The effects of microbial priming with reactive oxygen species: an *in vitro* and an *in vivo* approach

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ARPITA NATH

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Declaration of Independence

Herewith I certify that I have prepared and written my thesis independently and that I have not used any sources and aids other than those indicated by me. This dissertation has not yet been presented to any other examination authority in the same or a similar form and has not yet been published.

Berlin, 30th November 2022

Arpita Nath

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List of abbreviations

Ecc15	*Erwinia carotovora carotovora 15*
CFU	Colony-forming units
H_2O_2	Hydrogen peroxide
LC-MS	Liquid chromatography-mass spectrometry
LB	Lysogeny Broth
SYA	Standard sugar yeast agar
GO	Gene ontology
HPLC	High-performance liquid chromatography
PCA	Principal component analysis

Summary

Organisms need efficient ways to store environmental information so that they can easily use it in the future and react to their environment appropriately. This ability has usually been associated with organisms having a central nervous system. Recently more and more evidence has piled up suggesting organisms like plants, fungi and bacteria also possess the ability to store the encounters from the past and inform their future decisions based on these past encounters. This thesis deals with one such instance of microbes storing past stresses and using this information for improved survival in case of future stresses. This phenomenon has been termed “priming”, which refers to organisms’ ability to show heightened immune responses on a second exposure to stress. The first exposure to stress is a sub-lethal dose followed by a lethal dose, referred to as the challenge dose. Priming helps organisms survive stresses that would have otherwise been lethal, providing a considerable survival advantage, but nothing comes without a cost. Priming also comes at a cost since organisms spend energy in heightening their immune responses. Still, this cost is usually negligible compared to the survival benefits it provides in a stressful environment. Microbes are constantly present in settings such as wastewater treatment plants and antibiotic production units, where they are always under exposure to low doses of antibiotics. This exposure to low doses of antibiotics could arm them against the lethal doses of antibiotics, adding to an already growing problem of resistance emergence. The priming phenomenon has been tested in several microbial species and stresses such as pH, temperature, osmotic pressure, salt stress, antimicrobial peptides, etc. All the existing literature on priming deals with priming and challenge stress where microbes are in the same environment. This gives us a robust idea of priming being a generalised phenomenon but does not tell us if priming confers an advantage in natural settings. In nature, most microbes do not stay in the same environment and are dynamically jumping across environments. Among other stresses, microbes are constantly exposed to oxidative stress in nature, either because of the presence of reactive oxygen species (ROS) in antiseptics or ROS immune defences inside the host. ROS levels are tightly regulated in organisms by enzymes such as peroxidases and catalases since excess ROS can be lethal. When a host, such as an insect, is infected with microbes, ROS is the first line of defence to fight the infection.

In this thesis, ROS priming in an *in vitro* and *in vivo* setting was studied using a phytopathogen *Erwinia carotovora carotovora 15* (*Ecc15*), a causal agent of soft rot in crop plants. It was tested if *in vitro* priming with ROS, specifically hydrogen peroxide, leads to improved survival upon receiving a challenge. It was found that priming leads to enhanced survival upon *in vitro* challenge. The phenotypic markers associated with peroxide priming were then investigated using LC-mass spectrometry. Testing the advantage of primed bacteria inside the host

Drosophila melanogaster highlighted the need to consider costs associated with *in vitro* priming, leading to differential bacterial numbers in primed and non-primed treatments. These costs were then reduced from 50% to 4% by testing a lower range of priming concentrations before testing the advantages of priming with lower concentrations of ROS inside the host. It was established that the effect of priming inside the host differs with time and sex of the host, possibly due to sexual dimorphism in ROS amounts inside male and female *D. melanogaster*.

Zusammenfassung

Organismen benötigen effiziente Möglichkeiten zur Speicherung von Umweltinformationen, damit sie diese in der Zukunft problemlos nutzen und angemessen auf ihre Umwelt reagieren können. Diese Fähigkeit wird normalerweise mit Organismen in Verbindung gebracht, die über ein zentrales Nervensystem verfügen. In letzter Zeit häufen sich die Hinweise darauf, dass auch Organismen wie Pflanzen, Pilze und Bakterien die Fähigkeit besitzen, Begegnungen aus der Vergangenheit zu speichern und ihre zukünftigen Entscheidungen auf der Grundlage dieser vergangenen Begegnungen zu treffen. In dieser Arbeit geht es um einen solchen Fall, in dem Mikroben vergangene Belastungen speichern und diese Informationen für ein besseres Überleben im Falle künftiger Belastungen nutzen. Dieses Phänomen wird als "Priming" bezeichnet, was sich auf die Fähigkeit von Organismen bezieht, bei einer zweiten Stressbelastung eine verstärkte Immunreaktion zu zeigen. Die erste Stressbelastung ist eine sub-tödliche Dosis, gefolgt von einer tödlichen Dosis, der sogenannten Challenge-Dosis. Das Priming hilft den Organismen, Belastungen zu überleben, die andernfalls tödlich gewesen wären, und verschafft ihnen damit einen erheblichen Überlebensvorteil, aber nichts ist ohne Preis. Auch das Priming ist mit Kosten verbunden, da die Organismen Energie aufwenden, um ihre Immunreaktionen zu verstärken. Diese Kosten sind jedoch in der Regel vernachlässigbar im Vergleich zu dem Überlebensvorteil, den sie in einer stressigen Umgebung bieten. Mikroben sind ständig in Anlagen wie Kläranlagen und Produktionsanlagen für Antibiotika anzutreffen, wo sie stets niedrigen Dosen von Antibiotika ausgesetzt sind. Diese Exposition gegenüber niedrigen Dosen von Antibiotika könnte sie gegen die tödlichen Dosen von Antibiotika bewaffnen, was das bereits wachsende Problem der Resistenzbildung noch verschärft. Das Priming-Phänomen wurde bei verschiedenen Mikrobenarten und Stressfaktoren wie pH-Wert, Temperatur, osmotischer Druck, Salzstress, antimikrobielle Peptide usw. getestet. Die gesamte vorhandene wissenschaftliche Literatur über Priming befasst sich mit Priming und Challenge-Stress, bei dem sich die Mikroben in derselben Umgebung befinden. Dies vermittelt uns eine solide Vorstellung davon, dass Priming ein allgemeines Phänomen ist, sagt aber nichts darüber aus, ob Priming in natürlichen Umgebungen einen Vorteil verschafft. In der Natur bleiben die meisten Mikroben nicht in derselben Umgebung und wechseln dynamisch zwischen verschiedenen Umgebungen. Neben anderen Belastungen sind Mikroben in der Natur ständig oxidativem Stress ausgesetzt, entweder durch das Vorhandensein reaktiver Sauerstoffspezies (ROS) in Antiseptika oder durch die ROS-Immunabwehr des Wirts. Der ROS-Gehalt wird in Organismen durch Enzyme wie Peroxidasen und Katalasen streng reguliert, da ein Übermaß an ROS tödlich sein kann. Wenn ein Wirt z. B. ein Insekt mit Mikroben infiziert wird, ist ROS die erste Verteidigungslinie zur Bekämpfung der Infektion.

In dieser Arbeit wurde das ROS-Priming in einer In-vitro- und In-vivo-Umgebung mit dem Phytopathogen *Erwinia carotovora carotovora 15* (*Ecc15*), einem Erreger der Weichfäule bei Nutzpflanzen, untersucht. Es wurde getestet, ob das Priming mit ROS, insbesondere Wasserstoffperoxid in vitro zu einem verbesserten Überleben nach einer Herausforderung führt. Es wurde festgestellt, dass das Priming zu einem verbesserten Überleben nach einer In-vitro-Herausforderung führt. Die phänotypischen Marker, die mit dem Peroxid-Priming in Verbindung stehen, wurden anschließend mittels LC-Massenspektrometrie untersucht. Die Prüfung des Vorteils geprimter Bakterien im Wirtsorganismus *Drosophila melanogaster* machte deutlich, dass die mit dem In-vitro-Priming verbundenen Kosten berücksichtigt werden müssen, was zu einer unterschiedlichen Anzahl von Bakterien in geprimten und nicht geprimten Behandlungen führte. Diese Kosten wurden dann von 50 % auf 4 % gesenkt, indem ein geringerer Bereich von Priming-Konzentrationen getestet wurde, bevor die Vorteile des Primings mit niedrigeren ROS-Konzentrationen im Wirtsorganismus geprüft wurden. Es wurde festgestellt, dass die Wirkung des Primings im Inneren des Wirts von der Zeit und dem Geschlecht des Wirts abhängt, was möglicherweise auf den sexuellen Dimorphismus der ROS-Mengen im Inneren von männlichen und weiblichen *D. melanogaster* zurückzuführen ist.

Section 1

General Introduction

1.1 Memory, a trait of higher organisms?

Acquiring, processing, storing, and retrieving past information are important to react to future circumstances in a well-informed manner. The latter has been termed memory, an ability generally attributed to higher organisms (S. B. Klein 2015). This ability will affect the response of organisms to upcoming stimuli, helping them modulate decision-making based on their past environments. Memory retention can either be a short-term or a long-term phenomenon. The absence of this ability is known to lead to severe disorders, such as amnesia, in humans (Kandel, Dudai, and Mayford 2014). In higher organisms, the mechanisms acting behind memory formation are via changes in the membrane potential of neurons and persistent protein modifications (Axmacher et al. 2006; Sweatt 2016).

We use memory as a daily tool for efficient decision-making, but it is a quality we do not necessarily associate with simpler organisms such as bacteria, plants, and fungi. These simpler organisms lack a nervous system, which we consider the focal point of memory formation and processing. Nevertheless, is neuronal memory the only way of memory formation? Synthetic biologists have been trying to encode memory into simple systems by implementing simple on/off switches using Boolean functions (Yang et al., 2020). It has also been demonstrated that it is possible to encode memory in biofilms by encoding it into individual bacterial cells of the biofilm (C. K. Lee et al,. 2018).

Bacteria, for one, are not particularly known for their ability to memorise information. However, do simple organisms or organisms lacking a dedicated nervous system have this ability to store traces of their past? Memory mechanisms in bacterial cells are studied mainly in the context of bacterial immunity to foreign genetic materials. Examples include restriction-modification systems and, more recently, the CRISPR-Cas9 system (Doudna and Charpentier 2014; Du Toit 2015). Recently, there has been piling evidence that organisms such as bacteria, fungi and plants can remember past events (Casadesús and D'Ari 2002; Bruce et al. 2007; Thellier and Lüttge 2013). They even have the capacity to shape their responses to future environments based on past events, making their responses plastic. This kind of plasticity allows a remarkable adaptation of the physiological phenotype in response to the environment they are currently in (Sultan 2001). To cite one such example, persister cells, bacterial subpopulations with temporarily acquired antibiotic resistance in large bacterial biofilms, were shown to retain their persistent phenotype for up to four weeks after their withdrawal from the biofilm in *Escherichia coli* (Miyaue et al. 2018). The persister phenotype was "remembered" even when cells were in a different environment (Miyaue et al. 2018).

Furthermore, vaccinations also rely on a form of immunological memory. The duration of such memory can differ from a few days in the case of invertebrates (Kurtz and Franz 2003) to a

lifetime in the case of mammals (Banatvala, Van Damme, and Oehen 2000). In some cases, the immunological memory is even carried on to the offspring and is known as trans-generation immune priming (TGIP) (Tetreau et al. 2019; Wilson et al. 2021). Although there is the presence of memory-like phenomena in bacteria, one crucial issue that arises while talking about it is the inconsistency in the definition of "memory" used by experts across different fields. There is also an ambiguity whether the memory phenomenon is acquired protection due to an ongoing response or due to reactivation of memory cells being reactivated because of a "boost". Pradeu and Pasquier (Pradeu and Pasquier 2018) deal exclusively with the ambiguity in definitions of memory and tackle the concept based on five key features of memory, namely strength, speed, extinction, duration and specificity. They further mention that in the case of bacterial memory, the second response is stronger and quicker, quite specific and provides long-lasting protection. The major difference in bacterial memory compared to other organisms is that it is a population-level phenomenon rather than an individual one.

1.2 Priming phenomena in "simple" organisms

There are numerous other examples where research has demonstrated that organisms lacking a dedicated nervous system can recognise past stimuli when needed to make decisions about their current or future environments. For example, plants that have experienced heat stress in the past enable them to subsequently survive temperatures that would be otherwise lethal (Thellier and Lüttge 2013). This rapid response has been known for many years and is highly conserved across kingdoms (Borges et al. 2014). This ability of simpler organisms to recognise past stresses and use this information to heighten their immune defences on a second confrontation with similar stresses has been termed "**priming**" (Andrade-Linares, Lehmann, and Rillig 2016; Hilker et al. 2016; Rodríguez-Rojas et al. 2020). It is an adaptive defence strategy where the presence of mild stressors can "prime" organisms to the upcoming lethal (challenge) doses of stressors (Figure 1). A similar phenomenon is named "immune priming", where exposure to a non-lethal dose of a pathogen renders the individuals resistant to upcoming lethal infections (Kurtz 2005; Sheehan, Farrell, and Kavanagh 2020). This phenomenon has been observed across different taxa and is hypothesised to be present even in certain cell types in higher vertebrates (Kurtz 2004; Paust, Senman, and von Andrian 2010). For example, priming *Drosophila melanogaster* with non-lethal doses of *Streptococcus pneumoniae* protects against a second exposure to lethal concentrations of the same pathogen that would otherwise be lethal, with this protection persisting for the entire life of the fly (Pham et al. 2007). The term priming is used in various fields like psychology and neurobiology in a discipline-dependent manner but essentially means an improved response on a second encounter.

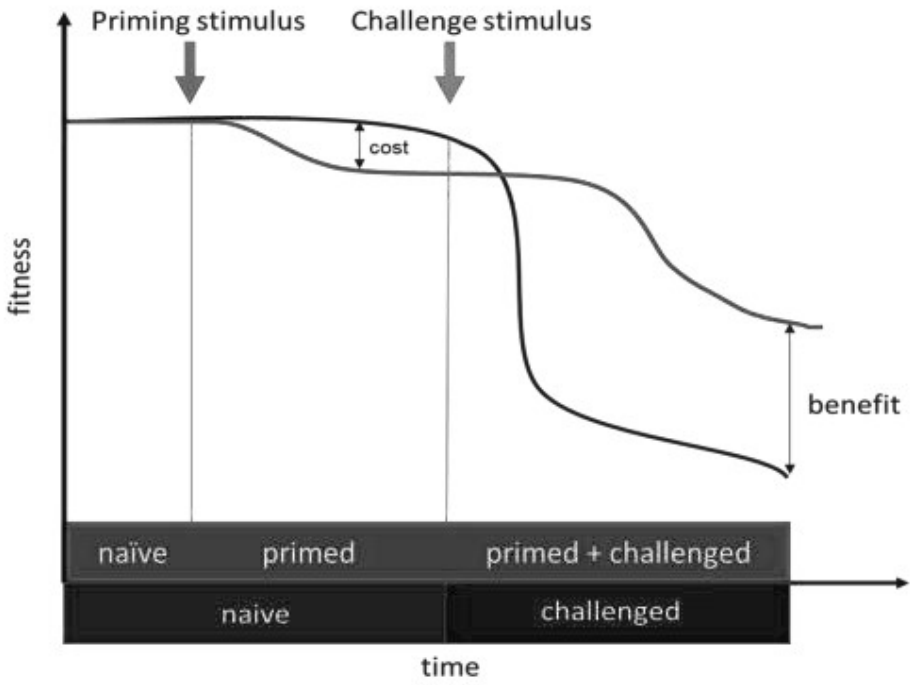

Figure 1: An illustration modified from Hilker *et al.,*2016 (Hilker et al. 2016) on how a priming stimulus provides a fitness advantage to the primed individuals after experiencing a challenge stimulus (lethal dose of the stressor). Although priming comes at a small fitness cost, this cost is significantly less compared to the fitness benefit it provides compared to a naïve bacterium. Primed stimulus refers to sub-lethal stress, and challenge stimulus refers to lethal stress.

In the case of priming, the stressors can be anything ranging from pathogens, antimicrobials, salt conditions, and drought conditions, depending on the environment the organism lives in. Upon perceiving stress, changes may occur at the physiological, transcriptional, metabolic, and epigenetic levels (Pecinka and Mittelsten Scheid 2012). For the purpose of this thesis, a "priming dose" will be used synonymously to a mild, non-lethal dose of a stressor, whereas a "challenge dose" would mean a lethal dose of the stressor.

The priming dose could be a form of stress in itself but can also work as an indicator of upcoming stress (Karban 2008). Based on the experimental evidence, bacterial priming can be a transient phenomenon that comes into action in stressful environments but does not last permanently over generations. For example, in the case of *E. coli*, it was shown to last for up to four divisions before the cells went back to their original state (Rodríguez-Rojas et al. 2020). The term used for bacteria exposed to non-lethal doses of stressors is "microbial priming". "Primed" bacteria have the ability to deal better with the anticipated changes in their immediate

environment, which gives them an edge compared to other bacteria who have never experienced such an environment beforehand.

It seems rather obvious that being primed comes at a small metabolic cost for the organism. This could be because of the mild stress that might lead to mortality or slower growth in a subpopulation, and the energy consumed to upregulate the defence mechanisms (Rodríguez-Rojas et al. 2020; van Hulten et al. 2006). This initial cost is compensated extremely well later compared to the loss of survival experienced by the naive organism upon challenge (Andrade-Linares et al., 2016). The next question that arises: Why do bacteria invest in a mechanism like priming when it comes at a cost? (van Hulten et al. 2006). Marieke van Hulten and colleagues (van Hulten et al. 2006) compared the effectiveness of priming with the direct defence to a variety of stressors in *Arabidopsis*. They showed that direct defence involves much higher costs compared to costs associated with the induction of priming; moreover, primed plants displayed the highest growth rates (van Hulten et al. 2006). To talk about the cost of priming in concrete numbers, Rodríguez-Rojas and colleagues (Rodríguez-Rojas et al. 2020) showed that the cost of priming *E.coli* with low doses of H_2O_2 accounted for 4% survival cost compared to naïve bacteria.

A meta-analysis conducted by Andrade-Linares *et al.* (2016) using data from 279 trials and 34 different studies confirmed an overall positive effect of priming irrespective of the model bacterium and the type of stressor used, establishing the universal nature of the priming phenomenon. They categorised various stresses into pH, osmotic, temperature, oxidative, growth depletion or physiological stresses and showed that the priming effect was evident across all the stress categories.

The priming agents can be either of the same or different nature. When the priming and challenge stress are of the same kind, it is termed “cis-priming”, whereas if the nature of stresses is different, it is referred to as “trans-priming” (Hilker et al. 2016). Trans-priming suggests that the phenomenon of priming does not just provide an advantage when experiencing the same stress again, but it also provides a cross-protection towards other stresses. It is also noteworthy that the improved response upon second exposure is not specific to the priming agent but rather prepares the organism to face a variety of stresses. The terms simply refer to the type of priming and challenge agents rather than any mechanisms involved. There is evidence of cis- and trans- priming from existing literature in the case of plants, insects, as well as bacteria. For instance, in cases of trans-priming, plants that have been primed with drought conditions become more resistant to antioxidants (X. Li et al. 2015), while exposure to high temperatures primes *E. coli* against low oxygen levels (Tagkopoulos, Liu, and Tavazoie 2008). Numerous examples of cis-priming in bacteria include

a study which reported that when *E. coli* was pre-exposed to 50 µM H_2O_2, it could survive subsequent exposure to 5 mM H_2O_2, whereas naïve cells could not (Demple and Halbrook 1983). The effect was re-established in a study led by Rodríguez-Rojas (Rodríguez-Rojas et al. 2020), where they further investigated the proteomic basis of memory of priming *E. coli* with H_2O_2 and how bacteria can save stable transcripts about recent stresses. They also experimentally showed that the priming effect is trans-generational, and the memory was maintained for up to four divisions in *E. coli* before returning to the basal levels. Priming response was shown to be causing proteome-wide adaptations in *E. coli*. In another example, priming *E. coli* with antimicrobial peptides, such as melittin and pexiganan, increases bacterial tolerance and helps them persist in the presence of otherwise lethal doses of respective antimicrobials (Rodríguez-Rojas et al. 2021).

1.3 Fluctuating lives of bacteria

Bacteria come across various stressors in their everyday life based on drastic fluctuations in their immediate environments (Smith and Casadevall 2022). Bacteria are abundant in environments such as soil and water, where they can meet with unforeseeable changes at any given moment. Bacteria are often transported into different environments by their hosts. They can find themselves in insect guts, with insects acting as vectors for them for further disease transmission (Perilla-Henao and Casteel 2016). On the other hand, antibiotics are continuously released into the environment because of incomplete metabolisation or disposal where they end up in soil, plants or water, and bacteria come into direct contact with them (Cycoń, Mrozik, and Piotrowska-Seget 2019). This could greatly harm us in the era of emerging antibiotic resistance. In addition to resistance evolution happening by genetic mutations, bacterial exposure to these antibiotics in a mild form (priming) can potentially also arm them against the lethal doses (challenge), creating superbugs with resistance to all the commonly used antibiotics. Resistance to disinfectants is another major problem, and it has been highly elevated in light of the ongoing pandemic (Tong et al. 2021; Z. Chen et al. 2021). Disinfectants are used to reduce the transmission of infectious diseases because of their ability to inhibit the growth of pathogens such as bacteria and viruses. The continuous exposure of bacteria to disinfectants has led to the development of adaptability and tolerance and has simultaneously reduced the killing efficiency of disinfectants (Cazares et al. 2020). In this context, it is imperative to unravel these memory responses in bacteria and understand how bacteria use them to arm themselves and emerge victorious in developing resistance. If priming has a role in facilitating resistance evolution, as has been demonstrated (Rodríguez-Rojas et al. 2020), it can have significant consequences for virulence evolution.

1.4 Primed bacteria inside a host

To date, even though there are have several reports of the ubiquitous effects of priming across microbial species and stressors, our knowledge is mainly acquired from *in vitro* or culture-based studies where the environment offers a significant level of experimental control; However, it is still artificial and fails to integrate the complexities of an *in vivo* environment. When a microbe enters the host, it must pass through several environments, from the point of entry to the final colonisation site (Kremer et al. 2013). The colonisation success depends on the ability of microbes to survive the environment of the colonisation site. Merrell and team (Merrell, Hava, and Camilli 2002) showed that pre-exposure to acidic stress renders *Vibrio fischeri* more successful in colonising the acidic host intestine. Another study that comes close to testing the consequences of priming in a more real-life environment is by Chen and colleagues (Chen et al. 2017), where they showed that priming *V. fischeri* to mildly acidic conditions (i.e., pH 6.5, host gut-like conditions) rather than the pH of seawater (i.e., pH 8, where the host squid *Euprymna scolopes* resides) renders them significantly greater resistance to the antimicrobial activities inside their host. This is hypothesised to happen because, on the first naïve exposure, *V. fischeri* cells tend to aggregate in acidic mucus on host organs, which supposedly helps them better survive harsh encounters with AMPs. Apart from that, we have limited knowledge of the priming effects that can be translated into environments other than the one in which the priming occurred. To state an example, if microbes have been primed with exposure to low doses of antimicrobials present in the soil, does this lead to enhanced survival when they experience high doses of antibiotics in the host gut? This could change the course of disease dynamics and hugely impact the development of antimicrobial resistance. So, the question remains, how does a previous exposure to a single antimicrobial affect the microbial response to the rest of the exposures to mixtures of antimicrobials in complex environments? This thesis tries to unravel how this cross-environmental priming works using a host-microbe pair and reactive oxygen species (ROS) as a stressor to further our understanding of the bacterial priming phenomena and mechanisms that might underlie it.

1.5 Microbial exposure to ROS

ROS represent a group of molecules derived from oxygen, formed by reduction and oxidation reactions or by electronic excitation. ROS such as superoxide anions ($O_{2}^{\cdot -}$), H_2O_2 and hydroxyl radicals (OH) are found endogenously in animal cells as well as bacterial cells. The defensive capabilities of bacteria suggest that they are well equipped to confront ROS that is not endogenous. In bacteria, ROS are produced as a by-product of the respiratory chain through the accidental transfer of electrons onto oxygen and as part of their innate immunity (Lambeth

2004; James A. Imlay 2003). Similarly, when bacteria infect an insect host, ROS are the first microbicidal line of defence they must deal with (E.-M. Ha et al. 2005; James A. Imlay 2013). Two molecules of superoxide can react to generate H_2O_2 in a reaction known as dismutation, which is accelerated by the enzyme superoxide dismutase (SOD). In the presence of iron, superoxide and H_2O_2 react to generate hydroxyl radicals (Figure 2). High ROS production levels are termed oxidative stress and can be detrimental to organisms because of their role in molecular damage or apoptosis (Kannan and Jain 2000). In humans, high levels of ROS production have been linked to various inflammatory and cardiovascular diseases (Okin and Medzhitov 2012; J. Zhang et al. 2016) and, more recently, it has been considered a hallmark of ageing and ageing related inflammation (termed inflammageing) (Shields, Traa, and Van Raamsdonk 2021; Alfadda and Sallam 2012; Bedard and Krause 2007).

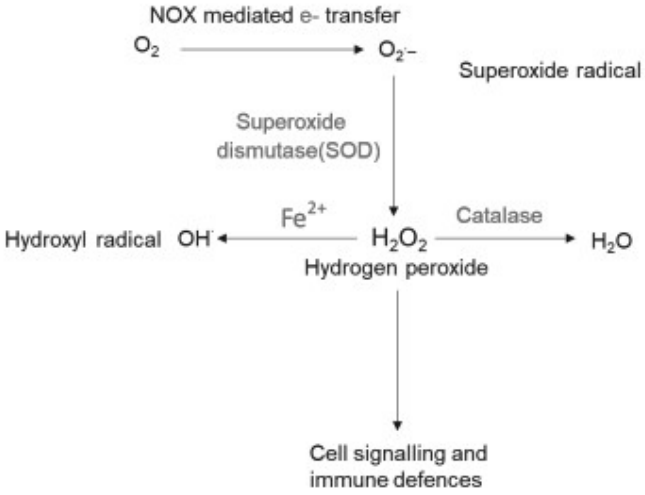

Figure 2: The production and interconversion of ROS. $O_2^{\cdot -}$ is formed from molecular O_2 by gaining a single electron from an NADPH oxidase (NOX) enzyme. Superoxide dismutase (SOD) enzymes convert two superoxide molecules into an H_2O_2 and a water (H_2O) molecule. H_2O_2 can undergo Fenton chemistry with Fe^{2+} to form $HO^{\cdot}$, which is highly reactive and can cause cellular damage. H_2O_2 can also modify redox-sensitive residues to change cellular signalling and can be reduced by catalase (Di Marzo, Chisci, and Giovannoni 2018).

1.6 H_2O_2, a ROS

H_2O_2 is the major ROS in the redox-dependent regulation of biological processes (Thannickal and Fanburg 2000; Reczek and Chandel 2015), as well as one of the major components of disinfectants (Lineback et al. 2018) and has the ability to diffuse through the semipermeable bacterial membranes (Fasnacht and Polacek 2021). The intracellular H_2O_2 is regulated through the protection of scavenging enzymes such as superoxide dismutase (SOD), catalases and peroxidases (James A. Imlay 2015). Such enzymes are found virtually in all

bacterial, animal and plant cells (James A. Imlay 2008). Catalase dismutates H_2O_2 to H_2O and O_2 or reduces H_2O_2 to H_2O by oxidising hydrogen-donating compounds (Chance, Sies, and Boveris 1979). Catalases are present in all the important sites of H_2O_2 production in the cellular environments (e.g., peroxisomes, mitochondria, cytosol, and chloroplast) of higher plants (Sharma and Ahmad 2014). H_2O_2 is ubiquitous in fresh and oceanic waters at levels in the low ranges (29-129 nM), enough to plausibly threaten bacteria and induce their stress responses (Meslé et al. 2017; Lesser 2006; Vermilyea, Dixon, and Voelker 2010; Yuan and Shiller 2001).

1.7 *D. melanogaster* immunity

D. melanogaster, as an invertebrate, lacks an adaptive immune system, and the immune functions are taken care of by a well-equipped innate (inborn) immunity to cope with various pathogens such as bacteria, viruses and fungi (Vodovar et al. 2005; Buchon et al. 2009). The *D. melanogaster* immune system has been extensively studied, and it possesses two arms of innate immunity, cellular response and humoral response, as reviewed in (Lemaitre and Hoffmann 2007). The cellular immune response encompasses mechanisms such as phagocytosis, cellular encapsulation, melanisation, and coagulation. The humoral response refers to the expression of antimicrobial peptides (AMPs) and ROS by cells triggered by the recognition of pathogens (Lemaitre and Hoffmann 2007). When a pathogen is able to breach through first physical barriers, like the cuticle and the mucus layer, it finds itself inside the host body, and the host must recognise the pathogen and mount a successful immune response in order to neutralise it (Lazzaro and Rolff 2011).

Inside hosts, such as insects, the first lines of defence against any infection are crucial, as they might determine the progression and outcome of infection. Therefore, the primary protection mechanism against invading pathogens must be strong in order to avoid further damage. In insects, as mentioned earlier, ROS produced by the joint action of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) and dual oxidases (DUOX) acts as a first line of the defence system by generating microbicidal ROS to deal with opportunistic pathogens (Ha et al. 2005). NOX enzymes evolved three billion years ago and are present across fungi, plants, and animals (Moghadam, Henneke, and Kolter 2021). The mammalian NOX family consists of 7 members (NOX1–5 and DUOX1-2), with DUOX 1 and 2 emerging from NOX5. DUOX enzymes were first described in mammals to be involved in thyroid hormone production by acting as a source of H_2O_2 (Dupuy et al. 1999), whereas the other NOX family members are known to produce only superoxide (Katsuyama, Matsuno, and Yabe-Nishimura 2012).

As many of the underlying mechanisms of the immune response are conserved across species, research on the fruit fly *D. melanogaster* has been a valuable tool for studying innate

immunity and host-pathogen interactions (Buchon, Silverman, and Cherry 2014). Notably, many physiological functions of ROS discovered in *D. melanogaster* can be extrapolated for deciphering and understanding analogous processes in humans, which could potentially lead to the development of novel therapeutic approaches in ROS-associated disease treatment. On top of that, the availability of an extensive database on fly research, such as Fly Base (https://flybase.org/), as well as fly stock centres such as Bloomington stock centre and Vienna *Drosophila* stock centre, are great collective resources to explore conserved mechanisms further.

1.7.1 ROS responses and mechanisms in *D. melanogaster*

In *D. melanogaster*, as opposed to mammals, only a single homolog of the two NADPH oxidases (NOX and DUOX) are present in different regions of the gut and act by different mechanisms for initiating ROS production (Dutta et al. 2015; Iatsenko, Boquete, and Lemaitre 2018). Lactate present in the microbiota leads to the activation of intestinal NOX (Iatsenko, Boquete, and Lemaitre 2018). On the other hand, DUOX is activated via sensing pathogen-derived uracil (K.-A. Lee et al. 2015). ROS play a role in direct pathogen killing as well as downstream signalling for antimicrobial peptide (AMP) activation (Chakrabarti and Visweswariah 2020). Apart from different mechanisms, ROS produced by DUOX and NOX plays different roles in host defences. DUOX-derived ROS have a major role in defence and regeneration upon ingesting a pathogen, whereas NOX-derived ROS is known to promote epithelial renewal during infection (Iatsenko, Boquete, and Lemaitre 2018).

Apart from ROS presence in the gut, H_2O_2 is also upregulated at the wounding sites, which is mediated by the upregulation of calcium ions (Razzell et al. 2013). Blocking H_2O_2 at the wounding site decreases haemocyte accumulation, suggesting that ROS signalling also plays a role in systemic wound responses (Krautz, Arefin, and Theopold 2014).

ROS are expressed in haemocytes in the haemocoel as well and show a biphasic response upon infection. As Myers *et al.* (2018) (Myers et al. 2018) demonstrate, a strong transient ROS signal is generated within haemocytes in the first hour of infection, while another delayed peak comes after ninety minutes from the cells that have engulfed bacteria. Another recent study showed that ROS production in haemocytes plays a bactericidal role against pathogens (Shaka et al. 2022). Haemocytes mount an initial transient ROS response followed by a stronger peak observed in phagocytes. The ROS responses in flies thus differ in their functions, activation mechanisms and the sequence of upregulation.

In specific gut regions, namely the foregut and the hindgut, basal levels of ROS are always present (Figure 3) and are produced by membrane-associated dual oxidase (DUOX) (Ha et

al. 2005; Buchon, Broderick, and Lemaitre 2013). These basal levels are known to maintain symbiosis between the host and the gut microbiota (Kim and Lee 2014), modulating microbial numbers. In order to keep damage by excess oxidative stress in check, the host has to keep ROS levels low by expressing redox enzymes such as an extracellular immune-regulated catalase (IRC) to regulate the excessive respiratory burst levels (Ha et al. 2005). The reduced expression of IRC in adult flies has been correlated with high mortality rates even after simply ingesting non-pathogenic bacteria (E.-M. Ha et al. 2005).

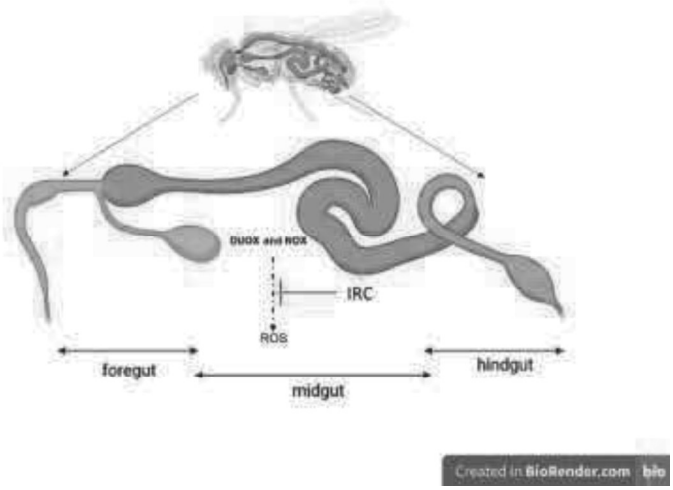

Figure 3: The gut of *D. melanogaster* is divided into three parts, with reactive oxygen species (ROS) present specifically in the foregut and hindgut regions of an adult gut (Broderick 2016). An active neutralisation with the help of immune-regulated catalase (IRC) maintains oxidative homeostasis in the gut to avoid oxidative stress.

Amongst many other roles, ROS are also believed to be involved in melanin production mediated by the enzyme phenoloxidase (Nam et al. 2012). Melanin is produced around invading pathogens in order to isolate and sequester the host and is also partaking in the wound healing process (Tang 2009). Following injuries or puncture wounds, studies in different model organisms reveal that summoning of different immune cells like haemocytes to the wounding site is almost always a consequence of rapid production of H_2O_2 at the site (Razzell et al. 2013; Yoo et al. 2011). Although wounding in wild flies is a common phenomenon in nature, it is likely that the hosts will more frequently come across pathogens in their gut via ingestion of food rather than a systemic infection via a puncture in the cuticle. Therefore, it was decided to use natural oral infections which flies suffer from by ingesting microbes present on their food sources to test the effects of primed microbes inside a host. Although ROS mechanisms need further investigations, they are known to play a direct role

in pathogen killing in case of natural infections, whereas there are contrasting reports of whether they kill directly in haemocytes (Shaka et al. 2022) or they are mere mediators of the signalling pathways in case of wound responses.

Interestingly, there is sexual dimorphism in the amount of ROS in *D. melanogaster* gut, with males having higher ROS throughout their lifetime compared to female flies (Regan et al. 2016). ROS levels were analysed using expression of DUOX, which is responsible for ROS production, in both young (7-day old) and old (42-day old) flies of both sexes. In another study, where *D. melanogaster* adults were subjected to oral infections with an opportunistic pathogen *Erwinia carotovora* subsp. *carotovora-15* (*Ecc15),* it did not harm the healthy host, but when the host's DUOX-dependent gut immunity was silenced using DUOX RNAi flies; male flies died faster in response to infections more than females (E. M. Ha et al. 2005; E.-M. Ha et al. 2009). These findings point toward males having a higher sensitivity to infections, despite having higher ROS compared to females (Regan et al. 2016). Apart from being important to test dimorphism in immune defences, these findings also give us a basis to include both male and female flies in our experiments to test whether bacterial survival differs between the two sexes based on differential amounts of ROS production.

1.8 *Erwinia carotovora subsp. carotovora-15 (Ecc15)*

Erwinia carotovora subsp. carotovora-15 or *Ecc15* is a naturally occurring opportunistic pathogen which increases uracil levels and leads to DUOX activation in *D. melanogaster* adults (K.-A. Lee et al. 2015; Joshi and Royet 2020). This Gram-negative, facultative anaerobe produces many enzymes to degrade plant cell wall to cause diseases (Das and Roychoudhury 2014). *Ecc15* is a phytopathogen causing soft rot to major plant crops such as potato, carrot, and pepper (Perombelon and Kelman 1980). H_2O_2 is produced in plant cells not only under normal conditions but also by oxidative stress, caused by factors like drought, chilling, UV radiation, wounding, and pathogen infection. So, *Ecc15,* as a plant pathogen, might be in contact with low levels of ROS being formed in the plants upon infection (Foyer and Noctor 2016). At the same time, it is one of the few plant pathogens that induce an oxidative burst in plants in order to be successful (Venisse, Gullner, and Brisset 2001). During oxidative bursts in plant-pathogen interactions, the first detectable ROS are superoxide anion ($O_2 \cdot^-$) and H_2O_2, which facilitate the initiation of infection (Torres, Jones, and Dangl 2006).

Ecc15 maintains an efficient cycle of plant-to-plant transmission by using insects belonging to Dipterans or Hymenopterans as vectors (Keesey et al. 2017). To deal with plant and insect hosts, *Ecc15* uses different virulence factors: pectolytic enzymes to degrade plants and *Erwinia* virulence factor (*evf*) to infect insect hosts (Basset et al. 2003). Adult *D. melanogaster* are a natural vector for *Ecc15* (Molina, Harrison, and Brewer 1974; Kloepper, Brewer, and

Harrison 1981). *Ecc15* macerates plant tissues leading to soft rot, and oozes out bacterial droplets, which act as an attractive food source for flies because of their affinity for rotting substrates (Perombelon and Kelman 1980). *Ecc15* also induces a robust whole-body immune response in *D. melanogaster* larvae (Basset et al. 2000) as well as adults (Buchon et al. 2009) in the case of oral infections compared to a weaker response on injections (Basset et al. 2000). The whole-body immune response involves a global AMP activation and upregulation of several signalling pathways. After a few hours of gut infection, *Ecc15* remains predominantly present in the foregut and parts of the hindgut (Basset et al. 2000). Studies show that *Ecc15* was able to induce DUOX-dependent ROS generation at 1–3.5 h following bacterial ingestion, mainly in the anterior midgut region (Basset et al. 2000).

Due to its surroundings, *Ecc15* comes across ROS species on a daily basis in its natural environment, including plant surfaces (Das and Roychoudhury 2014), and it also leads to ROS formation by infecting its vector species (Buchon et al. 2009), namely *D. melanogaster*, making it a suitable candidate to test the effects of microbial ROS priming. When studying biological mechanisms, it is of utmost importance that the host-parasite pair is compatible with our research question to reduce the external variations and have the system as close to natural conditions as possible. For testing priming in complex environments, *D. melanogaster* and *Ecc15* were used as the host-parasite pair for a number of reasons. As mentioned earlier, *D. melanogaster* is a natural vector for *Ecc15*, and *Ecc15* also induces ROS response upon infecting *D. melanogaster*, which is important for our work since ROS was used as priming stress. Being a natural vector means *Ecc15* does not have additional stress to adapt to the host environment. Although priming with ROS has been established in *E. coli* (Rodríguez-Rojas et al. 2020), it is not a natural pathogen of *D. melanogaster,* even though it leads to activation of antimicrobial peptides upon systemic infection (Armitage et al. 2014; Lemaitre, Reichhart, and Hoffmann 1997; Leulier et al. 2000). Additionally, the optimum temperature for *E. coli* growth under laboratory conditions is 37°C, which is more than ten degrees higher than the optimum temperature (25°C) for the host growth. This difference in growth temperature could provide additional stress for the pathogen to adapt to the host environment and interfere with the neutralisation by the host.

1.9 Objectives

The main objectives of this thesis are to test if *Erwinia carotovora carotovora 15* is primable with H_2O_2 and to find the underlying proteomic basis of such priming mechanisms. The next goal is to determine if *in vitro* priming helps bacterial survival in complex *in vivo* environments i.e., how well primed bacteria do when they encounter the stressor inside a host gut.

This thesis is written in the form of a monograph divided into four sections: introduction, materials and methods, followed by results and discussion.

The first set of experiments are *in vitro* experiments where *Ecc15* was primed with sub-lethal concentrations of H_2O_2 followed by challenge concentrations. The following question was asked: Does priming *Ecc15* with H_2O_2 *in vitro* confers a survival advantage upon an *in vitro* challenge?

For this, a full factorial design with the following treatments was used: i) only priming, where bacteria receive only the priming dosage of H_2O_2, providing us with the cost of priming; ii) only challenge, with bacteria receiving only the lethal dose of H_2O_2; iii) priming and challenge, where bacteria receive both the priming and challenge dose of H_2O_2; and lastly iv) control, where neither priming nor challenge is exerted on the bacterial cells. The parameter to quantify bacterial growth was the number of colony-forming units (CFU) per millilitre of bacterial culture (CFU/mL).

It was predicted that priming will confer a survival advantage upon challenge, and hence bacteria that have received first priming and then a challenge dose will have better survival compared to the bacteria that receive the challenge dose alone.

The next set of experiments deals with phenotypic markers of priming, where an overall proteomic response of *Ecc15* to H_2O_2 priming is summarised, and gene ontology analysis has been conducted to see if the signatures of stress differ across treatments.

Here, the following question was asked: Does priming with H_2O_2 lead to proteome-wide adaptations in *Ecc15*? Do those adaptations differ in the case of priming and challenge stress?

Further, it was tested if there is a survival advantage of primed microbes inside a host (*in vivo*) priming, where *D. melanogaster* was allowed to feed on primed *Ecc15,* and the survival of primed vs non-primed *Ecc15* was compared in the fly gut at several time points after feeding in order to test if the ROS primed bacteria have any advantage in the fly gut. Prior to *in vivo* priming, this section also deals with standardising the oral infection methods according to our experimental needs.

The main question was: Does priming *Ecc15 in vitro* with H_2O_2 provide a survival advantage inside the host? Does sexual dimorphism in host ROS have an influence on primed bacteria?

It was predicted that *in vitro* exposure of *Ecc15* to sub-lethal concentrations of H_2O_2, confers an advantage inside the host compared to non-primed bacteria. Hence, more colony forming units (CFUs) of *Ecc15* should be retrieved in case of host feeding on primed bacteria. Since

there is sexual dimorphism in the amounts of ROS, host sex-specific differences in numbers of primed vs non-primed bacteria were also predicted.

In summary, this thesis work enhances the understanding of the phenomenon of priming by *in vitro* priming using a single antimicrobial and test if it provides an advantage in a complex *in vivo* environment.

Section 2

Materials and Methods

2.1 Bacterial model: *Ecc15*

All experiments were performed using the Gram-negative bacterium *Erwinia carotovora carotovora 15* (*Ecc15*; a gift from Bruno Lemaitre). This strain is a known phytopathogen, and *D. melanogaster* is known to be one of the insect vectors of this pathogen (Molina, Harrison, and Brewer 1974; Kloepper, Brewer, and Harrison 1981). *Ecc15* (2141) was isolated on infected potato plants in 1978 from France. The rifampicin-resistant mutants of *Ecc15* (Ecc-15rif^{R}) were created by Basset and colleagues (Basset et al. 2000). *Ecc15* was cultured at 25 °C for all the experiments to match the temperature at which the host *D. melanogaster* is raised in laboratory conditions. Aliquots of *Ecc15* stored at −80°C were streaked on lysogeny broth (LB) agar (three agar plates) and incubated at 25°C for 24 h. One clone from each plate was picked into 100 mL of sterile LB broth in an Erlenmeyer flask (3 clones per 100 mL culture) and left to grow overnight (approximately 15 h) at 25°C and 200 rpm (MaxQ 6000, Thermo Scientific) for all the experiments. Since this *Ecc15* strain has rifampicin resistance (Muniz et al. 2007); it was always grown in liquid media and LB agar in the presence of rifampicin (100 µg/ml) unless mentioned otherwise.

2.2 Insect infection model: *D. melanogaster*

For *in vivo* experiments, insects from an outbred population of *D. melanogaster* (gift from Élio Sucena from Instituto Gulbenkian de Ciência, Portugal) were used. This population was established from 160 *Wolbachia*-infected fertilized females collected in Portugal (Martins et al. 2013) and then lab reared at a density of approximately 5000 flies, with non-overlapping generations. The populations were maintained at 25°C ± 1°C and 70% relative humidity on a 12:12-hours light-dark cycle (Percival Scientific, US) and fed on a sugar yeast agar (SYA) medium (1.5% agar, 5% sugar, 10% brewer's yeast, 3% nipagin, 0.3% propionic acid) (Bass et al. 2007). Fly rearing and all the experiments were always carried out in standard plastic food vials (95 x 25 mm).

They were allowed to grow for two generations on a standardised density of 100 flies per vial to obtain experimental flies. To do so, four purple grape juice agar plates (25 g agar-agar, 300 mL red grape juice, 21 mL 10% nipagin solution, 550 mL water (Wensing, Koppik, and Fricke 2017)) coated with a layer of baker’s yeast paste were placed inside the population cage to stimulate egg laying. The flies were allowed to lay eggs and the plates were removed from the cage 24 hours later, covered with lids, and placed in the incubator. They were allowed to further develop into larvae for 24 hours and then placed in groups of 100 individuals in plastic vials (95 x 25 mm) containing 7 mL of SYA medium. These individuals were left to develop for eight days under the light-dark, temperature and humidity conditions described above. Four

days after they had emerged as adults, they were placed in two embryo cages in groups of 600-800 adults and allowed to mate and lay eggs on a purple grape juice agar plate for 24 hours. Another 24 hours later, larvae were collected as described above and allowed to develop. Newly emerged adults were collected one day after emergence and placed in food vials in groups of five males and five females until they were used for the feeding assays.

2.3 H_2O_2 minimal inhibitory concentration (MIC) for *Ecc15*

Susceptibility testing is a way to know the concentrations of antimicrobials which are bacteriostatic for microbes. The minimum inhibitory concentration (MIC) is a measure used to determine bacterial susceptibility using the broth microdilution method. MIC value is read as the lowest concentration of the antimicrobial that acts as bacteriostatic and inhibits bacterial growth after overnight incubation. MIC was identified to test the sub-lethal H_2O_2 concentrations for *Ecc15*, to decide the priming concentrations. The methods followed were based on EUCAST (European committee on antimicrobial susceptibility testing) MIC determination guidelines. An exponential bacterial culture of OD 0.5 was used to test the MIC, the OD which will be used for further experiments. To test MIC, three independent overnight cultures were inoculated with three independent colonies of *Ecc15* each and incubated at 25 °C with shaking at 200 rpm (MaxQ 6000, Thermo Scientific) for ~15 hours. After overnight incubation, fresh exponential cultures were started from the overnight cultures where 100 ul of overnight culture was added to 10 ml of fresh LB media in 50 ml falcon tubes and allowed to incubate at 25 °C with shaking at 200 rpm. For MIC testing and all the further experiments with H_2O_2, the LB media was freshly prepared and stored in the dark to avoid reactive oxygen species (ROS) formation. The optical density (OD) of the exponential culture was measured using an Ultrospec10 classic spectrophotometer (Amersham) every 30 minutes until it reached OD 0.5, which took ~4-5 hours. MIC was determined by inoculation of 2 µl of exponential bacterial culture into a non-binding polypropylene V bottom 96-microwell plate (96 well, Th. Geyer, Germany) containing 198 µl of LB medium mixed with a range of concentrations of H_2O_2 (Sigma Aldrich, Germany) in different wells, starting from the highest and going towards the lowest concentration (32 µg/µl up to 0.0625 ug/ul). The plates were incubated at 25°C without shaking in a humid chamber to avoid evaporation of the contents of the plate. After ~18 hours of incubation, the plate was centrifuged for one minute at 420 rcf at 25°C to settle down all the bacterial growth in the V-shaped well bottoms. The bottom of the plate was then observed for bacterial pellets using a naked eye under natural light conditions. MIC was defined as the lowest concentration that inhibited visible bacterial growth after an overnight incubation. Additionally, another plate with the conditions mentioned above was prepared to place in a 96-well plate reader (Biotek Synergy, Germany) to obtain real-time growth curves of *Ecc15*

under above mentioned H_2O_2 concentrations. The only exception was that instead of a V-bottom plate, a flat bottom 96 well plate was used (Nunc, Denmark).

2.4 Generation time of *Ecc15* at 25°C

Generation time is the time taken for a bacterial population to double in numbers. It was essential to know the generation time to test the priming and challenge durations, and additionally to test memory of priming i.e., for how many generations does priming last. Since temperature can have a large effect on bacterial generation time, it was tested for *Ecc15* at 25°C , since it can differ from the commonly used growth temperature of 29°C (Quevillon-Cheruel et al. 2009). In order to obtain generation times, growth curve measurements were conducted in a 96-well plate reader over a time period of 24 hours. Bacterial growth curves were measured in flat-bottom 96-well micro-plates with anti-condensation lids (Nunc, Denmark). Three independent overnight cultures were inoculated with three independent colonies of *Ecc15* each and incubated at 25 °C with shaking at 200 rpm (MaxQ 6000, Thermo Scientific) for ~18 hours. All the overnight cultures were mixed together after overnight incubation. The optical density (OD) of the exponential cultures was measured using a spectrophotometer every 30 minutes until it reached OD 0.5 in ~4-5 hours. 2 µl of exponential culture of OD 0.5 was then inoculated into all the wells of a flat bottom 96-microwell plate (Nunc, Denmark) containing 198 µl of LB medium. To the last two columns, no bacterial culture was added and they were our medium contamination controls. The 96 well plate was then covered with anti-condensation lid and placed in a microplate reader (BioTek Synergy) to monitor the OD at 600 nm every 10 minutes with shaking the plate in between the readings. The plates were maintained at 25°C and shaken at slow speed for five seconds between every reading. The OD of each well was measured every 10 mins for 24 hours. The growth curve data was then extracted from the plate reader and further calculations of generation time during exponential growth phase were done by fitting the curves using a logarithmic equation. The experiment was repeated twice with 80 individual wells per plate used to calculate the generation time.

2.4.1 Calculating generation time

The generation time of exponentially growing *Ecc15* was calculated based on OD_{600} measurements at the beginning and end of exponential growth. Sigmoidal interpolation was carried out using Microsoft Excel to identify the beginning and end of exponential growth. Growth rates (r) were calculated by the formula r = (ln [OD2/OD1]) / (T2-T1) where OD refers to optical density and T refer to time and the doubling time corresponds to ln (2)/r. This method assumes that OD is directly proportional to the number of cells and takes OD measurements at fixed intervals. In this experiment, OD was measured every 10 minutes for 24 hours. The

growth was then plotted with lnOD on the y-axis and time on the x-axis to find exponentially growing parts of the curve. The generation time was then calculated using two points of the exponential growth area. This analysis of generation time was conducted in Microsoft Excel (version 2210).

2.5 *In vitro* experiments, high cost of priming

The following subsections deal with all the experiments carried out to establish *in vitro* priming doses followed by testing if priming has a survival advantage upon an *in vitro* challenge.
After establishing the MIC of *Ecc15* for H_2O_2, preliminary experiments were conducted *in vitro* to establish priming and challenge concentrations of H_2O_2. Further, it was tested if there is a survival advantage of priming upon challenge. Taking studies conducted by Rodríguez- Rojas and colleagues into account (Rodríguez-Rojas et al. 2020), where a priming dose ten-times lower than the MIC value was used, a concentration of 0.1 mM was tested. Additionally, studies show that average peroxide concentration in plant tissues are ~1 mM, which is relevant for plant pathogen *Ecc15* (Foyer and Noctor 2016). Following this logic, 0.1 mM, an in-between concentration of 0.5 mM and 1 mM were chosen for preliminary testing. Further, 5 mM was used as a challenge concentration, as this concentration was five-times more than the MIC and hence lethal for *Ecc15.* One can test higher challenge concentrations too, since it has been shown by (J A Imlay and Linn 1986) that priming provided a survival advantage to concentrations as high as 30 mM. These preliminary experiments described in the following sub-sections were carried out based on counting colony forming units upon priming and challenge treatments in falcon tubes.

2.5.1 *In vitro* priming assay: establishing priming concentrations

To test the priming doses in falcon tubes, the following treatment combinations were used: control, only challenge (5 mM) and priming + challenge (0.1 + 5, 0.5 + 5, 1 + 5 mM). This experiment lacked an only priming treatment; hence the design was not fully factorial. For this experiment, there were three biological replicates (falcon tubes) for each treatment and it was repeated one time following methods described in (Rodríguez-Rojas et al. 2020).
For bacterial culturing and sub-culturing, the methods described previously in section 1 were used. Additionally, OD was tested every 30-minutes following methods described in section 3. When the OD reached 0.5, 10 ml of OD 0.5 culture was added into each 50 ml falcon tube except for the negative control where only LB media was added. Meanwhile, a fresh stock of 20 mM H_2O_2 was prepared from the original concentration of 9.8 M. 0.1, 0.5- and 1-mM priming dose was added to the priming + challenge treatment falcon tubes. After adding the priming dose, all the falcon tubes were allowed to incubate for 30 minutes at 25°C with shaking at 200 rpm. After the 30 mins, the tubes were centrifuged for 10 minutes at 25°C at 4000 g. The

supernatant was removed without disturbing the pellet and replaced with 10 ml of LB medium. This step was to remove the excess H_2O_2 from the media. All the pellets were then resuspended in this fresh LB media by gentle mixing by hand and the bacterial cultures were allowed to recover for 60 minutes in the incubator at 25°C with shaking at 200 rpm. After the recovery time was over, 5 mM challenge dose was added to the treatments that required it (only challenge and priming + challenge). The bacterial cultures were allowed to undergo the challenge treatment by placing them in the incubator under the same conditions for 30 minutes, after which, 10 µl of pre-made catalase (4µg/ml) (Sigma Aldrich, Germany) was added to get rid of all the residual peroxide from the cultures. After letting the bacterial cultures incubate with catalase for 10 minutes in the incubator, they were placed on ice to inhibit further bacterial growth and used for quantifying the CFU's per ml in each treatment by plating serial dilutions as described in the following.

To estimate the number of viable bacteria (CFUs), all the falcon tubes were thoroughly mixed and 100 µl of bacterial culture from each falcon tube was added to the first column of 96-well plate which was pre-filled with 180 µl of LB media in all the columns except the first column. The bacterial cultures were then serially diluted from 1:10 until $1:10^8$ using a multichannel pipette. Per falcon tube, six rows of droplets of 5 µl of every dilution were plated onto square LB agar plates (120*120*17 mm). The plates were incubated upside down at 25ºC and the numbers of CFUs were counted after ~20 hours. Based on the CFU counts, viable bacterial cells per ml for each treatment were back-calculated using the mean CFU counts of the six droplets from the lowest countable dilution in the plate, which was usually between 10 and 60 CFUs per droplet.

2.5.1.1 Statistical analyses

All the statistical analyses in this thesis were performed in R version 4.0.4 (R Core Team 2020) and RStudio version 1.3.959-1 (Middlemist Red). All the percentage cost-related analysis was performed using Microsoft Excel. LC-MS data was analysed using Perseus 1.6.14 and GO analysis was carried out using Panther 17.0.

The following packages were used for plotting the data: "Growthcurver" (Sprouffske, 2016), "plyr", "dplyr", "ggplot2", "tidyverse" (Wickham 2011, 2014,2016,2018), "scales" (Wickham and Seidel 2020), "car" (John Fox, 2019), "DHARMa" (Florian Hartig,2017), "emmeans" (Russell Lenth,2018), "glmmTMB" (Brooks, 2017), "MASS" (Ripley,2002) and "lme4" (Douglas Bates,2015).

Here, a linear model was fitted to test if priming with different concentrations (0.1, 0.5 and 1 mM) of H_2O_2 leads to a survival benefit upon a challenge dose (5 mM).

Model 1: lm (CFUs) ~ treatment

Additionally, post-hoc multiple comparisons were performed using “emmeans”.

2.5.2 *In vitro* priming assay: survival advantage of priming upon challenge

Based on survival advantage provided upon *in vitro* challenge by different concentrations of H_2O_2 as mentioned in section 2.5.2, 1 mM was chosen as the priming concentration and further experiments of this section were carried with 1 mM as the priming concentration and 5 mM as the challenge concentration. In this set of experiments, following treatments were used: control, only priming, only challenge, priming + challenge, making a full factorial design. The same methods as described above in section 2.5.1 were followed except an additional “only priming” treatment to test costs associated with priming. To this only priming treatment, only the priming dose of 1 mM was added. Each treatment had three biological replicates (falcon tubes) and the whole experiment was replicated on four separate days. Additionally, three independent dilution series from each biological replicate (falcon tube) were carried out and these were called technical replicates. For each technical replicate, eight droplets of 5 µl of every dilution were plated onto LB agar. The plates were incubated upside down at 25ºC and the numbers of CFUs were counted after ~20 hours. Based on the CFU counts, viable bacterial cells per ml for each treatment were back-calculated using the mean CFU counts of eight droplets from the lowest countable dilution in the plate, which was usually between 10 and 60 CFUs per droplet.

2.5.2.1 Statistical analyses

It was tested if priming with 1 mM of H_2O_2 leads to an increase in survival when *Ecc15* receives an *in vitro* challenge dose (5 mM) of H_2O_2. To be able to test whether priming provides a survival advantage upon exposure to challenge, a linear model with natural log transformed bacterial load as the response variable was used. Presence or absence of priming and challenge, as well as their interaction, were included as factors, with experimental replicate and technical replicates included as random factors. The resulting model was the following:

Model 2: log (CFUs) ~ priming × challenge + (1| experimental replicate) + (1| technical replicate)

In this model, it was tested whether priming gave a survival advantage to *Ecc15* upon receiving challenge and if priming itself had a fitness effect on the bacteria when compared with the non-treated control. Additionally, post-hoc multiple comparisons were performed using “emmeans”.

2.6 Proteomic basis of *in vitro* priming

After establishing a survival advantage associated with priming upon receiving an *in vitro* challenge, the proteomic basis of *in vitro* priming and global ROS response of *Ecc15* was tested using liquid chromatograph-mass spectrometry (LC-MS). Mass spectrometry allows us to investigate changes at the protein level, which is relevant in case of priming since the memory formation has been associated with changes in gene expression (Rodríguez-Rojas et al. 2020). The standard protocol of mass spectrometry includes two critical steps before the data acquisition: protein digestion by proteases, typically trypsin, and a separation step (Y. Zhang et al. 2013). During the digestion step, proteolytic enzymes are used to break proteins into smaller peptides (Gundry et al. 2009) and then the digested samples are separated into different fractions via high-performance liquid chromatography (HPLC) so that they can be injected in the mass spectrometer in small quantities to allow for an improved signal acquisition. The protocol carried out to perform LC-MS has been described briefly.

2.6.1 *In vitro* priming assay for LC-MS sample preparation

For the sample preparation, an *in vitro* priming experiment similar to the one described in the section 2.5.1 was carried out with five independent biological replicates for each treatment: control, only priming, only challenge and priming + challenge. The main difference from the previous protocol was that this experiment was carried out with five independent overnight cultures which led to exponential cultures in order to have independent *Ecc15* populations. Another difference in the procedure was that the samples were collected after 10 minutes of exposure to the challenge dose. The rationale behind early collection of samples was to avoid excessive killing after the lethal challenge of 5mM. All the independent replicates of all treatments (five falcon tubes per treatment with 10 ml bacterial culture per falcon tube) were centrifuged at 10,000 x g for 2 minutes and the pellets were stored at -80°C in tubes containing 50 µl of urea denaturing buffer (6 M urea, 2 M thiourea, and 10 mM HEPES, pH 8.0) for further processing. After removing the pellets from -80°C, the cells were disrupted through 4 consecutive freeze-thaw cycles switching between -80°C for 20 minutes and a warm water bath (Eppendorf ThermoMixer C, 37°C) for 10 minutes to facilitate the protein extraction. The samples were processed for liquid chromatography-mass spectrometry (LC-MS) analyses based on Rodríguez-Rojas and Rolff (2020) with a few modifications and are summarised here. After four freeze-thaw cycles, the bacterial lysates were centrifuged at 5000 x g for 20 minutes and the supernatants were used as samples for the proteomic analysis.

2.6.2 *In vitro* priming assay: 10-minute challenge duration

As mentioned in section 2.6.1, the methods of sample collection for LC-MS differed from other *in vitro* assays (10 minutes of challenge exposure instead of 30 minutes). Since the exposure

to challenge was shortened to avoid excessive killing, a control experiment was carried out to test if priming provides a survival advantage to *Ecc15* upon an exposure to challenge dose for 10 minutes. To do so*, in vitro* priming assay as described in section 2.5.1 was carried out and CFU's were plated via serial dilutions.

2.6.2.2 Statistical analyses

The following packages were used for plotting the *in vitro* data: "plyr" (Wickham 2011), "dplyr" (Wickham et al. 2022), "ggplot2" (Wickham 2016), "tidyverse" (Wickham et al. 2019), "scales" (Wickham and Seidel 2020) and "car" (John Fox, 2019). "DHARMa" (Hartig, 2017) was used for model comparisons and post-hoc tests were conducted using "emmeans" (Lenth, 2018). "glmmTMB" (Brooks, 2017), "MASS" (Ripley,2002) and "lme4" (Bates,2015) were used to analyse the dataset.

To test whether priming with H_2O_2 gives a survival advantage to *Ecc15* after exposure to a challenge dose for 10 minutes, a linear model was used. The natural log of bacterial colony forming units were used as the dependent variable, priming treatment (primed with 1 mM or non-primed) and challenge treatment (challenged with 5 mM or non-challenged), as well as their interaction, were included as factors. Experimental replicate was included as a random factor.

Model 3: lmer (log (CFU) ~ priming × challenge + (1|experimental replicate))

2.6.3 Sample Digestion for LC-MS

All the buffers were freshly prepared for the experiment. The pellets were first resuspended in 50 µl of TE (10 mM Tris-HCl pH 8.0, 1mM EDTA) containing chicken lysozyme (0.1 mg/ml, Sigma Aldrich, Germany). To the resuspended pellets, 250 µl of denaturation buffer (6 M Urea/ 2 M Thiourea in 10mM HEPES pH 8.0) was added and the lysate obtained here was used for further protein digestion. Firstly, 1 µl of dithiothreitol 10 mM dissolved in 50mM ABC buffer was added to each sample followed by a 30-minute incubation step at room temperature. This was followed by the alkylation with 1 ul of a 55 mM iodoacetamide solution for 20 min to each sample followed by another incubation step of 20 mins at room temperature in the dark. The digestion step was initiated by the addition of lysyl endopeptidase (LysC, Wako, Japan) resuspended in 50 mM ABC buffer to each tube in a ratio of 1 µg per 50 µg of total proteins and incubated for 3 hours. This was followed by a sample dilution with four volumes of ABC buffer. Thereafter, 2 µl of freshly prepared 0.5 µg/µl trypsin solution (1 mg trypsin protease, LC-MS grade in 2 ml ABC buffer, Promega, USA) was added to all the samples that were incubated overnight at room temperature. All in-solution protein digestion steps were performed at room temperature and after addition of iodoacetamide the samples were kept in

the dark to avoid exposure to light. Next day, the digestion reactions were stopped by acidifying the samples by adding 7.5 µl of freshly prepared buffer A (2.5 ml acetonitrile, 1.5 ml TFA, 46 ml distilled water).

2.6.4 Peptide purification and elution

Before injecting the samples into the HPLC machine, the samples were subjected to a process of concentration and micro-purification as describe elsewhere. The first step was to prepare the in-house stage tips for sample loading. The StageTip purification tips were prepared following the methods previously described (Rappsilber, Mann, and Ishihama 2007; Rodríguez-Rojas and Rolff, 2020). We used a C18 reserve phase matrix disk (0.4 mm to 0.6 mm 3M^{TM} Empore C18 Extraction Discs), which was folded twice in order to make the discs tightly packed. Using a medical grade biopsy syringe, the disc was punched once, giving out four circular discs which were then introduced into a 200 µL filter-less tip and tightly packed by pressing the syringe multiple times. Each tip was then placed through the previously pierced cap of a 2 mL microcentrifuge tube, which played the role of tip holder and as a collection reservoir for the solutions coming out of the tips in all further steps. The tips were activated by adding 100 µL of liquid chromatography–mass spectrometry grade methanol and centrifuging for five minutes at 1,200 x g and 25 °C. The tips were then equilibrated by adding 200 µL of freshly prepared buffer A (5% acetonitrile and 0.3% of trifluoroacetic acid) and centrifuged for 5 minutes at 1,200 x g at 25 °C. The acidified samples were all added to their corresponding tips and centrifuged for 10 minutes at 5,000 x g. The tips were then washed by adding 200 µL of buffer A and centrifuging at 5,000 x g for 10 minutes. Prior to the LC-MS analyses, 100 µL of elution buffer B (300 µL TFA, 8 mL acetonitrile, 2 mL distilled water) was added to the samples, which were centrifuged for 10 minutes at 5,000 x g.

2.6.5 LC-MS data analysis

The sample runs and preliminary analysis was carried out at the Core facility BioSupraMol with the assistance of Dr. Benno Kuropka. For the analysis, dried peptides present on the stage tips were eluted with buffer B (80% acetonitrile and 0.3% of trifluoroacetic acid), vacuum dried using a speedvac machine and reconstituted in 0.1% trifluoroacetic acid and 5% acetonitrile solution. Approximately 0.5-2 µg of peptides were analysed by a reversed-phase nano liquid chromatography system (Ultimate 3000, Thermo Scientific) connected to a Q Exactive HF mass spectrometer (Thermo Scientific). Peptides were concentrated on to a trap column (PepMap100 C18, 3 µm, 100 Å, 75 µm i.d. x 2 cm, Thermo Scientific). After switching the trap column inline, LC separations were performed on a capillary column (Acclaim PepMap100 C18, 2 µm, 100 Å, 75 µm i.d. x 25 cm, Thermo Scientific) at an eluent flow rate of 300 nl/min at 40°C. Mobile phase A contained 0.1 % formic acid in water, and mobile phase

B contained 0.1% formic acid in 80 % acetonitrile, 20% water. Peptides were separated using a gradient of 5–44% B within 70 min and further increase to 95% B within 4 min, followed by a 7 min plateau before re-equilibration. Mass spectra were acquired in a data-dependent mode utilising a single MS survey scan (m/z 350–1650) with a resolution of 60,000 at m/z 200, and MS/MS scans of the 15 most intense precursor ions with a resolution of 15,000 at m/z 200 using an isolation window of 1.4 m/z. Higher-energy collisional dissociation MS/MS scans were performed with a normalised collision energy of 27. Only 2+ to 5+ charged precursors were selected for fragmentation. The dynamic exclusion time was set to 20 seconds. Automatic gain control (AGC) was set to $3x10^6$ for MS scans using a maximum injection time of 20 milliseconds. For MS2 scans the AGC target was set to $1x10^5$ with a maximum injection time of 25 milliseconds.

MS and MS/MS raw data were analysed with the MaxQuant software package (version 1.6.14) with the implemented peptide search engine (Andromeda) and label-free quantification (LFQ algorithm) (Tyanova, Temu, and Cox 2016). Data were searched against the reference proteome of *Ecc15* (4104 proteins, taxonomy 555, last modified October 2020) downloaded from the UniProt website. Default parameters were used for MaxQuant except the following: Label-free quantification was used with the match between runs option enabled. Filtering and statistical analysis was carried out using the software Perseus (version 1.6.14) (Tyanova et al. 2016). Protein hits from decoy database, potential contaminants and proteins that were identified exclusively by one site modification were excluded from the analysis. Only protein hits with measured intensity values from all the replicates were used for downstream analysis after removal of contaminants and reverse hits. Missing values were replaced from a normal distribution (imputation) using the default settings (width 0.3, down shift 1.8). Student's *t*-tests were performed using permutation-based FDR of 0.05.

2.6.5.5 Statistical analyses

The following R packages were used in the proteomics analyses and data representation: “stats” (R core team), “lme4” (Bates et al. 2014), “glmmTMB” (Brooks et al. 2017), “car” (Fox and Weisberg 2018), “pscl” (Jackman et al. 2015), “survival” (Therneau and Lumley 2014); and the following for plotting our data: “ggplot2” (Wickham 2016), “gplots” (Warnes et al. 2016), “RColorBrewer” (Neuwirth and Neuwirth 2011), “igraph” (Csardi 2013) and “plotrix” (Duursma, Levy, and Lemon 2009).

Additionally, a gene ontology (GO) analysis based on molecular functions of differentially regulated proteins was carried out in PANTHER 17.0 ((Mi et al. 2013).

2.7 *In vivo* experiments, high cost of priming

The following sub-sections contain experiments carried out using the host *D. melanogaster.* After establishing advantage of *in vitro* H_2O_2 priming upon an *in vitro* challenge in *Ecc15*, whether priming provides an advantage upon *in vivo* challenge was tested. Before testing primed bacteria in the host, the oral infection methods were modified from (Siva-Jothy et al. 2018). Standard methods and the introduced modifications will be described briefly in the following sub-sections. Following modifications in the protocol, *in vivo* priming assay where flies were allowed to feed on primed and non-primed bacteria were conducted and also described below.

2.7.1 Effect of starvation time and feeding time on host bacterial loads

Before conducting oral infections, hosts are starved to ensure maximum feeding. Whether starvation time and feeding time affect bacterial loads of the host was tested. The starvation time of 2 hours and 4 hours was tested. 2 hours is the standard starvation time used in protocols (Siva-Jothy et al. 2018; Troha and Buchon 2019; Zaidman-Rémy et al. 2006), where flies are starved for food and water followed by feeding over longer time periods of 18-24 hours (Siva-Jothy et al. 2018). However, in order to maintain consistency with *in vitro* experiments where the challenge duration was 30 minutes (section 2.5.1), a shorter feeding duration (30-minute challenge inside a host) unlike standard protocols was preferred. So, a 4-hour starvation was tested to observe if starving host for longer leads to countable bacterial loads upon shorter duration (30 minutes) of feeding. For feeding times, again, a standard feeding time of 4 hours feeding from the literature was tested (Troha and Buchon 2019), along with a feeding of 30 minutes to maintain consistency with *in vitro* experiments. The infecting bacterial solution was an OD 200 paste of *Ecc15* suspended in 5% sucrose solution**.** The experiment was repeated twice on separate days and the following methods were used.

2.7.1.1 Infecting bacterial solution

Bacterial culturing methods as described in section 1 were used. The bacterial cultures were allowed to grow overnight for ~15 hours. From these overnight cultures, exponential subcultures were started in the morning in three 2L conical flasks. To each conical flask, 190 ml of fresh LB media was added and 10 ml of bacterial culture was inoculated. A large amount of exponentially growing cultures were needed in order to have enough number of bacterial cells to create an OD 200 solution. The OD of exponentially growing cultures was checked every 30 minutes, until it reached OD 0.5, which took ~3-5 hours. After the OD reached 0.5, these cultures were mixed together and equal volumes (45 ml) were distributed across 50 ml falcon tubes. The tubes were then centrifuged at 2,500 x g for 10 minutes at 4 °C to pellet the bacteria. The supernatant was removed carefully without disturbing the pellet and spun again

under above mentioned conditions to ensure all the bacterial cells remaining in the supernatant to settle down in the pellet. All the pellets of separate falcon tubes were then combined into a single falcon tube by resuspending them in LB media and recombining them into a 50 ml falcon tube. This highly concentrated culture was then centrifuged again at 2,500 x g for 10 mins at 4°C. The supernatant was then discarded and the final pellet was re-suspended in 2 ml of 5% sucrose water solution. The OD of this highly concentrated pellet was checked by creating multiple serial dilutions and adjusted for the desired infection dose (OD 200 in this case) by adding required amounts of 5% sucrose solution.

2.7.1.2 Oral infection vials

Methods described in section 2 were used to obtain experimental flies. Five-day old adult flies were used for all the experiments. To increase the readiness for feeding, flies were starved for two and four hours prior to their exposure to the bacterial paste of *Ecc15* (Siva-Jothy et al. 2018). For starvation, flies were added by gently tapping them into an empty vial, with 5 flies of the same sex per vial and placed back in the incubator at 25°C. Meanwhile the infecting bacterial solution and infection vials were prepared. For preparation of infection vials, a round Whatman filter paper disc (Whatman™) equivalent to the diameter of food vial (25 mm) was carefully inserted into a standard plastic fly food vial (95 × 25 mm) using a self-made plunger system. This system consisted of a needleless injection syringe with glue tape added at the mouth. This glue tape was used to stick Whatman filter discs and release them carefully into the food vial. The filter discs covered the whole surface of food so that the flies have access only to the bacteria present on the filter disc. After the infection vials were ready, 100 µl of bacterial paste of OD 200 in 5% sucrose solution was added to the treatment vials whereas 100 µl of 5% sucrose solution was added to the control vials. Following were the treatment combinations: 2-hour starvation followed by a 30-minute feeding, 2-hour starvation followed by a 4-hour feeding, 4-hour starvation followed by a 30-minute feeding and 4-hour starvation followed by 4 hours feeding. For each treatment combination, 4 infection vials were prepared with 2 vials for male flies and 2 vials for female flies. Additionally, 8 control vials were prepared. All the infection vials were then allowed to absorb the microbial solution for 10 mins before addition of flies. To each infection vial, 5 flies of the same sex were added, covered with a plug and allowed to feed for 30 and 240 minutes by placing them in the incubator at 25°C and 60-70% relative humidity. In total, there were 10 flies per treatment combination per sex. Because the experiment was repeated twice, this gave a total sample size of 20 flies per treatment combination per sex.

2.7.1.3 Host surface sterilisation

To estimate the host bacterial load after treatments, the flies were surface sterilized immediately after the bacterial exposure time of 30 minutes or 4 hours. This was done by submerging them in 100 µL of 70% ethanol present in 1.5 ml centrifuge tubes for 30 s and gently mixing the ethanol using a pipette. The ethanol was then removed with a pipette and 100 µL of distilled water was added for another 30 s to wash the flies before proceeding for homogenisation. To validate the sterilisation technique, a control experiment (table 1) was performed where flies were anaesthetised with cold treatment for 30 minutes by placing them at 4°C. Following anaesthesia, they were carefully placed in a petri-dish with their body's upside down and a bacterial droplet of 5 µl was pipetted onto the abdominal surface. Treatment combinations as mentioned in table 1 were used.

Table 1: Treatment combinations used to validate the methods of fly surface sterilisation.

Treatment	n
Bacterial droplet (5 ul): No sterilisation	10
Bacterial droplet + ethanol + dH_2O: Sterilized	10
No bacteria no sterilisation	5
No bacteria plus sterilisation	5

After going through the respective treatments, flies were homogenised and plated (section 2.7.2.5) to test for the presence of countable number of CFU's in the treatments. Flies that were sterilized post bacterial droplets did not have any countable CFU's after plating whereas the flies that received just the bacterial droplets and no sterilisation had countable number of bacterial colonies.

2.7.1.4 Host homogenisation for bacterial load estimation

Post sterilisation, the flies were homogenised in an order independent of sex and treatment. One set of flies was homogenised after 30 minutes of feeding whereas the other set was homogenised after 4-hours of feeding. After each fly was individually surface sterilized, they were retrieved individually using sterilised forceps in a 1.5 mL microcentrifuge tube containing 100 µl of pre-chilled LB media and one stainless steel bead (Ø 3 mm, Retsch) on ice. The microcentrifuge tubes were then placed in a holder that had previously been chilled in the fridge at 4°C for at least 30 minutes to reduce further growth of bacteria. The holders were placed in a Retsch Mill (MM300) and the flies homogenised at a frequency of 20 Hz for 45 seconds. Then, the tubes were centrifuged at 420 rcf for one minute at 4ºC. After resuspending the solution thoroughly using a pipette, 100 microliters or as much as possible of the homogenate from each fly was pipetted into a 96-well plate and then serially diluted 1:10 until $1:10^5$. Per fly, five droplets of 5 µL of every dilution were plated onto square LB agar plates (120*120*17 mm). The plates were incubated upside down at 25ºC and the numbers of CFUs

were counted after ~20 hours. Individual bacterial loads per fly were back-calculated using the average of the five droplets from the lowest countable dilution in the plate, which was usually between 10 and 60 CFUs per droplet.

2.7.1.5 Statistical analyses

Here, a generalised linear model with a quasipoisson error structure was fitted to test if starvation time and feeding time have an effect on bacterial loads. The following model was tested:

Model 4 <- glm (CFUs per fly ~ feeding time * starvation time, family = quasipoisson)

Additionally, Levene test was used to test if the treatment groups have equal variances.

2.7.2 Effect of optical density and microbial growth medium on host bacterial loads

Standard protocols use OD 200 bacterial cultures mixed in a 5% sucrose solution (Siva-Jothy et al. 2018). However, under *in vitro* experimental conditions, an OD 0.5 was used. Additionally, the resuspension of bacterial culture in oral infections is always done in a sucrose solution (Siva-Jothy et al. 2018; Troha and Buchon 2019; Zaidman-Rémy et al. 2006) to make the infecting solution attractive for fly feeding. But the change of growth medium could be an additional stressor for bacteria since they were cultured throughout priming treatment in LB medium. The temperature of centrifugation while preparing infecting bacterial solution was changed from 4°C to 25°C to avoid cold shocks for *Ecc15*. Using the following methods, effect of optical density and growth medium on feeding was tested. For optical density, a bacterial culture of OD 0.5 (similar OD to *in vitro* experiments) and OD 2 were tested with both LB and sucrose solution as growth medium. Additionally, a control of OD 10 bacterial solution suspended in sucrose was used because of it having high bacterial density under standard conditions (sucrose solution). The following treatments were present: OD 0.5 in LB medium, OD 0.5 in sucrose solution, OD 2 in LB medium, OD 2 in sucrose solution and OD 10 in sucrose solution. Flies were allowed to feed on all the above-mentioned treatments for 30 minutes and 60 minutes except control flies, which were allowed to feed for 4 hours in line with the standard protocols.

2.7.2.1 Oral infection assay

For preparing infecting bacterial solution, methods described in section 2.7.2.1 were used with modifications. Since a large number of bacterial cells were not needed unlike previously mentioned, due to low OD's, bacteria were cultured in falcon tubes as described in section 2.1. After the OD reached 0.5, the cultures were combined and centrifuged at 2,500 x g for 10 minutes at 25 °C to pellet the bacteria. Once the pellets were obtained, supernatant was removed carefully without disturbing the pellets. The pellets were then combined and

resuspended in 5 ml LB media and OD of this pellet was checked by creating multiple serial dilutions and adjusted for the desired infection doses (OD 0.5 and 2) by diluting with required amounts of LB medium. Parallel to this, bacterial culture was also readjusted to OD 10 resuspended in sucrose to act as a control for this experiment. For oral infections in *D. melanogaster*, methods described in section 2.7.2.2 were used, including the same numbers of flies per treatment. Followed by a 4-hour starvation, flies were allowed to feed for 30 minutes and 60 minutes whereas control flies were allowed to feed for 240 minutes. The methods described in section 2.7.2.3 were used for sterilisation followed by methods mentioned in section 2.7.2.4 for homogenisation and bacterial load estimation.

2.7.3 Primed *Ecc15* inside the host *D. melanogaster*

It was tested if *Ecc15,* which has been primed *in vitro* with 1 mM of H_2O_2, has a survival advantage when present inside a host compared to non-primed *Ecc15*. *D. melanogaster* was allowed to feed on primed and non-primed *Ecc15* for 30, 60 and 240 minutes and then homogenised and plated to compare the bacterial loads across treatments. It was also tested if there is an effect of treatment (i.e., primed/non-primed) or sex of the host and whether this effect differs over time. The experiment was repeated three times on separate days. At the same time as the *in vivo* experiments were carried out, *in vitro* controls were simultaneously performed and are described in section 2.8. A simplified procedure of simultaneous *in vitro* and *in vivo* protocols is shown in figure 4.

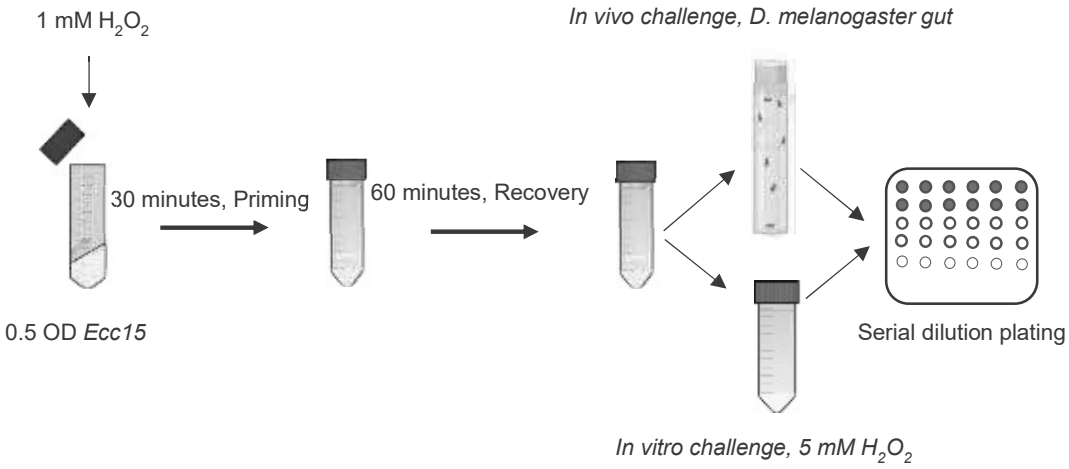

Figure 4: Simplified schematic of *in vivo* experiments and simultaneous *in vitro* controls. When performing oral infections for *in vivo* challenge, bacteria were primed *in vitro* for 30 minutes followed by a recovery phase of 60 minutes. After recovery, they were given an *in vivo* challenge inside the host. Simultaneously, *in vitro* challenge was carried out as a control to confirm the priming phenotype. After 30 minutes of challenge, both *in vivo* and *in vitro* treatments were then serially diluted and plated to estimated CFU counts.

2.7.3.1 Oral infection assay

Ecc15 was cultured based on methods described in section 2.1. An *in vitro* priming protocol as described in section 2.5.1 was carried out with three biological replicates (falcon tubes) per treatment. A priming concentration of 1 mM was added to the only priming treatment whereas non-primed bacteria (i.e., control) received equal volume of LB media. The procedure was similar until the recovery phase as described in section 2.5.1. After the recovery phase, primed and non-primed bacteria were added to infection vials to receive an *in vivo* challenge. The *in vitro* challenge protocol was carried out simultaneously to ensure priming phenotype under *in vitro* conditions as shown in simplified figure 4. For oral infections to test *in vivo* priming in *D. melanogaster*, methods described in section 2.7.2.2 were used with 10 flies per treatment per sex per time point. Flies were starved for 4 hours followed by a 30,60- and 240-minute feeding on primed and non-primed bacteria. The bacteria were primed based on methods described in section 2.5.1. Methods described previously in section 2.7.2.4 were used for homogenisation and bacterial load estimation.

2.7.3.2 Statistical analyses

Using a natural log transformed linear model, it was tested whether there is a significant difference in bacterial load of flies that were allowed to feed on primed *Ecc15* as compared to ones that fed on non-primed *Ecc15*. The response variable was bacterial load per fly, or CFU counts, extracted from each fly on homogenisation for each time point. Our experiment consisted of three time points: 30,60 and 240 minutes. Two-way interactions were included and experimental replicate was used as a fixed factor using the following model:

Model 5: log (CFUs per fly) ~ treatment * time + treatment * sex + time * sex + replicate

2.8 *In vitro* controls: differential number of bacteria in primed and non-primed treatments

Simultaneous to *in vivo* challenge via oral infections, *in vitro* experiments were conducted to confirm the priming phenotype. Several additional controls were conducted to test whether there are differential number of bacterial cells present in primed and non-primed treatments during different time points of the experiment. The following subsections describe *in vitro* control experiments and all of them were carried simultaneously with oral infections, hence replicated three times, except bacterial survival on filter discs (section xxx), which was replicated two times.

2.8.1 *In vitro* control: Do bacterial numbers differ across replicates in OD 0.5 bacterial cultures?

This experiment was carried out to test if the viable bacterial cells in exponentially growing OD 0.5 cultures differ across experimental replicates. To test that, the starting OD 0.5 cultures were serially diluted and plated followed by CFU counting to estimate CFUs per ml of each OD 0.5 culture using methods described elsewhere (section 2.5.1).

2.8.1.1 Statistical analyses

Here, a log transformed linear model was fitted to test if there are CFU differences in OD 0.5 cultures across different replicates performed on separate days using the following model:

Model 6 <- log (CFUs per ml ~ replicate)

2.8.2 *In vitro* control: Does priming stress lead to lower bacterial cells in primed treatments when compared with non-primed treatments?

This experiment was done to test if priming dose leads to some mortality and hence lower number of bacterial cells in priming treatments as compared to non-primed treatments. *Ecc15* underwent an *in vitro* priming procedure based on methods described in section 2.5.1. After the bacteria had been primed, it was followed by a recovery phase before undergoing an *in vivo* challenge via oral infections (section 2.7.3). Since it was after recovery that primed and non-primed *Ecc15* received an *in vivo* challenge via oral infections, both primed and non-primed bacteria were plated ten minutes prior to conclusion of recovery time to test if there are differential number of cells in the treatments. The plating was done ten minutes prior to the end of recovery time due to technical reasons. Differential number of cells could mean flies having an access to unequal viable CFU's of primed and non-primed bacteria. Both the treatments were serially diluted and plated using methods described in section 2.5.1.

2.8.2.1 Statistical analyses

Here, a natural log transformed linear model was used to test if the bacterial CFU's differ based on treatment and replicate using the following model:

Model 7 <- log (CFUs per ml ~ treatment * replicate)

2.8.3 *In vitro* control: Does *Ecc15* undergoing *in vivo* challenge have a survival advantage upon an *in vitro* challenge?

An *in vitro* experiment was carried out simultaneously to confirm whether *Ecc15* had a survival advantage upon challenge under *in vitro* conditions. To do so, methods described in section 2.5.1 were used, with three experimental replicates for each treatment. The treatments were plated via serial dilution plating and CFU's counts were carried out.

2.8.3.1 Statistical analyses

This control experiment was conducted to test if *Ecc15* cultures receiving an *in vivo* challenge show a priming phenotype upon receiving an *in vitro* challenge. It was tested this by fitting the following linear model:

Model 8 <- lm (CFUs per ml ~ treatment * replicate)

2.8.4 *In vitro* control: Do primed and non-primed bacteria survive differently on Whatman filter discs?

It was tested if bacteria survive differently on the surface of filter discs based on their priming status. For testing the differential survival of bacteria on Whatman filter discs, five additional infection vials were per treatment were prepared as described in section 2.7.2.2. These infection vials without any flies were then placed in incubator at 25° C along with oral infection vials for 30, 60 and 240 minutes. After each time point, the vials were taken out of the incubator, the filter discs were carefully removed using a sterilised tweezer and placed into a 50 ml falcon tube containing 2 ml of LB medium. The falcon tubes were vortexed at medium speed for 60 seconds in order to disrupt bacteria from the surface of filter discs and suspend them into the LB medium. The LB medium was diluted via serial dilutions 1:1 up to $1:10^8$ and plated to count CFU's per ml of primed and non-primed bacteria as described in section 2.5.1.

2.8.4.1 Statistical analyses

It was tested if there is a differential survival of primed and non-primed bacteria on filter discs at three different time points corresponding to *in vivo* oral infections: 30 minutes, 60 minutes and 240 minutes. This experiment, as previously mentioned, was repeated twice on two separate days and the data was analysed separately for each replicate using the following model:

Model 9 <- lm (CFU per ml ~ treatment * time)

2.8.5 Applying correction factors to host bacterial loads

Since all the control experiments described in section 2.8 suggested lower survival of primed bacteria as compared to non-primed, this mortality difference was controlled for differential numbers using post-hoc correction methods. Using control experiments mentioned above, it was possible to determine the differences in bacterial numbers between two treatments at several time points during *in vivo* experiments. However, it was after a recovery time that primed and non-primed *Ecc15* went through an *in vitro* and an *in vivo* challenge, it was decided to apply corrections to *in vivo* bacterial loads based on CFU differences at this time point. The correction factors were calculated by comparing the bacterial counts of primed and non-primed treatments using following formula: CFU per ml non-primed bacteria/ CFU per ml primed bacteria. The correction factors were then multiplied by CFUs of primed bacteria to account for lesser CFUs. Further, the correction factors were calculated individually for each replicate since analysis pointed to differential number of CFUs in the initial OD 0.5 cultures. The correction factors were applied to the bacterial loads obtained from section 2.7.4 and the data was analysed based on the corrected loads.

2.8.5.1 Statistical analyses

Using a natural log transformed linear model, it was tested whether there is a significant difference in corrected bacterial load of flies that were allowed to feed on primed *Ecc15* as compared to non-primed *Ecc15*. The response variable was corrected bacterial load per fly, or CFU counts, extracted from each fly on homogenisation for each time point and corrected using a correction factor. Our experiment consisted of three time points: 30,60 and 240 minutes. Two-way interactions were included and experimental replicate was used as a fixed factor using the following model:

Model 10: log (CFUs per fly) ~ treatment * time + treatment * sex + time * sex + replicate

2.9 *In vitro* experiments: low cost of priming

Since there was a substantial cost associated with the priming concentrations used in the previous set of *in vitro* experiments (section 2.5), where 1 mM was used as the priming concentration, another set of experiments was conducted to lower the costs imposed by priming experimentally. In order to do so, costs associated with priming for a range of H_2O_2 concentrations were tested by assessing their optical density and CFU counts upon receiving increasing doses of H_2O_2. It was then tested if priming with these concentrations of H_2O_2 leads to a survival advantage upon receiving a challenge dose. The following sections concern the experiments performed in order to test the H_2O_2 concentrations that might lead to a lower associated cost.

2.9.1 Costs of H_2O_2 *in vitro* priming: based on optical density

In order to test the priming costs associated with several concentrations of H_2O_2 on *Ecc15*, I first calculated the costs by following the optical density of *Ecc15* growth curves over 24 hours. This method provided a robust and easy approach to test microbial growth under different H_2O_2 concentrations by using optical density measurements at 600 nm as a proxy. Additionally, since Rodriguez-Rojas and colleagues (Rodríguez-Rojas et al. 2020) used an optical density-based approach to test the costs of priming in *Escherichia coli,* we wanted to compare the costs using the same methods for *Ecc15.*

Bacterial growth curves were measured in flat-bottom 96-well micro-plates with anti-condensation lids (Nunc, Denmark). Bacteria were cultured following the methods described in section 1. Fresh LB media and a fresh stock of 20 mM H_2O_2 were prepared and kept in a dark place until usage to avoid oxidation. Since previous *in vitro* experiments with 1mM priming concentration led to a substantial cost, a range of concentrations lower than 1 mM were tested. The following H_2O_2 concentrations were tested: 0.5 mM, 0.25 mM, 0.125 mM, 0.1 mM and 0.0625 mM with 16 independent wells for each concentration. To obtain these concentrations, LB (lysogeny broth) media was mixed with the appropriate amounts of H_2O_2 to make the highest concentration (0.5 mM) used in the experiment. In the 96-well plate, 200 µl of freshly made LB-H_2O_2 (0.5 mM) was added to the first two columns (16 wells). To all further wells, 100 ul of LB was added, and 100 ul from the first columns containing 0.5 mM was mixed multiple times using a multichannel pipette for serial dilutions until the concentration of 0.0625 mM was achieved. The excess volume of 100 µl was discarded from the last set of wells to have the final volume of 100 µl in all the wells. Separately, a final concentration of H_2O_2 at 0.1 mM was prepared by diluting with LB media. Additionally, a column of positive control was set-up with just bacterial culture and no H_2O_2. One µl of bacteria from the overnight culture of OD ~1 was inoculated using a multichannel pipette into all the wells of the 96-well plate except for the negative control, which was the control to confirm that the medium was not contaminated.

The plate was then placed in a microplate reader Synergy H1 (Biotek, Germany) and programmed to read kinetic readings OD_{600} every 10 mins with a short shaking of 5 seconds between readings for 24 hours at 25°C. The growth curves of *Ecc15* in the presence of different concentrations of H_2O_2 were tracked over a period of 24 hours, and the cost of priming by H_2O_2 was estimated from the parameters of the growth curves. All model parameters including: carrying capacity, initial population size, growth rate, doubling time and the empirical area under the curve, were calculated using the Growthcurver R package (Sprouffske and Wagner 2016).

2.9.1.1 Growth curve analyses

In order to obtain percentage costs associated with increasing concentrations of priming based on optical density, the changes in growth curves for 24 hours were followed. Data from 0 h to 24 h were used to obtain the following parameters for all the H_2O_2 concentrations. All the parameters, such as carrying capacity, generation time, area under the logarithmic curve and area under the empirical curve were obtained via R package Growthcurver (Sprouffske and Wagner 2016). The mean, and standard deviation for all the parameters for each of the concentrations were calculated for each H_2O_2 concentration. The area under the curve was used to compare the treatments and calculate associated costs. The mean area under the curve of non-treated control was compared with all the other treatments with different H_2O_2 concentrations, and the following formula was used to find the percentage cost of priming: 100- (area under the curve_control / area under the curve_peroxide concentration x100).

2.9.2 Costs of H_2O_2 *in vitro* priming: based on CFU counts

Although the costs of priming can be tested in a robust manner using an optical density-based method, it does not distinguish between live and dead bacteria, which could lead to wrong estimations. Additionally, growth curves were tracked for 24 hours, but *in vitro* experimental conditions call for shorter time frames (30 minutes); therefore, the costs were tested based on CFU counting. Using a CFU plating-based method, costs could be estimated reliably based on viable bacterial cells. It provided a closer estimate of the costs expected to be found under *in vitro* experimental conditions.

To test the cost of priming under *in vitro* experimental conditions, each priming concentration that had been tested in a 96-well plate was tested in a falcon tube with 2 replicates per concentration. Bacteria were cultured following the methods described in section 1. The three exponential cultures were then mixed together and equally distributed into falcon tubes with 10 ml per falcon tube, except in the negative control, where only LB media was added. From the freshly prepared stock of 20 mM H_2O_2, appropriate amounts of H_2O_2 were added to respective falcon tubes to make the final concentrations of H_2O_2. To make 0.5 mM in the falcon

tube, 250 µl of 20 mM H_2O_2 was added. Similarly, 125 µl, 62.5 µl, 31.25 µl and 50 µl of 20 mM H_2O_2 was added to make 0.25 mM, 0.125 mM, 0.0625 mM and 0.1 mM, respectively. These falcon tubes with bacterial cultures mixed respective concentrations of H_2O_2 were then incubated at 25 °C at 200 rpm for 30 minutes and the methods described in section 2.5.1 were followed with modifications. After 30 minutes of incubation with H_2O_2, the cells were allowed to recover from the stress for 60 minutes. After the recovery phase, all the treatments, including contamination control, were serially diluted from 1:10 to $1:10^6$ (20 µl of bacterial culture to 180 µl of LB) and plated as described in section 2.5.1. The colony-forming units (CFUs) were then counted the next day. The order of the treatments was randomised when plating and CFU counting was performed blind to the treatment.

2.9.2.1 Calculating cost based on CFU counts

In order to obtain costs associated with priming based on CFU count, the CFUs per ml of non-treated control were compared with the CFU counts when *Ecc15* received different concentrations of H_2O_2. The associated costs were then calculated using the following formula:100- (CFU per ml_control / CFU per ml_peroxide concentration x100).

2.9.3 *In vitro* experiments: lower cost of priming

After establishing the costs associated with different concentrations of H_2O_2, *in vitro* priming experiments were carried out to test if there was a survival advantage of priming with lower doses upon receiving a challenge dose. The following subsections contain experiments where survival advantage of priming upon challenge was tested for lower priming concentrations.

2.9.3.1 Survival advantage of lower concentrations of priming upon challenge

All the *in vitro* priming experiments were carried out in 50 ml falcon tubes. All the above-tested lower concentrations of H_2O_2 were used as priming concentrations (0.25, 0.125, 0.1 and 0.0625 mM) and it was tested whether they provide a survival advantage upon challenge (5 mM). For testing this, following treatment combinations were used: control, only priming (0.25, 0.125, 0.1 and 0.0625 mM), only challenge (5 mM) and priming + challenge (0.25 + 5, 0.125 + 5, 0.1 + 5 and 0.0625 + 5 mM). The experiment was replicated two times on separate days with two replicates per treatment.

To test the survival advantage upon challenge, methods described in 2.5.1 were used. In the falcon tubes with 10 ml bacterial culture, H_2O_2 according to the required priming concentrations (0.25 mM, 0.125 mM, 0.1 mM and 0.0625 mM) was added to the only priming and priming + challenge treatments. After adding the priming dose, all the falcons were allowed to incubate for 30 minutes at 25°C at 200 rpm, followed by a recovery phase. After the recovery time was over, desired challenge dose was added to only challenge and priming + challenge treatments. The bacterial cultures were allowed to undergo the challenge

treatment by placing them in the incubator for 30 minutes, after which 10 µl of pre-made catalase (4µg/ml) (Sigma Aldrich, Germany) was added to get rid of all the residual peroxide from the cultures. This was followed by plating the serial dilutions as described in section 2.5.1.

2.9.3.2 Statistical analyses

Pairwise comparisons were conducted to test if priming with 0.25, 0.125, 0.1 and 0.0625 mM of H_2O_2 leads to an increase in survival in case of an *in vitro* challenge (5 mM). All the priming + challenge treatment combinations were compared with only challenge treatment and used Bonferroni corrections for corrected *p*-value.

2.9.4 *In vitro* priming: 0.1 mM priming dose and 5 mM challenge dose

Based on costs and survival advantage provided upon *in vitro* challenge by different concentrations of H_2O_2, 0.1 mM was chosen as the priming concentration, and further experiments were carried out with 0.1 mM as the priming concentration and 5 mM as the challenge dose. These *in vitro* experiments had the following treatments: control, only priming, only challenge, and priming + challenge. Each treatment had three replicates (falcon tubes) and was replicated three times on separate days. The same methods as described above in section 2.5.1 were followed.

2.9.4.1 Statistical analyses

To test whether priming provides a survival advantage upon exposure to challenge, a linear model was used. Natural log transformed bacterial load was used as the response variable and presence or absence of priming and challenge, as well as their interaction, were included as factors, with experimental replicate included as a random factor. The resulting model was the following:

Model 11: log (CFUs) ~ priming × challenge + (1| experimental replicate)

In this model, it was tested whether priming gave a survival advantage to *Ecc15* upon receiving challenge and if priming itself had a fitness effect on the bacteria when compared with the non-treated control. Additionally, post-hoc multiple comparisons were performed using “emmeans”.

2.10 *In vivo* experiments: low cost of priming

Ecc15 that had been primed *in vitro* with 0.1 mM of H_2O_2, was tested for its survival advantage when present inside a host. In order to test this, oral infections using *D. melanogaster* as previously described were carried out. In order to test survival advantage inside a host, *D. melanogaster* was allowed to feed on primed and non-primed *Ecc15* for 30, 60 and 240 minutes and then homogenised and plated to compare the bacterial loads across treatments.

2.10.1 Oral infection assay

To obtain primed *Ecc15,* methods previously described in section 2.9.1 were used. After obtaining primed and non-primed *Ecc15*, methods described in section 2 were used to obtain experimental flies. Five-day-old flies were used for all the experiments, and this experiment was replicated twice on separate days. To increase the readiness for feeding, flies were starved for four hours (Siva-Jothy et al. 2018) prior to their exposure to primed and non-primed *Ecc15*. Infection methods described in 2.7.3 were followed. To each infection vial, 5 flies of the same sex were added, covered with a plug and allowed to feed for 30, 60 and 240 minutes by placing them in the incubator at 25°C and 60-70% relative humidity. In total, there were 10 flies per treatment per sex for each time point. Feeding was followed by surface sterilisation described in section 2.7.4. Homogenisation and plating as described in section 2.7.5 were carried out to estimate bacterial loads of flies.

2.10.1.1 Statistical analyses

Using a natural log-transformed linear model, it was tested whether there was a significant difference in the bacterial load of flies that were allowed to feed on primed *Ecc15* compared to non-primed *Ecc15*. The response variable was bacterial load per fly, or CFU counts, extracted from each fly on homogenisation for each time point. Our experiment consisted of three time points: 30,60 and 240 minutes, and each time point was analysed separately. A three-way interaction was not included since it was difficult to interpret. Additionally, the experimental replicate was not used as a random factor because the random effects were minimal, indicated by the “isSingular” warning. Treatment, corresponding to whether the flies fed on primed or non-primed bacteria, was added as a factor, and sex and experimental replicate were fitted as fixed factors.

Model 12: log (CFUs) ~ treatment * sex + experimental replicate

Section 3

Results

3.1 Hydrogen peroxide Minimal inhibitory concentration (MIC) for *Ecc15*

The MIC of hydrogen peroxide for *Ecc15* based on the broth microdilution method was confirmed to be 1 mM. This was further corroborated by examining growth curves of *Ecc15* under hydrogen peroxide concentrations ranging from 32 mM – 0.0625 mM using a plate reader (Figure S1).

3.2 Generation time of *Ecc15* at 25°C

Since *Ecc15* was grown at 25°C throughout the experiments, the doubling time for *Ecc15* at 25°C was tested. Based on two replicates of growth curves conducted in a 96-well plate with 80 individual wells per plate, the generation time of *Ecc15* under our experimental conditions was found to be 91.07 ± 6.54 minutes.

3.3 *In vitro* experiments, high cost of priming

3.3.1 *In vitro* priming assay: establishing priming concentrations

There was a significant effect of treatment on bacterial survival (table 2). Post-hoc comparisons indicated that all the priming + challenge treatments had a higher survival compared to the treatments that received only challenge (table S1). The highest survival advantage upon challenge was shown by bacteria primed with 1 mM hydrogen peroxide (Figure 5), which is why this dose was used for further experiments. Additionally, preliminary tests were carried out in 96-well plate reader (Figure S2).

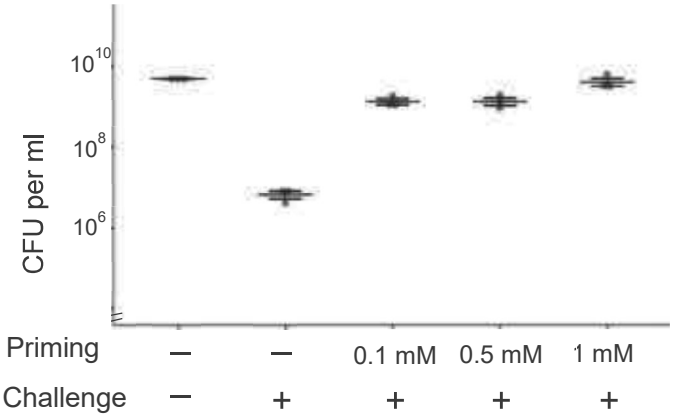

Figure 5: Testing several priming doses for their effect on bacterial survival upon challenge. Three priming doses and their survival advantage upon challenge were tested. (-) represents no hydrogen peroxide whereas (+) represents treatments wherever hydrogen peroxide was added. The concentrations mentioned above (0.1, 0.5 and 1 mM) were used as priming concentrations and 5 mM was used as a challenge concentration. Colony forming units (CFUs) per ml were estimated by plating out serial dilutions of each treatment. This experiment was performed once with three biological replicates (falcon tubes) for each treatemnt. Each dot is the mean bacterial load of eight droplets. Black lines show means and standard errors.

Table 2: Testing several priming doses for their effect on bacterial survival upon challenge. The significant factors are shown in bold.

Tested effect	*df*	*F*	*p-value*
treatment	4	37.80	**<0.0001**

3.3.2 Testing 1 mM H_2O_2 priming concentration for its survival advantage upon challenge

It was tested if *in vitro* priming with 1 mM H_2O_2 leads to survival advantage in *Ecc15* upon an *in vitro* challenge. The bacterial CFU's of following four treatments were compared: control, only priming, only challenge and priming + challenge. There was a statistically signifcant interaction between priming and challenge treatments (table 3).There was also a significant effect of an only challenge treatment. Post-hoc pairwise comparisons showed that the only challenge treatment differs significantly from the control treatment, suggesting that the only challenge concentration of 5 mM leads to a significant lethality as compared to non-treated *Ecc15* (table S2, Figure 6). Only challenge treatment also differed significantly from only priming treatment, suggesting high mortality when *Ecc15* experiences a challenge stress as compared to the priming stress. Additionally, bacteria that had been primed with H_2O_2 had higher survival upon challenge compared to bacteria that had only received a challenge dose, reflected by significant differences in an only challenge treatment compared with priming + challenge treatment. No signifcant differences in only priming treatment when compared with control (*df: 40.9, t: 2.502, p= 0.074*) were observed, suggesting priming does not lead to significant decrease in survival.

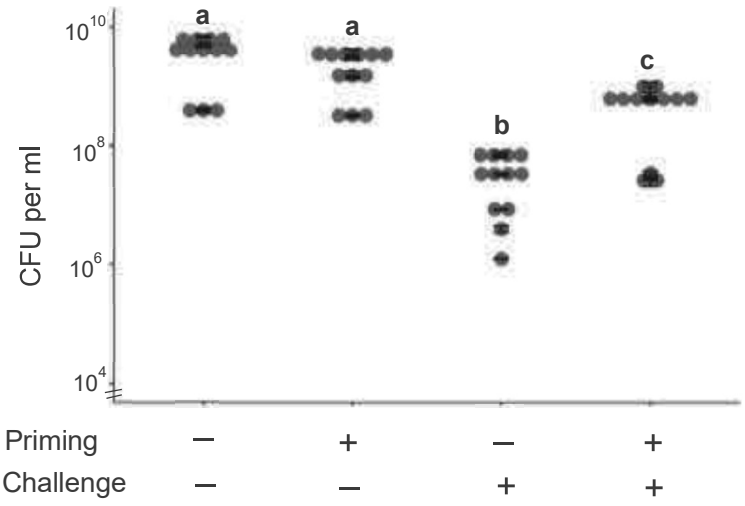

Figure 6: Differential survival of *Ecc15* primed with 1 mM of hydrogen peroxide upon receiving a 5 mM challenge dose. Primed populations had a survival advantage upon challenge compared to the *Ecc15* that received only the challenge doses of H_2O_2. (-) represents no addition of H_2O_2 whereas (+) represents the treatments where H_2O_2 was added. Colony forming units (CFUs) per ml were estimated by plating out serial dilutions of each treatment. The experiment was repeated four times on separate days. Each data point represents mean bacterial loads of three technical replicates. Black lines show means and standard errors and different letters represent treatments that differ significantly from each other.

Table 3: The effect priming and challenge with H_2O_2 on bacteria survival. 1 mM was used as the priming dose and 5 mM as the challenge dose. The significant factors are shown in bold.

Treatment	*df*	χ^2	*p*-value
Priming	1	6.26	**0.012**
Challenge	1	431.7424	**<0.0001**
Priming × Challenge	1	99.3012	**<0.0001**

3.4 Proteomic basis of *in vitro* priming

3.4.1 Does priming followed by a 10-min challenge exposure lead to higher *Ecc15* survival?

In the control *in vitro* experiment utilising the same conditions as those used to collect LC-MS samples, i.e., 10 minutes of exposure to a challenge dose of hydrogen peroxide, there was a significant interaction between the priming and challenge treatment (table 4). Post-hoc tests showed significantly higher survival of primed populations upon receiving a challenge as compared to the populations that received only a challenge dose (Figure 7, table S3). This data indicates that a 10-minute exposure to the challenge dose as opposed to a 30-minute exposure, as used in other *in vitro* experiments, is also capable of providing a survival advantage to the primed *Ecc15*. Additionally, only priming treatment differs significantly from control treatment, suggesting significant mortality associated with priming dose, which was found to be ~50%.

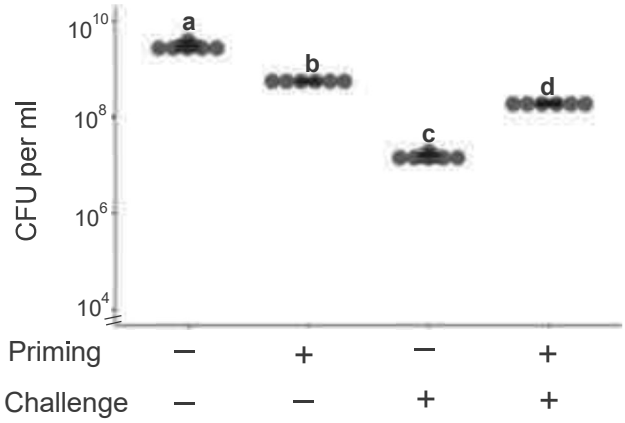

Figure 7: Survival advantage of priming (1 mM) upon challenge (5 mM) for 10 minutes. *Ecc15* primed with hydrogen peroxide shows improved survival on receiving a challenge dose (priming + challenge) of hydrogen peroxide compared to the treatment that receives just the challenge dose (only challenge). The exposure to challenge was just for 10 minutes instead of 30 minutes as shown in section 2.5. The experiment was performed as a control utilising same conditions used for LC-MS samples to test if priming provides a survival advantage upon challenge under an *in vitro* exposure to challenge for 10 minutes.

Table 4: The effects of priming and challenge, and their interaction, on the response variable bacterial CFUs. The experiment was carried out *in vitro* and simultaneous to preparation of samples for proteomics. Statistically significant factors are shown in bold.

Treatment	*df*	χ^2	*p*
Only priming	1	1193.7	**<0.0001**
Only challenge	1	11313.1	**<0.0001**
Priming × challenge	1	3638.5	**<0.0001**

3.4.2 Proteomics of the priming response

3.4.2.1 Global proteomics response

The quality of the mass-spectrometry derived dataset was examined by looking at reproducibility using Pearson correlation between replicates of the treatments (Figure S3). The correlation was based on label- free quantification (LFQ) intensities, a method used to determine relative number of proteins in biological samples. There was a tight positive linear correlation across all five replicates of the same treatment, lying between 0.992 ± 0.001.

The average number of proteins identified and matched to the *Ecc15* proteome were 1463.65 ± 9.93 proteins across all our treatments and replicates (table 5). In all the samples, the fraction of missing values was replaced by using a normal distribution of intensities close to the detection limit. This was done to make sure that the upregulated proteins are not removed from the dataset if they were not detected e.g., in the control. These proteins instead receive the lowest possible intensity value.

Table 5: Mean number of proteins detected in each treatment based on mean values across five replicates represented as mean ± standard error.

Treatment	Mean no. of proteins detected
Control	1452 ± 11.12
Priming	1494.6 ± 6.77
Challenge	1445.6 ± 34.50
Priming + challenge	1462.4 ± 12.70

The global proteomics dataset showed differential regulation corresponding to various branches of molecular activities, many of them corresponding to antioxidation, nucleic acid protection and damage repair mechanisms (Figure 8). The Gene Ontology (GO) analysis based on molecular functions of differentially regulated proteins suggested that the highest percentage of genes were involved in catalytic activity (54.4%), binding activity (25.8%) followed by structural molecule activity (9.2%) (table S4). Genes regulating transcription and

translation, such as initiation and elongation factors along with ATP dependent proteases such as serine proteases were also found to be differentially regulated.

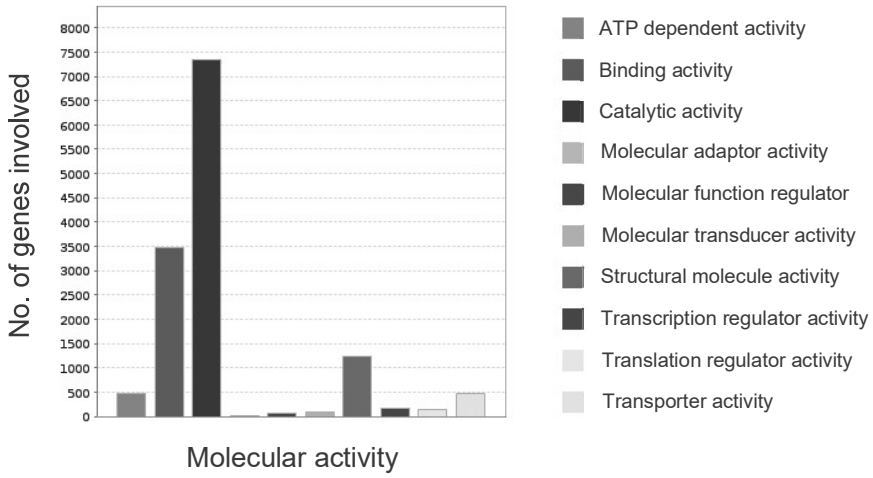

Figure 8: Representation of gene categories based on their molecular function from Gene ontology analysis (Panther 17.0). The categories here are an overall representation of differentially regulated genes irrespective of treatment categories.

Since catalytic activity proteins are the ones that show the maximum amount (54.4 %) of differential regulation, the categories of catalytic proteins involved in forming a global response against hydrogen peroxide stress were investigated (Figure 9, table S5).

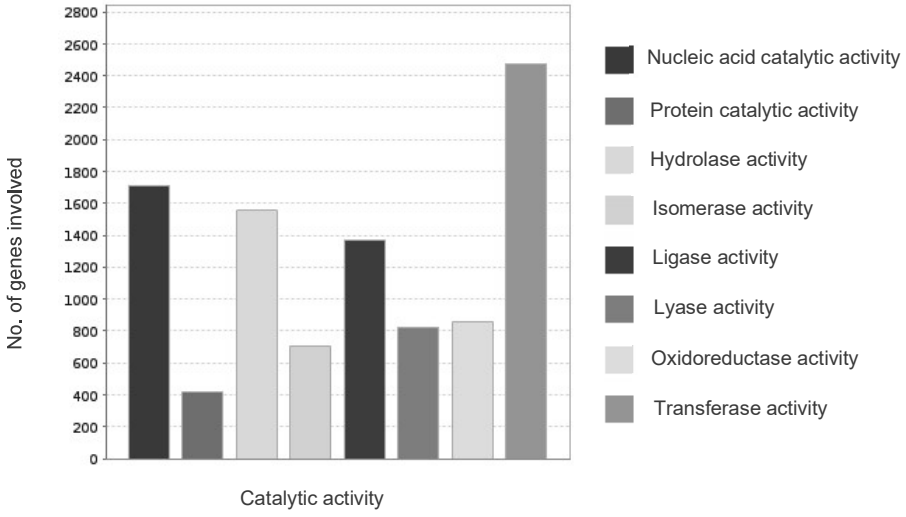

Figure 9: Representation of gene categories involved in catalytic activity based on their molecular function based on Gene ontology analysis (Panther 17.0). This suggests that the greatest number of genes are involved in transferase and nucleic acid catalysis followed by hydrolase and ligase enzymes.

Based on GO analysis, in the category of nucleic acid catalysis, 76% genes involved regulate RNA catalysis as opposed to DNA catalysis (23%). In the case of protein catalysis, 5.2% of the genes belong to the following categories mentioned in descending order of abundance: peptidases, protein kinases, phosphoprotein phosphatases, methyltransferases, protein disulphide reductases and ubiquitin like proteins.

3.4.2.2 Differential proteomics response based on priming and challenge status

In the principal component analysis (PCA), there was a clear segregation between "only challenge" treatment from "priming + challenge" treatment (Figure 10). However, control and only challenge treatments lie close together, suggesting that these two treatments are quite similar to each other. In case of only priming treatment, when compared with the priming + challenge treatment, close to no changes in proteome were observed based on their vicinity in PCA.

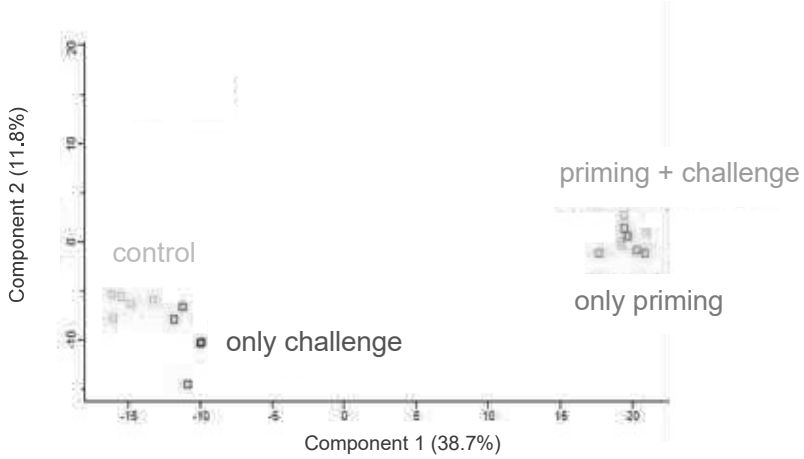

Figure 10: **PCA analysis representing the distribution of the treatment groups and replicates**. Principal component analysis (PCA) for five replicates of all the treatments. Each replicate is represented by a box. Treatments that are close or overlapping together suggests similar proteomes whereas treatments that are segregated from each other represents that the proteomes differ from each other.

Based on the PCA results, two sets of treatments were compared to test for significantly upregulated and downregulated proteins (control vs only priming and only challenge vs priming + challenge) (Figure 11). The control was compared with only priming in order to test for the proteomic changes induced by priming whereas only challenge was compared against priming + challenge to test which genes are differentially upregulated across these treatments which might lead to survival advantage of primed populations upon challenge. Additionally, control was compared with only challenge treatment to test for differential regulation caused by lethal doses of H_2O_2 (Figure S4).

When comparing the control treatment with only priming treatment, 58% proteins (880) were upregulated and 4% (60) proteins were downregulated. In only challenge treatment, 16.6% (250) proteins were upregulated and 15% (150) proteins were downregulated. When only challenge and priming + challenge treatment were compared, 6.66% (100) proteins were found to be upregulated whereas 10.66% (160) proteins were downregulated.

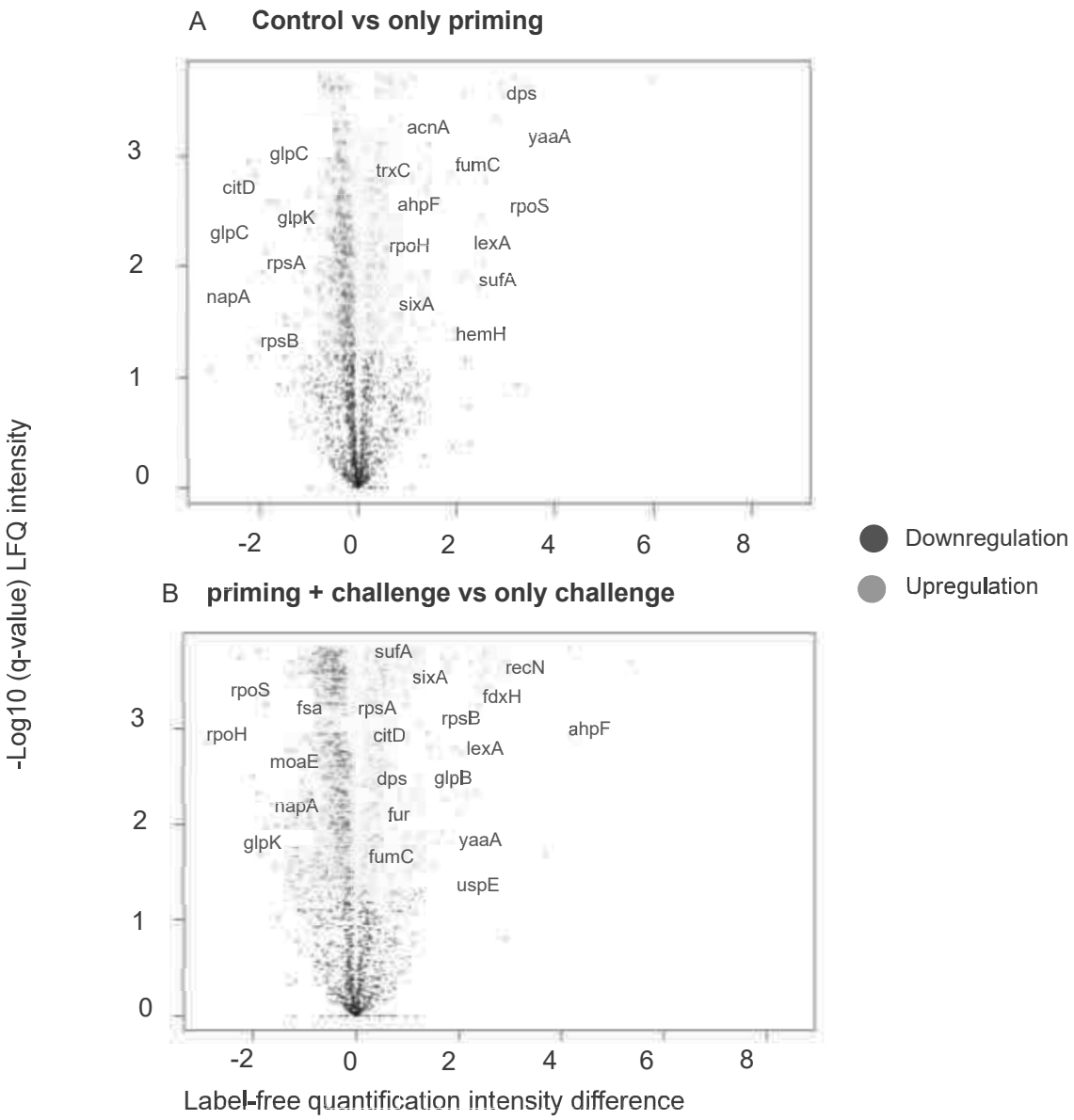

Figure 11: Volcano plot of –log q values (q value is a t-test value corrected for FDR) against the log2 fold-change of protein intensity measured by LC-MS using label-free quantification (LFQ) method. Figure A shows differentially regulated proteins when comparing controls with an only priming treatment. Figure B shows a comparison of only challenge treatment compared to the priming + challenge treatment. Black dots represent non-significantly expressed proteins, whereas orange and purple dots show significantly upregulated and downregulated proteins, respectively (Student's t-test q-value < 0.05). For improved visualisation of this plot, only proteins with a four-fold change, or above, are labelled with the gene names.

3.5 *In vivo* experiments, high cost of priming

3.5.1 Does starvation time and feeding time affect *D. melanogaster* bacterial loads?

Here, effect of starvation and feeding time on bacterial loads was tested. Flies were starved for 120 mins and 240 mins followed by a 30 min and 240 min feeding. There was a significant interaction of feeding time and starvation time (table 6). Post-hoc tests showed that any treatment combinations of starvation time and feeding time do not have an effect on bacterial loads (table S6). However, as seen in figure 12, starvation of 240 minutes leads to lesser variation in bacterial loads. This was confirmed by doing a Levene test to see if the treatment variances differ in order to test if starvation for longer leads to lesser variation in bacterial loads (*df*: 3, *F*: 2.77, $p = 0.046$). Additionally, there was a significant interaction of feeding time with sex of the host, suggesting bacterial loads upon feeding differ based on the sex of the host. However, post-hoc tests suggested no significant effect of sex based on feeding time.

Some flies had non-countable bacterial loads at each time point, either due to lack of feeding or bacterial loads below detectable limits, shown at the bottom of the figure 12. However, the proportion was low and did not differ according to the treatments and it was not statistically analysed.

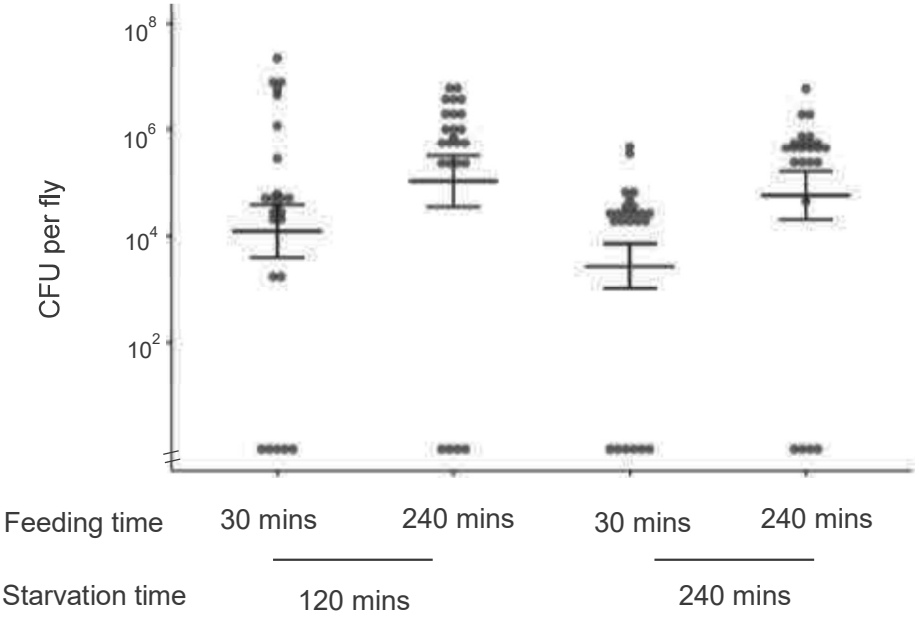

Figure 12: Two hour and four-hour starvation followed by 30 minutes and 240 minutes of feeding. Flies were starved in empty vials for 120 and 240 minutes and then placed in infection vials and allowed to feed on OD200 *Ecc15* for 30 and 240 minutes. Bacterial load estimation was done by homogenising and plating out the flies after each time point. The experiment was repeated two times with 10 flies per sex per condition per replicate. Each dot represents the bacterial load of an individual fly. Flies that had non-countable bacterial load were removed from the analysis. Black lines show means and standard errors.

Table 6: Effects of starvation time and feeding time were tested by fitting a linear model. The significant factors are shown in bold.

Tested effect	*df*	*p*
feeding time	1	0.88
starvation time	1	**0.0004**
sex	1	**0.017**
feeding time × starvation time	1	**0.003**
feeding time × sex	1	**<0.0001**
starvation time × sex	1	0.619
starvation time × feeding time × sex	1	0.097

3.5.2 Effect of OD and growth medium on host bacterial loads

With this experiment, it was tested if countable number of CFU's can be obtained upon homogenising the host when they have fed on bacterial culture of OD 0.5 and OD 2 suspended in an LB media rather than sucrose, i.e., conditions that are more similar to the *in vitro* experimental conditions (Figure 13). As seen in the figure X, flies that fed on OD 0.5 bacterial culture suspended in LB had countable number of CFU's. There is a proportion of flies at some time points where there were no countable number of CFU's, however this proportion seemed to decrease as the feeding time increased. Although there were some individuals with non-countable bacterial loads at 0.5 OD in LB media, after feeding for 30 minutes, the proportion decreased on longer feeding. Since these conditions are closest to the *in vitro* experimental conditions, so to test the effect of priming *in vivo*, OD 0.5 cultures resuspended in LB medium were used in the following experiments.

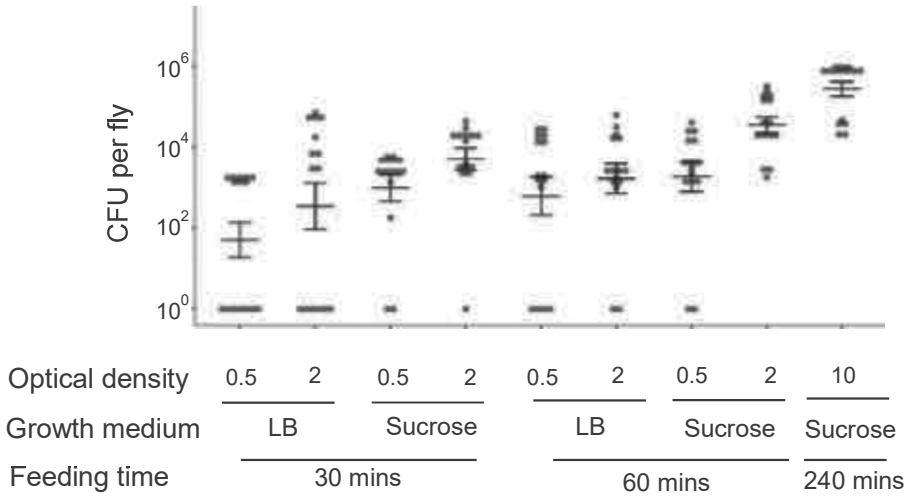

Figure 13: Testing effects of optical density, growth medium and feeding time on bacterial loads. Flies were starved in empty vials for 240 minutes and then placed in infection vials and allowed to feed on *Ecc15* for 30, 60 or 240 minutes. For each time point, two growth media were tested: LB and sucrose and OD 0.5 and 2 except at 240 minutes, where an OD 10 suspended in sucrose was used as a positive control. Bacterial load estimation was done by homogenising and plating out the flies after each time point. Each dot represents the bacterial load of an individual fly. The experiment was repeated once and there were 15 flies per optical density per growth medium for each of the feeding times. Flies that had non-countable bacterial load are also shown in the plot. Black lines show means and standard errors.

3.5.3 Primed *Ecc15* inside a host

Here, it was tested if priming with 1 mM hydrogen peroxide gives *Ecc15* a survival advantage upon oral exposure to a host. The host *D. melanogaster* were allowed to feed on primed and non-primed bacteria for 30, 60 or 240 minutes (Figure 14). There were signifcant interactions between treatment and time, time and sex, and treatment and sex, although the latter was borderline significant (table 7). An interaction between treatment and time suggests that bacterial loads differ over time with treatment. Post-hoc tests suggested that after 30 and 60 minutes of feeding, primed bacteria have significantly lower survival as compared to non-primed bacteria, however, after 240 minutes of feeding, primed and non-primed bacteria have no significant differences in survival (table S7).This suggests that time has a significant effect on the treatment, with bacterial load of flies with non-primed bacteria higher as compared to primed bacteria over time. Similarly, an interaction of time and sex was observed, suggesting significant differences in bacterial loads of male and female flies over time, however, a distinct pattern was not observed (Figure 15). There were few individuals without countable bacterial loads in both primed and non-primed treatments as shown in the figure 14 and 15. Since the

proportion of such individuals was very low and decreased over time, it was not statistically tested whether the proportion differed according to the treatments.

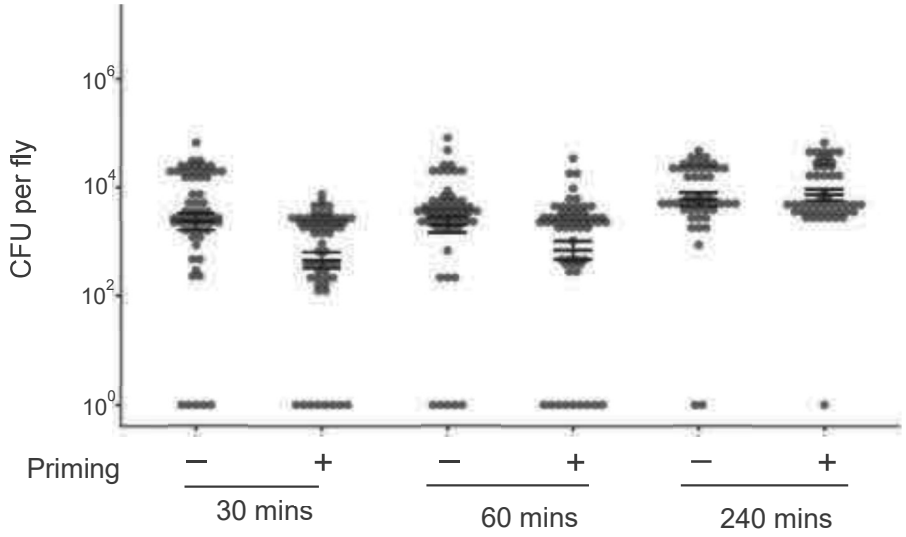

Figure 14: *In vivo* experiments where *D. melanogaster* were allowed to feed on primed and non-primed *Ecc15* for 30,60 and 240 minutes. (+) refers to bacterial load of flies that fed on *Ecc15* primed with 1 mM hydrogen peroxide and (-) refers to bacterial load of flies that fed on non-primed *Ecc15*. Bacterial load estimation was done by homogenising and plating out the flies after each time point and each dot represents the bacterial load of an individual fly. Flies that had non-countable bacterial loads were removed from the analysis. The data shown here is from three different replicates performed on separate days. Black lines show means and standard errors.

Table 7: *In vivo* analyses of three experimental replicates where *D. melanogaster* were allowed to feed on primed and non-primed *Ecc15* for 30,60 and 240 minutes. The significant factors are shown in bold.

Tested effect	*df*	*F*	*p*
treatment	1	26.30	**<0.0001**
time	2	37.40	**<0.0001**
sex	1	0.626	0.42
replicate	2	2.053	0.13
treatment × time	2	9.648	**<0.0001**
treatment × sex	1	4.078	**0.0443**
time × sex	2	6.818	**0.0012**

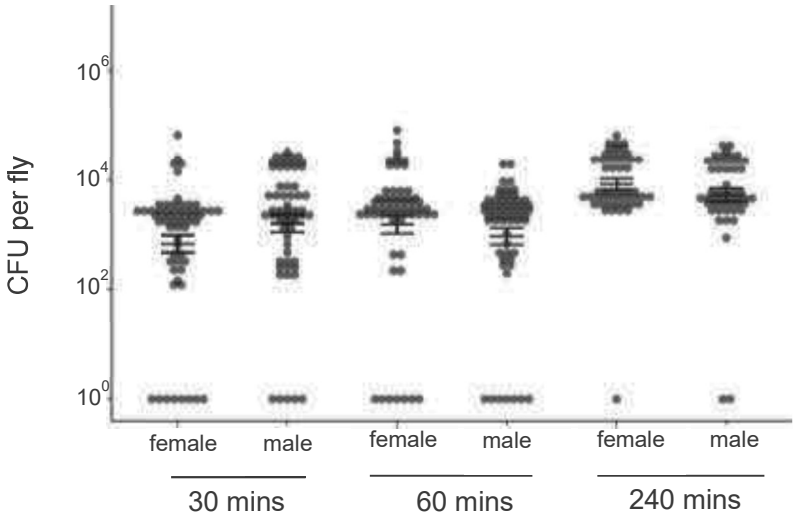

Figure 15: Bacterial loads over time based on the sex of the host. Bacterial load estimation was done by homogenising and plating out the flies after each time point and each dot represents the bacterial load of an individual fly. Flies that had non-countable bacterial loads were removed from the analysis. The data shown here is from three different replicates performed on separate days. Black lines show means and standard errors.

3.6 *In vitro* controls: differential number of bacteria in primed and non-primed treatments

3.6.1 Control experiment: Do bacterial numbers differ across experiments in OD 0.5 bacterial cultures?

Here, a significant difference in CFU counts based on the experimental replicate was observed, suggesting variable number of cells in OD 0.5 cultures depending on the replicate (*df: 2, F: 23.22, p:* ***0.0014***) (Figure 16). Post-hoc tests suggested that bacterial loads of replicate 1 and 2 differed significantly from replicate 3 (table S8).

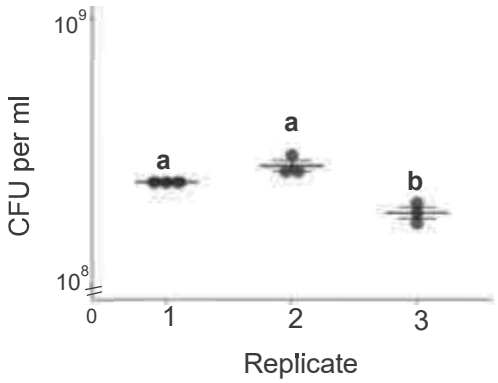

Figure 16: Bacterial numbers in OD 0.5 cultures across experimental replicates. Here it was tested whether the number of living cells in OD 0.5, measured as CFU, vary across different replicates. Colony forming units (CFUs) per ml were estimated by plating out serial dilutions of OD 0.5 cultures. The data shown is from three replicates performed on separate days. Black lines show means and standard errors. Each dot represents mean bacterial loads based on three technical replicates.

3.6.2 Control experiment: Differential numbers of CFU's in primed and non-primed treatments

Here, it was tested if there are differential number of viable cells in primed and non-primed treatments after they undergo an *in vitro* recovery procedure. This was done to test if host has access to same number of cells across treatments, since host will receive bacteria to feed at this time point (Figure 17). There was an interaction between treatment and replicate, suggesting that experimental replicate has an effect on treatment (table 8). Post-hoc tests showed that there are a greater number of cells in non-primed treatment as compared to primed treatment across all replicates (table S9).

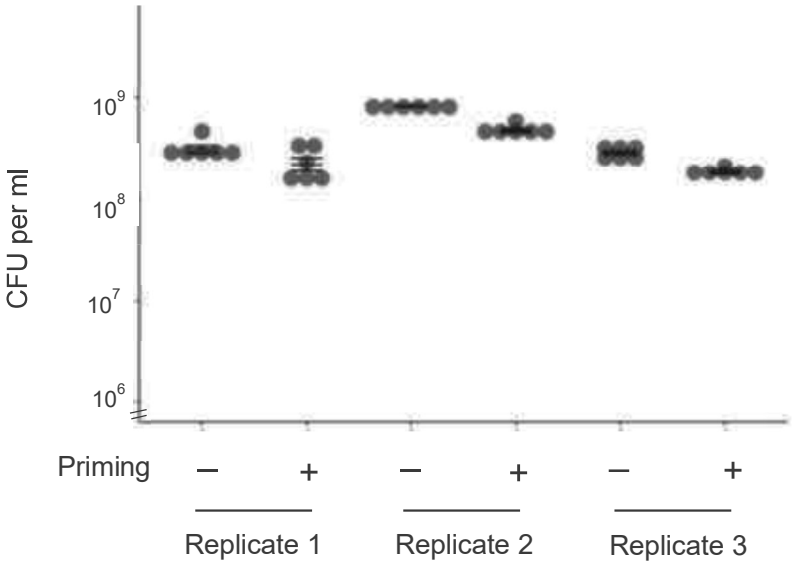

Figure 17: Differential viable cell numbers in primed and non-primed treatments after an *in vitro* recovery phase. This experiment was performed as a control to test whether there are differential number of cells in primed and non-primed treatments. (-) represents non-primed treatment whereas (+) represents cells that have been primed with 1 mM hydrogen peroxide. The treatments were plated 10-minutes prior to when the recovery phase was over. The experiment was repeated 3 times on separate days. Black lines show means and standard errors.

Table 8: Comparison of CFU counts of primed and non-primed treatments after recovery across experimental replicates. The significant factors are shown in bold.

Tested effect	*df*	*F*	*p*
treatment	1	83.152	**<0.0001**
replicate	2	181.103	**<0.0001**
treatment × replicate	2	18.356	**<0.0001**

3.6.3 Control experiment: Does *Ecc15* undergoing *in vivo* challenge have a survival advantage upon an *in vitro* challenge?

Simultaneous to i*n vivo* experiments, *in vitro* experiments were carried out to ensure if there was an improved survival of primed bacteria upon challenge under *in vitro* conditions. These experiments were carried out in the same way as described in section 2.5.1 (Figure 18). There was a significant interaction between priming and challenge (table 9). Post hoc tests for multiple comparisons suggested that control treatment differs significantly from only priming, only challenge and priming + challenge treatments (table S10). Control being different from

only priming treatment suggests that priming dose led to significant mortality. Additionally, only challenge treatment differs significantly from priming + challenge, suggesting survival advantage of priming upon challenge.

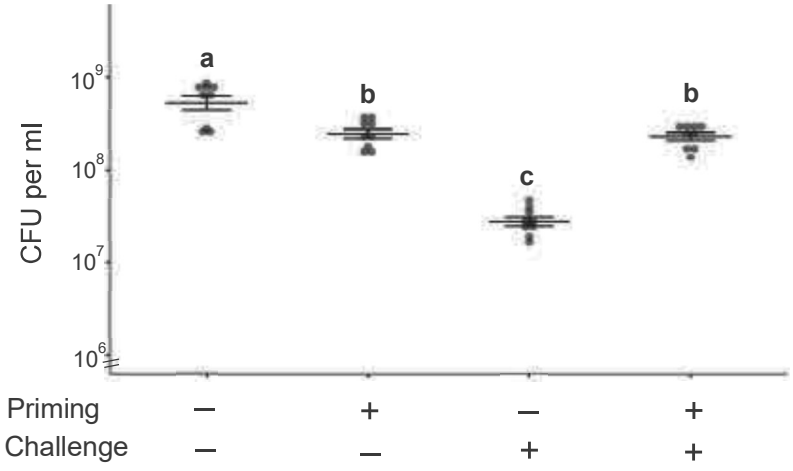

Figure 18: Control experiment: *In vitro* survival advantage of primed bacteria undergoing an *in vivo* challenge. Primed populations had a survival advantage upon challenge compared to the *Ecc15* that received only the challenge doses of H_2O_2. (-) represents no addition of H_2O_2 whereas (+) represents the treatments where H_2O_2 was added. Colony forming units (CFUs) per ml were estimated by plating out serial dilutions of each treatment. Each data point represents mean bacterial loads of three technical replicates. The experiment was repeated 3 times on separate days. Black lines show means and standard errors.

Table 9: Control experiment: *In vitro* survival advantage of primed bacteria undergoing an *in vivo* challenge. The significant factors are shown in bold.

Tested effect	*df*	*chisq*	*p*
Priming	1	61.84	**<0.0001**
Challenge	1	928.77	**<0.0001**
Priming × challenge	1	445.47	**<0.0001**

3.6.4 Control experiment: Is there differential survival of primed and non-primed bacteria on Whatman filter discs?

It was tested if *Ecc15* survives differentially on Whatman flter discs after 30, 60 and 240 minutes based on priming status. The data was analysed separately based on replicate (Figure 19). For the first experimental replicate there was a weak interaction between treatment and time (table 10). Additonally, there was a significant effect of treatment and time,

suggesting more non-primed bacteria on filter discs as compared to primed bacteria over time. Post-hoc tests suggested that CFU counts at 30 minutes are siginifcantly different from CFU counts at 60 and 240 minutes. However, CFU counts at 60 minutes do not differ significantly from CFU counts at 240 minutes (table S11).

There was no interaction between treatment and time in the second replicate but significant effects of treatment and time were observed (table 10). Additonally, post-hoc tests show that CFU counts at 30 minutes differs significantly from CFU counts at 60 and 240 minutes, but CFU counts at 60 minutes does not differ significantly from 240 minutes, just like the first replicate (table S11).

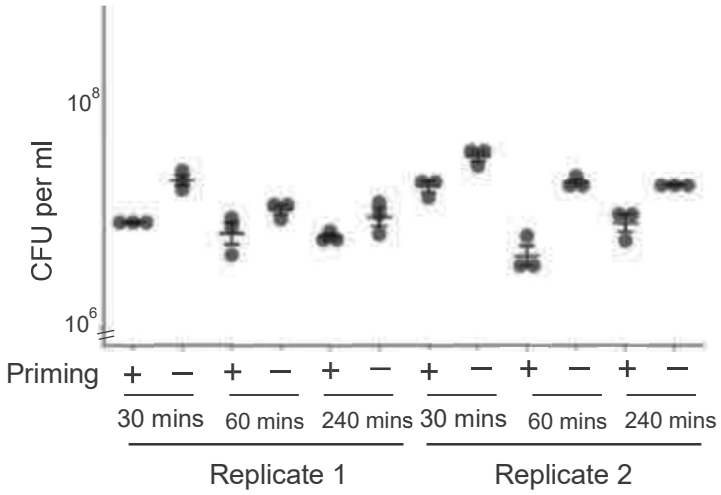

Figure 19: Survival of *Ecc15* primed with 1 mM of hydrogen peroxide as compared to non-primed bacteria on Whatman filter discs. CFUs retrieved from Whatman filter discs inoculated with primed or non-primed *Ecc15*, and left for 30, 60 or 240 minutes. (-) represents no addition of H_2O_2 whereas (+) represents the treatments where H_2O_2 was added. Colony forming units (CFUs) per ml were estimated by plating out serial dilutions of each treatment. The experiment was repeated 2 times on separate days. Black lines show means and standard errors.

Table 10: Differential survival of primed and non-primed bacteria on the surface of Whatman filter discs. The treatments here were primed and non-primed bacteria and survival was tested for 30, 60 and 240 minutes. The significant factors are shown in bold.

Replicate 1				Replicate 2			
Tested effect	*df*	*F*	*p*	Tested effect	*df*	*F*	*p*
treatment	1	30.94	**0.0001**	treatment	1	94.95	**<0.0001**
time	2	14.01	**0.0007**	time	2	37.16	**<0.0001**
treatment × time	2	4.13	**0.0433**	treatment × time	2	1.89	0.193

3.6.5 Applying correction factors for bacterial mortality differences due to priming to *in vivo* experiments

Given that the *in vitro* results in section 3.6.2 and 3.6.4 showed that there were fewer surviving bacteria in the priming treatment compared to the non-primed treatment, a correction factor was introduced to the number of bacteria retrieved from the *in vivo* experiment to account for mortality. The reasoning being that the flies exposed to non-primed bacteria have access to higher number of viable cells than the flies exposed to primed bacteria. The correction factors were calculated individually for the different experimental replicates (table 11), given that the number of CFUs also varied across experimental replicates (section 3.6.1). Bacterial loads of primed bacteria obtained from *in vivo* experiments (section 3.5.3) were multiplied with correction factors to account for lower survival. The *in vivo* analyses were then carried out again with the corrected bacterial load dataset.

Table 11: Correction factors calculated based on differences in CFUs of primed treatment and non-primed treatments calculated using the following formula: CFUs non-primed bacteria/ CFUs primed bacteria.

Experimental replicate	Correction factor
1	1.4
2	1.7
3	1.6

3.6.5.1 Corrected bacterial loads: Primed bacteria inside the host

Based on corrected bacterial loads, there were signifcant interactions between treatment and time, time and sex, and treatment and sex, although the latter was borderline significant (table 12). An interaction between treatment and time suggests that bacterial loads differ over time with treatment. Post-hoc tests suggested that after 30 and 240 minutes of feeding, primed bacteria have significantly lower survival as compared to non-primed bacteria, however, after 60 minutes of feeding, primed and non-primed bacteria have no significant differences in survival (table S12, Figure 20).This suggests that time has a significant effect on the treatment, with bacterial load of flies with non-primed bacteria higher as compared to primed bacteria over time. Similarly, an interaction of time and sex was observed, suggesting significant differences in bacterial loads of male and female flies over time, however, a distinct pattern was not observed. There were few individuals without countable bacterial loads in both primed and non-primed treatments as shown in the figure 14 and 15. Since the proportion of such individuals was very low and decreased over time, it was not statistically tested whether the proportion differed according to the treatments.

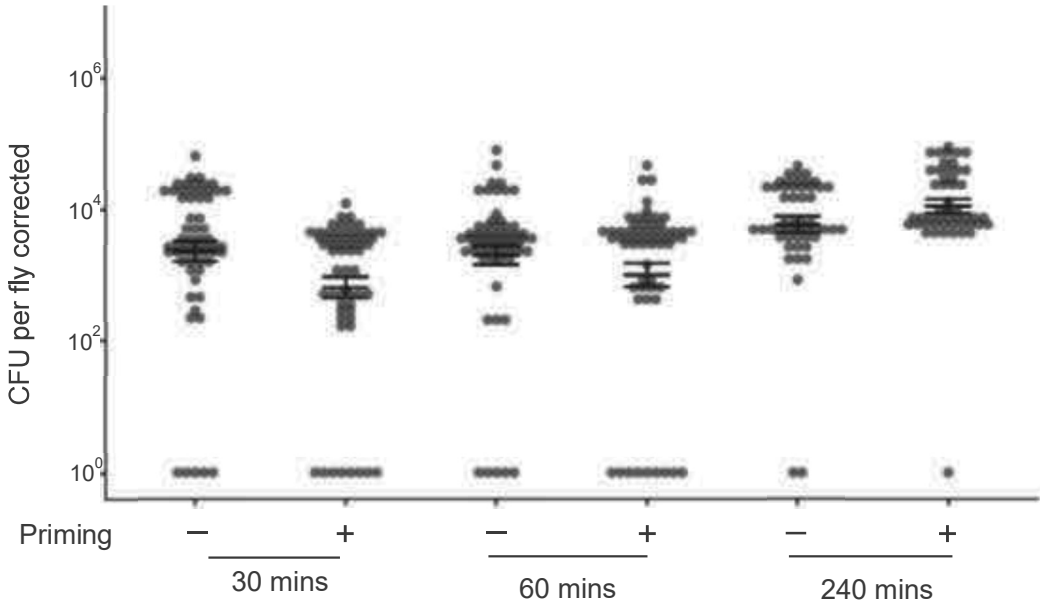

Figure 20: Corrected bacterial loads: *In vivo* experiments where *D. melanogaster* were allowed to feed on primed and non-primed *Ecc15* for 30,60 and 240 minutes. (+) refers to bacterial load of flies that fed on *Ecc15* primed with 1 mM hydrogen peroxide and (-) refers to bacterial load of flies that fed on non-primed *Ecc15*. Bacterial load estimation was done by homogenising and plating out the flies after each time point and adding a correction factor based on each replicate. Each dot represents the bacterial load of an individual fly. Flies that had non-countable bacterial load were removed from the analysis. The data shown here is from three different replicates performed on separate days. Black lines show means and standard errors.

Table 12: Corrected *in vivo* analyses of three replicates where *D. melanogaster* were allowed to feed on primed and non-primed *Ecc15* for 30,60 and 240 minutes. The significant factors are shown in bold.

Tested effect	*df*	*F*	*p*
treatment	1	26.30	0.12
time	2	37.40	**<0.0001**
sex	1	0.626	0.42
replicate	2	2.053	0.35
treatment × time	2	9.648	**<0.0001**
treatment × sex	1	4.078	**0.044**
time × sex	2	6.818	**0.0013**

3.7 *In vitro* experiments, low cost of priming

3.7.1 Cost of priming associated with low concentrations of H_2O_2 based on optical density

Based on three replicates of 96-well plates to test priming costs, it was found that 0.5 mM H_2O_2 causes lethality in *Ecc15*; hence, the growth curve is almost flat, not allowing to calculate the costs involved at that concentration (Figure S5 and S6). For all the other concentrations, it was possible to calculate the costs imposed by different concentrations of H_2O_2 in comparison with a non-treated control using the empirical area under the curve (auc_e) (table S13).

As predicted, the percentage costs of priming varied with the priming concentration used, with the highest costs imposed by the highest concentration of H_2O_2 (Figure 21). 0.25 mM of H_2O_2 led to an average cost of 34.6% based on three replicates, and 0.125,0.1 and 0.0625 mM of H_2O_2 led to 18.61%, 5.97% and 7.66%, respectively.

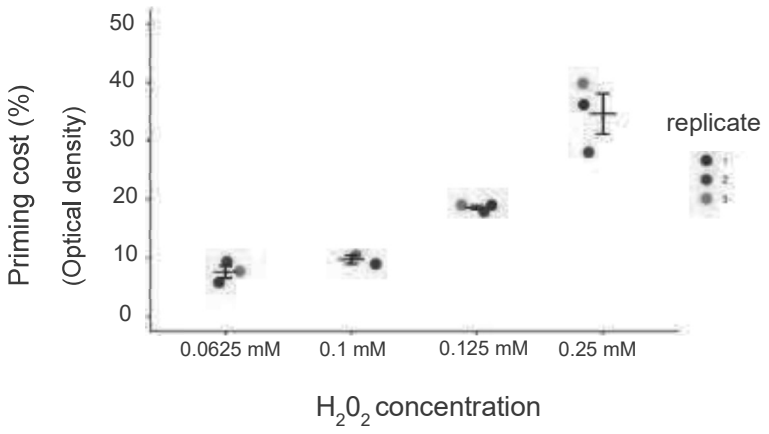

Figure 21: Optical density-based percentage costs conferred by increasing concentrations of H_2O_2 on *Ecc15*. *In vitro* experiments in a 96-well plate reader where the percentage costs conferred by different concentrations of H_2O_2 were calculated by tracking the optical density (OD) of *Ecc15* cultures over 24 hours. Growth curves were obtained for a period of 24 hours with OD600 readings every 10 minutes. To obtain the costs, the area under the curve after receiving different doses of H_2O_2 was compared to the non-treated control. The experiment was repeated three times on separate days, denoted by "replicate". Each dot represents a percentage cost based on the average area under the curve obtained by 16 individual wells of a 96-well plate. Dots have been jittered for easy visualisation. Black lines show means and standard errors.

3.7.2 Cost of priming with increasing concentrations of H_2O_2 based on CFU counts

Based on CFU count data from three replicates, it was found that the percentage costs were proportional to the concentration of H_2O_2 used. The average percentage cost of priming based on three replicates was found to be 23.5%, 6.45%, 4.90% and 1.61% for 0.25, 0.125, 0.1- and 0.0625-mM H_2O_2, respectively (Figure 22, table S14). These percentage cost estimates based on CFU data provided us with the likely costs of priming under our experimental conditions. For cost data based on individual experimental replicates, see supporting data.

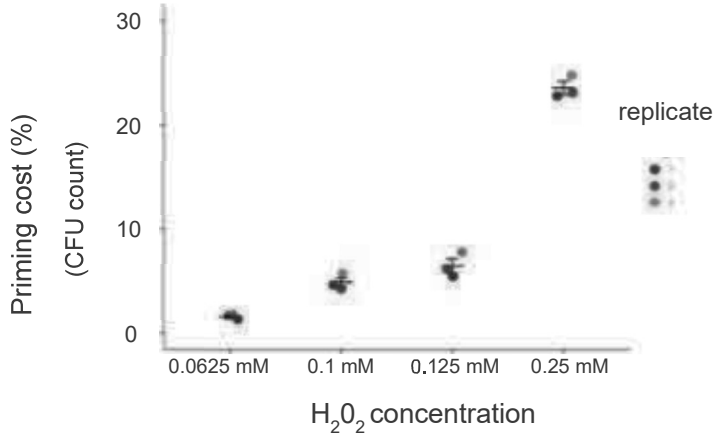

Figure 22: CFU count-based percentage costs conferred by increasing concentrations of H_2O_2 on *Ecc15.* *In vitro* experiments were conducted in falcon tubes where the percentage costs conferred by different concentrations of H_2O_2 were calculated based on CFUs of *Ecc15* cultures after receiving respective concentrations of H_2O_2. Replicate denotes that the experiment was repeated on three separate days. Data points have been jittered for easy visualisation. The percentage costs were calculated by comparing CFUs of respective concentrations with that of non-treated controls. Black bars represent means and standard errors.

3.7.3 Testing low cost H_2O_2 priming concentrations for their survival advantage upon challenge

After establishing priming concentrations that lead to a lower cost, here it was tested whether priming with lower cost imposing concentrations of H_2O_2 provides a survival advantage upon receiving a challenge dose of 5 mM when compared with the treatment that received only a challenge treatment (Figure 23). Pairwise comparisons of all the treatments that received a priming and challenge dose with the ones that received only a challenge dose were conducted. Considering overall costs and benefits associated with different priming and challenge concentrations of H_2O_2, 0.1 mM was chosen as a priming concentration to test for further *in vitro* and *in vivo* priming experiments, since it entails the least cost associated with priming

and provides a greater survival advantage upon challenge as compared to other tested priming concentrations (table S15).

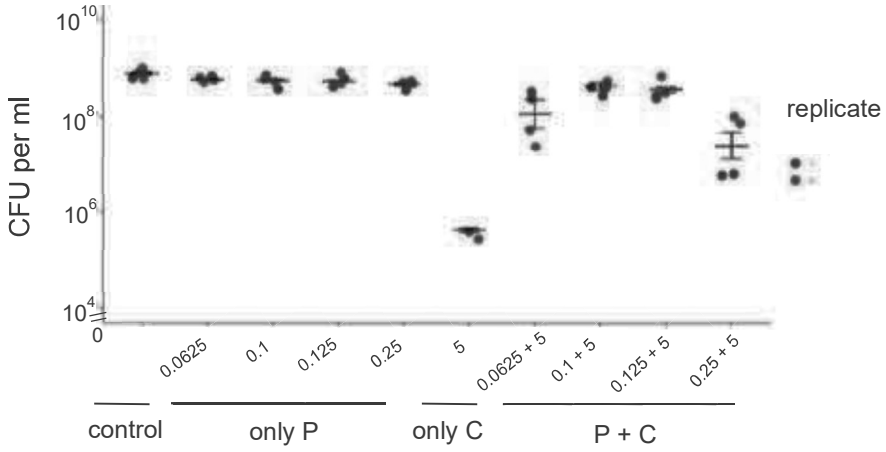

Figure 23: Differential survival of *Ecc15* primed with increasing concentrations of H_2O_2 upon receiving a challenge dose. Control represents neither priming nor challenge dose given to *Ecc15.* Only P represents only priming treatment, and only C represents only challenge treatment, whereas P + C represents priming + challenge treatments. All the doses on the x-axis are in millimolar (mM), and points have been jittered for easy visualisation. Different priming concentrations of H_2O_2 were tested under *in vitro* experimental conditions by CFU plating to observe if they provide a survival advantage upon challenge. 0.25 mM, 0.125 mM, 0.1 mM and 0.0625 mM were tested as the priming concentrations. The challenge concentration of 5 mM was used. Primed populations had a survival advantage upon challenge (P + C) compared to the bacteria that received only challenge concentration (only C) of 5 mM. The experiment was replicated twice on separate days. Black bars represent means and standard errors.

3.7.4 Testing 0.1 mM H_2O_2 as priming concentration for its survival advantage upon challenge

For next set of experiments, it was tested whether the lower cost-imposing concentration of 0.1 mM results in an increased survival upon challenge of 5 mM (Figure 24).

There was a statistically significant interaction between priming x challenge treatments suggesting that priming leads to advantage upon challenge (table 13) . Post-hoc tests showed that bacteria primed with H_2O_2 had higher survival after a challenge than bacteria that had only received a challenge (table S16).

There was no significant difference between our control and only priming treatments, suggesting that our priming treatment under *in vitro* experimental conditions does not lead to high fitness costs. Among other treatments, our only challenge treatment differs significantly from our control treatment, suggesting that our only challenge concentration of 5 mM leads to a significant lethality compared to non-treated *Ecc15*. Now, for the major comparison, there is a significant difference in our only challenge treatment compared to our priming + challenge treatment, suggesting that primed bacteria have a survival advantage upon receiving lethal doses of H_2O_2 when compared to bacteria that receive these lethal doses directly.

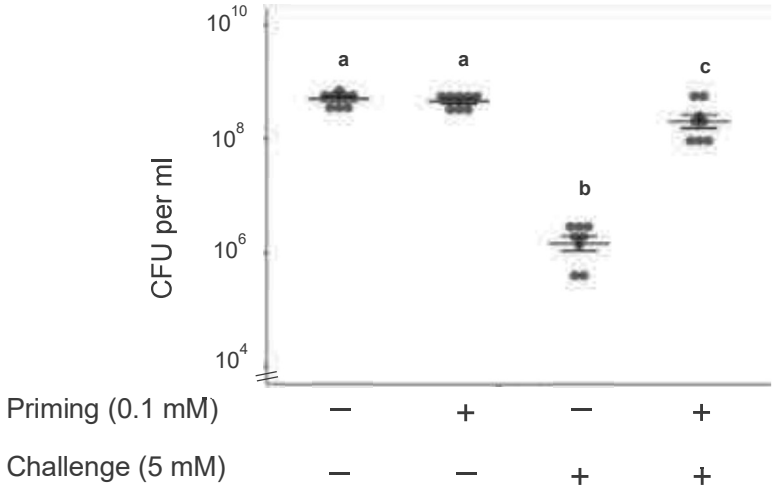

Figure 24: Differential survival of *Ecc15* primed with 0.1 mM of H_2O_2 upon receiving a challenge dose. Primed populations had a survival advantage upon challenge compared to the *Ecc15*, which received only the challenge dose of H_2O_2. Priming led to a small cost (4.9%) but did not significantly differ from our control treatment. (-) represents no addition of H_2O_2 whereas (+) represents the treatments where H_2O_2 was added. Colony forming units (CFUs) per ml were estimated by plating out serial dilutions of each treatment. The experiment was repeated 3 times on separate days. Black lines show means and standard errors, and different letters represent treatments that differ significantly.

Table 13: The effect of 0.1 mM as the priming dose of H_2O_2 and 5 mM as the challenge dose under our *in vitro* experimental conditions. The significant factors are shown in bold.

Treatment	***df***	***Chisq***	***p*-value**
Priming	1	0.1241	0.7
Challenge	1	454.7201	**<0.0001**
Priming × Challenge	1	168.9820	**<0.0001**

3.8 *In vivo* experiments, low cost of priming

3.8.1 Does a lower cost conferring priming dose lead to an increased survival upon in vivo challenge using host D. melanogaster?

Next, it was tested whether priming is advantageous when *Ecc15* primed with 0.1 mM H_2O_2 receives a challenge inside a host gut. The host *D. melanogaster* were allowed to feed on primed and non-primed bacteria for 30, 60 or 240 minutes (Figure 25).

With 30 minutes of feeding on primed and non-primed *Ecc15*, there were no significant differences in the bacterial loads in the case of both male and female flies, suggesting priming does not provide an advantage to *Ecc15* in a *D. melanogaster* gut under these experimental conditions with 30 minutes of feeding (table 14). Similarly, with 60 minutes of feeding on primed versus non-primed bacteria, any significant differences in bacterial loads of male and female *D. melanogaster* were not observed.

With 240 minutes of feeding, there was a significant interaction between treatment and sex (table 14), suggesting that sex plays a role in the effect of the treatment. Post-hoc tests show that the bacterial loads do not differ in female *D. melanogaster* depending on whether they were allowed to feed on primed or non-primed bacteria. On the other hand, multiple comparisons using “emmeans” suggested that the bacterial load of males that fed on primed bacteria differed significantly from the males that fed on non-primed bacteria (table S17).

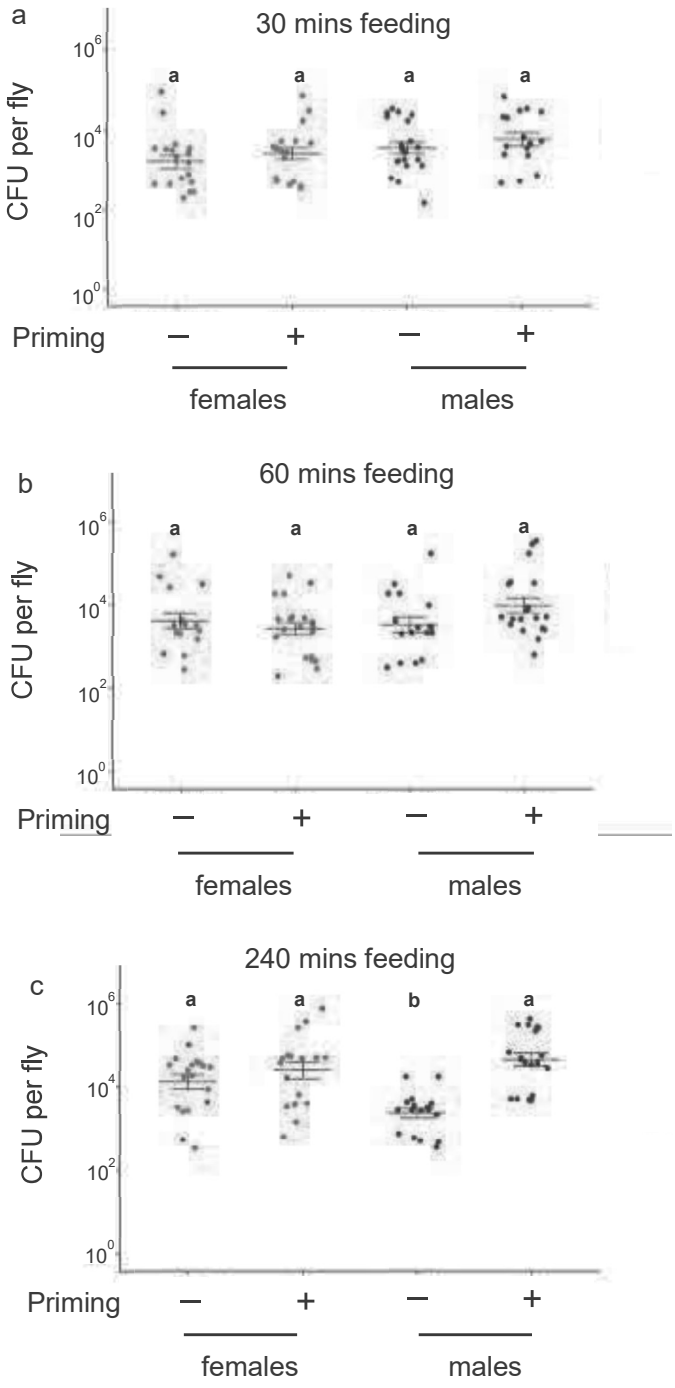

Figure 25: a-c *In vivo* experiments where *D. melanogaster* was allowed to feed on primed and non-primed *Ecc15* for 30,60 and 240 minutes. (+) refers to a bacterial load of flies that fed on primed *Ecc15,* and (-) refers to a bacterial load of flies that fed on non-primed *Ecc15*. Bacterial load estimation was done by homogenising and plating out the flies after each time point and each dot represents the bacterial load of an individual fly. Black lines show means and standard errors, and different letters represent treatments that differ from each other significantly.

Table 14: Effects of priming inside a host environment. *D. melanogaster* were allowed to feed for 30,60 and 240 minutes on primed and non-primed *Ecc15.* The significant factors are depicted in bold.

Tested effect	30 mins feeding				60 mins feeding				240 mins feeding			
	F	***df***	***resid df***	***p***	***F***	***df***	***resid df***	***p***	***F***	***df***	***resid df***	***p***
Treatment	0.82	1	67	0.36	0.4	1	67	0.50	1.35	1	69	0.24
Sex	2.36	1	67	0.12	0.1	1	67	0.75	10.44	1	69	**0.001**
Replicate	0.05	1	67	0.80	2.6	1	67	0.11	0.22	1	69	0.63
Treatment × Sex	0.002	1	67	0.98	3.4	1	67	**0.06**	9.43	1	69	**0.003**

3.9 Supporting data

3.9.1 Supporting figures

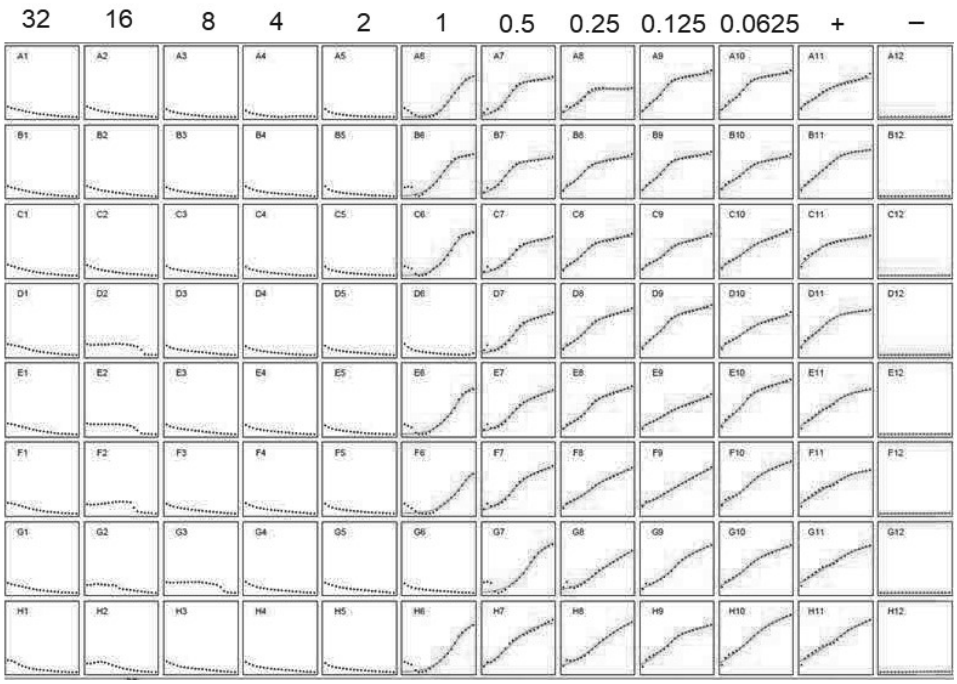

Figure S1: *Ecc15* killing curves in 96-well plate reader. This panel represents a graphical model fitting individual curves plotted by the Growthcurver R package. It shows the killing curves over 24 hours in the presence of a range of hydrogen peroxide concentrations mentioned above in the figure. All the concentrations are in millimolar (mM) and go from 32 mM to 0.0625 mM. (+) and (-) depict non-treated control and medium control, respectively.

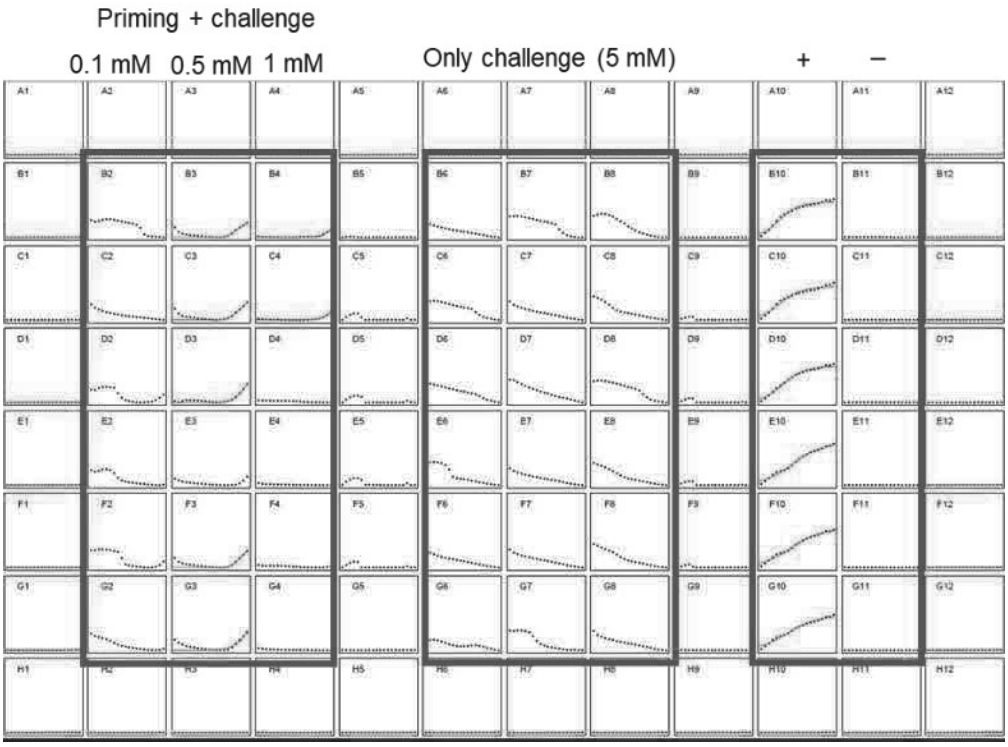

Figure S2: Establishing priming doses based on optical density**.** This panel represents a graphical model fitting individual curves plotted by the Growthcurver R package. It shows a priming assay conducted in a 96-well plate reader with the following treatments: only challenge and priming + challenge. (+) and (-) represent positive and negative control respectively. The first panel represents bacterial cultures that were primed with 0.1, 0.5 and 1 mM for 30 minutes followed by a challenge dose of 5 mM. Second panel represents cultures that received only a challenge dose of H_2O_2. The growth curves were measured for a period of 24 hours to test if priming has a survival advantage upon challenge. The primed + challenge populations started to grow after an initial mortality upon challenge, however the survival difference from an only challenge treatment could not be quantified because of absence of area under the curve.

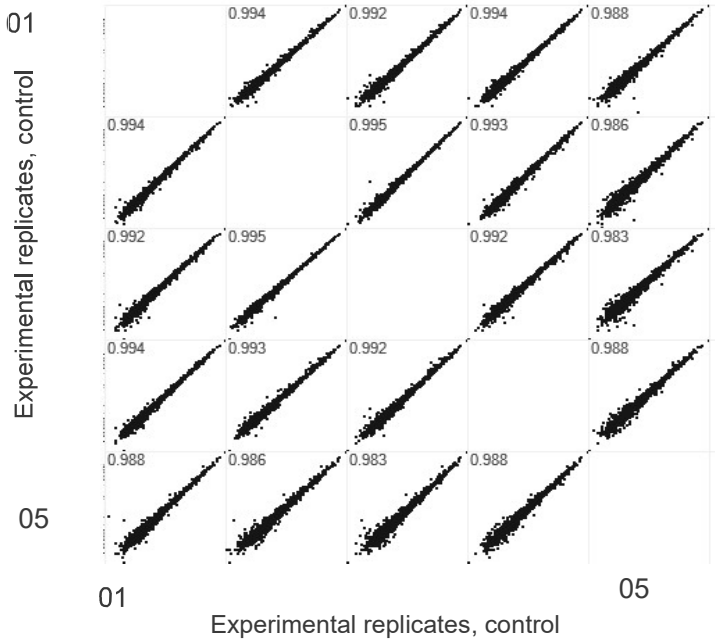

Figure S3: Pearson correlation to check the reproducibility across replicates of our experimental treatments in LC-MS. Only control samples here are shown as a representation.

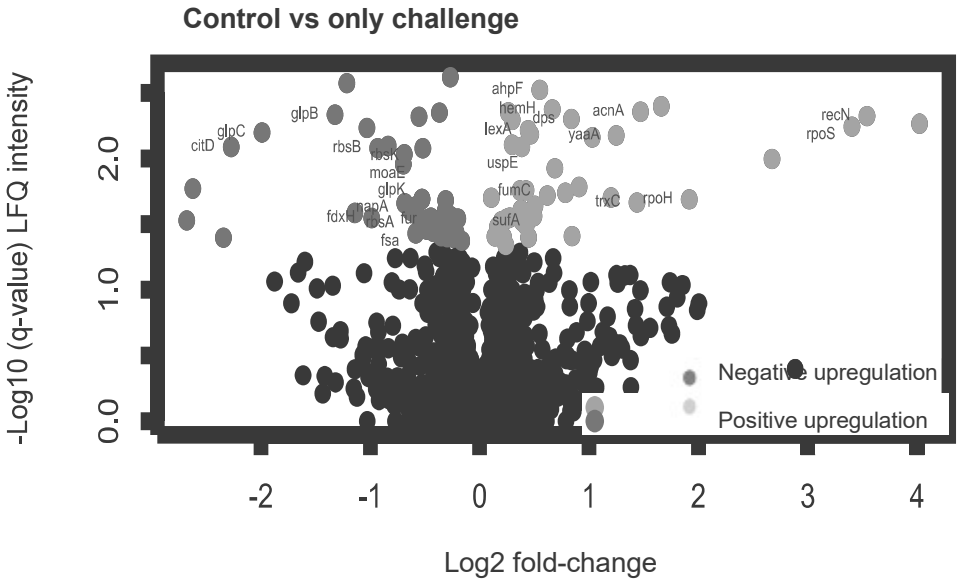

Figure S4: Volcano plot of –log q values against the log2 fold-change of protein intensity measured by LC-MS of only challenge treatment compared with control treatment. (Student's t-test q-value < 0.05). For improved visualisation of this plot, only proteins with a four-fold change, or above, are labelled. As it can be seen, there are not many proteins differentially upregulated between control and challenge treatments, also confirmed with the PCA analysis.

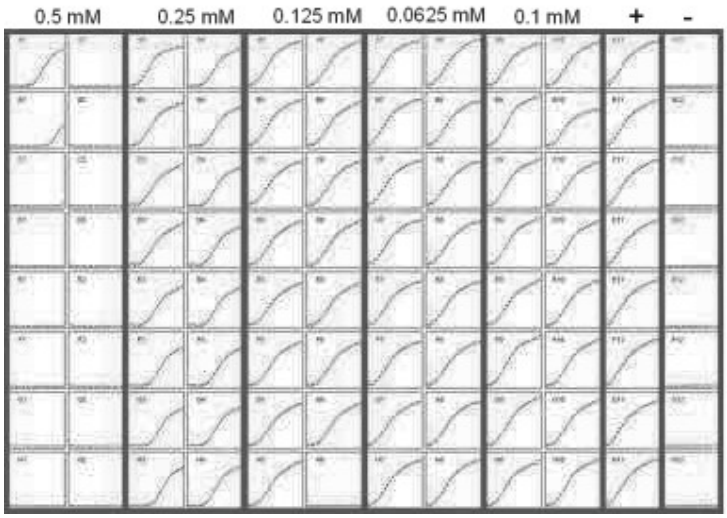

Figure S5: OD based cost of priming, replicate 1: This panel represents a graphical model fitting of individual curves plotted by the Growthcurver R package. There were 16 individual replicates for each concentration of H_2O_2, except for non-treated control (no H_2O_2 added) and negative control (only media) denoted by (+) and (-) respectively, where there were 8 replicates. All the concentrations were compared with the non-treated control to test the percentage costs.

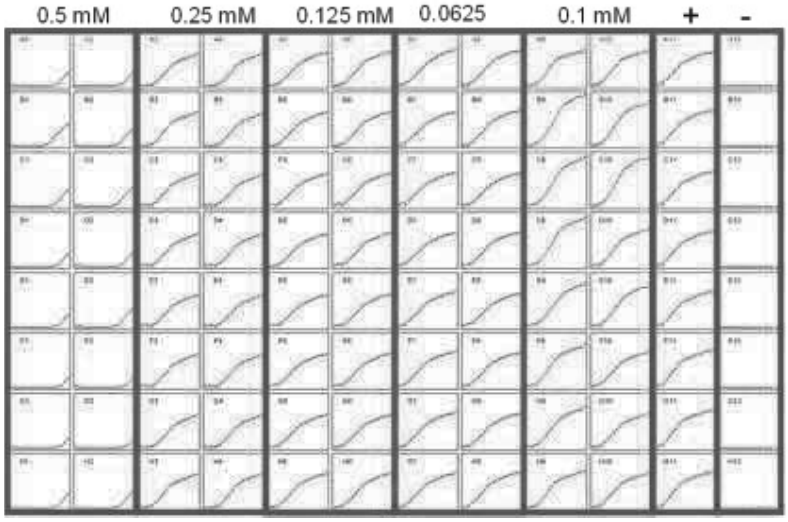

Figure S6: OD based cost of priming, replicate 2 This panel represents a graphical model fitting of individual curves plotted by the Growthcurver R package. There were 16 individual replicates for each concentration of H_2O_2, except for non-treated control (no H_2O_2 added) and negative control (only media) denoted by (+) and (-) respectively, where there were 8 replicates. All the concentrations were compared with the non-treated control to test the percentage costs.

3.9.2 Supporting tables

Table S1: Post-hoc comparisons for several priming doses for their effect on bacterial survival upon challenge. Three priming doses and their survival advantage upon challenge were tested. The concentrations mentioned in the figure (0.1, 0.5 and 1 mM) were used as priming concentrations and 5 mM was used as a challenge concentration. Significant figures are shown in bold.

Contrast	*df*	*t*	*p-value*
Control – only challenge	10	-12.07	**<0.0001**
Only challenge – priming(0.1 mM) + challenge	10	6.115	**0.0008**
Only challenge – priming(0.5 mM) + challenge	10	6.139	**0.0008**
Only challenge – priming(1 mM) + challenge	10	-11.02	**<0.0001**

Table S2: Post-hoc comparisons for 1 mM priming doses for their effect on bacterial survival upon 5 mM challenge. Three priming doses and their survival advantage upon challenge were tested. Significant figures are shown in bold.

Contrast	*df*	*t*	*p-value*
Control – only priming	41	2.502	0.0745
Control – only challenge	41	20.778	**<0.0001**
Control – priming + challenge	41	9.198	**<0.0001**
Only priming – only challenge	40	18.303	**<0.0001**
Only priming – priming + challenge	40	6.706	**<0.0001**
Only challenge – priming + challenge	40	-11.597	**<0.0001**

Table S3: Post-hoc comparisons for 1 mM priming doses for their effect on bacterial survival upon 5 mM challenge when challenge duration is 10-minutes. This experiment was conducted as a control for LC-MS sample collection. Significant figures are shown in bold.

Contrast	*df*	*t*	*p-value*
Control – only priming	39	25.16	**<0.0001**
Control – only challenge	39	30.72	**<0.0001**
Control – priming + challenge	39	28.87	**<0.0001**
Only priming – only challenge	39	5.40	**<0.0001**
Only priming – priming + challenge	39	3.567	**0.0051**
Only challenge – priming + challenge	39	-1.844	**0.0002**

Table S4: Percentage of genes involved in different functions based on their molecular functions as obtained by GO analysis. Analysis carried out using Panther 17.0.

Activity	**% of genes against total genes**
Transporter activity	3.6%
Translation regulator activity	1.0%
Transcription regulator activity	1.2%
Catalytic activity	54.4%
Molecular function regulator	0.6%
ATP- dependent activity	3.6%
Molecular transducer activity	0.6%
Molecular adaptor activity	0.1%
Structural molecule activity	9.2%
Binding activity	25.8%

Table S5: The categories and percentages of catalytic genes found to be upregulated in the global peroxide stress response. GO analysis was performed using Panther 17.0.

Activity	**% of genes involved against total genes**
Nucleic acid catalytic activity	17.3%
Protein catalytic activity	4.2%
Hydrolase activity	15.8%
Isomerase activity	7.1%
Ligase activity	13.8%
Lyase activity	8.3%
Oxidoreductase activity	8.7%
Transferase activity	25%

Table S6: Post-hoc comparisons of feeding time and starvation time on host bacterial loads. Flies were starved for 120 minutes or 240 minutes and then allowed to feed for 30 minutes or 240 minutes.

Feeding time × Starvation time	***df***	***z***	***p-value***
30 mins-120 mins – 240 mins 120 mins	40	1.207	0.62
240 mins-120 mins – 240 mins-240 mins	40	1.707	0.31
240 mins-120 mins – 30 mins 240 mins	40	2.279	0.10
30 mins-120 mins – 240 mins-240 mins	40	-0.442	0.97
30 mins-120 mins – 30 mins-240 mins	40	1.260	0.58
240 mins-240 mins – 30 mins-240 mins	40	1.757	0.29

Table S7: Post-hoc comparisons for bacterial loads of flies that were allowed to feed on primed and non-primed *Ecc15* for 30,60 or 240 minutes. Significant figures are shown in bold.

Contrast	***df***	***t***	***p-value***
Non primed – primed, 30 mins	294	6.311	**<0.0001**
Non primed – primed, 60 mins	294	2.646	**0.0086**
Non primed – primed, 240 mins	294	0.052	0.9589

Table S8: Post-hoc comparisons for differential number of viable cells in OD 0.5 cultures across three experimental replicates. Significant figures are shown in bold.

Tested combination	*df*	*t*	*p*
Replicate 1-Replicate 2	6	-2.606	**0.089**
Replicate 1- Replicate 3	6	4.151	**0.014**
Replicate 2 – Replicate 3	6	6.757	**0.012**

Table S9: Post-hoc comparisons for differential number of CFU's after in vitro recovery phase. Significant factors are shown in bold.

Tested combination	replicate	*df*	*t*	*p*
Non primed vs primed	1	30	2.465	**0.019**
Non primed vs primed	2	30	10.197	**0.001**
Non primed vs primed	3	30	3.132	**0.0039**

Table S10: Post-hoc comparisons for *in vitro* priming experiment carried out as a control for *in vivo* priming. The significant factors are depicted in bold.

Tested combination	*df*	*t*	*p*
Control – Only priming	30	7.864	**<0.0001**
Control – Only challenge	30	30.476	**<0.0001**
Control – Priming + challenge	30	8.492	**<0.0001**
Only priming – Only challenge	30	22.611	**<0.0001**
Only priming – Priming + challenge	30	0.627	0.9225
Only challenge – Priming + challenge	30	-21.984	**<0.0001**

Table S11: Post-hoc comparisons for two experimental replicates analysed separately where differential survival of primed and non-primed bacteria on filter discs was tested. Significant factors are shown in bold.

Replicate 1				Replicate 2			
Tested comparison	*df*	*t*	*p*	Tested comparison	*df*	*t*	*p*
30 min – 60 min	12	3.547	**0.0104**	30 min – 60 min	12	8.051	**<0.0001**
30 min – 240 min	12	4.342	**0.0025**	30 min – 240 min	12	6.698	**0.0001**
60 min – 240 min	12	0.796	0.7127	60 min – 240 min	12	-1.353	0.3949

Table S12: Post-hoc comparisons in bacterial loads of flies with primed or non-primed bacteria at 30,60 or 240 minutes. The analysis was carried out after correcting the host bacterial loads to account for mortality caused by priming. Significant factors are shown in bold.

Contrast	***df***	***t***	***p-value***
Non primed – primed, 30 mins	294	6.311	**<0.0001**
Non primed – primed, 60 mins	294	2.646	0.5468
Non primed – primed, 240 mins	294	0.052	**0.0465**

Table S13: Cost summary based on OD,The percentage cost of priming based on the different concentrations of H_2O_2 compared to the non-treated control. The cost for 0.5 mM could not be obtained. For 0.25 mM, the cost imposed on *Ecc15* is 36.11%, which is substantially high. In this replicate, the costs involved are directly proportional to the concentration of H_2O_2, with 0.0625 mM of H_2O_2 imposing the least fitness cost on *Ecc15.*

H_2O_2 concentration	Percentage cost plate 1	Percentage cost plate 2	Percentage cost plate 3	Average percentage cost
0.25 mM	36.11	28.03	39.77	34.6
0.125 mM	18.99	17.81	19.04	18.61
0.1 mM	9.05	-1.56	10.44	5.97
0.0625 mM	5.85	9.45	7.69	7.66

Table S14: Cost summary based on CFU plating This table summarises the percentage fitness cost of different H_2O_2 concentrations on *Ecc15* compared to the non-treated control. The first three columns show the data from 3 different replicates, and the last column shows the average cost based on three replicates. The data was collected by plating out the treatments and doing the CFU count to compare the bacterial mortality caused by different concentrations of H_2O_2.

H_2O_2 conc.	Cost replicate 1	Cost replicate 2	Cost replicate 3	Average cost
0.25 mM	22.8	23.1	24.85	23.5
0.125 mM	5.4	6.19	7.78	6.45
0.1 mM	4.7	4.32	5.69	4.90
0.0625 mM	1.34	1.69	1.80	1.61

Table S15: Post-hoc multiple comparisons to test if H_2O_2 concentrations causing lower costs lead to a survival advantage upon challenge. Significant factors are shown in bold.

Contrast	*df*	*t*	*p-value*
Only challenge – Priming (0.0625 mmM) + challenge	27	2.384	0.3
Only challenge – Priming (0.1 mM) + challenge	27	5.131	**0.0008**
Only challenge – Priming (0.125 mM) + challenge	27	4.219	**0.006**
Only challenge – Priming (0.25 mM) + challenge	27	0.651	**0.05**

Table S16: Post-hoc comparisons for *in vitro* priming experiment where 0.1 mM was used as a priming concentration and 5 mM was used as a challenge concentration. The significant factors are depicted in bold.

Contrast	*df*	*t*	*p-value*
Control – only priming	26	0.352	0.984
Control – only challenge	26	21.324	**<0.0001**
Control – priming + challenge	26	3.293	0.014
Only priming – only challenge	26	20.972	**<0.0001**
Only priming – priming + challenge	26	2.940	**0.0032**
Only challenge – priming + challenge	26	-18.032	**<0.0001**

Table S17: Post-hoc comparisons where male and female D. melanogaster were allowed to feed on primed and non-primed bacteria for 240 minutes. Significant factors are shown in bold.

Contrast	*df*	*t*	*p-value*
Non-primed female – primed male	69	-1.164	0.651
Non-primed female – non-primed male	69	3.231	**0.010**
Non-primed female – primed male	69	-2.306	0.106
Primed female – non-primed male	69	4.338	**0.0003**
Primed female – primed male	69	-1.112	0.683
Non-primed male – primed male	69	-5.507	**<0.0001**

Section 4

Discussion and Outlook

4.1 Discussion

The work presented in this thesis approaches the concept of microbial priming from both an *in vitro* and an *in vivo* perspective. This work reinforces the robust nature of *in vitro* microbial priming. It was shown that the bacterium *Ecc15* can be primed with hydrogen peroxide, and this priming gives it a survival advantage on experiencing lethal ROS conditions. We then focussed on the proteome-wide perturbations that *Ecc15* undergoes upon priming. After that, we go a step further and investigate the effects of microbial priming inside a host environment while also taking host sexual dimorphism into account. In the following sections, I will briefly discuss each of the findings and their implications and conclude the thesis by giving future perspectives on some of the open-end questions this thesis brings forward.

4.2 H_2O_2 MIC for *Ecc15* is similar to *E. coli.*

The MIC assay was performed to assess sub-lethal and lethal H_2O_2 concentrations for *Ecc15*. The MIC of hydrogen peroxide for *Ecc15* was found to be 1 mM. This is in line with 1 mM MIC of hydrogen peroxide for *E. coli* (Rodríguez-Rojas et al. 2020). The sensitivity of different microbial species towards hydrogen peroxide differs and is also determined by their Gram status. *E. coli* and *Ecc15* are Gram-negative bacteria belonging to the *Enterobacter* genus, so they might have common defence mechanisms against H_2O_2 (McEvoy, Thurn, and Chatterjee 1987). Another Gram-negative bacterium, *P*seudomonas *aeruginosa* shows an H_2O_2 MIC value of 4 mM (Zubko and Zubko 2013). Among all the Gram-positive bacteria tested, *Staphylococcus aureus* shows the highest sensitivity to H_2O_2 with 200 µM as the inhibitory concentration (Zubko and Zubko 2013). Hydrogen peroxide is widely used in antiseptics due to its potency and broad-spectrum activity (McDonnell 2014). However, the activity depends mainly on the concentration and the MIC of the targeted microorganism (Murdoch et al. 2016).

4.3 The generation time of *Ecc15* varies according to the growth temperature

Temperature plays an important role in microbial growth, and the variation in generation time with temperature has been well characterised (Billing 1974). Since *Ecc15* was cultured at 25°C throughout this thesis in order to maintain consistency with host growth temperature, it was essential to establish generation time under experimental conditions employed in the thesis. The generation time of *Ecc15* at 25°C was found to be 91.07 ± 6.54 minutes. It has been observed that *Ecc15* cannot grow well above the temperatures of 30°C and has a prolonged growth below 18°C (Billing 1974). In previous studies, *Ecc15* is cultured at different temperatures ranging from 25°C-30°C (Vieira et al. 2020; Buchon et al. 2009). On the contrary, *E. coli* can grow at temperatures ranging from 21 – 41 °C (Ferrer et al. 2003), with the optimum growth temperature being 37°C (Noor et al. 2013). It is important to consider that generation

time *in vitro* may not reflect the generation times when *Ecc15* is present in a host (plants or *D. melanogaster*), where the source of nutrition and temperature conditions could differ. However, it has been shown using apple plants under field conditions that the growth rates of *Ecc15 in vivo* did not differ from *in vitro* conditions (Eden-Green and Billing 1972).

4.4 *In vitro* priming experiments, high cost of priming

4.4.1 Priming with different doses gives different survival advantage upon challenge

Before testing whether *in vitro* priming has a survival advantage upon an *in vitro* challenge, a priming dose was established based on preliminary experiments. To establish the priming dose, the following doses of H_2O_2 were tested for their survival advantage upon challenge: 0.1 mM, 0.5 mM and 1 mM. The range of doses to test was decided based on the MIC value, with doses lower than and including MIC included in the experiments.

Based on methods from (Rodríguez-Rojas et al. 2020) *Ecc15* was primed for 30 minutes, followed by a recovery time of 60 minutes in the absence of any hydrogen peroxide stress. After recovery, the challenge stress was applied for 30 minutes. A gap separates the priming and challenge stimulus; this is the period where the information about the priming experience is stored as stable transcripts until a challenge appears (Rodríguez-Rojas et al. 2020). This information storage for future use has been termed “memory”, which could last up to a few generations before resetting the phenotype to a naïve state in the case of *E. coli* primed with H_2O_2 (Rodríguez-Rojas et al. 2020). In the case of plants, this memory has been shown to last for several months (Karban and Shiojiri 2009; Haukioja 1991; Nykänen and Koricheva 2004). Since *Ecc15* has a generation time of 91.07 ± 6.54 minutes, some cells in culture are likely to be doubling when they receive a challenge after a priming dose for 30 minutes and a recovery dose for 60 minutes.

A survival advantage of priming with 0.1 mM, 0.5 mM and 1 mM H_2O_2 was observed; however, this advantage differed with the priming dose. Multiple comparisons showed that the survival advantage upon challenge was highest with 1 mM priming dose. This suggests that the survival advantage of priming works over a range of concentrations. However, it is interesting to observe that survival benefit upon challenge is highest for the priming dose of 1 mM. It could be that 1 mM priming dose being higher compared to the other tested doses, triggers the immune system strongly leading to a more elevated response upon challenge. It would be interesting to explore if survival advantage follows a specific pattern based on priming doses.

Priming leads to an induced response, which is upregulated when needed, being a cost-saving strategy compared to a constitutive approach (Zangerl 2003; Karban 2008). Priming has also been referred to as a condition of readiness (Conrath, Pieterse, and Mauch-Mani 2002; Frost

et al. 2008). It is supposed to be active against occasionally occurring stresses that do not lead to changes in the genome and are mostly known to protect the organism via phenotypic changes (Ashapkin et al. 2020).

4.4.2 Priming with hydrogen peroxide (1 mM) results in higher *Ecc15* survival upon an *in vitro* challenge (5 mM)

One of the main objectives of this thesis was to test whether priming *Ecc15* with hydrogen peroxide *in vitro* gives it higher survival upon an *in vitro* challenge with a higher dose. It was demonstrated via testing a range of concentrations *in vitro* that hydrogen peroxide priming provides a survival advantage to *Ecc15* upon an *in vitro* challenge. The survival advantage was quantified to be 99% in priming + challenge treatment when compared with an only challenge treatment when *Ecc15* was primed with 1 mM H_2O_2 followed by a 5 mM challenge dose. However, there was also ~50% mortality associated with priming dose as observed by comparing an only priming treatment with controls, the effect was deemed as non-significant upon analysis. This mortality due to priming is termed as the “cost” of priming and it was decreased experimentally in later sections of the thesis. The advantage of ROS priming observed in *Ecc15* upon challenge solidifies the fact that microbial priming could be a prevalent phenomenon (Andrade-Linares, Lehmann, and Rillig 2016). These results are in agreement with prior studies where hydrogen peroxide priming has been successfully demonstrated in *E. coli,* with challenge doses ranging from 5 mM to 30 mM (J A Imlay and Linn 1986; Rodríguez-Rojas et al. 2020). However, there are a vast array of stressors, and it remains to be seen how general the priming phenomenon towards different stress categories is. The *in vitro* priming concentrations used in the experiments were similar to the average concentration of hydrogen peroxide in leaf discs of plants (1 mM), which can go up to 10 mM under stress conditions, relevant for plant pathogen *Ecc15* (Foyer and Noctor 2016).

It remains to be seen if there is an advantage of priming if there is an increase or decrease the time duration of exposure to priming stimulus. What is the cut-off value of exposure time at which it is still considered priming and not acclimation, with the former being a subsequent exposure and the latter being a chronic exposure to stress? What happens in the absence of a recovery phase? Does the recovery phase provide time to undergo required phenotypic changes to form memory markers for the upcoming challenge? There are plenty of questions along these lines that need to be answered in order to make our understanding of the priming phenomenon better. Duration and intensity of priming stress could also be the decisive factors in how long organisms maintain the memory of priming.

4.5 Proteome-wide changes upon hydrogen peroxide stress

Microbes are exposed to numerous stressors every day and there are likely to be some categories of stressors that are more prevalent than others, for which it is advantageous to mount constitutive responses rather than an “on-demand” response. Adaptations are usually intrinsically wired in the genome for such constantly occurring stresses since a constitutive response against less-prevalent stresses could be very costly to maintain. To avoid these costs due to less prevalent stresses, bacteria store information about them via activation of stable transcripts or proteins rather than hardwiring it into the genome (Veening, Smits, and Kuipers 2008; Ronin et al. 2017). To test for markers underlying priming, proteome wide changes experienced by *Ecc15* upon peroxide stress were tested via quantitative LC- mass spectrometry for the following treatments: control, only priming, only challenge and priming + challenge.

In the proteomics dataset, just under 1500 *Ecc15* proteins were detected to be differentially regulated proteins across all treatments from the total of 4,104 proteins available for *Ecc15* in the UniProt database. The PCA suggested that the control treatment and only challenge treatment do not differ from each other, which could be due to sudden exposure to a high level of ROS, which gives no time for the bacteria to respond. This might be due to the short exposure (10 minutes) to challenge stimulus, not allowing for significant changes in the proteome and hence being close to the control treatment in PCA. Another reason could be the high toxicity caused by challenge stress, as indicated by ~99% mortality in *Ecc15* under *in vitro* conditions both after 10- and 30-minutes of challenge leading to stalling of cell activity and making cells unresponsive (Heo, Kim, and Kang 2020). This high mortality was the initial reason for using 10-minutes as a challenge duration to avoid mortality of the whole population and thereby no viable cells to test proteomic changes. Similarly, the priming treatment overlaps with the priming + challenge treatment indicating that the proteomic changes induced at priming are all maintained when cells receive a challenge dose. These changes might help cells survive the challenge better as compared to only challenge treatment. Additionally, it could also be that there was not enough time for proteomic changes in priming + challenge treatments as the challenge was applied for 10-minutes, as previously mentioned, possibly explaining the vicinity of these two treatments.

The GO analysis showed that the differentially regulated proteins could be categorised based on their molecular function into these three categories: catalytic activity, nucleic acid protection and damage repair pathways. Under basal oxidative stress conditions, redox defences are constitutively active to protect bacteria from damage by constantly scavenging chemically reactive oxygen or by maintaining a reducing environment (Reniere 2018). When the ROS

levels are elevated, bacteria produce ROS detoxifying enzymes such as catalases, peroxidases and superoxide dismutases in order to protect nucleic acids, enzymes and proteins from oxidation (Mishra and Imlay 2012). Another quick response is to upregulate DNA protection and repair proteins such as Dps (DNA protection under stationary phase). All the detoxifying enzymes such as catalases, peroxidases and superoxide dismutases were found to be upregulated in ROS receiving treatments. The most abundant gene category in catalytic activity belonged to transferases, detoxification enzymes that protect cells from reactive electrophiles.

The magnitude of the response to hydrogen peroxide depends not only on the magnitude of the stimulus but also on the microbial growth phase. The response to oxidative stress differs when cells are in the logarithmic phase compared to the stationary phase, since cells in the logarithmic phase are more sensitive towards ROS (Thorpe et al. 2004). For example, stationary-phase bacterial cultures of *Bacillus subtilis* have been shown to be completely viable after treatment with 10 mM hydrogen peroxide whereas the same concentration reduces viability of exponential-phase cultures to 0.01% (Dowds 1994). A similar phenomenon has also been observed in *E. coli* (Eisenstark et al. 1996). In the experiments conducted in this thesis, cells were growing exponentially making them more sensitive towards ROS stress. It could be possible that cells in stationary phase are able to survive much higher doses of H_2O_2.

In case of the only priming treatment, a downregulation of RpoS was observed (also known as katF). However, in case on an only challenge treatment, an upregulation of RpoS was observed. It is an alternate sigma factor controlling a large array of oxidative stress genes in many bacterial species including *Ecc15* (Mukherjee et al. 1998). RpoS is a 342 amino acid, 38 kDa protein and it has been deemed essential in *Ecc15* to cope with hydrogen peroxide toxicity (Santander et al. 2014). It has also been established that inactivation of the *RpoS* gene leads to enhanced sensitivity to hydrogen peroxide (Mukherjee et al. 1998). *Erwinia* species and *E. coli* are closely related evolutionarily, both being Enterobacteria, and hence many biochemical and genetic regulatory pathways could be conserved between them (Gardner and Kado 1972). The nucleotide sequence of *RpoS* structural gene in *Ecc15* is 81% similar to *E. coli* (Calcutt et al. 1998). RpoS is known to control hundreds of genes, which are absent in case of RpoS mutants (Schellhorn 2020). It controls some of these genes by direct regulation and others by dual regulation in combination with OxyR (Eisenstark et al. 1996; Loewen and Hengge-Aronis 1994). One point to note is that RpoS helps the bacterial cells in dealing with oxidative stress in stationary phase whereas OxyR is a key player in log phase (Calcutt et al. 1998). RpoS is involved in antioxidant activities, iron level regulation and iron-sulphur cluster assemblies and upregulation of proteases in stationary phase. Therefore, the reason for

downregulation in an only priming treatment could be to avoid conflict with OxyR, which is active in exponential growth phase. It is known from *E. coli* that RpoS is rapidly degraded during exponential phase with a half-life of 2 minutes but it is very stable during stationary phase (Zhou and Gottesman 1998). This downregulation of RpoS in case of only challenge treatment might suggest that priming helps bacterial cells deal with oxidative stress while staying in an exponential phase whereas, in case of only challenge treatment, where cells receive just the lethal dose, they tend to enter a stationary phase to avoid further damage.

The oxyR regulon plays a key role in antioxidant defense in replicating bacteria by activating the expression of genes such as catalases and other detoxifying enzymes in response to high levels of hydrogen peroxide. It also activates proteins involved in DNA repair and iron homeostasis (Figure 26) (Cabiscol, Tamarit, and Ros 2000; Flores-Cruz and Allen 2011; Storz et al. 1990; James A. Imlay 2008). OxyR works as an on "on-off" switch and in the absence of oxidative stress, it is in an "off" form (Dubbs and Mongkolsuk 2012). As soon as the levels of reactive oxygen species are elevated, it oxidizes a Cys-199 residue to sulfenic acid and hence activates oxyR which directly stimulates all the genes involved in defense (Dubbs and Mongkolsuk 2012). At least two dozen genes have been known so far to be a part of oxyR regulon (Åslund et al. 1999). OxyR is fine-tuned to respond to low doses of hydrogen peroxide, since sub-micromolar levels of intracellular hydrogen peroxide can be damaging to DNA and enzymes (Park, You, and Imlay 2005; Jang and Imlay 2007).

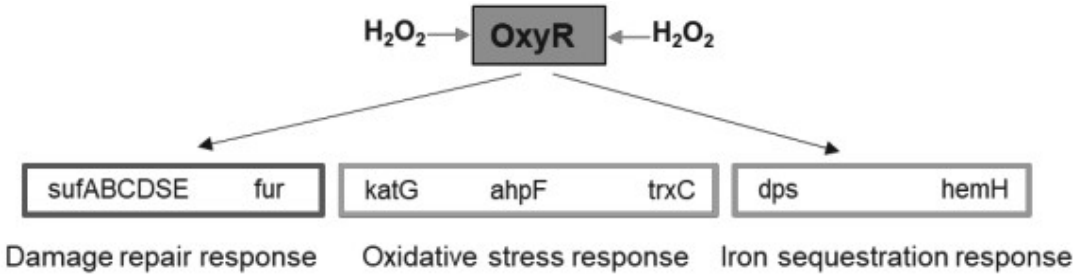

Figure 26: A summary of oxyR regulon and a major oxidative stress proteins upregulated by oxyR regulon among 2 dozen proteins. It plays a direct role in oxidative stress response and also in iron sequestration to avoid Fenton reactions, hence bringing hydrogen peroxide levels down. All these proteins were found four-fold upregulated in all our treatments except controls. The function of oxyR can be categorized into oxidative stress response, damage repair and iron sequestration response. The figure was modified from (Cornelis et al. 2011).

In *E. coli*, oxyR gets activated when hydrogen peroxide levels in the cytoplasm exceed ~0.2 µM (Park, You, and Imlay 2005). In our experiments, an upregulation of oxyR was observed even in the case of sub-lethal hydrogen peroxide concentrations (1 mM) confirming quick and

rapid oxyR response. As confirmed from the other studies, oxyR elevates the synthesis of KatG catalase and ahpC peroxidase to almost 10-fold to quickly bring down intracellular hydrogen peroxide concentration to non-damaging levels (Jang and Imlay 2007). An upregulation of katG and ahpC in all the treatments receiving hydrogen peroxide was confirmed. oxyR upregulates a protein known as Dps which sequesters loose iron and thus plays a role in supressing Fenton reaction mediated damage to DNA and to proteins (Grant et al. 1998; Ilari et al. 2002; Park, You, and Imlay 2005). oxyR also induces the SufABCDSE protein mediated iron-sulphur clusters assembly which helps to repair the clusters that have been oxidized (Djaman, Outten, and Imlay 2004). This group of proteins is not synthesized under normal conditions and are activated by oxyR only under hydrogen peroxide stress. Suf proteins form a complex in order to supply enzymes to iron-sulphur clusters. Iron-sulphur clusters are responsible for critical pathways such as respiration, which are disrupted by peroxide stress, and Suf system replaces these clusters for normal functioning of the cell (Ezraty et al. 2013; James A. Imlay 2008).

Dps, a ferritin class protein, strongly supresses DNA damage by sequestering unincorporated iron (James A. Imlay 2013). As summarized in Figure S, all the key proteins that lie in oxyR regulon mentioned in the figure were found to be upregulated in response to high intracellular levels of hydrogen peroxide.

YaaA is known to be another key element in fighting peroxide stress and it was found to be upregulated across all our treatments receiving hydrogen peroxide. Other studies confirmed that yAA is a key element in hydrogen peroxide stress in *E. coli* and it is activated in an oxyR dependent manner in presence of 1 mM hydrogen peroxide, which happens to be our priming dose (Zheng et al. 2001; Liu, Bauer, and Imlay 2011). YaaA plays a role in minimizing damage both to the DNA and to the proteins by binding to the faulty sites and acting as a damage repair enzyme (Liu, Bauer, and Imlay 2011). It does not seem to play much of a role during routine bacterial growth, but the levels are upregulated only during hydrogen peroxide stress (Prahlad et al. 2020).

These results are in line with previous studies where proteins helping in memory formation to survive challenge doses of hydrogen peroxide were tested five minutes after H_2O_2 challenge in *E. coli* (Rodríguez-Rojas et al. 2020). Proteins such as KatG, AhpF or RecA were found to be present even 150 minutes after the priming stress whereas proteins such as GrxA, YaaA and XthA, SufA, SufS, AcrA-AcrB had completely declined at this point after initial induction. It was shown by Rodríguez-Rojas and colleagues (2020) that induction of defences in primed *E. coli* upon challenge is mainly mediated by the scavenging proteins such as KatG and AhpCF. Additionally, they observed that even the low priming dose, which did not change any

growth rates in rich medium, led to major changes across the whole proteome in *E. coli*. The changes in proteome were maintained up to four-divisions before going back to the naïve state, confirming induction of phenotypic responses being an “on-demand” phenomenon. Our dataset strengthens the fact that ROS response is multifaceted with OxyR controlled genes acting as key antioxidation factors.

Proteomics gives an overall proteome reshuffling upon stress. However, this approach could be combined with transcriptomics to help us understand the changes occurring at the RNA level. Mass spectrometry provides us with several advantages, the most obvious being the observation of the proteome, which no other methods currently can provide. Connecting both transcriptome and proteome may aid in our understanding of gene regulatory networks and observe changes happening at both RNA and protein levels. A reason why transcriptomics and proteomics provide us with different outputs is that transcriptomics reveals RNA expression at a particular moment, so it is a representation of what an individual is expressing, but it is known that a lot of RNA that gets transcribed never turns into proteins (Tan et al. 2009). While many studies point out that the correlation between transcriptome and proteome is relatively low (Bonaldi et al. 2008; Butter et al. 2013; J. J. Li, Bickel, and Biggin 2014; Casas-Vila et al. 2017), combining them can provide us with a better understanding of specific molecular mechanisms. The cons of proteomics are that the current methods are unable to detect membrane proteins of microbes, because of their inability to aggregate and precipitate (Chandramouli and Qian 2009). Additionally, there is a lack of a database for relatively less studied species. Even after the disadvantages mentioned above, since the proteins are generally the direct effectors of the cell, directly studying their variation could provide new insights into molecular mechanisms not detected by transcriptomics studies. Current techniques of proteomics come with a detection limit of 6 miilion proteins (Timp and Timp 2020). Proteins also have very different half-lives (Mathieson et al. 2018); therefore, proteomics encompasses all the proteins present after all the interferences and regulations and should reflect actual phenotypic differences.

4.5.1 10-minute challenge duration is enough for survival advantage of priming

In case of sample preparation for LC-MS experiment, an *in vitro* control was simultaneously carried out to confirm the priming phenotype under modified experimental conditions (10 minutes of challenge instead of 30 minutes). It was confirmed that priming provides a survival advantage upon a 10-minute challenge, as opposed to 30-minute challenge duration used in previous experiments. This could point towards the fact that even a short challenge exposure is enough to reap survival advantages of priming. Similar survival advantage was also found in *E. coli* upon 5 minutes of challenge (Rodríguez-Rojas et al. 2020).

4.6 *In vivo* experiments, high cost of priming

4.6.1 Longer starvation does not lead to higher bacterial loads

After priming *Ecc15* under *in vitro* conditions, and observing that priming does provide a survival advantage upon challenge *in vitro*, the survival advantages of priming when *Ecc15* is inside a host were tested. However, the published methods for oral infections with bacteria in *D. melanogaster* use bacterial culturing conditions that are quite different from the methods used to test priming *in vitro*, i.e., the flies are allowed to starve for variable durations and feed on bacteria for 4 hours or longer (Siva-Jothy et al. 2018; Regan et al. 2016; Buchon et al. 2009). Additionally, the bacteria are resuspended in sucrose solution and offered to the fly at an OD of 200 (Siva-Jothy et al. 2018). These methods are employed to test the effects of infection on the host, however, the protocols needed to be modified to suit the experimental needs of this thesis, since the objective and time frames here is different. Since the *in vitro* challenge was applied for 30-minutes, in order to maintain consistency, a 30-minute feeding time was tested.

To do that, the starvation and feeding conditions were modified from Siva-Jothy et al. (2018). A 2-hour and a 4-hour starvation period was tested, followed by a 30 minutes and 4-hours of feeding. It was predicted that longer starvation will ensure higher feeding in the host. It has been shown using food colouring that longer starvation times (8-hours) lead to more food intake compared to shorter starvation times (2-hours) (Itskov et al. 2014).On the contrary, a significant increase in feeding upon prolonging the starvation period from 2-hours to 4-hours was not observed. However, a four-hour starvation led to less-variable bacterial loads as opposed to a two-hour starvation. Additionally, this experiment confirmed that it is possible to extract countable number of CFU's from the host upon a 30-minute feeding.

4.6.2 Effects of optical density and growth media on feeding

Ecc15, being an entomopathogen, proliferates in high numbers in the parenchyma of infected plants (Davidsson et al. 2013; Slack et al. 2017). Bacterial cells come out as an ooze which is a combination of bacteria and sugars (Slack et al. 2017). This bacterial ooze of *Ecc15* acts as an attractive food source for flies because of its sugary composition. For similar reasons, oral infections in *D. melanogaster* are carried out by mixing bacteria with a sucrose solution (Gordesky-Gold et al. 2008). Additionally, to make sure that flies have access to a lot of bacteria, a highly dense microbial paste of OD ~200 is used. However, this is far from the *in vitro* conditions under which the experiments were conducted. To circumvent the difference in conditions, a low optical density (OD 0.5) microbial solution mixed in LB as well as sucrose was tested. The results demonstrated that it was possible to extract countable number of bacterial CFU's from flies upon homogenisation when they fed on a bacterial culture of lower

OD suspended in LB media. There were a proportion of flies with non-countable bacterial loads under low OD conditions, however this proportion decreased when they were allowed to feed for longer periods of time.

4.6.3 Primed microbes inside a host, 1 mM priming

Since *in vitro* studies provide an excellent level of experimental control, it is easy to do priming experiments under *in vitro* conditions, as indicated by a plethora of such studies (Rodríguez-Rojas et al. 2020; J A Imlay and Linn 1986; H. M. Nguyen et al. 2020). It was also shown in section 3.3 that an *in vitro* priming leads to survival advantage upon an *in vitro* challenge. However, it is also common knowledge that microbes keep moving across environments quite quickly, so they will not necessarily experience challenge in the same environment they were primed in (J. Nguyen, Lara-Gutiérrez, and Stocker 2021). The first question that comes to mind in such a scenario is, does priming still give an advantage when priming and challenge happen in different environments? Apart from being in places such as wastewater treatment plants and soil, microbes are also carried around to different environments by insects acting as vectors (Heck 2018). An insect-microbial model hence provides a natural setting to test effects of priming in ecological settings.

When it comes to an insect host, microbes do not necessarily face similar amounts of stress, owing to the sexual dimorphism in the immune responses of males and females (S. L. Klein and Flanagan 2016). Sex is an important variable that affects the functions of an immune system along with other factors such as age, nutrition, environment and reproductive status (Regan et al. 2016). Despite a large amount of studies taking sex-specific differences into account, only 10% of immunity studies analyse their data by taking sex into consideration (Beery and Zucker 2011; Nystrand and Dowling 2020). Host *D. melanogaster* shows strong sexual dimorphism in innate immunity at baseline, upon infection and over a lifetime (Regan et al. 2016; Kopp, Duncan, and Carroll 2000; Teder and Tammaru 2005). ROS producing dual oxidases (Duox) are higher in 7 and 42 day old males when compared with females of the same ages (Regan et al. 2016).

An exhaustive survey was conducted by Belmonte et al. (2020) by taking peer-reviewed immunity studies of both sexes into account. They tested the effect of sexual dimorphism on behavioural, epithelial, cellular and systemic immunity for larval and adult stages of *D. melanogaster* (Belmonte et al. 2020).

Additionally, males and females show different gut pathologies upon ageing and over their lifetime, with males being more susceptible to gut infections and xenobiotics (Regan et al. 2016). It has been shown that there is sexual dimorphism in the size of the gut in male versus

female *D. melanogaster* (Hudry, Khadayate, and Miguel-Aliaga 2016). It has been shown by Hudry and colleagues (2016) that wild-type female midguts are longer than male midguts at day 3, 5 and 20 of their lifetimes. Additionally, masculinisation of the intestinal stem cells (ISCs) leads to the shrinking of the female midgut (Hudry, Khadayate, and Miguel-Aliaga 2016). This could mean that the amount of time microbes stay in contact with the gut in the case of male and female flies differs, leading to different infection trajectories.

A few studies have compared sex differences in gut metabolism (Hudry, Khadayate, and Miguel-Aliaga 2016), and physiology (Regan et al. 2016; Belmonte et al. 2020), most work focusses on females and nothing is known about sex dimorphisms in ROS production or survival upon oral infections.

After standardizing the feeding conditions, primed *Ecc15* was given a challenge inside the host, using oral infections. Here, the host *D. melanogaster* was fed with primed and non-primed bacteria and the bacterial loads were quantified via homogenisation 30-min, 60-min and 240-min post feeding. Statistical analysis suggested a significant interaction of treatment with time, suggesting bacterial loads of primed and non-primed bacteria differed with time. As previously mentioned, duration and intensity of ROS response is host gut upon infection has not been well characterised. It could be that intensity of ROS formation differs over time potentially explaining an interaction of treatment and time. A significant interaction between treatment and sex was also observed, suggesting bacterial load differences in treatments based on the sex of the host, which could be explained due to sexual dimorphism in ROS amounts owing to higher DUOX in males as compared to females (Regan et al. 2016). However, contrary to the prediction there was no effect of treatment (primed vs non-primed) on bacterial loads of the host. Although, the survival advantage of priming upon challenge was observed under *in vitro* experiments carried simultaneously. Additional *in vitro* controls (section 3.6) were carried out to test if there is bacterial mortality upon priming. The results demonstrated that there were lesser number of viable cells in primed treatments as compared to non-primed treatments. Among additional factors such as complex host ROS responses, this could explain why there was no advantage of priming observed *in vivo*, since flies had differential number of viable cells available to them for feeding.

4.7 *In vitro* control experiments show differential number of bacteria in primed and non-primed treatments

In vitro control experiments (section 3.6) suggested lower CFU's in primed treatment as compared to non-primed treatment, suggesting priming itself has a cost, confirmed by significant differences in control and "only priming" treatments under *in vitro* conditions, which suggested that the priming dose used (1 mM) led to ~50% associated cost. Additional *in vitro*

controls included CFU plating after a recovery period and bacterial survival on filter discs. All these experiments suggested substantial cost that priming entails.

The main role of priming is to improve future fitness with ideally minimal resource investment and low incurred costs. Priming is also expected to reduce the costs of responding to a future stressful event. The response to a priming stimulus could be expected to be associated with some costs since it requires changes in a regulatory network that need to be kept active until a challenge event, in order to store the stress information. However, extensive information of costs associated with microbial priming could not be found in other studies except (Rodríguez-Rojas et al. 2020). In nature, the cost associated with priming could differ for different stresses. Since costs of induced defense should not be outweighed by the benefits of enhanced protection, the costs of priming in nature should be generally very low. There are many studies showing costs of immune priming in invertebrates (Sadd and Schmid-Hempel 2009; Contreras-Garduño et al. 2014).There are a few studies where costs of priming have been studied in plants, yet the cost phenomenon overall remains poorly documented, partly due to lack of studies conducted under natural conditions. It has been shown that although primed plants grow faster than non-primed plants, they have lower clonal reproduction (Yip et al. 2019; van Hulten et al. 2006). Fortunately, *in vitro* experiments provide a great deal of experimenter's control. This allowed us, as shown in the later part of this thesis, to experimentally lower the costs associated with priming, so that the priming stress itself in not lethal and does not lead to high mortality among primed populations.

4.7.1 Correction factors to deal with bacterial mortality differences due to priming

Since priming costs led to lower bacterial numbers in primed treatment as compared to non-primed treatment, this means that flies having access to the primed bacteria would receive a lower live dose of bacteria than flies given the non-primed bacteria. Therefore, correction factors were applied to account for mortality caused by priming. Since costs could potentially conceal any effects of treatment, correction factors were calculated individually for each experimental replicate, based on costs of priming. These correction factors were then multiplied by the bacterial loads of primed treatments of *in vivo* experiments (section 3.6.5) to create a dataset with corrected bacterial loads. This dataset was reanalysed using corrected bacterial loads instead of initial bacterial loads. The analysis based on corrected dataset reflected similar patterns of interactions as non-corrected bacterial loads, with treatment showing an interaction with both time and sex. The interaction between treatment and time suggests that the effect of treatment differs over time, which could be due to the variable amounts of ROS formation inside the host. It is tempting to predict that ROS amounts after infection do not remain constant but appear in peaks, leading to more ROS at certain time

points. An interaction of treatment with sex suggests sex specific differences in ROS amounts in male and female *D. melanogaster,* which have been previously explored (Regan et al. 2016).However, a significant effect of treatment was not observed. This could either be due to absence of any effect of priming, or it could be due to the costs masking any effect. To test whether the absence of a treatment effect is not due to significant costs, in the following experiments priming costs were reduced *in vitro* by decreasing the priming dose of hydrogen peroxide.

4.8 *In vitro* experiments, low cost of priming

4.8.1 Reduced priming costs based on optical density

With the aim of reducing the costs associated with priming, a range of lower doses (0.25, 0.125, 0.1 and 0.0625 mM) of hydrogen peroxide were tested and the associated percentage cost was calculated. The cost calculation was done based on optical density of bacterial cultures in a plate reader (Rodríguez-Rojas et al. 2020). This method was employed in a study conducted by Rodríguez-Rojas and colleagues where they found the percentage cost associated with hydrogen peroxide priming in *E. coli* to be 4%. Following these methods, a range of concentrations were tested and the associated costs were calculated. These tests based on optical density showed that the costs of priming can be reduced substantially (from 50% to 5%) depending on the concentration of hydrogen peroxide used. This is in line with previously mentioned study where priming *E. coli* with hydrogen peroxide entailed ~4% cost, which was considered to be a negligible cost (Rodríguez-Rojas et al. 2020).

4.8.2 Reduced cost of priming based on CFU counts

However, the *in vitro* experiments in this thesis were carried out by estimating bacterial numbers by CFU counts. The OD and CFU counts based methods could have distinct costs based on growth and detection conditions. Since OD based method takes both viable and non-viable bacteria into account. To confirm if the costs can be reduced under *in vitro* experimental conditions too, cost associated with a range of concentrations were calculated based on priming and challenge treatments being applied and the CFUs being plated out. This confirmed that costs could be reduced from 50% to 4.9% by decreasing priming concentrations of hydrogen peroxide.

It has been shown that hydrogen peroxide has two different modes of microbial killing, depending on the concentration (Uhl et al. 2015; J A Imlay and Linn 1986). At lower concentrations (1 mM), it leads to killing by damaging the DNA, whereas at higher concentrations (>10 mM), the damage is caused to all the macromolecules (J A Imlay and Linn 1986). It could be that 1 mM H_2O_2 leads to enough DNA damage in *Ecc15,* leading to

significant mortality. This damage caused by 1 mM H_2O_2 could be due to low concentrations of peroxide destroying iron-sulphur clusters, leading to compromised respiration (Stiban, So, and Kaguni 2016).

4.8.3 H_2O_2 concentrations with lower costs lead to a survival advantage upon challenge

The concentrations leading to lower associated costs were used as priming doses and tested for their survival advantage upon challenge. 0.1 mM was the concentration that provided highest cost/benefit ratio with ~4.9% cost and a significant survival benefit upon challenge. Interestingly, 0.0625 mM H_2O_2 led to a lower cost of priming (1.61%), however, the survival advantage upon challenge was lower compared to 0.1 mM. The reason could be that 0.0625 mM is a concentration low enough to not appear threatening to *Ecc15*, leading to lesser or no upregulation in immune defences. Upon testing if priming *Ecc15 in vitro* with 0.1 mM hydrogen peroxide leads to a survival advantage upon an *in vitro* challenge, it was established that there is a significant survival advantage of priming when *Ecc15* receives an *in vitro* challenge. This suggests that microbes are able to remember a range of concentrations of priming stress and use it to their survival advantage when a challenge stress appears. This also suggests that primability does not depend on a specific concentration of priming stress, which is beneficial since microbes come across a gradient of stresses in nature.

4.9 *In vivo* low cost of priming

4.9.1 *Ecc15* primed with 0.1 mM H_2O_2 inside a host

After *Ecc15* was primed with 0.1 mM hydrogen peroxide, the effects of priming were again tested inside a host. The results demonstrated that bacterial loads differ with time, with no significant differences among primed and non-primed treatments at 30 and 60 minutes. However, after 240 minutes of feeding, an interaction between treatment and sex was observed, with bacterial loads of primed bacteria being significantly higher in male hosts as compared to non-primed bacteria. This pattern was not observed in female hosts where there were no significant differences in bacterial loads of flies with primed and non-primed bacteria. ROS response to infections has been studied in *D. melanogaster* larvae and is known to be biphasic in case of hemocytes (Myers et al. 2018). The intensity and duration of ROS production in gut are unclear, however it could be that a short transient peak is reached when bacteria are ingested and a stable response acts to clear the infection over a longer period of time, possibly explaining the survival advantage upon 240 minutes post feeding as opposed to 30- and 60-minutes post feeding.

This set of *in vivo* experiments strengthened the fact that it is very important to consider sexual dimorphism in immune defences in experiments. A sex-specific effect of priming was observed

where primed bacteria had a survival advantage compared to non-primed bacteria when they were inside a male host. It is known that male *D. melanogaster* have higher DUOX over lifetime, as compared to females, leading to higher ROS production in males (Regan et al. 2016). It could be that higher ROS in males acts as a trigger for lethal environment, leading *Ecc15* to upregulate stronger defences in males as compared to females. This could have several implications for the host and their strategies to deal with an infection. A sex-specific effect of priming means that microbes survive better inside a particular sex, predisposing that sex to higher bacterial loads, which could ultimately lead to higher host mortality. From the microbial perspective, priming could help microbes survive the immune defences of a particular sex better, making their colonisation easier. This could support the hypothesis that sex-specific differences could lead to evolution favouring pathogens with sex-specific virulence (Úbeda and Jansen 2016).

An interesting point of discussion is how infection methods affect sex-specific immune responses. In the *in vivo* experiments, oral infections as an infection route were used to test differences between primed and non-primed bacteria inside a host. Oral infections were chosen since ROS is involved more directly in immunity inside the host gut. However, recent evidence has shown that ROS are also involved in bactericidal responses in host haemocoel (Shaka et al. 2022). Additionally, H_2O_2 acts as the universal wound signal, hence expected to be upregulated at wounded sites (Moreira et al. 2010).This makes it the next logical step to test primed bacteria inside a host via the route of systemic infections by injecting *Ecc15* into the host. It also provides greater experimental control regarding the number of bacteria injected inside the host. It has been characterized that ROS has a biphasic mode of killing in hemocytes, with a transient but heterogenous immediate response followed by a stable delayed response upon infection (Myers et al. 2018). If *Ecc15* has been primed with H_2O_2, a survival advantage upon systemic infections is expected. However, since the intensity of H_2O_2 could differ in both phases, one phase can lead to better survival compared to the other. Additionally, a sex specific effect of systemic infections cannot be neatly explored, however reports show that males are more resistant to systemic infections with some bacterial species (Belmonte et al. 2020; Buchanan, Meiklejohn, and Montooth 2018; Duneau et al. 2017).This approach could combine the effects of priming on bacteria upon infecting the host systemically and sex-specific immune differences upon systemic infections.

Another interesting approach could be to use *D. melanogaster* larvae instead of adults for the oral infection experiments. Unlike *D. melanogaster* adults, larvae constantly feed (Sewell, Burnet, and Connolly 1974); this behaviour could lead to robust and less variable bacterial loads upon homogenisation.

4.10 Concluding remarks

The work presented in this thesis approaches the concept of microbial priming from both an *in vitro* and an *in vivo* perspective. It was shown that the bacterium *Ecc15* can be primed with hydrogen peroxide, and this priming gives it a survival advantage upon experiencing lethal ROS conditions. We then established the global proteomic response to ROS stress by identifying regulators such as oxyR to play a major role in antioxidation mechanisms. *In vivo* priming, using *D. melanogaster* as a host showed that survival advantages of priming upon an *in vivo* challenge depend on duration of *in vivo* challenge and the sex of the host. We established that primed bacteria had a survival advantage upon challenge when they were present inside male *D. melanogaster*.

Although, there is substantial evidence of priming under *in vitro* conditions, there is a lack of priming studies under natural settings. This is mainly due to interference of biotic and abiotic factors in natural settings. All the instances of priming shown in plants have been mostly conducted under green-house settings (Karban 2011). To understand the phenomenon fully, more studies in natural settings are essential to observe impacts of priming. This thesis, provides a direction and a explores a compatible system to study priming in nature, while trying to circumvent interfering factors.

Altogether, this thesis provides evidence of effects of cross environmental priming, providing a basis to probe the phenomenon further. In this study, we mostly explored priming from a phenomenological level and therefore, our conclusions could be in part limited due to it. We recognize the need to go deep into the mechanistic underpinnings to fully understand the stochastic nature of the phenomenon and that would be the next natural step for this project. In the next section I will discuss some potential ways going forward by giving future perspectives on some of the open-end questions this thesis brings forward.

4.11 Future perspectives

In this thesis, I exclusively dealt with the phenomenological basis of microbial priming and whether the survival advantage is also present in more complex environments than the one in which bacteria were primed. This leads to an exciting set of open questions about the phenomenological and mechanistic basis of priming, some of which will be posed in the following sections.

4.9.1 Microbial priming: an individual or a population level phenomenon?

In the case of microbial priming, we still need to gain knowledge whether priming works at an individual bacterial cell level or if it is a population-level phenomenon, i.e., a phenomenon

where all the cells of the same bacterial species present together in same space and time participate. It could be that in a homogenous bacterial population, a small fraction of cells are being primed, just like in bacterial persistence, as shown by Balaban and team (Balaban et al. 2004). In case it works at an individual level, how is the information exchanged between bacterial cells? Experimentally, it has been hard to address at an individual level in the absence of correct tools. More recently, the arrival of microfluidic systems has the ability to enable the study of microbial priming at an individual level. Single-cell experiments can be combined with fluorescent markers to be analysed in a mother machine (Zaslaver et al. 2006). Microfluidics has already helped us understand non-heritable antibiotic resistance or persistence (Balaban et al. 2004). Following a single cell over multiple divisions, we might be able to establish the duration of priming response or "memory of priming".

4.9.2 Memorising and forgetting priming responses

We currently have a handful of instances of memory of priming in bacteria. In one such study, priming in *E. coli* was shown to last up to 4 divisions (Rodríguez-Rojas et al. 2020), but we do not know if that is the case in all the microbial species. Additionally, the degree of specificity in the memory of priming responses is unknown to us. We also do not know how these memory mechanisms differ depending on the stress category and if the type of stress plays a role in how long the memory lasts. It could be that microbes perceive some stresses as more dangerous compared to others, in which case, it makes sense to maintain the memory markers for a bit longer compared to less threatening stresses. Another point we need to consider is that all the microbes have variable generation times in nature depending on the life cycle and life history, ranging from minutes to days (Gibson et al. 2018), which could have a role to play in the duration of memory of priming. Another factor deciding the length of the memory phase could be the stability of the regulatory molecules involved. Having more information on how long the memory of priming lasts in case of different stresses and different microbial species will help disentangle if the memory is specific to the stress category, microbe, half-life of regulatory molecules and generation time.

Since memory responses do not last forever, it also raises another question: How is this information forgotten? It is also worth finding how the primed state is reset back to a naïve state after the stress has been removed in microbes. In case of plants, it has been established that the process of "forgetting" happens through an epigenetic reset. A specific example of cold stress can be presented, where exposure to cold is remembered throughout the life span of winter plants, whereas the memory is lost in the progeny via an epigenome reset (Paszkowski and Grossniklaus 2011; Iwasaki and Paszkowski 2014).

4.9.3 Five dimensions of immunological memory

Since priming is a highly stochastic phenomenon and not as black and white as we might make it to be, we can go back to an extensive review on immunological memory by Pradeu and du Pasquier (Pradeu and Pasquier 2018) where they mention five key dimensions involved in the formation of immunological memory. These five pillars are SSSED namely: *speed, strength, specificity, extinction* and *duration*. Speed represents how rapid the second immune response activation upon challenge is compared to the first response. Strength represents the quantitative and qualitative increase in strength on the second exposure compared to the first. Specificity relates to whether the second response is specific towards a particular stress or shows a more generalised protection phenomenon. Extinction signifies whether there was an extinction phase of immune responses, i.e., if the second response is heightened due to the persistence of immunity from the first response or if the second response is a reactivation of immune responses. Lastly, duration, as the name suggests measures the time the response to the second exposure lasts. All the key dimensions can exist in the form of gradients and can be variable in the extent to which they play a role in achieving a primed phenotype. As Pradeu and du Pasquier put forward, if priming is a multidimensional concept, all the dimensions must be considered when studying it. Clearly defining the dimensions and having a common consensus will help design rigorous experiments. A priming response could either be a result of the high speed or high strength of response, and disentangling these dimensions might be a better way to understand the phenomenon of priming.

4.9.4 Microbial communities in nature

The basic idea of priming is that there is an initial response that leads to a primed state, which helps protect cells better when they are in an even more harmful environment. Since microbes occur in nature as a norm in heterogeneous groups of different species, the primed phenotype may increase competitiveness among these groups based on their primability (Rillig et al. 2015). Varying degrees of primability may increase the probability of survival of certain groups better than those that do not respond timely to stress. In these scenarios, it is essential to consider the effects of dynamic environmental changes at the level of microbial groups or communities (Rillig et al. 2015). In a microbial community, the stress response can also be varied based on the presence or absence of physiological response pathways or varied stress tolerance. Some microbes may be able to be primed better by a particular stressor, while others may not be. It can also differ based on the location of microbial species, for example, if they are present inside a biofilm, they might not be able to be exposed to the priming stress, and if the lethal stress experienced later is able to disrupt the biofilm, they could succumb to

the stress. Microbial priming studies have yet to take the presence of bacterial communities and their interactions in nature into consideration. It is important to study how these interactions or the presence of competitively superior or inferior microbial species effects priming behaviour. Diving into studying priming in microbial community situations might help us establish how group dynamics affect priming in a realistic setting.

4.9.5 Role of priming in microbial transmission

The process of spreading microbes is called transmission. The microbial transmission could happen between an insect – plant system or an insect-to-insect transmission (Heck 2018). This means that microbes constantly jump from the environment to a host and from one host to another. This jumping from host to host comes with a varying degree of immune defences faced by the microbe. Being inside one host with lower immune defences could potentially prime the microbe when it finds itself inside a host that has a stronger immune defence. If priming helps them survive the more potent immune response better, this could potentially lead to increased transmission and increased risk of diseases.

To pose a specific example, the bacterial model used in this thesis is *Ecc15*, which is a plant pathogen, and the transmission happens via insects. *Ecc15* is the main cause of diseases like soft rot in major crops. If ROS priming improves the ability of *Ecc15* to survive on plants, and overcome plant ROS defences better, it could help *Ecc15* in colonising plants better and eventually increase disease transmission.

Another interesting approach is to test if predisposing microbes to insect immune defences helps them survive better when they are inside a host. This could be achieved by preparing a homogenate of an insect host, *D. melanogaster*, in our case, and exposing microbes to this homogenate in an *in vitro* setting. The host could then be allowed to feed on these microbes to see if they have better survival inside the host as compared to non-primed bacteria.

4.9.6 Unifying the term “priming”

The importance of priming studies has been dealt with briefly in the introduction of this thesis. However, here, I would like to reflect on the obstacles that might hinder further understanding of the priming phenomenon. There is a need for a common framework of terms in priming studies since the usage of different terms for describing the phenomenon of priming makes it harder to find and merge the findings together. We could try to surpass field-specific terms and unify the concepts so they can be easily extrapolated across various fields of research. While reading previously published reports on priming, the ambiguity in using different terms to describe priming is evident. Priming is often defined using terms such as cross-protection, predictive response strategy or acquired stress resistance. Additionally, as shown by Hilker

and colleagues (Hilker et al. 2016) in their exhaustive review on priming, terms like adaptation, acclimation or systemic acquired resistance (SAR) are also used interchangeably with priming. It is easy to equate “priming” with “adaptation” but the two terms are conceptually different. Adaptation refers explicitly to the adaptive genotypic changes to cope successfully with a new environment. In contrast, priming refers to the coping with stressful environments by introducing phenotypic changes. Similarly, “priming” tends to be replaced with the word “acclimation”. Acclimation is a process that happens after chronic exposure of an individual to a new environment (Demmig-Adams et al. 2008). It is essentially an organism trying to adjust to a sustained environmental change, whereas priming comes from subsequently occurring, occasional stressful events. In the presence of a plethora of words to describe the same phenomenon, it is not straightforward to find all the studies essentially talking about the same phenomenon under an umbrella of different terms. A common framework of terms will help to find and compare priming studies and make it possible to reproduce them. My observation currently deals only with microbial priming since, in plant biology and invertebrate biology (Milutinović and Kurtz 2016), usage of the term “priming” seems to be a common practice to describe the predictive response strategies. However, in the case of microbial studies, this ambiguity persists and makes it challenging to find studies from already sparse reports. Adopting the term “priming” in the case of the predictive response strategy of microbes, will make the field more uniform in terms of accessibility of existing studies and promote reproducibility.

References

Alfadda, Assim A., and Reem M. Sallam. 2012. “Reactive Oxygen Species in Health and Disease.” *Journal of Biomedicine & Biotechnology* 2012: 936486. https://doi.org/10.1155/2012/936486.

Andrade-Linares, Diana R., Anika Lehmann, and Matthias C. Rillig. 2016. “Microbial Stress Priming: A Meta-Analysis.” *Environmental Microbiology* 18 (4): 1277–88. https://doi.org/10.1111/1462-2920.13223.

Armitage, Sophie A. O., Wei Sun, Xintian You, Joachim Kurtz, Dietmar Schmucker, and Wei Chen. 2014. “Quantitative Profiling of Drosophila Melanogaster Dscam1 Isoforms Reveals No Changes in Splicing after Bacterial Exposure.” *PLOS ONE* 9 (10): e108660. https://doi.org/10.1371/journal.pone.0108660.

Ashapkin, Vasily V., Lyudmila I. Kutueva, Nadezhda I. Aleksandrushkina, and Boris F. Vanyushin. 2020. “Epigenetic Mechanisms of Plant Adaptation to Biotic and Abiotic Stresses.” *International Journal of Molecular Sciences* 21 (20): 7457. https://doi.org/10.3390/ijms21207457.

Åslund, Fredrik, Ming Zheng, Jon Beckwith, and Gisela Storz. 1999. “Regulation of the OxyR Transcription Factor by Hydrogen Peroxide and the Cellular Thiol—Disulfide Status.” *Proceedings of the National Academy of Sciences* 96 (11): 6161–65. https://doi.org/10.1073/pnas.96.11.6161.

Axmacher, Nikolai, Florian Mormann, Guillen Fernández, Christian E. Elger, and Juergen Fell. 2006. “Memory Formation by Neuronal Synchronization.” *Brain Research Reviews* 52 (1): 170–82. https://doi.org/10.1016/j.brainresrev.2006.01.007.

Balaban, Nathalie Q., Jack Merrin, Remy Chait, Lukasz Kowalik, and Stanislas Leibler. 2004. “Bacterial Persistence as a Phenotypic Switch.” *Science* 305 (5690): 1622–25. https://doi.org/10.1126/science.1099390.

Banatvala, Jangu, Pierre Van Damme, and Stephan Oehen. 2000. “Lifelong Protection against Hepatitis B: The Role of Vaccine Immunogenicity in Immune Memory.” *Vaccine* 19 (7): 877–85. https://doi.org/10.1016/S0264-410X(00)00224-3.

Bass, Timothy M., Richard C. Grandison, Richard Wong, Pedro Martinez, Linda Partridge, and Matthew D. W. Piper. 2007. “Optimization of Dietary Restriction Protocols in Drosophila.” *The Journals of Gerontology: Series A* 62 (10): 1071–81. https://doi.org/10.1093/gerona/62.10.1071.

Basset, Alan, Ranjiv S. Khush, Anne Braun, Louis Gardan, Frédéric Boccard, Jules A. Hoffmann, and Bruno Lemaitre. 2000. “The Phytopathogenic Bacteria Erwinia Carotovora Infects Drosophila and Activates an Immune Response.” *Proceedings of the National Academy of Sciences* 97 (7): 3376–81. https://doi.org/10.1073/pnas.97.7.3376.

Basset, Alan, Phoebe Tzou, Bruno Lemaitre, and Frédéric Boccard. 2003. “A Single Gene That Promotes Interaction of a Phytopathogenic Bacterium with Its Insect Vector, Drosophila Melanogaster.” *EMBO Reports* 4 (2): 205–9. https://doi.org/10.1038/sj.embor.embor730.

Bates, Douglas, Martin Mächler, Ben Bolker, and Steve Walker. 2014. “Fitting Linear Mixed-Effects Models Using Lme4.” *ArXiv Preprint ArXiv:1406.5823*.

Bedard, Karen, and Karl-Heinz Krause. 2007. “The NOX Family of ROS-Generating NADPH Oxidases: Physiology and Pathophysiology.” *Physiological Reviews* 87 (1): 245–313. https://doi.org/10.1152/physrev.00044.2005.

Beery, Annaliese K., and Irving Zucker. 2011. “Sex Bias in Neuroscience and Biomedical Research.” *Neuroscience & Biobehavioral Reviews* 35 (3): 565–72. https://doi.org/10.1016/j.neubiorev.2010.07.002.

Belmonte, Rebecca L., Mary-Kate Corbally, David F. Duneau, and Jennifer C. Regan. 2020. “Sexual Dimorphisms in Innate Immunity and Responses to Infection in Drosophila Melanogaster.” *Frontiers in Immunology* 10. https://www.frontiersin.org/articles/10.3389/fimmu.2019.03075.

Billing, Eve. 1974. “The Effect of Temperature on the Growth of the Fireblight Pathogen, Erwinia Amylovora.” *Journal of Applied Bacteriology* 37 (4): 643–48. https://doi.org/10.1111/j.1365-2672.1974.tb00488.x.

Bonaldi, Tiziana, Tobias Straub, Jürgen Cox, Chanchal Kumar, Peter B. Becker, and Matthias Mann. 2008. “Combined Use of RNAi and Quantitative Proteomics to Study Gene Function in Drosophila.” *Molecular Cell* 31 (5): 762–72. https://doi.org/10.1016/j.molcel.2008.07.018.

Borges, Andrés A., David Jiménez-Arias, Marino Expósito-Rodríguez, Luisa M. Sandalio, and José A. Pérez. 2014. “Priming Crops against Biotic and Abiotic Stresses: MSB as a Tool for Studying Mechanisms.” *Frontiers in Plant Science* 5. https://www.frontiersin.org/articles/10.3389/fpls.2014.00642.

Broderick, Nichole A. 2016. “Friend, Foe or Food? Recognition and the Role of Antimicrobial Peptides in Gut Immunity and Drosophila-Microbe Interactions.” *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 371 (1695): 20150295. https://doi.org/10.1098/rstb.2015.0295.

Brooks, Mollie E, Kasper Kristensen, Koen J van Benthem, Arni Magnusson, Casper W Berg, Anders Nielsen, Hans J Skaug, Martin Machler, and Benjamin M Bolker. 2017. “GlmmTMB Balances Speed and Flexibility among Packages for Zero-Inflated Generalized Linear Mixed Modeling.” *The R Journal* 9 (2): 378–400.

Bruce, Toby J. A., Michaela C. Matthes, Johnathan A. Napier, and John A. Pickett. 2007. “Stressful ‘Memories’ of Plants: Evidence and Possible Mechanisms.” *Plant Science* 173 (6): 603–8. https://doi.org/10.1016/j.plantsci.2007.09.002.

Buchanan, Justin L, Colin D Meiklejohn, and Kristi L Montooth. 2018. “Mitochondrial Dysfunction and Infection Generate Immunity–Fecundity Tradeoffs in Drosophila.” *Integrative and Comparative Biology* 58 (3): 591–603. https://doi.org/10.1093/icb/icy078.

Buchon, Nicolas, Nichole A. Broderick, and Bruno Lemaitre. 2013. “Gut Homeostasis in a Microbial World: Insights from Drosophila Melanogaster.” *Nature Reviews Microbiology 2013 11:9* 11 (9): 615–26. https://doi.org/10.1038/nrmicro3074.

Buchon, Nicolas, Nichole A. Broderick, Mickael Poidevin, Sylvain Pradervand, and Bruno Lemaitre. 2009. “Drosophila Intestinal Response to Bacterial Infection: Activation of Host Defense and Stem Cell Proliferation.” *Cell Host & Microbe* 5 (2): 200–211. https://doi.org/10.1016/j.chom.2009.01.003.

Buchon, Nicolas, Neal Silverman, and Sara Cherry. 2014. “Immunity in Drosophila Melanogaster--from Microbial Recognition to Whole-Organism Physiology.” *Nature Reviews. Immunology* 14 (12): 796–810. https://doi.org/10.1038/nri3763.

Butter, Falk, Ferdinand Bucerius, Margaux Michel, Zdenka Cicova, Matthias Mann, and Christian J. Janzen. 2013. “Comparative Proteomics of Two Life Cycle Stages of Stable Isotope-Labeled Trypanosoma Brucei Reveals Novel Components of the Parasite’s Host Adaptation Machinery.” *Molecular & Cellular Proteomics: MCP* 12 (1): 172–79. https://doi.org/10.1074/mcp.M112.019224.

Cabiscol, E., J. Tamarit, and J. Ros. 2000. “Oxidative Stress in Bacteria and Protein Damage by Reactive Oxygen Species.” *International Microbiology: The Official Journal of the Spanish Society for Microbiology* 3 (1): 3–8.

Calcutt, Michael J, Michelle Becker-Hapak, Martin Gaut, James Hoerter, and Abraham Eisenstark. 1998. “The RpoS Gene of Erwinia Carotovora: Gene Organization and Functional Expression in E. Coli.” *FEMS Microbiology Letters* 159 (2): 275–81. https://doi.org/10.1111/j.1574-6968.1998.tb12872.x.

Casadesús, Josep, and Richard D’Ari. 2002. “Memory in Bacteria and Phage.” *BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology* 24 (6): 512–18. https://doi.org/10.1002/BIES.10102.

Casas-Vila, Nuria, Alina Bluhm, Sergi Sayols, Nadja Dinges, Mario Dejung, Tina Altenhein, Dennis Kappei, Benjamin Altenhein, Jean-Yves Roignant, and Falk Butter. 2017. “The Developmental Proteome of Drosophila Melanogaster.” *Genome Research*, April. https://doi.org/10.1101/gr.213694.116.

Cazares, Adrian, Matthew P. Moore, James P. J. Hall, Laura L. Wright, Macauley Grimes, Jean-Guillaume Emond-Rhéault, Pisut Pongchaikul, et al. 2020. “A Megaplasmid Family Driving Dissemination of Multidrug Resistance in Pseudomonas.” *Nature Communications* 11 (1): 1370. https://doi.org/10.1038/s41467-020-15081-7.

Chakrabarti, Sveta, and Sandhya S. Visweswariah. 2020. “Intramacrophage ROS Primes the Innate Immune System via JAK/STAT and Toll Activation.” *Cell Reports* 33 (6): 108368. https://doi.org/10.1016/j.celrep.2020.108368.

Chance, B., H. Sies, and A. Boveris. 1979. “Hydroperoxide Metabolism in Mammalian Organs.” *Physiological Reviews* 59 (3): 527–605. https://doi.org/10.1152/physrev.1979.59.3.527.

Chandramouli, Kondethimmanahalli, and Pei-Yuan Qian. 2009. “Proteomics: Challenges, Techniques and Possibilities to Overcome Biological Sample Complexity.” *Human Genomics and Proteomics : HGP* 2009 (December): 239204. https://doi.org/10.4061/2009/239204.

Chen, Fangmin, Benjamin C. Krasity, Suzanne M. Peyer, Sabrina Koehler, Edward G. Ruby, Xiaoping Zhang, and Margaret J. McFall-Ngai. 2017. “Bactericidal Permeability-Increasing Proteins Shape Host-Microbe Interactions.” *MBio* 8 (2): e00040-17. https://doi.org/10.1128/mBio.00040-17.

Chen, Zhongli, Jinsong Guo, Yanxue Jiang, and Ying Shao. 2021. “High Concentration and High Dose of Disinfectants and Antibiotics Used during the COVID-19 Pandemic Threaten Human Health.” *Environmental Sciences Europe* 33 (1): 11. https://doi.org/10.1186/s12302-021-00456-4.

Conrath, Uwe, Corné M. J. Pieterse, and Brigitte Mauch-Mani. 2002. “Priming in Plant–Pathogen Interactions.” *Trends in Plant Science* 7 (5): 210–16. https://doi.org/10.1016/S1360-1385(02)02244-6.

Contreras-Garduño, J., M. C. Rodríguez, M. H. Rodríguez, A. Alvarado-Delgado, and H. Lanz-Mendoza. 2014. “Cost of Immune Priming within Generations: Trade-off between Infection and Reproduction.” *Microbes and Infection* 16 (3): 261–67. https://doi.org/10.1016/j.micinf.2013.11.010.

Cornelis, Pierre, Qing Wei, Simon Andrews, and Tiffany Vinckx. 2011. “Iron Homeostasis and Management of Oxidative Stress Response in Bacteria.” *Metallomics : Integrated Biometal Science* 3 (June): 540–49. https://doi.org/10.1039/c1mt00022e.

Csardi, Maintainer Gabor. 2013. “Package ‘Igraph.’” *Last Accessed* 3 (09): 2013.

Cycoń, Mariusz, Agnieszka Mrozik, and Zofia Piotrowska-Seget. 2019. “Antibiotics in the Soil Environment—Degradation and Their Impact on Microbial Activity and Diversity.” *Frontiers in Microbiology* 10. https://www.frontiersin.org/articles/10.3389/fmicb.2019.00338.

Das, Kaushik, and Aryadeep Roychoudhury. 2014. “Reactive Oxygen Species (ROS) and Response of Antioxidants as ROS-Scavengers during Environmental Stress in Plants.” *Frontiers in Environmental Science* 2. https://www.frontiersin.org/articles/10.3389/fenvs.2014.00053.

Davidsson, Pär, Tarja Kariola, Outi Niemi, and Tapio Palva. 2013. “Pathogenicity of and Plant Immunity to Soft Rot Pectobacteria.” *Frontiers in Plant Science* 4. https://www.frontiersin.org/articles/10.3389/fpls.2013.00191.

Demmig-Adams, B., M. R. Dumlao, M. K. Herzenach, and W. W. Adams. 2008. “Acclimation.” In *Encyclopedia of Ecology*, edited by Sven Erik Jørgensen and Brian D. Fath, 15–23. Oxford: Academic Press. https://doi.org/10.1016/B978-008045405-4.00001-X.

Demple, Bruce, and James Halbrook. 1983. “Inducible Repair of Oxidative DNA Damage in Escherichia Coli.” *Nature* 304 (5925): 466–68. https://doi.org/10.1038/304466a0.

Di Marzo, Noemi, Elisa Chisci, and Roberto Giovannoni. 2018. “The Role of Hydrogen Peroxide in Redox-Dependent Signaling: Homeostatic and Pathological Responses in Mammalian Cells.” *Cells* 7 (10): 156. https://doi.org/10.3390/cells7100156.

Djaman, Ouliana, F. Wayne Outten, and James A. Imlay. 2004. “Repair of Oxidized Iron-Sulfur Clusters in Escherichia Coli*.” *Journal of Biological Chemistry* 279 (43): 44590–99. https://doi.org/10.1074/jbc.M406487200.

Doudna, Jennifer A., and Emmanuelle Charpentier. 2014. “The New Frontier of Genome Engineering with CRISPR-Cas9.” *Science* 346 (6213): 1258096. https://doi.org/10.1126/science.1258096.

Dowds, B. C. 1994. “The Oxidative Stress Response in Bacillus Subtilis.” *FEMS Microbiology Letters* 124 (3): 255–63. https://doi.org/10.1111/j.1574-6968.1994.tb07294.x.

Du Toit, Andrea. 2015. “What CRISPR Memories Are Made Of.” *Nature Reviews Microbiology* 13 (4): 185–185. https://doi.org/10.1038/nrmicro3457.

Dubbs, James M., and Skorn Mongkolsuk. 2012. “Peroxide-Sensing Transcriptional Regulators in Bacteria.” *Journal of Bacteriology* 194 (20): 5495–5503. https://doi.org/10.1128/JB.00304-12.

Duneau, David F., Hannah C. Kondolf, Joo Hyun Im, Gerardo A. Ortiz, Christopher Chow, Michael A. Fox, Ana T. Eugénio, J. Revah, Nicolas Buchon, and Brian P. Lazzaro. 2017. “The Toll Pathway Underlies Host Sexual Dimorphism in Resistance to Both Gram-Negative and Gram-Positive Bacteria in Mated Drosophila.” *BMC Biology* 15 (1): 124. https://doi.org/10.1186/s12915-017-0466-3.

Dupuy, Corinne, Renée Ohayon, Alexander Valent, Marie-Sophie Noël-Hudson, Danielle Dème, and Alain Virion. 1999. “Purification of a Novel Flavoprotein Involved in the Thyroid NADPH Oxidase: CLONING OF THE PORCINE AND HUMAN CDNAs *.” *Journal of Biological Chemistry* 274 (52): 37265–69. https://doi.org/10.1074/jbc.274.52.37265.

Dutta, Devanjali, Adam J. Dobson, Philip L. Houtz, Christine Gläßer, Jonathan Revah, Jerome Korzelius, Parthive H. Patel, Bruce A. Edgar, and Nicolas Buchon. 2015. “Regional Cell-Specific Transcriptome Mapping Reveals Regulatory Complexity in the Adult Drosophila Midgut.” *Cell Reports* 12 (2): 346–58. https://doi.org/10.1016/j.celrep.2015.06.009.

Duursma, Elisa Biancotto, Ofir Levy, and Maintainer Jim Lemon. 2009. “Package ‘Plotrix.’”

Eden-green, S. J., and Eve Billing. 1972. “Fireblight: Occurrence of Bacterial Strands on Various Hosts under Glasshouse Conditions.” *Plant Pathology* 21 (3): 121–23. https://doi.org/10.1111/j.1365-3059.1972.tb01741.x.

Eisenstark, A., M. J. Calcutt, M. Becker-Hapak, and A. Ivanova. 1996. “Role of Escherichia Coli Rpos and Associated Genes in Defense against Oxidative Damage.” *Free Radical Biology and Medicine* 21 (7): 975–93. https://doi.org/10.1016/S0891-5849(96)00154-2.

Ezraty, Benjamin, Alexandra Vergnes, Manuel Banzhaf, Yohann Duverger, Allison Huguenot, Ana Rita Brochado, Shu-Yi Su, et al. 2013. “Fe-S Cluster Biosynthesis Controls Uptake of Aminoglycosides in a ROS-Less Death Pathway.” *Science* 340 (6140): 1583–87. https://doi.org/10.1126/science.1238328.

Fasnacht, Michel, and Norbert Polacek. 2021. “Oxidative Stress in Bacteria and the Central Dogma of Molecular Biology.” *Frontiers in Molecular Biosciences* 8: 671037. https://doi.org/10.3389/fmolb.2021.671037.

Ferrer, Manuel, Tatyana N. Chernikova, Michail M. Yakimov, Peter N. Golyshin, and Kenneth N. Timmis. 2003. “Chaperonins Govern Growth of Escherichia Coli at Low Temperatures.” *Nature Biotechnology* 21 (11): 1267–1267. https://doi.org/10.1038/nbt1103-1266b.

Flores-Cruz, Zomary, and Caitilyn Allen. 2011. “Necessity of OxyR for the Hydrogen Peroxide Stress Response and Full Virulence in Ralstonia Solanacearum ▿.” *Applied and Environmental Microbiology* 77 (18): 6426–32. https://doi.org/10.1128/AEM.05813-11.

Fox, John, and Sanford Weisberg. 2018. *An R Companion to Applied Regression*. Sage publications.

Foyer, Christine H, and Graham Noctor. 2016. “Stress-Triggered Redox Signalling: What’s in PROSpect?” *Plant, Cell & Environment* 39 (5): 951–64. https://doi.org/10.1111/pce.12621.

Frost, Christopher J., Mark C. Mescher, John E. Carlson, and Consuelo M. De Moraes. 2008. “Plant Defense Priming against Herbivores: Getting Ready for a Different Battle.” *Plant Physiology* 146 (3): 818–24. https://doi.org/10.1104/pp.107.113027.

GARDNER, J. M., and C. I.YR 1972 KADO. n.d. “Comparative Base Sequence Homologies of the Deoxyribonucleic Acids of Erwinia Species and Other Enterobacteriaceae.” *International Journal of Systematic and Evolutionary Microbiology* 22 (4): 201–9. https://doi.org/10.1099/00207713-22-4-201.

Gibson, Beth, Daniel J. Wilson, Edward Feil, and Adam Eyre-Walker. 2018. “The Distribution of Bacterial Doubling Times in the Wild.” *Proceedings of the Royal Society B: Biological Sciences* 285 (1880): 20180789. https://doi.org/10.1098/rspb.2018.0789.

Gordesky-Gold, Beth, Natasha Rivers, Osama M. Ahmed, and Paul A.S. Breslin. 2008. “Drosophila Melanogaster Prefers Compounds Perceived Sweet by Humans.” *Chemical Senses* 33 (3): 301–9. https://doi.org/10.1093/chemse/bjm088.

Grant, R. A., D. J. Filman, S. E. Finkel, R. Kolter, and J. M. Hogle. 1998. “The Crystal Structure of Dps, a Ferritin Homolog That Binds and Protects DNA.” *Nature Structural Biology* 5 (4): 294–303. https://doi.org/10.1038/nsb0498-294.

Gundry, Rebekah L., Melanie Y. White, Christopher I. Murray, Lesley A. Kane, Qin Fu, Brian A. Stanley, and Jennifer E. Van Eyk. 2009. “Preparation of Proteins and Peptides for Mass Spectrometry Analysis in a Bottom-up Proteomics Workflow.” *Current Protocols in Molecular Biology* Chapter 10 (October): Unit10.25. https://doi.org/10.1002/0471142727.mb1025s88.

Ha, Eun Mi, Chun Taek Oh, Yun Soo Bae, and Won Jae Lee. 2005. “A Direct Role for Dual Oxidase in Drosophila Gut Immunity.” *Science* 310 (5749): 847–50. https://doi.org/10.1126/SCIENCE.1117311/SUPPL_FILE/HA.SOM.PDF.

Ha, Eun-Mi, Kyung-Ah Lee, You Yeong Seo, Sung-Hee Kim, Jae-Hong Lim, Byung-Ha Oh, Jaesang Kim, and Won-Jae Lee. 2009. “Coordination of Multiple Dual Oxidase–Regulatory Pathways in Responses to Commensal and Infectious Microbes in Drosophila Gut.” *Nature Immunology* 10 (9): 949–57. https://doi.org/10.1038/ni.1765.

Ha, Eun-Mi, Chun-Taek Oh, Ji-Hwan Ryu, Yun-Soo Bae, Sang-Won Kang, In-hwan Jang, Paul T. Brey, and Won-Jae Lee. 2005. “An Antioxidant System Required for Host Protection against Gut Infection in Drosophila.” *Developmental Cell* 8 (1): 125–32. https://doi.org/10.1016/j.devcel.2004.11.007.

Haukioja, E. 1991. “Induction of Defenses in Trees.” *Annual Review of Entomology* 36: 25–42.

Heck, Michelle. 2018. “Insect Transmission of Plant Pathogens: A Systems Biology Perspective.” *MSystems* 3 (2): e00168-17. https://doi.org/10.1128/mSystems.00168-17.

Heo, Sukyeong, Suree Kim, and Dongmin Kang. 2020. “The Role of Hydrogen Peroxide and Peroxiredoxins throughout the Cell Cycle.” *Antioxidants* 9 (4): 280. https://doi.org/10.3390/antiox9040280.

Hilker, Monika, Jens Schwachtje, Margarete Baier, Salma Balazadeh, Isabel Bäurle, Sven Geiselhardt, Dirk K. Hincha, et al. 2016. “Priming and Memory of Stress Responses in Organisms Lacking a Nervous System.” *Biological Reviews* 91 (4): 1118–33. https://doi.org/10.1111/BRV.12215.

Hudry, Bruno, Sanjay Khadayate, and Irene Miguel-Aliaga. 2016. “The Sexual Identity of Adult Intestinal Stem Cells Controls Organ Size and Plasticity.” *Nature* 530 (7590): 344–48. https://doi.org/10.1038/nature16953.

Hulten, Marieke van, Maaike Pelser, L. C. van Loon, Corné M. J. Pieterse, and Jurriaan Ton. 2006. "Costs and Benefits of Priming for Defense in Arabidopsis." *Proceedings of the National Academy of Sciences* 103 (14): 5602–7. https://doi.org/10.1073/pnas.0510213103.

Iatsenko, Igor, Jean-Philippe Boquete, and Bruno Lemaitre. 2018. "Microbiota-Derived Lactate Activates Production of Reactive Oxygen Species by the Intestinal NADPH Oxidase Nox and Shortens Drosophila Lifespan." *Immunity* 49 (5): 929-942.e5. https://doi.org/10.1016/j.immuni.2018.09.017.

Ilari, Andrea, Pierpaolo Ceci, Davide Ferrari, Gian Luigi Rossi, and Emilia Chiancone. 2002. "Iron Incorporation into Escherichia Coli Dps Gives Rise to a Ferritin-like Microcrystalline Core*." *Journal of Biological Chemistry* 277 (40): 37619–23. https://doi.org/10.1074/jbc.M206186200.

Imlay, J A, and S Linn. 1986. "Bimodal Pattern of Killing of DNA-Repair-Defective or Anoxically Grown Escherichia Coli by Hydrogen Peroxide." *Journal of Bacteriology* 166 (2): 519–27. https://doi.org/10.1128/jb.166.2.519-527.1986.

Imlay, James A. 2003. "Pathways of Oxidative Damage." *Annual Review of Microbiology* 57: 395–418. https://doi.org/10.1146/annurev.micro.57.030502.090938.

Imlay, James A. 2008. "Cellular Defenses against Superoxide and Hydrogen Peroxide." *Annual Review of Biochemistry* 77: 755–76. https://doi.org/10.1146/annurev.biochem.77.061606.161055.

Imlay, James A 2013. "The Molecular Mechanisms and Physiological Consequences of Oxidative Stress: Lessons from a Model Bacterium." *Nature Reviews Microbiology 2013 11:7* 11 (7): 443–54. https://doi.org/10.1038/nrmicro3032.

Imlay, James A 2015. "Transcription Factors That Defend Bacteria Against Reactive Oxygen Species." *Annual Review of Microbiology* 69 (1): 93–108. https://doi.org/10.1146/annurev-micro-091014-104322.

Itskov, Pavel M., José-Maria Moreira, Ekaterina Vinnik, Gonçalo Lopes, Steve Safarik, Michael H. Dickinson, and Carlos Ribeiro. 2014. "Automated Monitoring and Quantitative Analysis of Feeding Behaviour in Drosophila." *Nature Communications* 5 (1): 4560. https://doi.org/10.1038/ncomms5560.

Iwasaki, Mayumi, and Jerzy Paszkowski. 2014. "Epigenetic Memory in Plants." *The EMBO Journal* 33 (18): 1987–98. https://doi.org/10.15252/embj.201488883.

Jackman, Simon, Alex Tahk, Achim Zeileis, Christina Maimone, Jim Fearon, Zoe Meers, Maintainer Simon Jackman, and MASS Imports. 2015. "Package 'Pscl.'" *Political Science Computational Laboratory* 18 (04.2017).

Jang, Soojin, and James A Imlay. 2007. "MICROMOLAR INTRACELLULAR HYDROGEN PEROXIDE DISRUPTS METABOLISM BY DAMAGING IRON-SULFUR ENZYMES." *The Journal of Biological Chemistry* 282 (2): 929–37. https://doi.org/10.1074/jbc.M607646200.

Joshi, Manish, and Julien Royet. 2020. "Uridine Catabolism Breaks the Bonds of Commensalism." *Cell Host & Microbe* 27 (3): 312–14. https://doi.org/10.1016/j.chom.2020.02.008.

Kandel, Eric R., Yadin Dudai, and Mark R. Mayford. 2014. "The Molecular and Systems Biology of Memory." *Cell* 157 (1): 163–86. https://doi.org/10.1016/j.cell.2014.03.001.

Kannan, null, and null Jain. 2000. “Oxidative Stress and Apoptosis.” *Pathophysiology: The Official Journal of the International Society for Pathophysiology* 7 (3): 153–63. https://doi.org/10.1016/s0928-4680(00)00053-5.

Karban, Richard. 2008. “Plant Behaviour and Communication.” *Ecology Letters* 11 (7): 727–39. https://doi.org/10.1111/j.1461-0248.2008.01183.x.

Karban,Richard 2011. “The Ecology and Evolution of Induced Resistance against Herbivores.” *Functional Ecology* 25 (2): 339–47. https://doi.org/10.1111/j.1365-2435.2010.01789.x.

Karban, Richard, and Kaori Shiojiri. 2009. “Self-Recognition Affects Plant Communication and Defense.” *Ecology Letters* 12 (6): 502–6. https://doi.org/10.1111/j.1461-0248.2009.01313.x.

Katsuyama, Masato, Kuniharu Matsuno, and Chihiro Yabe-Nishimura. 2012. “Physiological Roles of NOX/NADPH Oxidase, the Superoxide-Generating Enzyme.” *Journal of Clinical Biochemistry and Nutrition* 50 (1): 9–22. https://doi.org/10.3164/jcbn.11-06SR.

Kim, Sung Hee, and Won Jae Lee. 2014. “Role of DUOX in Gut Inflammation: Lessons from Drosophila Model of Gut-Microbiota Interactions.” *Frontiers in Cellular and Infection Microbiology* 3 (JAN): 116. https://doi.org/10.3389/FCIMB.2013.00116/BIBTEX.

Klein, Sabra L., and Katie L. Flanagan. 2016. “Sex Differences in Immune Responses.” *Nature Reviews Immunology* 16 (10): 626–38. https://doi.org/10.1038/nri.2016.90.

Klein, Stanley B. 2015. “What Memory Is.” *WIREs Cognitive Science* 6 (1): 1–38. https://doi.org/10.1002/wcs.1333.

Kloepper, J. W., J. W. Brewer, and M. D. Harrison. 1981. “Insect Transmission OfErwinia Carotovora Var.Carotovora AndErwinia Carotovora Var.Atroseptica to Potato Plants in the Field.” *American Potato Journal* 58 (4): 165–75. https://doi.org/10.1007/BF02854416.

Kopp, Artyom, Ian Duncan, and Sean B. Carroll. 2000. “Genetic Control and Evolution of Sexually Dimorphic Characters in Drosophila.” *Nature* 408 (6812): 553–59. https://doi.org/10.1038/35046017.

Krautz, Robert, Badrul Arefin, and Ulrich Theopold. 2014. “Damage Signals in the Insect Immune Response.” *Frontiers in Plant Science* 5. https://www.frontiersin.org/articles/10.3389/fpls.2014.00342.

Kurtz, Joachim. 2004. “Memory in the Innate and Adaptive Immune Systems.” *Microbes and Infection*, Forum in Immunology: Toll-like receptors, 6 (15): 1410–17. https://doi.org/10.1016/j.micinf.2004.10.002.

Kurtz. 2005. “Specific Memory within Innate Immune Systems.” *Trends in Immunology* 26 (4): 186–92. https://doi.org/10.1016/j.it.2005.02.001.

Kurtz, Joachim, and Karoline Franz. 2003. “Evidence for Memory in Invertebrate Immunity.” *Nature* 425 (6953): 37–38. https://doi.org/10.1038/425037a.

Lambeth, J. David. 2004. “NOX Enzymes and the Biology of Reactive Oxygen.” *Nature Reviews Immunology* 4 (3): 181–89. https://doi.org/10.1038/nri1312.

Lazzaro, Brian P., and Jens Rolff. 2011. “Immunology. Danger, Microbes, and Homeostasis.” *Science (New York, N.Y.)* 332 (6025): 43–44. https://doi.org/10.1126/science.1200486.

Lee, Calvin K., Jaime de Anda, Amy E. Baker, Rachel R. Bennett, Yun Luo, Ernest Y. Lee, Joshua A. Keefe, et al. 2018. “Multigenerational Memory and Adaptive Adhesion in Early Bacterial Biofilm Communities.” *Proceedings of the National Academy of Sciences* 115 (17): 4471–76. https://doi.org/10.1073/pnas.1720071115.

Lee, Kyung-Ah, Boram Kim, Jinhyuk Bhin, Do Hun Kim, Hyejin You, Eun-Kyoung Kim, Sung-Hee Kim, Ji-Hwan Ryu, Daehee Hwang, and Won-Jae Lee. 2015. “Bacterial Uracil Modulates Drosophila DUOX-Dependent Gut Immunity via Hedgehog-Induced Signaling Endosomes.” *Cell Host & Microbe* 17 (2): 191–204. https://doi.org/10.1016/j.chom.2014.12.012.

Lemaitre, Bruno, and Jules Hoffmann. 2007. “The Host Defense of Drosophila Melanogaster.” *Annual Review of Immunology* 25: 697–743. https://doi.org/10.1146/annurev.immunol.25.022106.141615.

Lemaitre, Bruno, Jean-Marc Reichhart, and Jules A. Hoffmann. 1997. “Drosophila Host Defense: Differential Induction of Antimicrobial Peptide Genes after Infection by Various Classes of Microorganisms.” *Proceedings of the National Academy of Sciences* 94 (26): 14614–19. https://doi.org/10.1073/pnas.94.26.14614.

Lesser, Michael P. 2006. “Oxidative Stress in Marine Environments: Biochemistry and Physiological Ecology.” *Annual Review of Physiology* 68: 253–78. https://doi.org/10.1146/annurev.physiol.68.040104.110001.

Leulier, F., A. Rodriguez, R. S. Khush, J. M. Abrams, and B. Lemaitre. 2000. “The Drosophila Caspase Dredd Is Required to Resist Gram-Negative Bacterial Infection.” *EMBO Reports* 1 (4): 353–58. https://doi.org/10.1093/embo-reports/kvd073.

Li, Jingyi Jessica, Peter J. Bickel, and Mark D. Biggin. 2014. “System Wide Analyses Have Underestimated Protein Abundances and the Importance of Transcription in Mammals.” *PeerJ* 2: e270. https://doi.org/10.7717/peerj.270.

Li, Xiangnan, Henrik Topbjerg, Dong Jiang, and Fulai Liu. 2015. “Drought Priming at Vegetative Stage Improves the Antioxidant Capacity and Photosynthesis Performance of Wheat Exposed to a Short-Term Low Temperature Stress at Jointing Stage.” *Plant and Soil*, April. https://doi.org/10.1007/s11104-015-2499-0.

Lineback, Caitlinn B., Carine A. Nkemngong, Sophie Tongyu Wu, Xiaobao Li, Peter J. Teska, and Haley F. Oliver. 2018. “Hydrogen Peroxide and Sodium Hypochlorite Disinfectants Are More Effective against Staphylococcus Aureus and Pseudomonas Aeruginosa Biofilms than Quaternary Ammonium Compounds.” *Antimicrobial Resistance & Infection Control* 7 (1): 154. https://doi.org/10.1186/s13756-018-0447-5.

Liu, Yuanyuan, Sarah C. Bauer, and James A. Imlay. 2011. “The YaaA Protein of the Escherichia Coli OxyR Regulon Lessens Hydrogen Peroxide Toxicity by Diminishing the Amount of Intracellular Unincorporated Iron.” *Journal of Bacteriology* 193 (9): 2186–96. https://doi.org/10.1128/JB.00001-11.

Loewen, P. C., and R. Hengge-Aronis. 1994. “The Role of the Sigma Factor Sigma S (KatF) in Bacterial Global Regulation.” *Annual Review of Microbiology* 48: 53–80. https://doi.org/10.1146/annurev.mi.48.100194.000413.

Martins, Nelson E., Vitor G. Faria, Luis Teixeira, Sara Magalhães, and Élio Sucena. 2013. “Host Adaptation Is Contingent upon the Infection Route Taken by Pathogens.” *PLOS Pathogens* 9 (9): e1003601. https://doi.org/10.1371/journal.ppat.1003601.

Mathieson, Toby, Holger Franken, Jan Kosinski, Nils Kurzawa, Nico Zinn, Gavain Sweetman, Daniel Poeckel, et al. 2018. “Systematic Analysis of Protein Turnover in Primary Cells.” *Nature Communications* 9 (1): 689. https://doi.org/10.1038/s41467-018-03106-1.

McDonnell, Gerald. 2014. “The Use of Hydrogen Peroxide for Disinfection and Sterilization Applications.” In *PATAI’S Chemistry of Functional Groups*, 1–34. John Wiley & Sons, Ltd. https://doi.org/10.1002/9780470682531.pat0885.

McEvoy, James L., Kerry K. Thurn, and Arun K. Chatterjee. 1987. “Expression of the E. Coli LexA+ Gene in Erwinia Carotovora Subsp. Carotovora and Its Effect on Production of Pectin Lyase and Carotovoricin*.” *FEMS Microbiology Letters* 42 (2–3): 205–8. https://doi.org/10.1111/j.1574-6968.1987.tb02073.x.

Merrell, D. Scott, David L. Hava, and Andrew Camilli. 2002. “Identification of Novel Factors Involved in Colonization and Acid Tolerance of Vibrio Cholerae.” *Molecular Microbiology* 43 (6): 1471–91. https://doi.org/10.1046/j.1365-2958.2002.02857.x.

Meslé, Margaux M., Jacob P. Beam, Zackary J. Jay, Brynna Bodle, Eric Bogenschutz, and William P. Inskeep. 2017. “Hydrogen Peroxide Cycling in High-Temperature Acidic Geothermal Springs and Potential Implications for Oxidative Stress Response.” *Frontiers in Marine Science* 4. https://www.frontiersin.org/articles/10.3389/fmars.2017.00130.

Mi, Huaiyu, Anushya Muruganujan, John T. Casagrande, and Paul D. Thomas. 2013. “Large-Scale Gene Function Analysis with the PANTHER Classification System.” *Nature Protocols* 8 (8): 1551–66. https://doi.org/10.1038/nprot.2013.092.

Milutinović, Barbara, and Joachim Kurtz. 2016. “Immune Memory in Invertebrates.” *Seminars in Immunology* 28 (4): 328–42. https://doi.org/10.1016/j.smim.2016.05.004.

Mishra, Surabhi, and James Imlay. 2012. “Why Do Bacteria Use so Many Enzymes to Scavenge Hydrogen Peroxide?” *Archives of Biochemistry and Biophysics* 525 (2): 145–60. https://doi.org/10.1016/j.abb.2012.04.014.

Miyaue, Saki, Erika Suzuki, Yoko Komiyama, Yu Kondo, Miki Morikawa, and Sumio Maeda. 2018. “Bacterial Memory of Persisters: Bacterial Persister Cells Can Retain Their Phenotype for Days or Weeks after Withdrawal from Colony-Biofilm Culture.” *Frontiers in Microbiology* 9 (JUN): 1396. https://doi.org/10.3389/FMICB.2018.01396/BIBTEX.

Moghadam, Zohreh Mansoori, Philipp Henneke, and Julia Kolter. 2021. “From Flies to Men: ROS and the NADPH Oxidase in Phagocytes.” *Frontiers in Cell and Developmental Biology* 9. https://www.frontiersin.org/articles/10.3389/fcell.2021.628991.

Molina, J. J., M. D. Harrison, and J. W. Brewer. 1974. “Transmission OfErwinia Carotovora Var.Atroseptica ByDrosophila Melanogaster Meig. I. Acquisition and Transmission of the Bacterium.” *American Potato Journal* 51 (8): 245–50. https://doi.org/10.1007/BF02851435.

Moreira, Severina, Brian Stramer, Iwan Evans, Will Wood, and Paul Martin. 2010. “Prioritization of Competing Damage and Developmental Signals by Migrating Macrophages in the Drosophila Embryo.” *Current Biology* 20 (5): 464–70. https://doi.org/10.1016/j.cub.2010.01.047.

Mukherjee, Asita, Yaya Cui, Weilei Ma, Yang Liu, Akira Ishihama, Abraham Eisenstark, and Arun K. Chatterjee. 1998. “RpoS (Sigma-S) Controls Expression of RsmA, a Global Regulator of Secondary Metabolites, Harpin, and Extracellular Proteins in Erwinia Carotovora.” *Journal of Bacteriology* 180 (14): 3629–34.

Muniz, Carlos Acosta, Danielle Jaillard, Bruno Lemaitre, and Frédéric Boccard. 2007. “Erwinia Carotovora Evf Antagonizes the Elimination of Bacteria in the Gut of Drosophila Larvae.” *Cellular Microbiology* 9 (1): 106–19. https://doi.org/10.1111/j.1462-5822.2006.00771.x.

Murdoch, L.e., L. Bailey, E. Banham, F. Watson, N.m.t. Adams, and J. Chewins. 2016. “Evaluating Different Concentrations of Hydrogen Peroxide in an Automated Room Disinfection System.” *Letters in Applied Microbiology* 63 (3): 178–82. https://doi.org/10.1111/lam.12607.

Myers, Amber L., Caitlin M. Harris, Kwang-Min Choe, and Catherine A. Brennan. 2018. “Inflammatory Production of Reactive Oxygen Species by Drosophila Hemocytes Activates Cellular Immune Defenses.” *Biochemical and Biophysical Research Communications* 505 (3): 726–32. https://doi.org/10.1016/j.bbrc.2018.09.126.

Nam, Hyuck-Jin, In-Hwan Jang, Hyejin You, Kyung-Ah Lee, and Won-Jae Lee. 2012. “Genetic Evidence of a Redox-Dependent Systemic Wound Response via Hayan Protease-Phenoloxidase System in Drosophila.” *The EMBO Journal* 31 (5): 1253–65. https://doi.org/10.1038/emboj.2011.476.

Neuwirth, Erich, and Maintainer Erich Neuwirth. 2011. “Package ‘RColorBrewer.’” CRAN 2011-06-17 08: 34: 00. Apache License 2.0.

Nguyen, Hung Manh, Mikael Kim, Peter J. Ralph, Lázaro Marín-Guirao, Mathieu Pernice, and Gabriele Procaccini. 2020. “Stress Memory in Seagrasses: First Insight Into the Effects of Thermal Priming and the Role of Epigenetic Modifications.” *Frontiers in Plant Science* 11. https://www.frontiersin.org/articles/10.3389/fpls.2020.00494.

Nguyen, Jen, Juanita Lara-Gutiérrez, and Roman Stocker. 2021. “Environmental Fluctuations and Their Effects on Microbial Communities, Populations and Individuals.” *FEMS Microbiology Reviews* 45 (4): fuaa068. https://doi.org/10.1093/femsre/fuaa068.

Noor, Rashed, Zahidul Islam, Saurab Munshi, and Farjana Rahman. 2013. “Influence of Temperature on Escherichia Coli Growth in Different Culture Media.” *Journal of Pure and Applied Microbiology* 7 (June).

Nykänen, Heli, and Julia Koricheva. 2004. “Damage-Induced Changes in Woody Plants and Their Effects on Insect Herbivore Performance: A Meta-Analysis.” *Oikos* 104 (2): 247–68. https://doi.org/10.1111/j.0030-1299.2004.12768.x.

Nystrand, M., and D. K. Dowling. 2020. “Effects of Immune Challenge on Expression of Life-History and Immune Trait Expression in Sexually Reproducing Metazoans—a Meta-Analysis.” *BMC Biology* 18 (1): 135. https://doi.org/10.1186/s12915-020-00856-7.

Okin, Daniel, and Ruslan Medzhitov. 2012. “Evolution of Inflammatory Diseases.” *Current Biology: CB* 22 (17): R733-740. https://doi.org/10.1016/j.cub.2012.07.029.

Park, Sunny, Xiaojun You, and James A. Imlay. 2005. “Substantial DNA Damage from Submicromolar Intracellular Hydrogen Peroxide Detected in Hpx- Mutants of Escherichia Coli.” *Proceedings of the National Academy of Sciences of the United States of America* 102 (26): 9317–22. https://doi.org/10.1073/pnas.0502051102.

Paszkowski, Jerzy, and Ueli Grossniklaus. 2011. “Selected Aspects of Transgenerational Epigenetic Inheritance and Resetting in Plants.” *Current Opinion in Plant Biology* 14 (2): 195–203. https://doi.org/10.1016/j.pbi.2011.01.002.

Paust, Silke, Balimkiz Senman, and Ulrich H. von Andrian. 2010. “Adaptive Immune Responses Mediated by Natural Killer Cells.” *Immunological Reviews* 235 (1): 286–96. https://doi.org/10.1111/j.0105-2896.2010.00906.x.

Pecinka, Ales, and Ortrun Mittelsten Scheid. 2012. “Stress-Induced Chromatin Changes: A Critical View on Their Heritability.” *Plant and Cell Physiology* 53 (5): 801–8. https://doi.org/10.1093/pcp/pcs044.

Perilla-Henao, Laura M., and Clare L. Casteel. 2016. “Vector-Borne Bacterial Plant Pathogens: Interactions with Hemipteran Insects and Plants.” *Frontiers in Plant Science* 7. https://www.frontiersin.org/articles/10.3389/fpls.2016.01163.

Perombelon, M C M, and A Kelman. 1980. “Ecology of the Soft Rot Erwinias.” *Annual Review of Phytopathology* 18 (1): 361–87. https://doi.org/10.1146/annurev.py.18.090180.002045.

Pham, Linh N., Marc S. Dionne, Mimi Shirasu-Hiza, and David S. Schneider. 2007. “A Specific Primed Immune Response in Drosophila Is Dependent on Phagocytes.” *PLOS Pathogens* 3 (3): e26. https://doi.org/10.1371/journal.ppat.0030026.

Pradeu, Thomas, and Louis Pasquier. 2018. “Immunological Memory: What’s in a Name?” *Immunological Reviews* 283 (May): 7–20. https://doi.org/10.1111/imr.12652.

Prahlad, Janani, Yifeng Yuan, Jiusheng Lin, Chou-Wei Chang, Dirk Iwata-Reuyl, Yilun Liu, Valérie de Crécy-Lagard, and Mark A. Wilson. 2020. “The DUF328 Family Member YaaA Is a DNA-Binding Protein with a Novel Fold.” *The Journal of Biological Chemistry* 295 (41): 14236–47. https://doi.org/10.1074/jbc.RA120.015055.

Quevillon-Cheruel, Sophie, Nicolas Leulliot, Carlos Acosta Muniz, Michel Vincent, Jacques Gallay, Manuela Argentini, David Cornu, Frédéric Boccard, Bruno Lemaître, and Herman van Tilbeurgh. 2009. “Evf, a Virulence Factor Produced by the Drosophila Pathogen Erwinia Carotovora, Is an S-Palmitoylated Protein with a New Fold That Binds to Lipid Vesicles *.” *Journal of Biological Chemistry* 284 (6): 3552–62. https://doi.org/10.1074/jbc.M808334200.

Razzell, William, Iwan Robert Evans, Paul Martin, and Will Wood. 2013. “Calcium Flashes Orchestrate the Wound Inflammatory Response through DUOX Activation and Hydrogen Peroxide Release.” *Current Biology* 23 (5): 424–29. https://doi.org/10.1016/j.cub.2013.01.058.

Reczek, Colleen R., and Navdeep S. Chandel. 2015. “ROS-Dependent Signal Transduction.” *Current Opinion in Cell Biology* 33 (April): 8–13. https://doi.org/10.1016/j.ceb.2014.09.010.

Regan, Jennifer C, Mobina Khericha, Adam J Dobson, Ekin Bolukbasi, Nattaphong Rattanavirotkul, and Linda Partridge. 2016. “Sex Difference in Pathology of the Ageing Gut Mediates the Greater Response of Female Lifespan to Dietary Restriction.” Edited by Andrew Dillin. *ELife* 5 (February): e10956. https://doi.org/10.7554/eLife.10956.

Reniere, Michelle L. 2018. “Reduce, Induce, Thrive: Bacterial Redox Sensing during Pathogenesis.” *Journal of Bacteriology* 200 (17): e00128-18. https://doi.org/10.1128/JB.00128-18.

Rillig, Matthias C., Jens Rolff, Britta Tietjen, Jeannine Wehner, and Diana R. Andrade-Linares. 2015. “Community Priming—Effects of Sequential Stressors on Microbial Assemblages.” *FEMS Microbiology Ecology* 91 (5): fiv040. https://doi.org/10.1093/femsec/fiv040.

Rodríguez-Rojas, Alexandro, Desiree Y. Baeder, Paul Johnston, Roland R. Regoes, and Jens Rolff. 2021. “Bacteria Primed by Antimicrobial Peptides Develop Tolerance and Persist.” *PLOS Pathogens* 17 (3): e1009443. https://doi.org/10.1371/journal.ppat.1009443.

Rodríguez-Rojas, Alexandro, Joshua Jay Kim, Paul R. Johnston, Olga Makarova, Murat Eravci, Christoph Weise, Regine Hengge, and Jens Rolff. 2020. “Non-Lethal Exposure to H2O2 Boosts Bacterial Survival and Evolvability against Oxidative Stress.” *PLoS Genetics* 16 (3). https://doi.org/10.1371/JOURNAL.PGEN.1008649.

Ronin, Irine, Naama Katsowich, Ilan Rosenshine, and Nathalie Q Balaban. 2017. “A Long-Term Epigenetic Memory Switch Controls Bacterial Virulence Bimodality.” Edited by Michael Laub. *ELife* 6 (February): e19599. https://doi.org/10.7554/eLife.19599.

Sadd, Ben M., and Paul Schmid-Hempel. 2009. “A Distinct Infection Cost Associated with Trans-Generational Priming of Antibacterial Immunity in Bumble-Bees.” *Biology Letters* 5 (6): 798–801. https://doi.org/10.1098/rsbl.2009.0458.

Santander, Ricardo D., Mercedes Monte-Serrano, José J. Rodríguez-Herva, Emilia López-Solanilla, Pablo Rodríguez-Palenzuela, and Elena G. Biosca. 2014. “Exploring New Roles for the RpoS Gene in the Survival and Virulence of the Fire Blight Pathogen Erwinia Amylovora.” *FEMS Microbiology Ecology* 90 (3): 895–907. https://doi.org/10.1111/1574-6941.12444.

Schellhorn, Herb E. 2020. “Function, Evolution, and Composition of the RpoS Regulon in Escherichia Coli.” *Frontiers in Microbiology* 11. https://www.frontiersin.org/articles/10.3389/fmicb.2020.560099.

Sewell, D., B. Burnet, and K. Connolly. 1974. “Genetic Analysis of Larval Feeding Behaviour in Drosophila Melanogaster.” *Genetical Research* 24 (2): 163–73. https://doi.org/10.1017/s0016672300015196.

Shaka, Maria, Aranzazu Arias-Rojas, Alexandra Hrdina, Dagmar Frahm, and Igor Iatsenko. 2022. “Lipopolysaccharide -Mediated Resistance to Host Antimicrobial Peptides and Hemocyte-Derived Reactive-Oxygen Species Are the Major Providencia Alcalifaciens Virulence Factors in Drosophila Melanogaster.” *PLOS Pathogens* 18 (9): e1010825. https://doi.org/10.1371/journal.ppat.1010825.

Sharma, Iti, and Parvaiz Ahmad. 2014. “Chapter 4 - Catalase: A Versatile Antioxidant in Plants.” In *Oxidative Damage to Plants*, edited by Parvaiz Ahmad, 131–48. San Diego: Academic Press. https://doi.org/10.1016/B978-0-12-799963-0.00004-6.

Sheehan, Gerard, Gemma Farrell, and Kevin Kavanagh. 2020. “Immune Priming: The Secret Weapon of the Insect World.” *Virulence* 11 (1): 238–46. https://doi.org/10.1080/21505594.2020.1731137.

Shields, Hazel J., Annika Traa, and Jeremy M. Van Raamsdonk. 2021. “Beneficial and Detrimental Effects of Reactive Oxygen Species on Lifespan: A Comprehensive Review of Comparative and Experimental Studies.” *Frontiers in Cell and Developmental Biology* 9. https://www.frontiersin.org/articles/10.3389/fcell.2021.628157.

Siva-Jothy, Jonathon A., Arun Prakash, Radhakrishnan B. Vasanthakrishnan, Katy M. Monteith, and Pedro F. Vale. 2018. “Oral Bacterial Infection and Shedding in Drosophila melanogaster.” *JoVE (Journal of Visualized Experiments)*, no. 135 (May): e57676. https://doi.org/10.3791/57676.

Slack, Suzanne M., Quan Zeng, Cory A. Outwater, and George W. Sundin. 2017. “Microbiological Examination of Erwinia Amylovora Exopolysaccharide Ooze.” *Phytopathology®* 107 (4): 403–11. https://doi.org/10.1094/PHYTO-09-16-0352-R.

Smith, Daniel F. Q., and Arturo Casadevall. 2022. “Disaster Microbiology—a New Field of Study.” *MBio* 0 (0): e01680-22. https://doi.org/10.1128/mbio.01680-22.

Sprouffske, Kathleen, and Andreas Wagner. 2016. “Growthcurver: An R Package for Obtaining Interpretable Metrics from Microbial Growth Curves.” *BMC Bioinformatics* 17 (April): 172. https://doi.org/10.1186/s12859-016-1016-7.

Stiban, Johnny, Minyoung So, and Laurie S. Kaguni. 2016. “Iron-Sulfur Clusters in Mitochondrial Metabolism: Multifaceted Roles of a Simple Cofactor.” *Biochemistry. Biokhimiia* 81 (10): 1066–80. https://doi.org/10.1134/S0006297916100059.

Storz, G., L. A. Tartaglia, S. B. Farr, and B. N. Ames. 1990. “Bacterial Defenses against Oxidative Stress.” *Trends in Genetics: TIG* 6 (11): 363–68. https://doi.org/10.1016/0168-9525(90)90278-e.

Sultan, Sonia. 2001. “Phenotypic Plasticity for Plant Development, Function and Life History.” *Trends in Plant Science* 5 (January): 537–42. https://doi.org/10.1016/S1360-1385(00)01797-0.

Sweatt, J. David. 2016. “Neural Plasticity and Behavior – Sixty Years of Conceptual Advances.” *Journal of Neurochemistry* 139 (S2): 179–99. https://doi.org/10.1111/jnc.13580.

Tagkopoulos, Ilias, Yir-Chung Liu, and Saeed Tavazoie. 2008. “Predictive Behavior within Microbial Genetic Networks.” *Science (New York, N.Y.)* 320 (5881): 1313–17. https://doi.org/10.1126/science.1154456.

TAN, KAR-CHUN, SIMON V. S. IPCHO, ROBERT D. TRENGOVE, RICHARD P. OLIVER, and PETER S. SOLOMON. 2009. “Assessing the Impact of Transcriptomics, Proteomics and Metabolomics on Fungal Phytopathology.” *Molecular Plant Pathology* 10 (5): 703–15. https://doi.org/10.1111/j.1364-3703.2009.00565.x.

Tang, Huaping. 2009. “Regulation and Function of the Melanization Reaction in Drosophila.” *Fly* 3 (1): 105–11. https://doi.org/10.4161/fly.3.1.7747.

Teder, Tiit, and Toomas Tammaru. 2005. “Sexual Size Dimorphism within Species Increases with Body Size in Insects.” *Oikos* 108 (2): 321–34. https://doi.org/10.1111/j.0030-1299.2005.13609.x.

Tetreau, Guillaume, Julien Dhinaut, Benjamin Gourbal, and Yannick Moret. 2019. “Trans-Generational Immune Priming in Invertebrates: Current Knowledge and Future Prospects.” *Frontiers in Immunology* 10 (August): 1938. https://doi.org/10.3389/fimmu.2019.01938.

Thannickal, V. J., and B. L. Fanburg. 2000. “Reactive Oxygen Species in Cell Signaling.” *American Journal of Physiology. Lung Cellular and Molecular Physiology* 279 (6): L1005-1028. https://doi.org/10.1152/ajplung.2000.279.6.L1005.

Thellier, M., and U. Lüttge. 2013. “Plant Memory: A Tentative Model.” *Plant Biology* 15 (1): 1–12. https://doi.org/10.1111/j.1438-8677.2012.00674.x.

Therneau, Terry M, and Thomas Lumley. 2014. “Package ‘Survival.’” *Survival Analysis Published on CRAN* 2: 3.

Thorpe, Geoffrey W., Chii S. Fong, Nazif Alic, Vincent J. Higgins, and Ian W. Dawes. 2004. “Cells Have Distinct Mechanisms to Maintain Protection against Different Reactive Oxygen Species: Oxidative-Stress-Response Genes.” *Proceedings of the National Academy of Sciences of the United States of America* 101 (17): 6564–69. https://doi.org/10.1073/pnas.0305888101.

Timp, Winston, and Gregory Timp. 2020. “Beyond Mass Spectrometry, the next Step in Proteomics.” *Science Advances* 6 (2): eaax8978. https://doi.org/10.1126/sciadv.aax8978.

Tong, Chaoyu, Hong Hu, Gang Chen, Zhengyan Li, Aifeng Li, and Jianye Zhang. 2021. “Disinfectant Resistance in Bacteria: Mechanisms, Spread, and Resolution Strategies.” *Environmental Research* 195 (April): 110897. https://doi.org/10.1016/j.envres.2021.110897.

Torres, Miguel Angel, Jonathan D.G. Jones, and Jeffery L. Dangl. 2006. “Reactive Oxygen Species Signaling in Response to Pathogens.” *Plant Physiology* 141 (2): 373–78. https://doi.org/10.1104/pp.106.079467.

Troha, Katia, and Nicolas Buchon. 2019. “Methods for the Study of Innate Immunity in Drosophila Melanogaster.” *WIREs Developmental Biology* 8 (5): e344. https://doi.org/10.1002/wdev.344.

Tyanova, Stefka, Tikira Temu, Pavel Sinitcyn, Arthur Carlson, Marco Y. Hein, Tamar Geiger, Matthias Mann, and Jürgen Cox. 2016. “The Perseus Computational Platform for Comprehensive Analysis of (Prote)Omics Data.” *Nature Methods* 13 (9): 731–40. https://doi.org/10.1038/nmeth.3901.

Úbeda, Francisco, and Vincent A. A. Jansen. 2016. “The Evolution of Sex-Specific Virulence in Infectious Diseases.” *Nature Communications* 7 (1): 13849. https://doi.org/10.1038/ncomms13849.

Uhl, Lionel, Audrey Gerstel, Maialène Chabalier, and Sam Dukan. 2015. “Hydrogen Peroxide Induced Cell Death: One or Two Modes of Action?” *Heliyon* 1 (4): e00049. https://doi.org/10.1016/j.heliyon.2015.e00049.

Veening, Jan-Willem, Wiep Klaas Smits, and Oscar P. Kuipers. 2008. “Bistability, Epigenetics, and Bet-Hedging in Bacteria.” *Annual Review of Microbiology* 62: 193–210. https://doi.org/10.1146/annurev.micro.62.081307.163002.

Venisse, Jean-Stéphane, Gabor Gullner, and Marie-Noëlle Brisset. 2001. “Evidence for the Involvement of an Oxidative Stress in the Initiation of Infection of Pear by Erwinia Amylovora 1.” *Plant Physiology* 125 (4): 2164–72. https://doi.org/10.1104/pp.125.4.2164.

Vermilyea, Andrew W., Taylor C. Dixon, and Bettina M. Voelker. 2010. “Use of H218O2 To Measure Absolute Rates of Dark H2O2 Production in Freshwater Systems.” *Environmental Science & Technology* 44 (8): 3066–72. https://doi.org/10.1021/es100209h.

Vieira, Filipe J. D., Pol Nadal-Jimenez, Luis Teixeira, and Karina B. Xavier. 2020. “Erwinia Carotovora Quorum Sensing System Regulates Host-Specific Virulence Factors and Development Delay in Drosophila Melanogaster.” *MBio* 11 (3): e01292-20. https://doi.org/10.1128/mBio.01292-20.

Vodovar, Nicolas, Marisa Vinals, Peter Liehl, Alan Basset, Jeril Degrouard, Paul Spellman, Frédéric Boccard, and Bruno Lemaitre. 2005. “Drosophila Host Defense after Oral Infection by an Entomopathogenic Pseudomonas Species.” *Proceedings of the National Academy of Sciences of the United States of America* 102 (32): 11414–19. https://doi.org/10.1073/pnas.0502240102.

Warnes, Maintainer Gregory R, Ben Bolker, Lodewijk Bonebakker, Robert Gentleman, and Wolfgang Huber. 2016. “Package ‘Gplots.’” *Various R Programming Tools for Plotting Data*.

Wensing, Kristina U., Mareike Koppik, and Claudia Fricke. 2017. “Precopulatory but Not Postcopulatory Male Reproductive Traits Diverge in Response to Mating System Manipulation in *Drosophila Melanogaster*.” *Ecology and Evolution* 7 (23): 10361–78. https://doi.org/10.1002/ece3.3542.

Wickham, Hadley. 2011. “The Split-Apply-Combine Strategy for Data Analysis.” *Journal of Statistical Software* 40 (April): 1–29. https://doi.org/10.18637/jss.v040.i01.

Wickham 2016. *Ggplot2: Elegant Graphics for Data Analysis*. springer.

Wickham, Hadley, Mara Averick, Jennifer Bryan, Winston Chang, Lucy D’Agostino McGowan, Romain François, Garrett Grolemund, et al. 2019. “Welcome to the Tidyverse.” *Journal of Open Source Software* 4 (43): 1686. https://doi.org/10.21105/joss.01686.

Wickham, Hadley, Romain François, Lionel Henry, Kirill Müller, and RStudio. 2022. “Dplyr: A Grammar of Data Manipulation.” https://CRAN.R-project.org/package=dplyr.

Wilson, Kenneth, David Grzywacz, Jenny S. Cory, Philip Donkersley, and Robert I. Graham. 2021. “Trans-Generational Viral Transmission and Immune Priming Are Dose-Dependent.” *Journal of Animal Ecology* 90 (6): 1560–69. https://doi.org/10.1111/1365-2656.13476.

Yang, Chih-Yu, Maja Bialecka-Fornal, Colleen Weatherwax, Joseph W. Larkin, Arthur Prindle, Jintao Liu, Jordi Garcia-Ojalvo, and Gürol M. Süel. 2020. “Encoding Membrane-Potential-Based Memory within a Microbial Community.” *Cell Systems* 10 (5): 417-423.e3. https://doi.org/10.1016/j.cels.2020.04.002.

Yip, Eric C., John F. Tooker, Mark C. Mescher, and Consuelo M. De Moraes. 2019. “Costs of Plant Defense Priming: Exposure to Volatile Cues from a Specialist Herbivore Increases Short-Term Growth but Reduces Rhizome Production in Tall Goldenrod (Solidago Altissima).” *BMC Plant Biology* 19 (1): 209. https://doi.org/10.1186/s12870-019-1820-0.

Yoo, Sa Kan, Taylor W. Starnes, Qing Deng, and Anna Huttenlocher. 2011. “Lyn Is a Redox Sensor That Mediates Leukocyte Wound Attraction in Vivo.” *Nature* 480 (7375): 109–12. https://doi.org/10.1038/nature10632.

Yuan, Jinchun, and Alan M Shiller. 2001. “The Distribution of Hydrogen Peroxide in the Southern and Central Atlantic Ocean.” *Deep Sea Research Part II: Topical Studies in Oceanography*, Trace Chemical Species in the Water Column and Atmosphere of the Atlantic Ocean, 48 (13): 2947–70. https://doi.org/10.1016/S0967-0645(01)00026-1.

Zaidman-Rémy, Anna, Mireille Hervé, Mickael Poidevin, Sébastien Pili-Floury, Min-Sung Kim, Didier Blanot, Byung-Ha Oh, Ryu Ueda, Dominique Mengin-Lecreulx, and Bruno Lemaitre. 2006. “The Drosophila Amidase PGRP-LB Modulates the Immune Response to Bacterial Infection.” *Immunity* 24 (4): 463–73. https://doi.org/10.1016/j.immuni.2006.02.012.

Zangerl, Arthur R. 2003. “Evolution of Induced Plant Responses to Herbivores.” *Basic and Applied Ecology* 4 (1): 91–103. https://doi.org/10.1078/1439-1791-00135.

Zaslaver, Alon, Anat Bren, Michal Ronen, Shalev Itzkovitz, Ilya Kikoin, Seagull Shavit, Wolfram Liebermeister, Michael G. Surette, and Uri Alon. 2006. “A Comprehensive Library of Fluorescent Transcriptional Reporters for Escherichia Coli.” *Nature Methods* 3 (8): 623–28. https://doi.org/10.1038/nmeth895.

Zhang, Jixiang, Xiaoli Wang, Vikash Vikash, Qing Ye, Dandan Wu, Yulan Liu, and Weiguo Dong. 2016. “ROS and ROS-Mediated Cellular Signaling.” *Oxidative Medicine and Cellular Longevity* 2016: 4350965. https://doi.org/10.1155/2016/4350965.

Zhang, Yaoyang, Bryan R. Fonslow, Bing Shan, Moon-Chang Baek, and John R. Yates. 2013. “Protein Analysis by Shotgun/Bottom-up Proteomics.” *Chemical Reviews* 113 (4): 2343–94. https://doi.org/10.1021/cr3003533.

Zheng, Ming, Xunde Wang, Lori J. Templeton, Dana R. Smulski, Robert A. LaRossa, and Gisela Storz. 2001. “DNA Microarray-Mediated Transcriptional Profiling of the Escherichia Coli Response to Hydrogen Peroxide.” *Journal of Bacteriology* 183 (15): 4562–70. https://doi.org/10.1128/JB.183.15.4562-4570.2001.

Zhou, Yanning, and Susan Gottesman. 1998. “Regulation of Proteolysis of the Stationary-Phase Sigma Factor RpoS.” *Journal of Bacteriology* 180 (5): 1154–58.

Zubko, Elena I., and Mikhajlo K. Zubko. 2013. “Co-Operative Inhibitory Effects of Hydrogen Peroxide and Iodine against Bacterial and Yeast Species.” *BMC Research Notes* 6 (1): 272. https://doi.org/10.1186/1756-0500-6-272.