinear least squares regression of selected *S. aureus* CFU in *T. molitor*

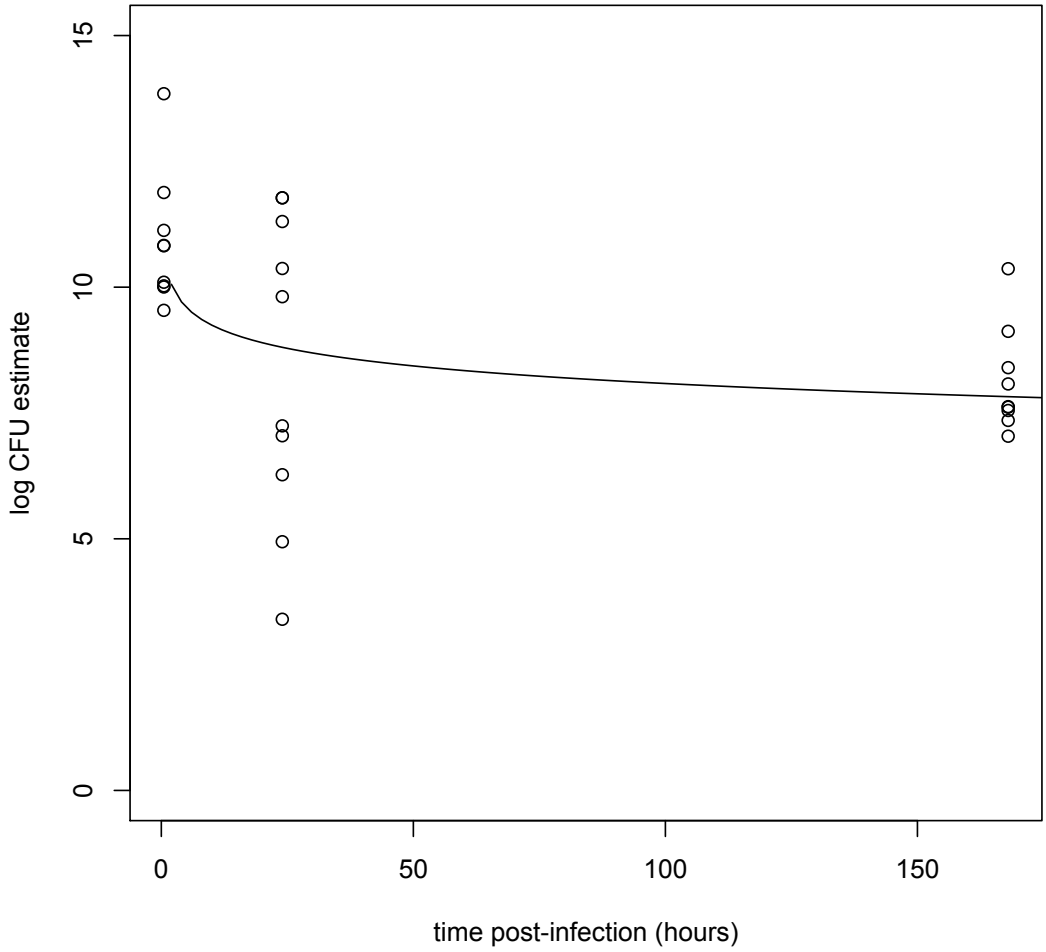
In analysis of data contributing to Chapter 3, nonlinear least-squares regression was used to fit a model of the form

$$\log(\boldsymbol{CFU\ estimate}) \sim \boldsymbol{a} + \boldsymbol{b} * \log(\boldsymbol{time})$$

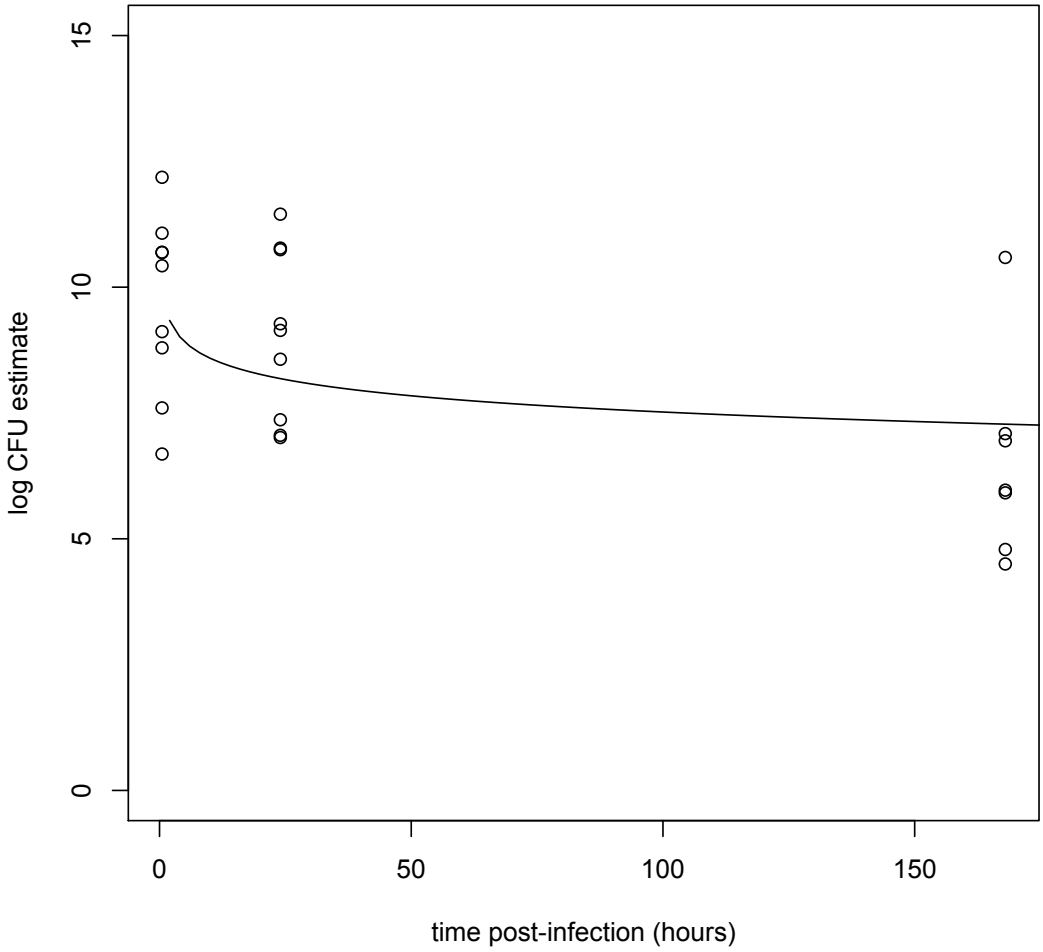
to the CFU estimates of selected bacteria extracted from infected T. molitor 30 minutes, 24 hours and 7 days after infection. The ***b*** parameter of this model describes the decay of the model (presented in Figure 3.4.2.2). The data from each bacterial treatment are plotted below in Figures A.3a on a normal time scale, with the fitted model modified appropriately.



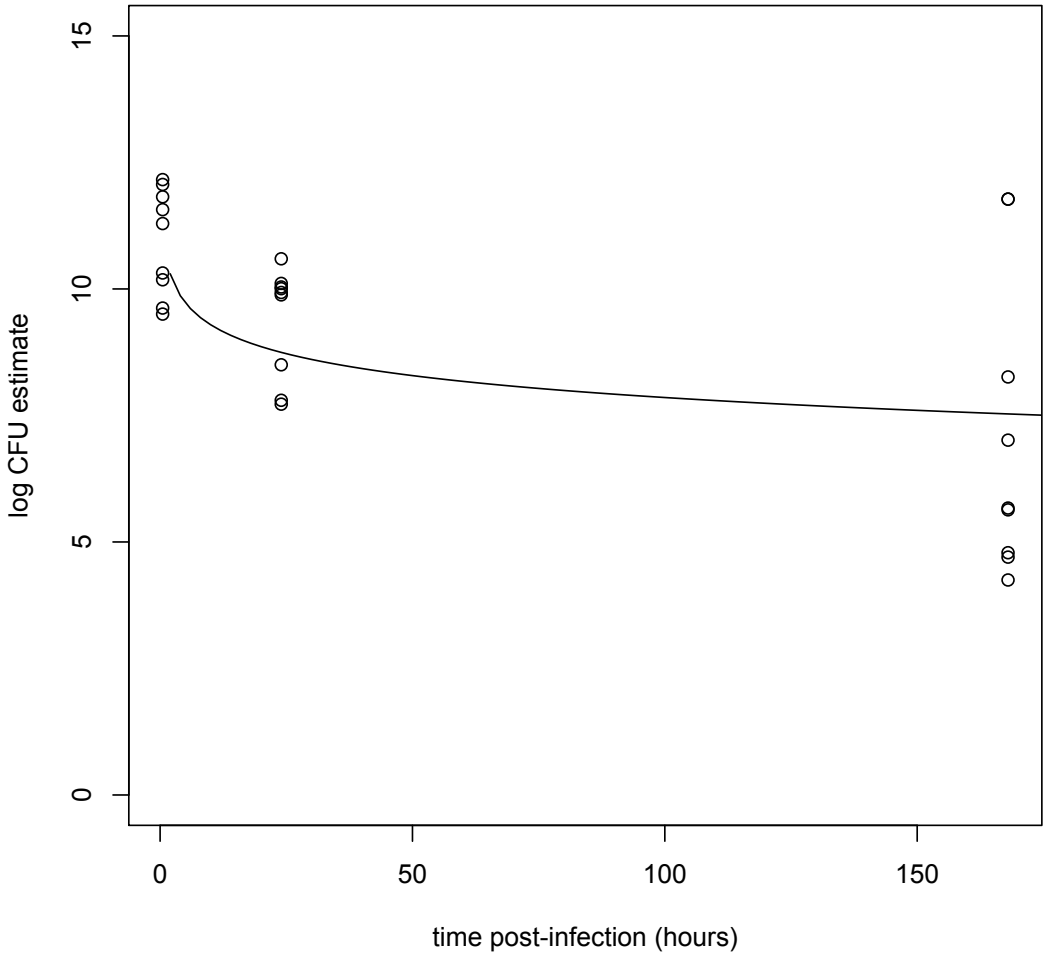
Figures A.3a. Ancestral *S. aureus* (day 0) persistence in *T. molitor*



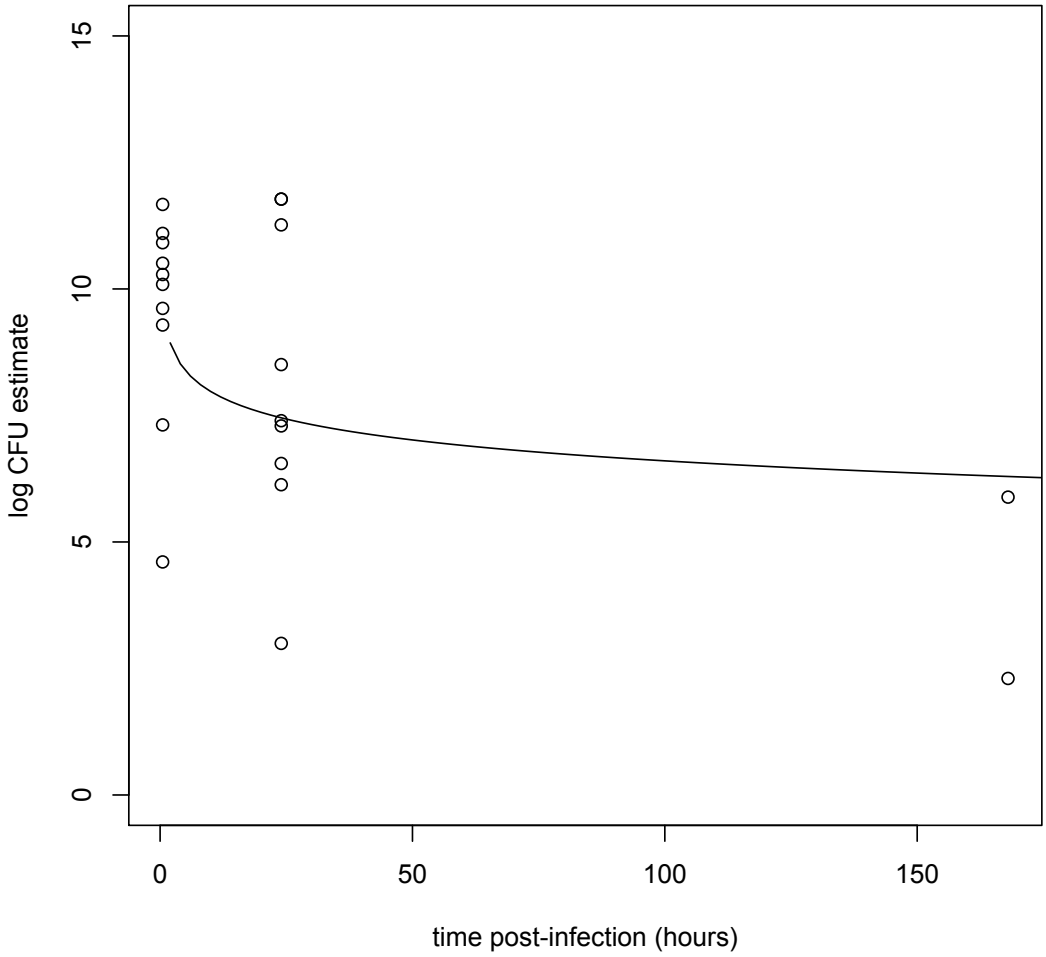
Figures A.3b. Melittin-selected *S. aureus* (day 28) persistence in *T. molitor*



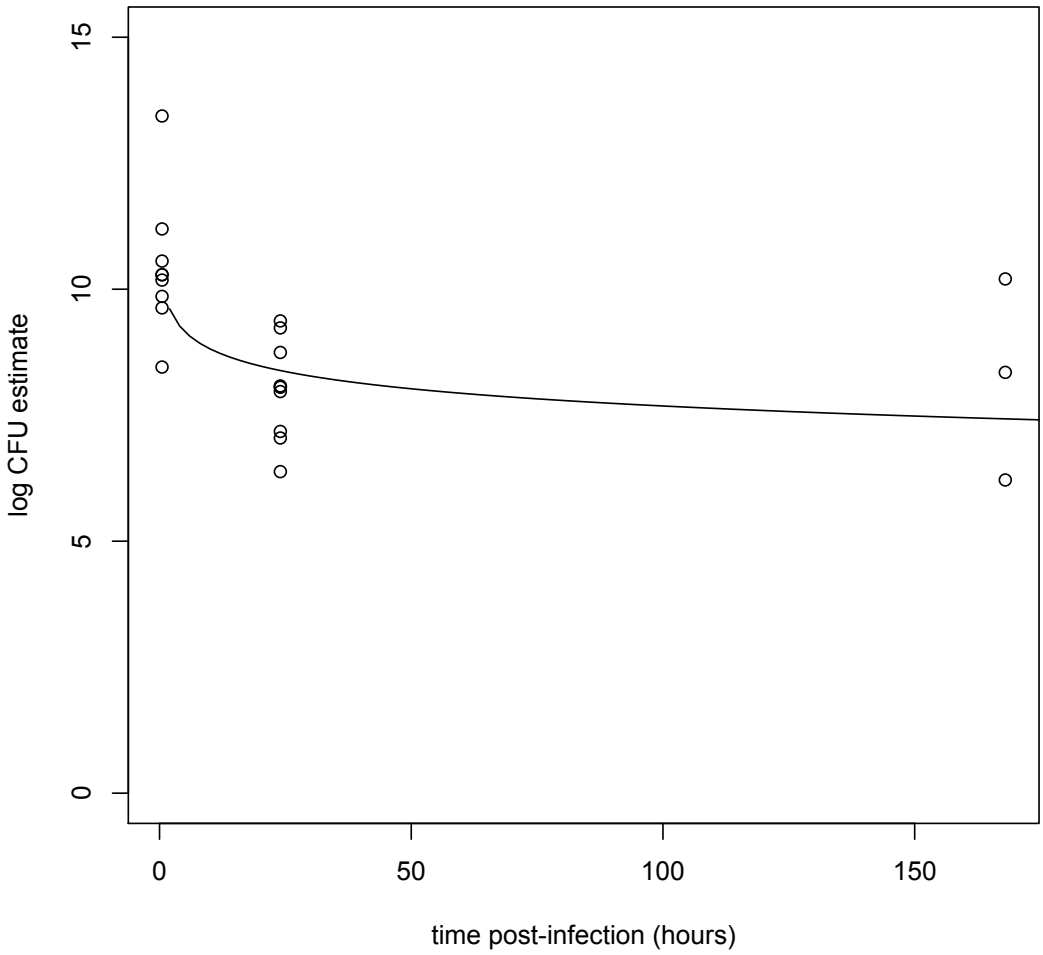
Figures A.3c. Pexiganan-selected *S. aureus* (day 14) persistence in *T. molitor*



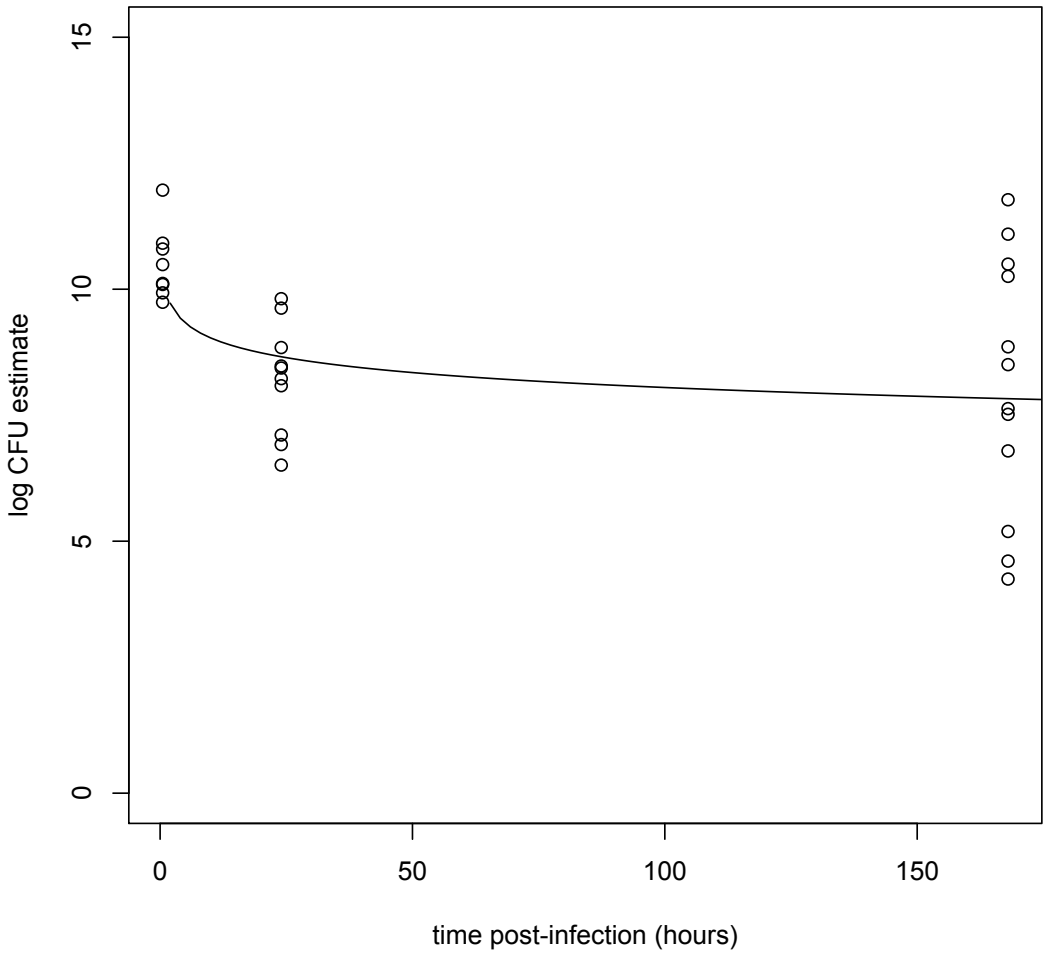
Figures A.3d. Pexiganan-selected *S. aureus* (day 28) persistence in *T. molitor*



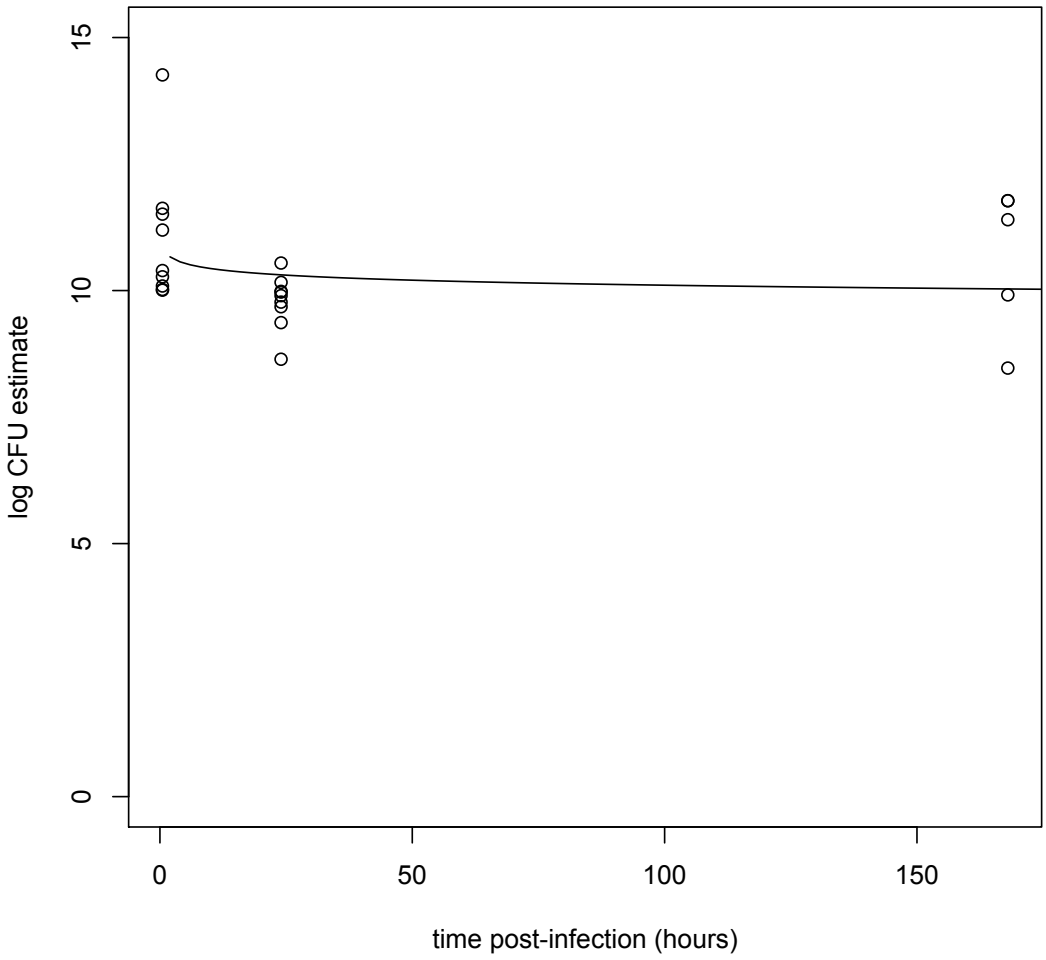
Figures A.3e. pgml-selected *S. aureus* (day 14) persistence in *T. molitor*



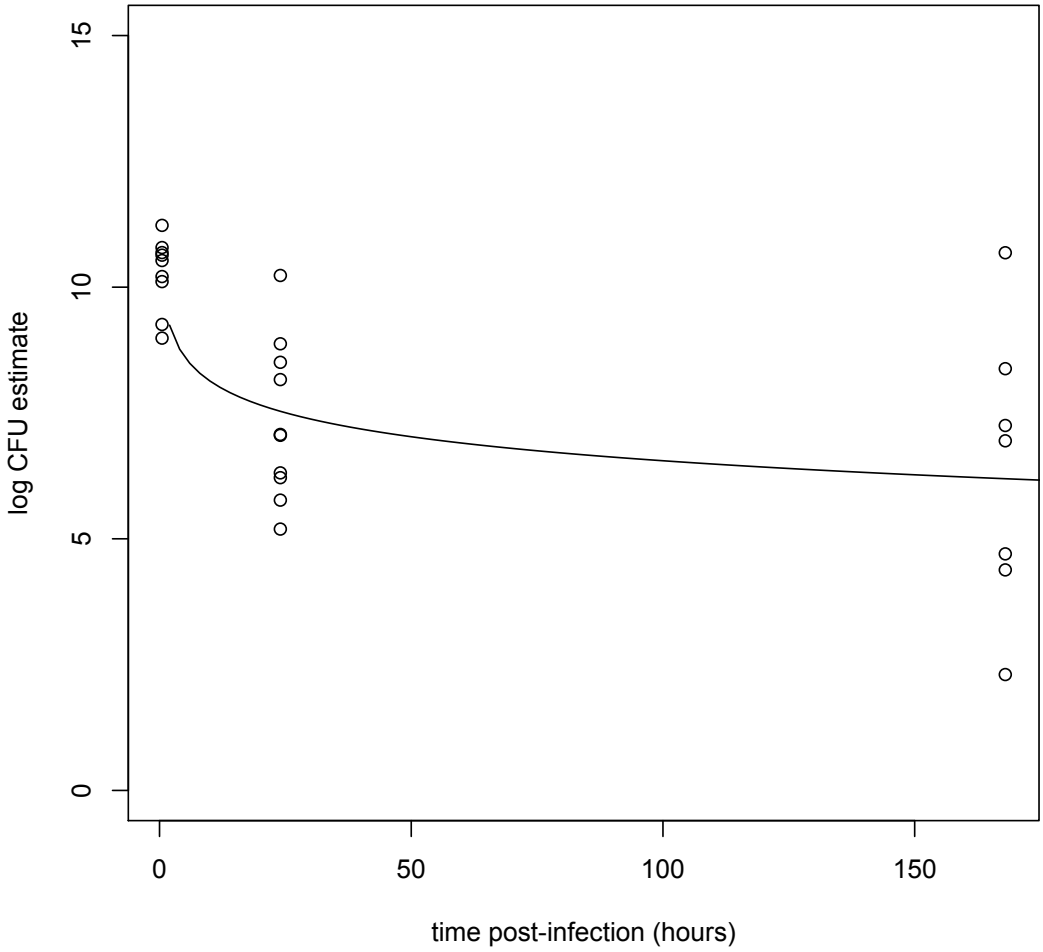
Figures A.3f. Unselected-selected *S. aureus* (day 14) persistence in *T. molitor*



Figures A.3g. Unselected-selected *S. aureus* (day 28) persistence in *T. molitor*



Figures A.3h. Streptomycin-selected *S. aureus* (day 28) persistence in *T. molitor*



Figures A.3i. Unselected-selected *S. aureus* (day 14) persistence in *T. molitor*

Appendix 4. Second attempt to measure costs of autopathological immunity in *T. molitor*

The results of the survival experiment presented in Chapter 4 were weakened by the effect of suspending bacterial cellular material in supernatant. This experiment was attempted a second time, but after washing the bacterial cultures and re-suspending them in an equal volume of sterile PBS. Dosage was not varied in this second experiment. Beetles were reared from the same stock as in Chapter 4, in which a microsporidian infection had previously been noted (see Figure 4.4.2.3). Survival curves of the beetles in this second experiment are presented in Figure A.4.1.

Unfortunately survival in all treatment groups was decreased relative to those in the first experiment. Pupal mass of the beetles that went into the repeated experiment was much diminished relative to the masses of the beetles used for the original experiment (Figure A.4.2), indicating poor general condition. No attempt was made to quantify microsporidia at either point, but it is plausible that the infection had worsened in the second experiment, which would account for poor condition of the beetles.

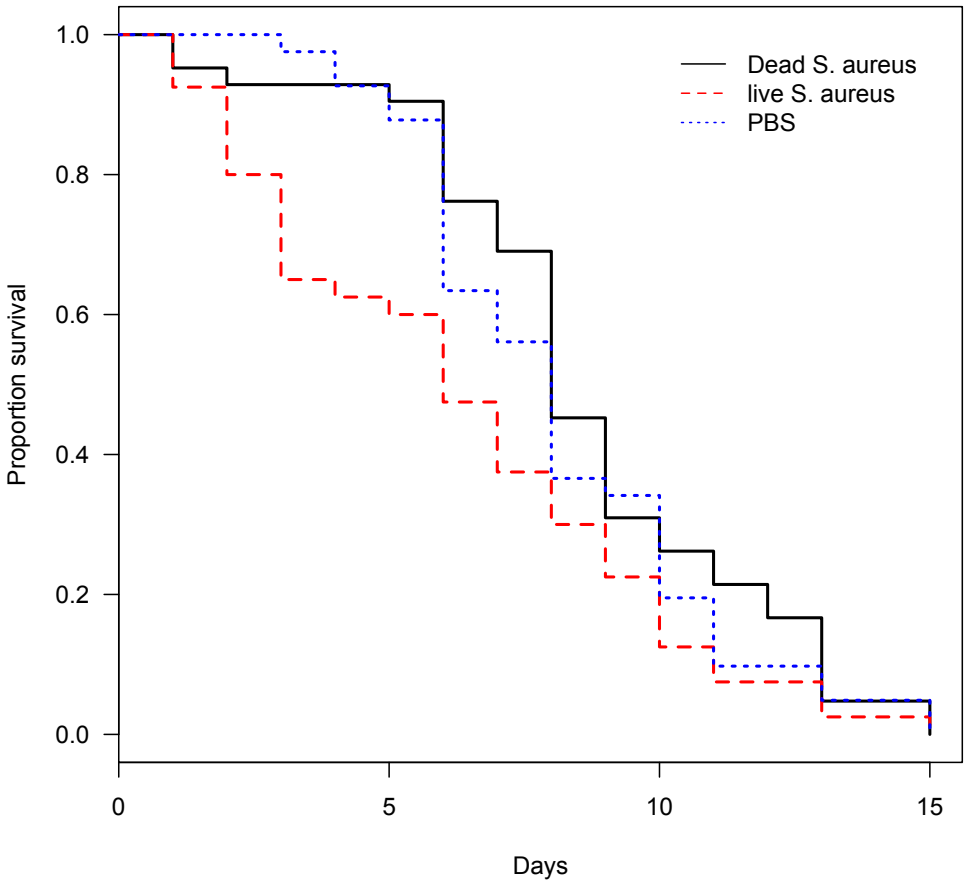


Figure A.4.1. Survival curves of T. molitor inoculated with equal doses of dead and live *S. aureus*

Beetles were inoculated with dead (heat-shocked) and live *S. aureus*, which had been washed and re-suspended in a volume of PBS equal to the volume of growth medium they grew in. Maximum beetle survival in all groups was much less than *T. molitor*'s the normal expected lifespan, suggesting poor condition.

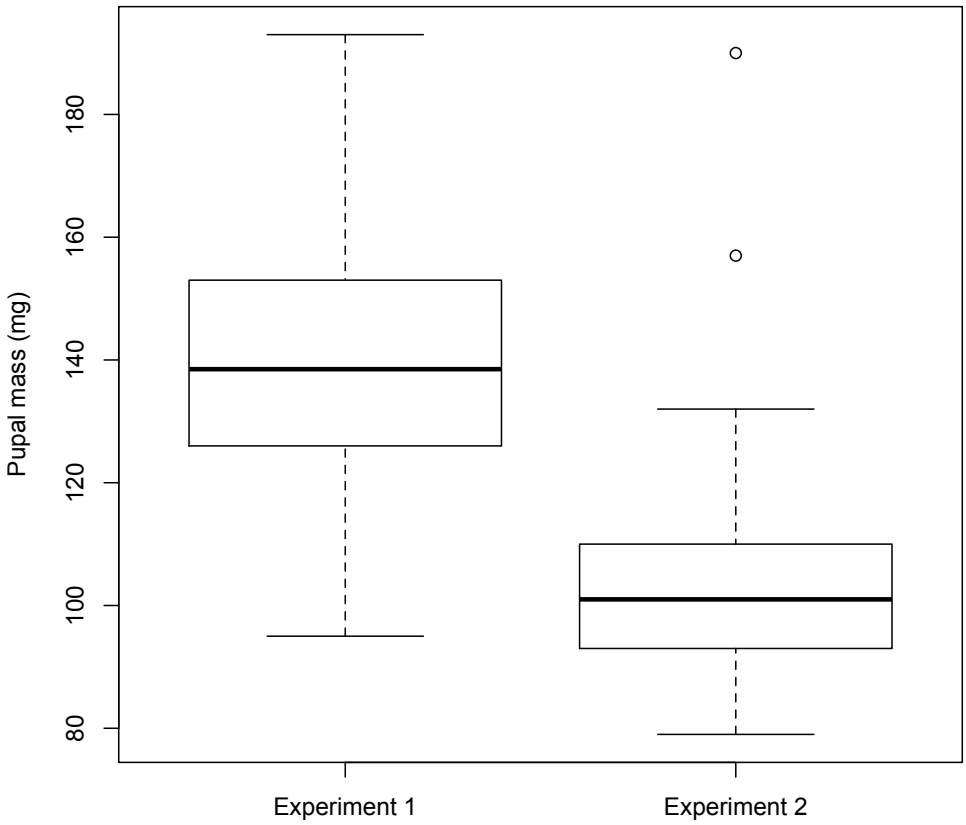


Figure A.4.2. Comparison of beetle pupal masses used for two post-immunopathology survival experiments

Pupal masses of beetles used for survival study 1 (Chapter 4, Figure 4.4.1.1) were considerably greater than the masses of those used for survival 2 (Figure A.4.1), consistent with poor general beetle condition.

Appendix 5. Full list of immune-induced mRNA sequences identified by SSH in Tenebrio molitor larvae after infection with Staphylococcus aureus JLA 513

The SSH transcriptomic analysis produced numerous BLAST hits that were assigned gene ontologies (Conesa et al., 2005). Gene ontologies (GOs) and each sequence's most significant BLASTx hit were used to classify the sequences by proposed function. The classification categories are based on those of (Altincicek et al., 2008a). The full list of BLASTx alignments are presented in Table A.5 with GOs.

Table A.5. Transcriptomic (SSH) characterisation of T. molitor's immune response to S. aureus infection: Gene Ontologies (GOs). Sequences are assigned to a certain proposed group based on what is known of their best BLAST hit and GO descriptions. GOs are automatically classified as biological process (BP), molecular function (MF) or cellular component (CC)

Proposed group	Best BLAST hit	GO group	GO description
Immune defence and effectors	laccase 1	BP	oxidation reduction
		BP	iron ion transport
		MF	copper ion binding
		MF	iron ion transmembrane transporter activity
		MF	oxidoreductase activity
Immune defence and effectors	transferrin	BP	cellular iron ion homeostasis
		BP	iron ion transport
		CC	extracellular region
		MF	ferric iron binding
Immune defence and effectors	tyrosine hydroxylase	BP	catecholamine biosynthetic process
		BP	oxidation reduction
		BP	tyrosine metabolic process
		MF	tyrosine 3-monooxygenase activity
		MF	iron ion binding
Immune defence and effectors	tyrosine hydroxylase	BP	developmental pigmentation
		BP	adult locomotory behavior
		BP	regulation of dopamine metabolic process
		BP	male courtship behavior
		BP	oxidation reduction
		BP	dopamine biosynthetic process
		BP	tyrosine metabolic process
		MF	tyrosine 3-monooxygenase activity
		MF	iron ion binding
Immune defence and effectors	dopa decarboxylase	BP	catecholamine biosynthetic process
		BP	histidine metabolic process
		BP	L-phenylalanine metabolic process
		BP	tryptophan metabolic process
		BP	tyrosine metabolic process
		BP	alkaloid biosynthetic

			process
		BP	indole biosynthetic process
		MF	pyridoxal phosphate binding
		MF	aromatic-L-amino-acid decarboxylase activity
Immune defence and effectors	multicopper oxidase	BP	oxidation reduction
		BP	iron ion transport
		MF	copper ion binding
		MF	iron ion transmembrane transporter activity
		MF	oxidoreductase activity
Immune defence and effectors	transferrin	BP	cellular iron ion homeostasis
		BP	iron ion transport
		CC	extracellular region
		MF	ferric iron binding
Immune defence and effectors	beta- -glucan recognition protein 2	BP	regulation of innate immune response
		CC	extracellular region
		MF	bacterial cell surface binding
Immune defence and effectors	multicopper oxidase	BP	oxidation reduction
		MF	oxidoreductase activity
		MF	copper ion binding
Immune defence and effectors	phenylalanine hydroxylase	BP	oxidation reduction
		BP	L-phenylalanine catabolic process
		BP	tryptophan biosynthetic process
		BP	tyrosine biosynthetic process
		BP	L-phenylalanine biosynthetic process
		MF	phenylalanine 4-monooxygenase activity
		MF	iron ion binding
		MF	amino acid binding
Immune defence and effectors	antibacterial peptide	CC	extracellular region
Immune defence and effectors	Coleoptericin (antimicrobial peptide)	BP	defense response to bacterium
		BP	innate immune response
		CC	extracellular region
Immune defence and effectors / stress	stress-induced-phosphoprotein 1	BP	encapsulation of foreign target

		BP	compound eye photoreceptor cell differentiation
		BP	protein folding
		BP	tyrosine phosphorylation of STAT protein
		BP	periodic partitioning
		BP	hindgut morphogenesis
		BP	open tracheal system development
		BP	humoral immune response
		BP	equator specification
		BP	germ-line stem cell division
		BP	ovarian follicle cell stalk formation
		BP	defense response to virus
		BP	Regulation of haemocyte differentiation
		BP	stem cell maintenance
		BP	somatic stem cell division
		BP	lamellocyte differentiation
		BP	primary sex determination
		BP	eye-antennal disc morphogenesis
		BP	imaginal disc-derived leg morphogenesis
		BP	Haemocyte proliferation
		BP	border follicle cell migration
		BP	STAT protein nuclear translocation
		BP	imaginal disc-derived wing morphogenesis
		BP	ommatidial rotation
		BP	cytokinesis
		BP	cellular defense response
		BP	signal transduction
		CC	Golgi apparatus
		CC	nucleus
		MF	Janus kinase activity
		MF	protein binding
		MF	non-membrane spanning protein tyrosine kinase activity
Immune defence and effectors	transferrin	BP	cellular iron ion homeostasis
		BP	iron ion transport
		CC	extracellular region

		MF	ferric iron binding
Immune defence and effectors	transferrin	BP BP CC MF	cellular iron ion homeostasis iron ion transport extracellular region ferric iron binding
Immune defence and effectors	ferritin heavy chain	BP BP BP CC CC MF MF MF	cellular iron ion homeostasis oxidation reduction iron ion transport Golgi apparatus intracellular ferritin complex ferrous iron binding oxidoreductase activity ferric iron binding
Immune defence and effectors	laccase 1	BP MF MF	oxidation reduction oxidoreductase activity copper ion binding
Immune defence and effectors	multicopper oxidase	BP BP BP MF MF MF	oxidation reduction electron transport iron ion transport laccase activity copper ion binding iron ion transmembrane transporter activity
Immune defence and effectors	transferrin	BP BP CC MF	cellular iron ion homeostasis iron ion transport extracellular region ferric iron binding
Immune defence and effectors	tyrosine hydroxylase	BP BP BP MF	cellular amino acid and derivative metabolic process cellular aromatic compound metabolic process cellular amine metabolic process "oxidoreductase activity
Immune defence and effectors	tyrosine hydroxylase	BP BP BP MF MF	catecholamine biosynthetic process oxidation reduction tyrosine metabolic process tyrosine 3-monooxygenase activity iron ion binding

Immune defence and effectors	tyrosine hydroxylase	BP BP BP MF MF	catecholamine biosynthetic process oxidation reduction tyrosine metabolic process tyrosine 3-monooxygenase activity iron ion binding
Immune defence and effectors	tyrosine hydroxylase	BP BP BP MF MF	catecholamine biosynthetic process oxidation reduction tyrosine metabolic process tyrosine 3-monooxygenase activity iron ion binding
Proteases	masquerade-like serine proteinase homologue	BP MF	proteolysis serine-type endopeptidase activity
Proteases	hemolymph proteinase 5 hemolymph proteinase 5	MF MF	serine-type peptidase activity peptidase activity
Proteases	trypsin-like proteinase	BP MF	proteolysis serine-type endopeptidase activity
Proteases	bleomycin hydrolase	BP MF	proteolysis cysteine-type endopeptidase activity
Proteases	clipa6 protein	BP MF	proteolysis serine-type endopeptidase activity
Proteases	proprotein convertase subtilisin kexin type furin	BP CC CC CC MF	proteolysis Golgi stack plasma membrane integral to Golgi membrane serine-type endopeptidase activity
Proteases	serine protease	BP MF	proteolysis serine-type endopeptidase activity
Catabolism	fatty acyl- reductase 1	MF	catalytic activity
Metabolism	amp dependent ligase	BP BP MF MF MF	transport acyl-carrier-protein biosynthetic process cofactor binding ligase activity acyl carrier activity

Metabolism	nad beta subunit	BP CC MF MF	metabolic process membrane binding NAD(P) transhydrogenase activity
Metabolism	carnitine o-acyltransferase synaptosomal-associated protein 29	BP BP BP MF MF	fatty acid metabolic process acyl-carrier-protein biosynthetic process glycerolipid metabolic process carnitine O-palmitoyltransferase activity protein binding
Metabolism	fatty acyl- reductase 1	BP CC CC MF MF	lipid metabolic process peroxisome membrane binding oxidoreductase activity
Metabolism	sphingolipid delta 4 desaturase c-4 hydroxylase protein des2	BP BP BP CC CC CC MF MF	spermatogenesis fatty acid biosynthetic process oxidation reduction plasma membrane integral to membrane mitochondrion stearoyl-CoA 9-desaturase activity sphingolipid delta-4 desaturase activity
Metabolism	quinone oxidoreductase	BP BP MF MF	oxidation reduction electron transport zinc ion binding NADPH:quinone reductase activity
Metabolism	fatty acyl- reductase cg5065-like	BP MF MF	metabolic process binding catalytic activity
Metabolism	amp dependent ligase	BP MF	metabolic process catalytic activity
Metabolism	nadh-ubiquinone oxidoreductase 24 kDa	BP BP CC MF MF	"mitochondrial electron transport electron transport mitochondrial respiratory chain complex I NAD or NADH binding NADH dehydrogenase

			activity
Metabolism	phosphoglyceromutase	BP MF	glycolysis "2
Ribosomal protein	60s acidic ribosomal protein p2	BP BP CC MF	translational elongation ribosome biogenesis ribosome structural constituent of ribosome
Signaling	lung seven transmembrane receptor	BP CC MF	signal transduction integral to membrane receptor activity
Stress-associated proteins	cytochrome p450	MF	metal ion binding
Stress-associated proteins	protein vac14 homolog	BP BP BP CC CC CC CC MF MF MF	response to osmotic stress signal transduction interspecies interaction between organisms microsome vacuolar membrane endoplasmic reticulum endosome membrane receptor activity kinase activator activity protein binding
Transcription and protein biogenesis	transcription initiation factor tfiid subunit 2	MF MF	zinc ion binding metallopeptidase activity
Transcription and protein biogenesis	-like protein subfamily b member 11	BP MF MF	protein folding heat shock protein binding unfolded protein binding
Transcription and protein biogenesis	tbc1 domain family member 19	BP CC MF	regulation of Rab GTPase activity intracellular Rab GTPase activator activity
Transcription and protein biogenesis	eukaryotic translation initiation factor 3 subunit b	BP CC CC MF MF MF	regulation of translational initiation eukaryotic translation initiation factor 3 complex ribosome RNA binding translation initiation factor activity nucleotide binding
Transcription and protein biogenesis	nuclear rna export factor 1	BP CC	mRNA transport nucleus

Transcription and protein biogenesis	translation elongation factor 2	BP CC MF MF MF MF	regulation of translational elongation ribosome translation elongation factor activity GTP binding protein binding GTPase activity
Transcription and protein biogenesis	histone transcription regulator	BP BP BP CC MF MF MF	chromatin modification gastrulation "regulation of transcription nuclear chromatin transcription regulator activity protein binding chromatin binding
Transcription and protein biogenesis	piwi	MF	nucleic acid binding
Transcription and protein biogenesis	arylsulfatase b	BP MF	metabolic process sulfuric ester hydrolase activity
Transcription and protein biogenesis	transposable element p transposase (p-element transposase)	MF	nucleic acid binding
Transcription and protein biogenesis	microphthalmia-associated transcription factor	BP BP CC MF	compound eye morphogenesis regulation of transcription nucleus transcription regulator activity
Transcription and protein biogenesis	dolichyl-diphosphooligosaccharide protein glycotransferase	BP CC MF	protein amino acid N-linked glycosylation lipid particle transferase activity
Transport proteins	nadh dehydrogenase subunit 4l	BP BP BP BP CC CC MF	"mitochondrial electron transport NADH to ubiquinone" ubiquinone biosynthetic process sodium ion transport proton transport respiratory chain mitochondrion NADH dehydrogenase (ubiquinone) activity
Transport proteins	organic cation	BP	transmembrane transport
Transport proteins	amino acid transporter	BP	amino acid transport

		CC	integral to membrane
Transport proteins	pleckstrin homology domain family f (with fyve domain) member 2	CC MF	transport vesicle zinc ion binding
Transport proteins	amp dependent ligase	BP BP MF MF MF	transport acyl-carrier-protein biosynthetic process cofactor binding ligase activity acyl carrier activity
No proposed group	peroxisome biogenesis factor 1	BP BP BP BP BP CC CC CC MF MF MF MF MF MF MF	microtubule-based peroxisome localization auxin biosynthetic process protein import into peroxisome matrix regulation of transcription multicellular organismal development cytosol peroxisomal membrane nucleus zinc ion binding "ATPase activity protein C-terminus binding protein complex binding lipid binding DNA binding ATP binding
No proposed group	serine threonine-protein phosphatase 4 regulatory subunit 2	BP CC MF	mitotic cell cycle protein phosphatase 4 complex protein phosphatase regulator activity
No proposed group	gtp cyclohydrolase i isoform b	BP BP BP BP BP BP BP BP BP	tetrahydrofolate biosynthetic process compound eye pigmentation embryonic pattern specification tetrahydrobiopterin biosynthetic process pteridine biosynthetic process cuticle pigmentation one-carbon metabolic process preblastoderm mitotic cell

			cycle
		BP	larval chitin-based cuticle development
		BP	folic acid biosynthetic process
		CC	cytoplasm
		MF	GTP cyclohydrolase I activity
No proposed group	-like protein subfamily c member 22	BP	"liquid clearance
		BP	"regulation of tube length
		BP	extracellular matrix organization
		BP	endocytosis
		CC	early endosome
		CC	plasma membrane
		CC	cytoplasmic vesicle
		MF	heat shock protein binding
No proposed group	chaperone protein dnaj	BP	spermatogenesis
		BP	protein folding
		BP	"DNA damage response
		BP	androgen receptor signaling pathway
		BP	sperm motility
		BP	response to heat
		BP	signal transduction
		CC	cytoplasm
		CC	membrane
		MF	metal ion binding
		MF	low-density lipoprotein receptor binding
		MF	heat shock protein binding
		MF	ATP binding
		MF	unfolded protein binding
No proposed group	ubiquitin c	BP	axon guidance
		BP	ER-associated protein catabolic process
		BP	positive regulation of transcription
		BP	regulation of synaptic plasticity
		BP	induction of apoptosis by extracellular signals
		BP	anti-apoptosis
		BP	positive regulation of ubiquitin-protein ligase activity during mitotic cell cycle
		BP	long-term strengthening of neuromuscular junction

		BP	anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process
		BP	negative regulation of ubiquitin-protein ligase activity during mitotic cell cycle
		BP	ribosome biogenesis
		CC	nucleoplasm
		CC	endosome membrane
		CC	cytosolic small ribosomal subunit
		MF	transcription regulator activity
		MF	protein binding
		MF	structural constituent of ribosome
No proposed group	gtp cyclohydrolase i	BP	tetrahydrofolate biosynthetic process
		BP	compound eye pigmentation
		BP	embryonic pattern specification
		BP	tetrahydrobiopterin biosynthetic process
		BP	pteridine biosynthetic process
		BP	cuticle pigmentation
		BP	one-carbon metabolic process
		BP	preblastoderm mitotic cell cycle
		BP	larval chitin-based cuticle development
		BP	folic acid biosynthetic process
		CC	cytoplasm
		MF	GTP cyclohydrolase I activity
No proposed group	rab gdp-dissociation inhibitor	BP	neurotransmitter secretion
		BP	regulation of GTPase activity
		BP	vesicle-mediated transport
		BP	protein transport
		CC	synaptic vesicle
		MF	Rab GDP-dissociation inhibitor activity
No proposed group	serine threonine-protein kinase	BP	auxin biosynthetic process

	tao1		
		BP	protein amino acid phosphorylation
		BP	apoptosis
		BP	transmembrane receptor protein serine/threonine kinase signaling pathway
		BP	serine family amino acid metabolic process
		MF	receptor signaling protein serine/threonine kinase activity
		MF	ATP binding
No proposed group	anopheles stephensi	BP	oxidation reduction
		MF	oxidoreductase activity
		MF	copper ion binding
No proposed group	maternal expression at isoform b	BP	auxin biosynthetic process
		BP	cytoplasmic mRNA processing body assembly
		BP	"nucleobase
		CC	cytoplasmic mRNA processing body
		MF	SUMO binding
		MF	ATP-dependent RNA helicase activity
		MF	RNA binding
		MF	ATP binding

Appendix 6. Model selection for survival analysis (accelerated failure time model)

A range of models were fitted to the survival data presented in Chapter 3. The models with their respective residuals are presented in Figure A.6. The Weibull distribution was chosen as the best fit based on Akaike's information criterion.

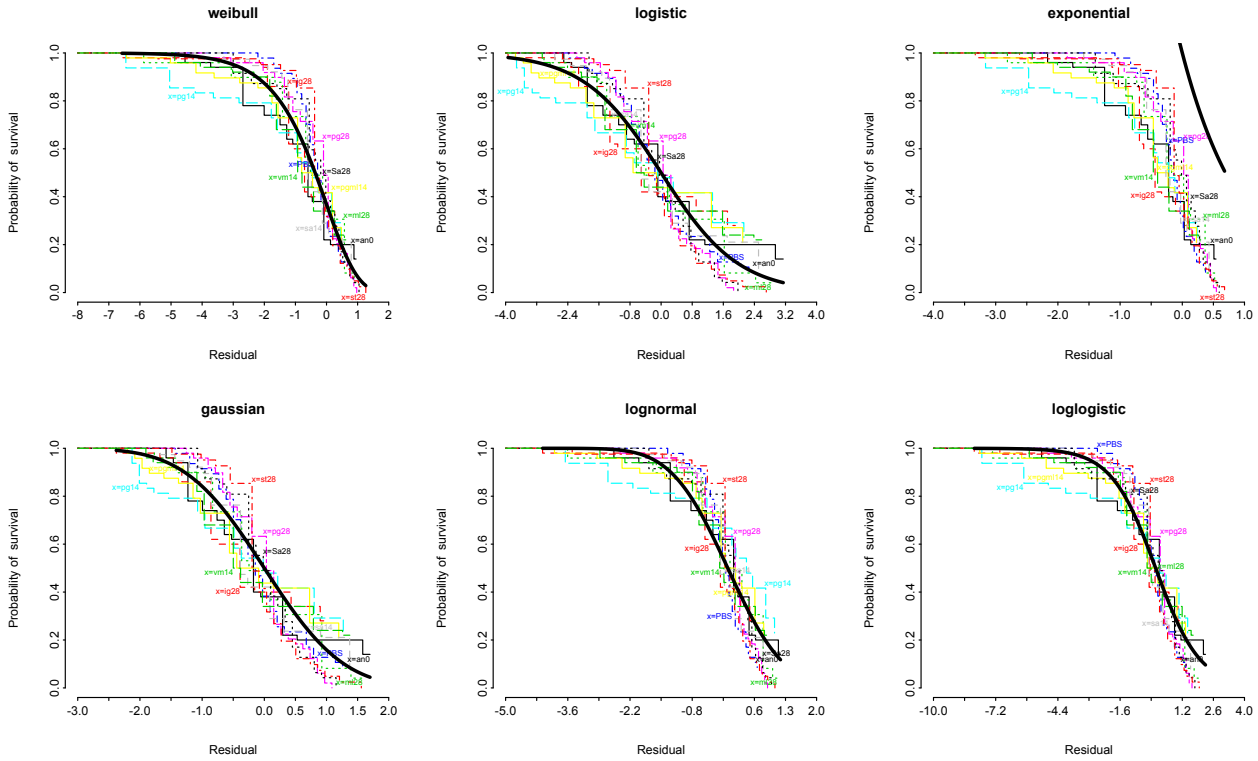


Figure A.6. Model selection for accelerated failure time analysis, showing fitted model (black lines) and residuals (coloured lines). The Weibull distribution was chosen as the best-fitting model (Akaike's information criterion values: Weibull = 3328.198, logistic = 3416.745, exponential = 3566.084, gaussian = 3412.853, lognormal = 3445.279, loglogistic = 3377.702; Equivalent degrees of freedom for all treatments = 12)

Appendix 7. Estimated densities of *S. aureus* injected into *T. molitor* (Chapter 3)

T. molitor were injected with bacteria that had been selected for AMP resistance, antibiotic resistance, unselected serially cultured controls, or the ancestor of all these strains. There was some variation in the injected densities of these bacteria, shown in the tables below. Table A.7.1 shows the densities of *S. aureus* injected to beetles whose survival was monitored. Table A.7.2 shows the densities of *S. aureus* injected into *T. molitor* before monitoring persistence in the beetle.

Table A.7.1. Injected CFU estimates of *S. aureus* strains injected into *T. molitor* for survival study (Chapter 3)

Strain	Injected CFU estimate
Ancestor	**1.57E+07**
Iseganan 28	**2.06E+07**
Melittin	**2.18E+08**
Pexiganan 14	**1.19E+07**
Pexiganan 28	1.18E+07
pgml 14	**1.34E+07**
Unselected 14	**1.34E+07**
Unselected 28	1.16E+07
Streptomycin	**6.54E+07**
Vancomycin	**1.21E+07**

Table A.7.2. Injected CFU estimates of *S. aureus* strains injected into *T. molitor* for persistence study (Chapter 3)

Strain	Injected CFU estimate
Ancestor (2 separate days)	7.20E+06
	1.67E+07
Iseganan 28	4.64E+07
Melittin	7.00E+06
Pexiganan 14	4.70E+08
Pexiganan 28	9.40E+06
pgml 14	1.17E+07
Unselected 14	2.08E+08
Unselected 28	1.16E+07
Streptomycin	1.28E+07
Vancomycin	1.11E+07

Appendix 8. Stipulations of qPCR primer design.

qPCR primers were designed using the standard Primer3 input, with the following stipulations:

- 90-110 bps product
- Minimum primer tm = 59c, optimum tm = 60c, maximum tm = 61c
- Maximum self-complimentarity = 4
- Maximum 3' complimentarity = 1
- Return >20 sequences