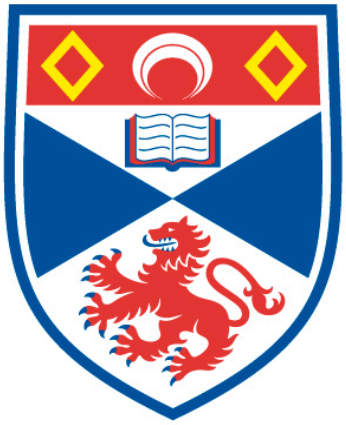
ESTABLISHING GALLERIA MELLONELLA, THE LARVAE OF THE GREATER WAX MOTH, AS A MODEL FOR MYCOBACTERIAL INFECTIONS TO ASSESS EXISTING AND NOVEL TREATMENTS

Frances der Weduwen

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



2020

Establishing *Galleria mellonella*, the larvae of the greater wax moth, as a model for Mycobacterial infections to assess existing and novel treatments

Frances der Weduwen

University of
St Andrews

This thesis is submitted in partial fulfilment for the degree of

Doctor of Philosophy (PhD)

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December 2019

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I, Frances der Weduwen, do hereby certify that this thesis, submitted for the degree of PhD, which is approximately 79,000 words in length, has been written by me, and that it is the record of work carried out by me, or principally by myself in collaboration with others as acknowledged, and that it has not been submitted in any previous application for any degree.

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Abstract

Galleria mellonella, the larvae of the greater wax moth, are cheap, practical and readily-available organisms which can be utilised for a comprehensive range of experimental protocols. They are valuable *in vivo* intermediaries between *in vitro* studies and models which require greater investments of time, equipment, and ethical approval. Larvae exhibit cellular and humoral immune responses with structural and functional similarities to mammals. They are ideal for high-throughput screens of pathogens, antimicrobials, toxicity tests, and novel treatments.

In this thesis, *G. mellonella* was used with a range of *Mycobacterium* species and strains. Mycobacteria represent a unique challenge as they are intrinsically resistant to antibiotics, "hide" from immune responses, and interact directly with immune cells. *Mycobacterium tuberculosis*, the causative agent in tuberculosis, has infected billions of humans and is notoriously difficult to study due to its slow generation time and ability to infect immunocompetent humans. Hence, using related Mycobacterial species allows assessment of this genus with minimal risk and inconvenience. Using these bacteria in tandem with a simple, easily used *in vivo* model allows for a greater range of experiments in a standard laboratory.

When this thesis commenced, Mycobacteria had not been used with a *Galleria* model in the modern era, Therefore, *M. fortuitum, M. marinum* and *M. aurum* were assessed with *G. mellonella* and confirmed to cause larval death. Various anti-Mycobacterials were assessed, alongside uncommon treatment combinations such as β

lactams and efflux pump inhibitors. The immune response to infection and treatment was evaluated – the number of immune cells, their ability to phagocytose Mycobacteria, and the formation of melanised nodules were all affected by infection and the antibiotics used to treat them.

This work demonstrates the suitability of *G. mellonella* as a model for Mycobacterial infections and indicates how they may be used in further research efforts.

Contents

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Abbreviations

°C – degrees Centigrade

µg – microgram

µL – microlitre

µm – micrometre

ADC – albumin dextrose catalase [media supplement]

AIDS – acquired immunodeficiency syndrome

AMK – amikacin

AMP – antimicrobial peptide

AMX – amoxicillin

ATP – adenosine triphosphate

BCG – Bacillus Calmette-Guérin

BDQ – bedaquiline

BMI – body mass index

c.f.u – colony forming units

CAP – capreomycin

CDC – Centre for Disease Control

CEC – cefaclor

CEF – cephalothin

CIP – ciprofloxacin

CPZ – chlorpromazine

DEX – dexamethasone

DNA – deoxyribonucleic acid

dO_2 – dissolved O_2

ELISA – enzyme linked immunosorbent assay

EMB – ethambutol

EPI – efflux pump inhibitor

FITC – fluorescein isothiocyanate

FLU – flupenthixol

g – gram

g – relative centrifugal force

GI – gastrointestinal tract

h – hour/hours

HEPES – hydroxyethylpiperazineethanesulphonic acid

HIV - human immunodeficiency virus

INH – isoniazid

KAN – kanamycin

kg – kilogram

L – litre

LEV – levofloxacin

LPS – lipopolysaccharide

MDR-TB – multi-drug resistant tuberculosis

mg – milligram

MIC – minimum inhibitory concentration

min – minute/minutes

mL – millilitre

mm – millimetre

mM – millimolar

MOX – moxifloxacin

mRNA – messenger RNA

NCTC – National Collection of Type Cultures

OD – optical density

OFX – ofloxacin

PAS – *para*-aminosalicylic acid

PaβN – Phe-Arg-β-naphthylamide

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PIP - piperacillin

PTU – phenylthiourea

PZA – pyrazinamide

RIF – rifampicin

RNA – ribonucleic acid

RND – resistance, nodulation and cell division

ROS – reactive oxygen species

SEM – standard error of the mean

TB – tuberculosis

TDZ – thioridazine

TFPZ – trifluoperazine

tRNA – transfer RNA

TZO – tazobactam

UV – ultraviolet

v/v – volume per volume

VPL – verapamil

WHO – World Health Organisation

XDR-TB – extremely drug resistant tuberculosis

β – beta

Acknowledgements

This thesis represents more to me than I could ever put into words, both academically and emotionally. There have been so many people who have played a part in preparing this text, all of whom deserve my gratitude and I wish I could list them here. However, if teaching undergraduate students has taught me anything, it is the importance of being concise and precise.

I will always be grateful to my supervisor, Peter Coote, who from my interview onwards has always been a source of confidence, positivity and cheerful determination. Even when my larvae were mysteriously dying by the dozen or every experiment seemed to be failing, his assurance that we would find a solution was always a comfort and reminded me that there is always another day and another chance to try again.

There are so many people who made my time in labs a pleasure rather than a chore: Amy, Jonas, Liz, James, Stef, Maiko and Shirley to name but a few. Guest appearances from Selma, Angus and Chris brightened up our rainy Scottish lunches. In the wider community, Faith and all of the teaching staff stopped me becoming boring and let me exercise my desire to boss around sizeable groups of people. Mhairi and Calum recognised my yearning to stand in front of large crowds and make a fool of myself – thank you for all of the amazing opportunities you have given me, and the endless stories our adventures have created! Equally, a huge thanks to Jacqueline, Cathy, Val, and so many of the academics I've worked with over the years who have represented the best of the University and helped me make the most of my time here.

From the other side of town, my thanks to all of the history crew who tolerated my endless science facts and taught me the value of a well-placed question in a seminar. Marc, Drew, Edwin, Graham, Saskia, Forrest, Laura, Sanda, Nora, Andrew, Jane, and many more.

It is difficult to pinpoint the moments in your life that will lead to great events, but I know that without my family I would never have found my way to St Andrews. In particular, I am indebted to Emma, John L., John E. and Catherine. My parents, Andrew and Helen, and my sister, Flo, have always been my greatest allies and encouraged me to become the best I can be, even if they may not have always understood what I was doing all day. I am also grateful to my Dutch family – Timo, Anne-Marie, Dagmar, and Victor – who have welcomed me with kindness and huge quantities of HEMA treats.

Last but not least, my best friend and the love of my life, Arthur. We've already written so many chapters together and there are so many more to come. It will be an honour to look back at this thesis in a few decades with you and remember the time we had in St Andrews.

Post script – approximately 100,000 larvae were used in the production of this thesis.

This work was funded by the School of Biology at the University of St Andrews, through the PhD Apprentice Scheme 2015.

Conferences, publications, and public engagement events

Conferences

- **Oral presentation** - "Wax moth larvae as a model for Mycobacteria infections – a rapid, low cost and efficient model for antibiotic efficacy testing", Antimicrobial Resistance and One Health Conference, 29–30th August 2017, Maynooth University, Co. Kildare, Ireland
- **Oral presentation** - "Wax moth larvae as a model for Mycobacteria infections – a rapid, low cost and efficient model for antibiotic efficacy testing", School of Biology Postgraduate Conference, January 2018, University of St Andrews, UK
- **Poster** – "Wax moth larvae as a model for Mycobacteria infections – a rapid, low cost and efficient model for antibiotic efficacy testing", The 5th Young Microbiologist Symposium on Microbe Signalling, Organisation and Pathogenesis, 27-28th August 2018, Riddel Hall, Belfast, UK
- **Poster** – "Evaluation of greater wax moth larvae, *Galleria mellonella*, as a novel *in vivo* model for non-tuberculosis Mycobacteria infections and antibiotic treatments", SULSA's Antimicrobial Resistance Conference, 26-27th April 2018, Technology and Innovation Centre, Glasgow, UK
- **Poster** – "An *in vivo* invertebrate infection model using *Galleria mellonella,* the larvae of the greater wax moth, for rapid, low-cost antimicrobial efficacy testing", School of Biology Postgraduate Conference, January 2017, University of St Andrews, UK

Publications

- **First author** – "Evaluation of greater wax moth larvae, *Galleria mellonella*, as a novel *in vivo* model for non-tuberculosis Mycobacteria infections and antibiotic treatments", Entwistle F, Coote PJ (2018). *J Med Microbiol,* 67(4); 585-97. Portions of **Chapter Two, Three**, and **Five** are represented in this publication, which in included at the end of this thesis
- **Co-author** – "Effective immunosuppression with dexamethasone phosphate in the *Galleria mellonella* larva infection model resulting in enhanced virulence *of Escherichia coli* and *Klebsiella pneumoniae*", Torres MP, Entwistle F, Coote PJ (2016). *Med Microbiol*

Immunol, 205(4); 333-43. This work was completed separately from the main topic of this PhD, although similar techniques were used in **Chapter Five**

- Designed and produced an infographic representing our group's current research as part of a greater project for the Biomedical Sciences Research Complex, University of St Andrews, May 2016

Public Engagement Events and Awards

- **PhD Apprenticeship** – hand-selected to be an apprentice within the School of Biology, allowing 25% of the working week to be spent teaching, demonstrating, and leading classes. Spaces on this scheme are only allocated to students who are capable of managing a difficult workload with a high level of responsibility
- **Awarded** the the University of St Andrews School of Biology Public Engagement and Outreach Award in the summer of 2018
- **Nominated** for the Principal's Medal (2019) by the Head of School. The medal awarded to students who displayed exceptional endeavour and achievement during their time at St Andrews
- **Cell Block Science** – first participant in a scheme which brings STEM research topics into Scottish prisons., and engagements were in HMPYOI Cornton Vale women's prison, HMP Shotts maximum-security men's prison, HMP Low Moss men's prison, and HMP Perth men's prison. The CBS was awarded The Herald's Higher Education Partnership Award, and my lectures were observed by representatives from the Wellcome Trust, who awarded the scheme £147,000 to continue the project
- **Participant and organiser for 3 Minute Thesis** – presented in the 2018 and 2016 competitions, and organised and compered the 2017 competition
- **Participant for The XX Factor** – part of the Women in Science Festival at the University of St Andrews for 2016, 2017,and 2018.
- **Awarded** – People's Choice Award for The XX Factor 2017, in conjunction with hosting a solo research table at Explorathon
- **Stand-up comedy –** participant in Bright Club, a stand-up comedy evening for researchers at the University
- **Lecturer –** wrote and presented lectures for the Open Association ("Infection in the community: An introduction of epidemiology", 2018) and Retirement is Opportunity

groups ("From mummies to maggots: Tuberculosis through history to the modern age", 2018 and 2019) in the University of St Andrews

- **Lead demonstrator –** designed and hosted practical laboratory classes on a "zombie apocalypse" to teach epidemiology and ELISA testing for students between the ages of 14 and 17. Sessions have been provided for various international summer schools and visiting students from under-represented communities hosted by the Sutton Trust in 2016, 2017 and 2018

Chapter One

1.0 Mycobacteria and *Galleria mellonella*

This chapter will give an overview to the background of this thesis, with particular focus on Mycobacteria and *Galleria mellonella.*

1.1 The Mycobacteria family in the wider bacterial community

1.1.1 Bacteria and the Actinobacteria phylum

In our day-to-day lives, very few of us ever consider that we are constantly exposed to bacteria. From the bacteria that ferment so many of the foods we eat and the bacteria in our intestines which help us digest it, to the millions of bacteria that live on our skin, in the soil, and in the water around us, we share our world with an estimated 5×10^{30} bacterial cells[1]. Even within our own bodies, the number of bacterial cells equals the number of mammalian cells[2].

Yet the vast majority of bacteria we regularly come into contact with are non-pathogenic and cause us no harm. Less than 1% of the total number of microbial species on the planet are human pathogens[3]. Our microbiome contains a range of bacterial phyla and families, with the Firmicutes and Bacteroidetes phyla dominating the gut flora[4] and Actinobacteria on the skin surface[5]. The Actinobacteria phylum contains a wide range of bacteria, most of which are Gram-positive and found in soil, where they can behave similarly to fungi by producing mycelia and symbiotically living on the roots of plants[6]. The vast majority of Actinobacteria are saprophytic, deriving their nutrient from dead and decaying organic matter. These types of bacteria are the focus of huge interest within research because soil bacteria, in particular the *Streptomyces* genus, are an excellent source of antibiotic compounds[7]. Many antibiotics have been discovered by assessing the secondary metabolites of bacteria, which are often produced to eradicate or

supress the growth of other microorganisms growing within their vicinity. As much as 66% of all naturally derived antibiotics have been found in Actinobacteria species[8], and Actinobacteria have been a source for antifungal, anti-parasite, and anticancer drugs.

Nevertheless many species within the Actinobacteria phylum are harmful pathogens, and cause infections of plant, animal, and mammalian hosts. Those widely responsible for pathogenicity are *Gordonia, Myocardia*, and *Rhodococcus*, which can cause plant diseases like rot of sweet potato and human diseases such as paratuberculosis, allergic pneumonias, actinomycosis, mycetomas, and abscesses[9]. The pathogenicity of various Actinobacteria is often linked to the peculiar combinations of fungal and bacterial characteristics exhibited by many species within the phylum, which leads to difficulties in treatment and diagnosis.

Much of this is due to features that are shared with many other individual species from the Actinobacteria phylum – abnormal cell membranes[6], an innate and highly adaptive resistance to a range of antibiotics[10], peculiar growth morphologies or behaviours[6], and an ability to interact directly or indirectly with the immune system of a host[11,12]. Many have growth patterns that would be much more familiar to a mycologist, producing the long, filamentous hyphae usually associated with fungi[13]. Others have the standard morphology stereotypical to the images of bacteria from school textbooks – rod shaped capsules, small spheres, or chains of cocci – but perhaps with atypical cell walls compared to other bacteria.

1.1.2 The cell wall – Gram-positive, Gram-negative, and acid-fast

All bacteria have the plasma membrane that is ubiquitous to all living cells, but they also have another layer of protection from the environment – the cell wall. Most bacteria can be broadly divided into two categories: Gram negative or Gram positive. A protocol for Gram staining will allow a researcher to categorise a bacteria into either category. In brief, a primary

stain of crystal violet is flooded across a fixed bacterial sample, followed by an iodine solution, which binds the crystal violet to any bacteria that have taken up this stain. The sample is then washed with ethanol or acetone, lifting any unbound stain from the sample before the counter stain (usually safranin) is added. Any bacteria that did not bind to the primary stain should bind to the counter stain. Gram-negative bacteria will be stained pink by the safranin, and Gram-positive bacteria will be stained purple from the crystal violet[14] (see **Figure 1.1)**. For Gram-negative bacteria, the cell wall is a thick sandwich of the plasma membrane, periplasmic space, a layer of peptidoglycan, another periplasmic space, and an outer membrane with lipopolysaccharides that extend out into the surroundings. Gram-positive bacteria simply have a thick layer of peptidoglycan surrounding their plasma membrane, but no outer membrane and no periplasm (see **Figure 1.2**[15]).

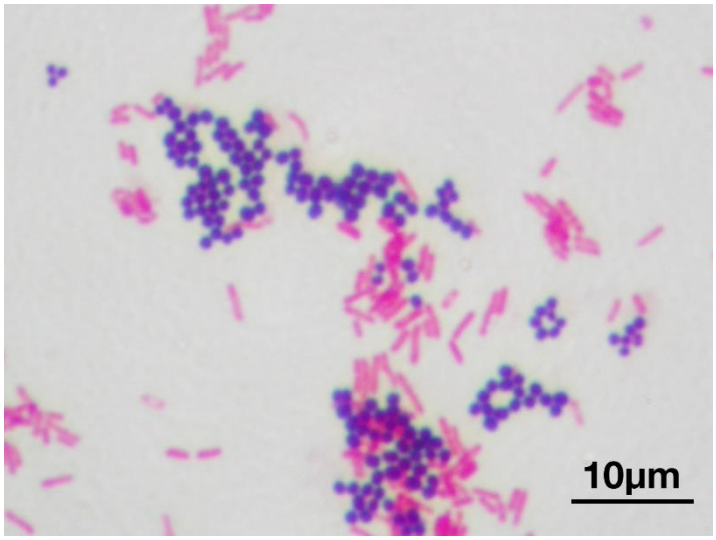

Figure 1.1 – Image showing Gram-negative (pink) *Escherichia coli* and Gram-positive (purple) *Staphylococcus aureus* with 1,000X magnification. Image by Wikimedia Commons user Y tambe, licenced under the Creative Commons Attribution-Share Alike 3.0 Unported, 2.5 Generic, 2.0 Generic and 1.0 Generic license.

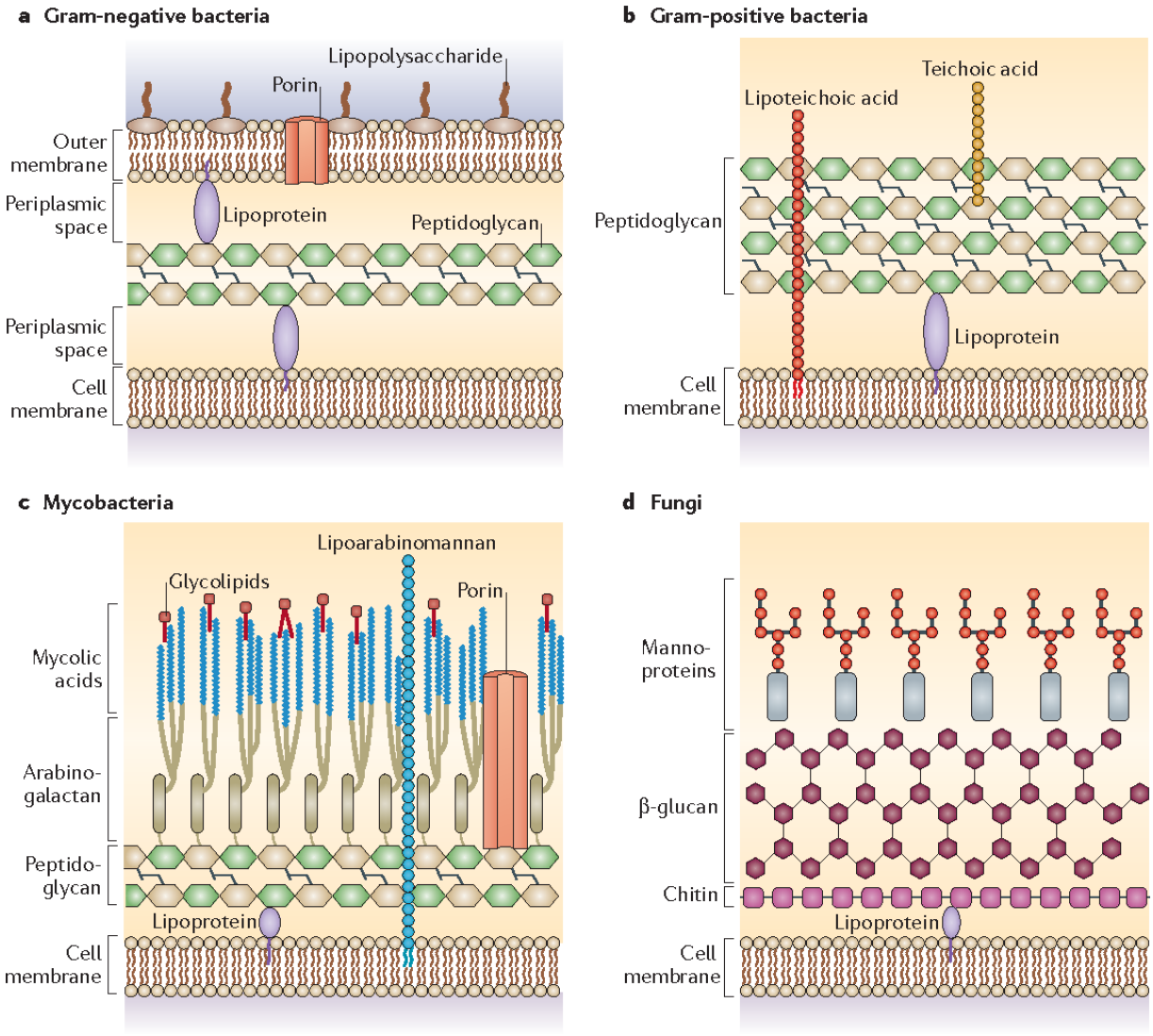

Figure 1.2 – Diagrams to show the differences in cell wall structures between different bacterial types and fungi. Gram-negative (a) bacteria and Gram-positive (b) bacteria both share some similarities with Mycobacteria (c), but Mycobacteria also shares parallels to fungal structures (d). Figure used with agreement from Springer Nature (License Number 4640270644416) from Brown *et al.* (2015)

Some bacteria cannot be placed into these categories because they resist the alcohol decolourisation stages in the Gram protocol or the stains are poorly absorbed and can lead to misidentification. The term for these bacteria is "acid-fast" because the alternative staining protocols used for these bacteria takes advantage of the retention of a stain during decolourisation with an acid alcohol solution. These bacteria have a thin layer of peptidoglycan attached to their plasma membrane, much like Gram-positive bacteria, but then a layer of arabinogalactan and a layer of mycolic acids. There is a final outer layer of polypeptides, phenolic lipids, and mycolic acids (see **Figure 1.2**). This outer layer, often described as "waxy", is integral to the staining of these bacteria. Most methods for staining acid-fast bacteria use a primary stain that is soluble in the bacteria's lipid-rich outer layer, such as carbol fuchsin, and that will be retained by the bacteria in the decolourising stage[16]. Gram-positive and Gram-negative bacteria both lose the primary stain when the sample is washed with an acid alcohol but will take up the secondary stain (often methylene blue), leaving a slide where acid-fast bacteria are stained pink and any other bacteria are stained blue.

1.1.3 Mycobacteria

Very few bacteria are acid-fast, however all species within the genus *Mycobacterium* can be identified with this type of staining. This is very helpful for diagnostics, as a simple histological stain can reduce the number of potential infectious agents to only a small range of species. For Mycobacteria, identifying the bacteria responsible for the infection is imperative, as species from the *Mycobacterium* genus are conspicuous from other Actinobacteria in their pathogenicity. Some species produce toxins which cause painful, necrotic ulcers in the extremities of patients (*Mycobacterium ulcerans*[17]), others present a constant threat to cattle herds and British wildlife (*Mycobacterium bovis*[18]), and many are dangerous to immune-

compromised patients (*Mycobacterium chimaera*[19], *Mycobacterium avium*[20], *Mycobacterium avium paratuberculosis*[21]). *Mycobacterium leprae*, the causative bacterium in Hansen's disease, is still incredibly stigmatised despite having been shown to be easily cured and almost entirely non-contagious between humans[22]. As the prevalence of leprosy is less than one case per 10,000 population, leprosy is no longer regarded as a public health problem[23]

However, one species, *Mycobacterium tuberculosis,* is responsible for more deaths in human history than any other microbial pathogen[24] and continues to be a significant burden on patients and health providers alike. Infections with *M. tuberculosis* can cause a variety of diseases and symptoms based on the point of infection (Pott's disease[25], tubercular meningitis[26], renal tuberculosis[27], hepatic tuberculosis[28], genitourinary tuberculosis[29], pleural tuberculosis[30]), although the general diagnosis is usually tuberculosis and the organ most susceptible to infection is the lung[31] (pulmonary tuberculosis).

The incidence of *M. tuberculosis* infections immediately sets Mycobacteria apart from other bacteria, not just in the Actinobacteria phylum but also within the domain of bacteria. Mycobacteria are idiosyncratic bacteria for a number of reasons. As described previously, their cell walls are uncommon compared to most bacteria, and confer a high resistance to many antibiotics[32]. Many Mycobacteria grow more slowly than other bacteria. *Mycobacterium tuberculosis* takes around 15 hours to divide[33], *M. ulcerans* can take over 48 hours[34], but *M. leprae* can take 14 days[35]. In comparison, *Escherichia coli* has a doubling time of 20 minutes[36], and most *Streptomyces* take 4 – 6 hours[37]. This slow growth rate is linked to the pathogenicity of many Mycobacteria, as patients can develop a well-established, chronic infection well before any symptoms are detected. Tuberculosis can be a latent infection for a patients entire life[38], but active infections usually emerge after 2 to 5 years of incubation[39], but leprosy can be

dormant for up to 20 years[40]. Obviously, this impacts directly on detection, treatment, clearance, and recovery from these infections.

1.2 Mycobacteria

1.2.1 Mycobacteria in the modern world

As mentioned, *M. tuberculosis* is responsible for more human deaths than any other microbe[24]. In the modern world, the World Health Organisation (WHO) suggests that around 1.8 billion people are infected with TB, with 10 million new cases in 2017 and 1.6 million deaths[41]. Tuberculosis is of particular concern to people infected with HIV, as the immunosuppression associated with the virus increases the likelihood of catching tuberculosis and the risk that it will progress to an active infection[42]. TB is currently the leading killer of patients with HIV, with 300,000 deaths in 2017[41].

Another aspect of TB infections is the increasing incidence of multi-drug resistant strains. As we approach a "post-antibiotic era", *M. tuberculosis* strains which are resistant to conventionally used anti-Mycobacterial drugs are now reported in almost all countries surveying for drug resistance[43] with 558,000 new cases in 2017. These cases are predominantly from Russian, Eastern Europe and Central Asia. Extensively drug resistant TB cases were reported in 123 WHO member states in 2016, and "totally drug resistant" cases have been described in Italy[44], India[45], Iran[46] and South Africa[47].

Historically TB has been regarded as a disease of poverty, but the epidemiology of TB and its incidence in a modern population is far more complex. Many aspects of "modern" lifestyles – living in dense urban communities[48], increased representation of individuals from high-risk countries within these communities[49], high levels of drug use and homelessness[50], a decline in

well-balanced nutrition within some groups[51], increased diagnosis of diabetes[52], and the emergence of HIV/AIDS[53] – are intrinsic to the increased reports of TB cases in previously "low-risk" cities and countries[54,55]. This has become a considerable burden on prison populations[56] and homeless centres[50]. In the developing world TB can still be described as a disease of poverty, but again, this is an oversimplification of a far more complex condition. Malnourishment and a low BMI[57], increased usage of tobacco[58], but most importantly the HIV/AIDS pandemic[53], all contributed to developing countries suffering a huge resurgence of new TB cases in the 1990s[59]. Once treatment for HIV/AIDS became more available for areas where TB is endemic, the incidence of TB decreased dramatically[60,61].

1.2.1.1 Non-tuberculous Mycobacteria in the community

In terms of other species of *Mycobacterium*, they generally do not receive the same attention as tuberculosis, both in terms of research and in the public domain, yet there are many species which cause harm to immunocompetent and immunosuppressed patients.

One species of *Mycobacterium* which is severely neglected and causes clear trauma to patients is *Mycobacterium ulcerans,* which is the third most common mycobacterial infection of humans after tuberculosis and leprosy[62] and produces mycolacetones which cause tissue necrosis (see **Figure 1.3 D**). The ulcers are painless, continue to grow unless treated, and are not associated with any pre-existing conditions or immunosuppression[63]. Extreme cases can lead to the patient's bones, muscles and tendons being exposed. Despite having such disfiguring symptoms, there is still no clear mode of transmission, although changes in local water sources such as flooding, building dams, agricultural irrigation, and irrigation in parks/golf courses have been linked to outbreak clusters[63]. There are also no clear methods of reducing the risk of acquisition, and no recommended treatment plan.

In areas where *M. ulcerans* is endemic, mainly West African countries, clinical test for suspected *M. ulcerans* infections are routinely ordered and the risk of *M. ulcerans* infections is

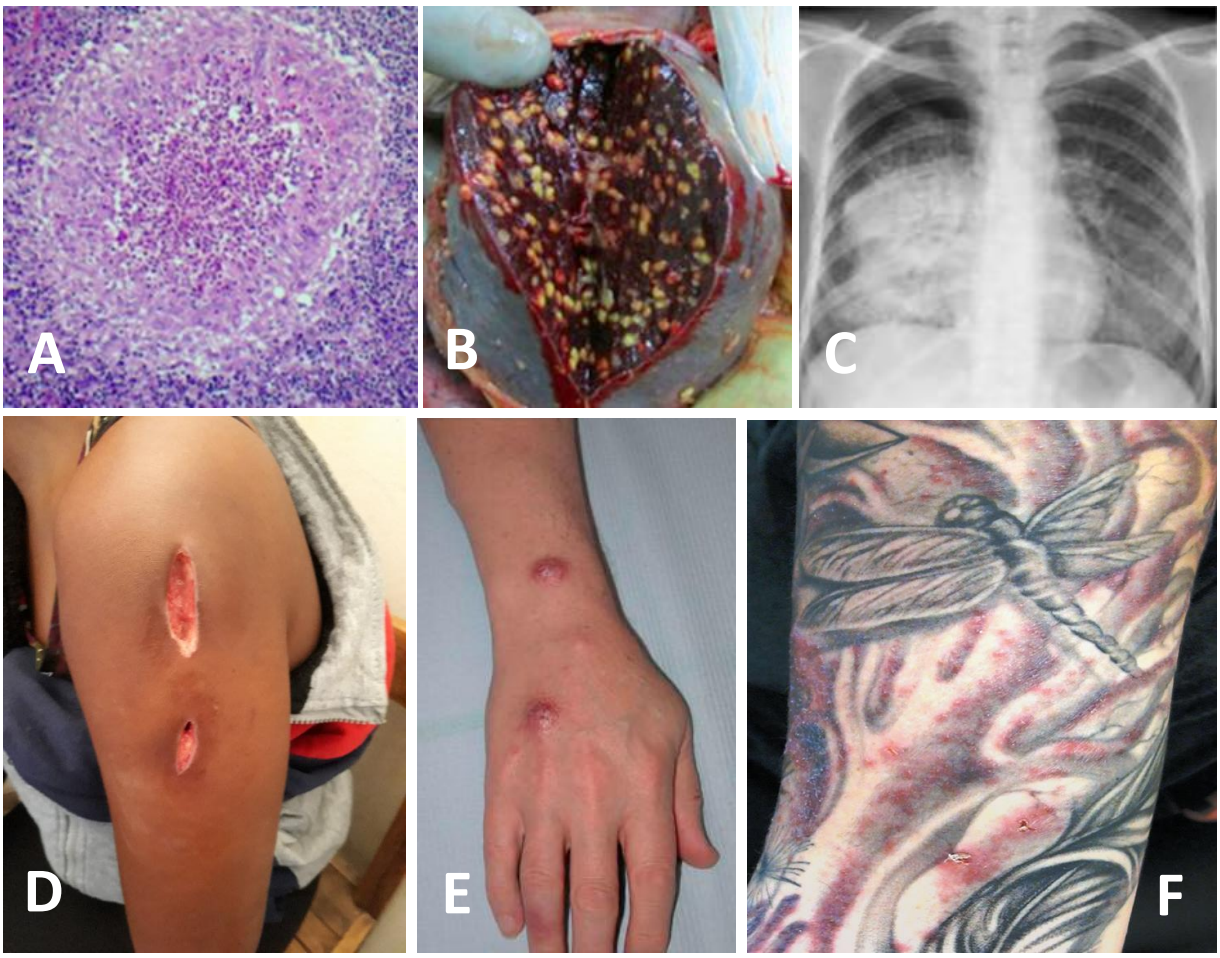

Figure 1.3 – Examples of various types of Mycobacterial infection. (A) An hematoxylin and eosin stained section through a small tuberculous granuloma, isolated from a patient with tuberculosis of the appendix. (B) Tuberculosis of the spleen showing numerous, small granulomas described as miliary, meaning the granulomas look like millet seeds. (C) A chest x-ray of a patient with pulmonary TB, showing dense opacity in both lungs. (D) Painless, necrotising Buruli ulcers on the upper arm, caused by an infections with *Mycobacterium ulcerans*. (E) "Fish-tank" granulomas from infection with *M. marinum*. (F) Cutaneous infection with *M. chelonae* after application of black tattoo ink diluted with tap water.

well-recognised. However, patients in countries where *M. ulcerans* is uncommon may have to undergo multiple debridement procedures, punch biopsies, skin grafts, assessment by various clinicians and specialists, and months of drug therapy before their lesions are healed[64].

In the late 1970s and early 1980s, doctors in American began to follow a rapidly emerging epidemic of deaths among "the 4 H's" – haemophiliacs, Haitians, heroin users, and homosexuals. During this time, the second most common infection (after *Pneumocyctis jirovecii* fungal pneumonia) seen in gay men being treated at the Los Angeles Medical Centre at the University of California was avian tuberculosis, caused by infections with *Mycobacterium avium*[65]. The increased incidence of avian tuberculosis in these communities was recognised by many clinical groups[66–69], with an increase from 32 reported cases of disseminated avian tuberculosis in humans before the HIV/AIDS crisis to over 41,000 cases reported to the CDC by the end of 1999[70]. Eventually, avian tuberculosis was one of the secondary diseases used by the Centre for Disease Control to locate patients who had a primary infection with HIV across America, with almost a quarter of AIDS patients infected with *M. avium* by the 1990s[65]. *Mycobacterium avium* still represents a high risk to HIV-positive people[71]. Disseminated infections with *M. avium* are still very common bacterial infections, occurring in about 10 – 20% of adults with AIDS in the developed world[72].

Mycobacterium avium is also the most commonly isolated non-tuberculous *Mycobacterium* from the lungs of patients with cystic fibrosis[73]. Among this patient community, *M. abscessus* is also of concern, as it seems to be capable of transmission between humans and accelerates the decline in lung function, increasing the likelihood that a transplant is needed and raising patient mortality[74]. *Mycobacterium abscessus* is of increasing concern in many treatment settings as it grows quite rapidly (particularly in comparison to *M. tuberculosis*), can establish an infection in

many tissue types and organs, and has an innate resistance to many antibiotics[75]. As with many Mycobacterial infections, patients who lack a robust immune system are at particular risk, but *M. abscessus* can also be acquired after tattooing, acupuncture, non-surgical cosmetic medical treatments (such as vitamin, collagen, and homeopathic injections into subcutaneous fat), at hot springs and spas, and during surgeries[76]. *Mycobacterium abscessus* is found in soil and water and can be acquired from the environment, but the majority of clinical cases seem to be from human-to-human transmission, fomites, aerosols, and contaminated surgical instruments and equipment between patients[76,77].

The transmission of Mycobacterial infections between patients in a healthcare setting is certainly an under-reported and mis-diagnosed issue. A number of reviews on cardiac-surgery patients identified *Mycobacterium chimaera* as infecting a number of patients from compromised heart-lung machines used during open chest heart surgery[78–80], with up to half of the patients in the European and North American case clusters dying. Clearly, such a high mortality rate is a concern to both patients and medical staff, but these issues are compounded by a lack of awareness of how Mycobacteria can be associated with nosocomial infections. *Mycobacterium* blood samples are not usually ordered for surgical patients who are immunocompetent[81], so the ambiguous symptoms of fevers, joint and muscle pains, loss of energy, and weight loss can be difficult to associate with the causative bacteria.

A similar concern has arisen in immunocompetent people who had tattoos with ink contaminated with *Mycobacterium chelonae*[82,83], with ink purchased from multiple companies and in distinct, sperate geographical areas. *Mycobacterium chelonae* has also infected surgical patients who had undergone face-lifts and mammoplasty procedures using contaminated gentian violet skin-marking solutions (the gentian violet stock held at the supplying pharmacy

was also contaminated)[84], patients who underwent liposuction using implements that were cleaned using washing up liquid and tap water[85], people who received grey tattoos where black ink had been diluted with tap water[86] (see **Figure 1.3 F**), and podiatry patients administered with lidocaine from a needle-free jet injector kept in a mixture of contaminated water and disinfectant[87]. These cases clearly show the ability of this Mycobacteria to survive in potable water, but also to survive in solutions containing surfactants, preservatives, and disinfectants. This is not unexpected, considering the robust Mycobacterial cell membrane (see **Section 1.1.1**), Mycobacteria are often early populators of biofilms[88–90], and that they have been shown to have increased resistance to disinfectants[91], with some species showing a tolerance for high water temperatures[92].

Water-borne infections from *M. marinum* are also well documented in the literature, and are often associated with contact with fish[93,94]. Historically, *M. marinum* was also commonly acquired from swimming pools[95], giving cutaneous infections the name "swimming pool granulomas", but this term has been rendered almost obsolete now that proper chlorination of pools is more common[96,97], and people are more aware of bacterial contamination of pools. "Fish tank granuloma" is now more commonly used to describe these surface infections, which can take several months to present, and may require surgery to remove stubborn granulomas[98] (see **Figure 1.3 E**).

Much like *M. ulcerans,* lesions from *M. marinum* are usually painless, but can heal without medical intervention[99]. *Mycobacterium marinum* has an optimum growth temperature of 30 ^{o}C, with very little to no growth on plates at 37 ^{o}C[99], which indicates why growth on hands, feet, legs and arms are more common[100] and infections in in organs are not commonly seen in immunocompetent humans. However, it is common for infections to spread to tendons, bones,

and the cushioning between tendons and bones[101], or for bacteria to spread to lymph nodes close to the point of infection[101].

As with many other Mycobacterial species described here, a key limitation of diagnosing *M. marinum* infections is that the presentation is non-specific, and unless the patient discloses that they own an aquarium[102] or work with fish then diagnosis may be significantly delayed[99] as standard tests will not include Mycobacteria. Considering that the incubation period for *M. marinum* averages to several weeks[101], and up to 9 months[96], it may not be immediately obvious that symptoms are caused by an exposure which could have occurred many months ago. In terms of *M. marinum* infections in an immunocompromised host, confusion in the diagnostic process can allow time for infections to become systemic[100,103–107], and mis-diagnosis can lead to patient death, especially if immunosuppressants are prescribed[108].

Examples of disseminated *M. marinum* infections have also been reported in a baby boy, where the baby was being washed in the same bathtub where the family aquarium was also cleaned[107], an elderly man who was taking prednisone and azathioprine (both drugs are immunosuppressants)[100], an elderly man suffering from steroid-dependent chronic obstructive lung disease, coronary artery disease, and congestive heart failure who was also taking prednisone and azathioprine[108], and a middle aged man with diabetes who had received a kidney transplant 8 years before infection[109]. Of course, there are many more examples of disseminated infections with *M. marinum*[109], often associated with steroid use, chemotherapy, or AIDS.

Mycobacterium fortuitum shares many similarities to *M. marinum* and other Mycobacteria skin infections in terms of presentation in an immunocompetent host[110,111], although *M. fortuitum* has the additional characteristic of growing more rapidly than many other

Mycobacterial species[112]. *Mycobacterium fortuitum* and *M. abscessus* were commonly isolated from the respiratory samples of patients infected with non-tuberculous Mycobacteria[113]. Infections with *M. fortuitum* have also been linked to water sources, including footbaths[114–116], hospital water tanks, reservoirs, and municipal water systems[117]. However the most commonly observed infections seem to arise from catheters[118,119]. surgical incisions, and other nosocomial sources or medical devices[119–121].

The three Mycobacteria used in this work at *M. fortuitum, M. marinum* and *M. aurum.* **Table 1.1** shows the similarities and differences these bacteria have in relation to *M. tuberculosis*.

Table 1.1 – Similarities and differences between the non-tuberculous Mycobacteria used in this thesis and *M. tuberculosis*

Species	Strains	Similarities to *Mycobacterium tuberculosis*	Differences to *Mycobacterium tuberculosis*
Mycobacterium fortuitum	NCTC 10394 NCTC 8573 R365371Q	• Can survive intracellularly within macropahges[122] • The GC content of both genomes are similar – 66.19% for *M. fortuitum* and 66.5% for *M. tuberculosis*[123] • Similar cell wall profiles[124], efflux pumps[125] and drug resistance patterns[126] • Requires a long period of antibiotic treatment and can cause symptomatic and non-symptomatic infections[127,128] • Can produce granulomas in infected hosts[127,129–131], including humans[128,132] • Capable of growing at 37°C[128]	• Grows much more rapidly – around 8 hours for *M. fortuitum*[88] compared to 18 to 54 hours for *M. tuberculosis*[133] • Little similarity between genomes – 18.7% of DNA sequences aligned with the *M. tuberculosis* reference genome[123] • Can infect healthy people[110,111,113–117] (as can *M. tuberculosis*), but infects far fewer people per year
Mycobacterium marinum	Strain M NCTC 2275 R356933F	• Can survive intracellularly within macrophages[134] • Share a large number of orthologous genes with *M. tuberculosis*[135,136], particularly enriched in DNA metabolism such a recombination, repair, integration and protection • Similar cell wall profiles[124,137], virulence genes[136,138] and drug resistance patterns[139] • Requires a long period of antibiotic treatment[128] • Can produce granulomas in infected hosts[140,141], including humans[128,142–144] • Can produce latent infections[140] • Capable of growing at 37°C[145]	• Has a larger genome size than *M. tuberculosis* (around 6.6 Mb compared to 4.4 Mb[144]) and a larger number of genes[135] • Produces yellow pigments when exposed to light (photochromic)[146] • Can infect healthy people[93–95,101] (as can *M. tuberculosis*), but infects far fewer people per year
Mycobacterium aurum	NCTC 10437	• Can survive intracellularly inside macropahges[147] • Shows a similar cell wall profile[148], drug resistance patterns, intracellular therapeutic targets and gene organisation with *M. tuberculosis*[147] • Capable of growing at 37°C[147]	• Incapable of causing infections in an immune-competent host[149,150] • Grows more rapidly than *M. tuberculosis*[147] – around 2.5 hours • Cannot survive in low oxygen environments, or when acidity is high and nutrient levels are low[151] – essentially the conditions within a tuberculosis granuloma • Produces orange pigments when growing[151]

1.2.2 Mycobacteria through history

1.2.2.1 In the pre-antibiotic era

The *Mycobacterium* genus likely originated in the Jurassic period[152] and the slow mutation rate of *M. tuberculosis*[153] allows researchers to trace ancestors of modern *M. tuberculosis* to an antecedent which may have infected early hominids 3,000,000 years ago[154], meaning that Mycobacteria have been infecting humans since we first evolved. Modern *M. tuberculosis* is comparatively young in evolutionary terms, originating 15,000-20,000 years ago[155], but palaeopathological studies have isolated biomarkers for *M. tuberculosis* in humans remains many thousands of years old[156–159] and TB infections have been confirmed in Ancient Egyptian[160–162], Roman[163], Chinese[164] and South American[165–167] remains, and many other palaeopathological remains[168]. TB is clearly described in Ancient Greek[169–172], Roman[172,173], Arabic[174] and Chinese[175] writings, and tuberculosis is even referred to in the Bible by its ancient Hebrew name, schachepheth[176].

It is therefore unsurprising, considering the high incidence of TB and its prevalence across the globe, that treatments of the illness were an obsession for generations of physicians. It would be impractical to list the full variety of cures touted for *M. tuberculosis* throughout an extensive shared history[177–179], but it has produced some remarkably unusual "cures". Of course, the almost ubiquitous practice of bloodletting was a popular treatment in Europe and, later, America, but other "cures" included licking certain types of limestone rocks or eating a wolf's liver boiled in wine (Pliny the Elder, 23-79 AD), consuming plenty of garlic, drinking elephants blood, or bathing in human urine (Celsus, c. 25 BC – c. 50 AD), inducing a variety of purges[180], sealing patients into airless rooms[180] to prevent exposure to fresh air, dosing patients

with digitalis[181], and the evocative "Royal Touch" (originating with Saint Louis IX of France, r. 1226–1270 AD) where the touch of a French or English king was considered curative.

Surprisingly, many texts suggest a hearty diet, bed rest and relocating to locations with clean, dry air. Considering these recommendations are practically indistinguishable from those prescribed in modern sanatoria well into the 20th century, physicians seemed well aware that malnutrition[182], poor ventilation[183] and exposure to unclean air[184] reduced the life expectancy of patients with TB.

Attitudes to TB changed dramatically in the 19th century, almost solely due to the pioneering work of Robert Koch (1843-1910) presented in his 1882 lecture to the Berlin Physiological Society, *Die Ätiologie der Tuberkulose*. Although Koch was not the first to culture *M. tuberculosis* in the lab nor use a stain to identify them[185], he identified the bacteria using Paul Ehrlich's new methylene blue staining protocol, infected a number of guinea pigs with the bacteria, and was then able to re-isolate the bacteria both from re-culturing serum samples from the guinea pigs, but also from stained histological samples. It was at this same lecture that Koch presented his celebrated "Four Postulates". The lecture marked a watershed in medical science – attendees were reportedly completely silent when Koch concluded his lecture, and Paul Ehrlich later described the evening as "*the most awe-inspiring scientific event [he had] ever attended*"[185]. His research was widely circulated not only with the scientific community but also within the public sphere, and left a deep and lasting impression – most impressively, shrines were erected in his honour in Japan[185]. Demonstrating that TB was caused by a contagious external unit, a "germ", changed public perceptions of TB from a "*vague phantom*" to a "*unseen killer*"[186] which needed eliminating.

As the public became more uncomfortable with TB in the community and came to terms with the infectious nature of the disease, they turned to a service that had been offered well before Koch's discovery as a means of isolating and "treating" infected patients – the sanatorium. The first sanatorium was established in 1836 by the British general practitioner George Bodington (1799-1882) at Driffold House in Sutton Coldfield[180]. Bodington criticised the attitude that "*consumptives were closely confined from a fear of the evil influence of cold fresh air [...] thus forcing them to breathe over and over again the same foul air contaminated with the diseased effluvia of their own persons*"[180], instead implementing a regimen of nutritious foods, exercise in the fresh air, and "*the stimulus of a proper quantity of wine*". He reported his treatment in an 1840 essay to the Lancet, *On the treatment and cure of pulmonary consumption*, including the positive recoveries of five patients to whom he had applied his regimen.

An obvious benefit from sanatoria is that infected members of the community were isolated from those who they might otherwise infect while being given the rest and nourishment that they may not have received at home, especially in the state-sponsored sanatoria that became popular in the early 20th century. However, good food, long walks and cold showers may make a person healthier and more capable of managing their infection, but they do not constitute a cure. A five-year study of over 4,000 patients in 1920s Lancashire showed that patients who were sputum-negative but culture-positive (indicating a latent TB infection) and treated at home had a 38% mortality rate, whereas those treated in a sanatorium had a 14% mortality rate. Patients with active TB (smear-positive and culture-positive) had mortality rates of 61% in a sanatorium and 81% if treated at home. The ability for sanatoria to improve mortality is impressive; however, an American study showed that when patients left the sanatorium 60% died of tuberculosis within 6 years. A true cure for TB, like so many other illnesses, was only to be made possible by the dawn of the antibiotic era.

The research generally regarded as bringing us into the antibiotic age is Alexander Fleming's accidental discovery of penicillin, followed by Howard Florey and Ernest Chain's purification protocol that allowed for the mass production and distribution of penicillin in 1945. Nevertheless, the idea of directly trying to kill the microorganism infecting a TB patient had been established only shortly after Koch demonstrated that TB was caused by a bacterium. In 1885, an Italian doctor named Arnaldo Cantani noted that *M. tuberculosis* loses virulence when in a decomposing corpse. Perhaps in consideration of Louis Pasteur's 1877 work showing that the growth of one microorganism could be inhibited by the presence of another microorganism, Cantani made a truly extraordinary leap by producing cultures of the saprotroph "*Bacterium thermos"* and exposing the lungs of TB patients to these cultures through an aerosol[187,188]. Sputum smears for these patients became positive for *B. thermos*, negative for *M. tuberculosis,* and the patients reported that their condition was improved. There is evidence that other physicians[189,190] tried to apply this treatment to their own patients, although with some small adjustments such as adding peppermint essence to the culture media to disguise the smell of the bacteria, yet this procedure was clearly not destined for mainstream medicine. However, this form of "replacement therapy" has been applied to other diseases – a prominent modern example would be the so-called "faecal transplant" for patients whose digestive microbiota have been damaged by chemotherapy or harmful bacterial infections such as *Clostridium difficile*[191].

Only a few years later in 1888, the Romanian physician Victor Babeș presented research which indicated that the metabolic products of some saprophytic bacteria (such as staphylococci) inhibited the growth of *M. tuberculosis*, whereas other products promoted growth (including streptococci and pneumococci)[187]. The role of other microorganisms in the microbiome of a patient is now a well-established consideration in research and treatment

studies, and was utilised again in 1912 by Gustave Rappin who showed that filtered cultures of *Bacillus subtilis* prevented the development of tuberculosis when injected into infected rabbits[187], and also by Albert Vaudremer who showed in 1913 that *M. tuberculosis* cultured with filtered *Aspergillus fumigates* became non-pathogenic[187]. Vaudremer even went on to "vaccinate" rabbits with these non-pathogenic *M. tuberculosis* cultures – a month later the rabbits were injected with fresh, pathogenic *M. tuberculosis* but failed to develop TB. He did extend his investigations into a human model, injecting over 200 patients in various hospitals and sanatoria with his *A. fumigates* extracts, but he deemed the results too unpredictable to merit further study.

1.2.2.2 The advent of antibiotic treatments for TB

Selman Waksman's group at Rutgers University were also especially interested in saprophytic bacteria, more specifically the Actinomycetes, and eventually isolated the first antibiotic effective against *M. tuberculosis* using these bacteria as a source of antibiotic compounds. Actinomycetes grow in the soil and may produce up to 10,000 antimicrobial compounds (45% of all known bioactive microbial metabolites) and 8,700 antibiotics[192] (see **Section 1.1.1**). Waksman's group initially isolated an antibiotic called actinomycin, which was especially effective against Gram-positive bacteria but proved extremely toxic when injected into lab animals[193]. In 1942, the group isolated another antibiotic sourced from Actinomycete cultures – streptothricin[194]. Initially this compound seemed promising – it was effective against a range of Gram-negative and Gram-positive bacteria and was much less toxic than actinomycin *in vivo*.

In vitro studies carried out in Waksman's labs indicated that streptomycin was highly effective against a range of bacteria including *M. tuberculosis*[195], although this seminal paper on

streptomycin is remarkable in that tuberculosis is barely mentioned. In the words of Frank Ryan "*if streptomycin were to prove the cure for tuberculosis, it had arrived into the world unannounced*"[196]. Several months later the Waksman lab produced the pivotal paper in cementing streptomycin as the first effective antibiotic for preventing the growth of *M. tuberculosis* and related organisms[197]. The human strain, H37-Rv, was especially sensitive. Streptomycin had been shown to have low toxicity in rats and mice[198], allowing for Feldman and Hinshaw of the Mayo Clinic to test the effectiveness of streptomycin against *M. tuberculosis* infections in guinea pigs[199] and demonstrate its efficacy *in vivo.* The results were more than impressive enough to warrant clinical application, so in the winter of 1944 treatment began on the first human patient[200]. The patient was a 21-year-old female with advanced pulmonary tuberculosis who had been receiving treatment in a sanatorium for a year. The conditions of this first trial are a marker of how desperate researchers were to find a treatment for TB – there was no data on a possible therapeutic dose or at what point streptomycin would become toxic. Doses had to be adjusted regularly to provide relief from side effects but also factor in that the supplies of streptomycin were so low that treatment could not be continuous[187].

Although streptomycin represented a huge leap forwards in the treatment of tuberculosis, resistance to this drug was quick to emerge[201]. Waksman himself spoke on the subject at a meeting of the American Medical Association in 1947, stating "*The use of streptomycin in the treatment of tuberculosis presents certain challenging problems to the microbiologist, the chemist and the clinician. Most significant of these are the development by the tubercle organism of resistance to the streptomycin, and the drug's ineffectiveness against certain forms of tuberculosis. In order to overcome these limitations an intensive search must be made for other agents that would be more effective alone or when combined with streptomycin*". This was the year that streptomycin was made available to the civilian population at large, although Waksman was

wary in his belief that streptomycin was simply a bacteriostatic agent, and only prevented the multiplication of bacteria but could not clear an infection. In fact, streptomycin is now considered to be bactericidal[202], although Waksman's other concerns about resistance, the damage the medication could cause to the eight cranial nerve and patients' hearing[203], and the requirement for streptomycin to be administered via injection were still well founded.

1.2.2.3 Recognising the value of existing compound libraries and synthesised drugs

The first chemotherapeutic drug, created well before the discovery of penicillin, was Salvarsan, which was used to treat patients with syphilis. This drug was synthesised in the laboratories of Paul Ehrlich and was based on the structure of one of the dye derivatives that his lab was investigating. His group worked to find compounds with biological activity, and to then optimise these lead compounds via systematic modification to their chemical structure – this style of drug development is still in wide use over 100 years later. However, the Ehrlich lab was not alone in developing libraries of compounds, and many went untested for potential antimicrobial activity. One such compound was *para*-aminosalicylic acid (PAS), which was first synthesised in 1902, but PAS was not used in an antibiotic study until the early 1940s. A Danish chemist, Jörgen Lehmann noticed that salicylate increased the metabolic activity of *M. tuberculosis*, so looked to inhibit or block metabolism by using a drug with a similar structure. PAS was in the library of compounds he tested, and was found to be the most effective, and also effective at treating *M. tuberculosis* infections in guinea pigs and rats. Importantly, the drug could be given orally, a key advantage over streptomycin. Experiments in humans showed that PAS combined with streptomycin was a very efficient treatment[204], and particularly effective for cases of extrapulmonary and intestinal tuberculosis.

The success of these two treatments encouraged further investigations into other synthesised compounds, The next great leap in tuberculosis treatment shared many similarities to PAS – isoniazid (INH) was initially synthesised in 1912, but only used as part of a great study into pyridine derivatives after nicotinamide was found to have anti-tuberculous activity in animal models of TB[205]. Oddly, isoniazid was discovered in multiple labs simultaneously – three separate pharmaceutical companies, Hoffman La Roche, Farbenfabriken Bayer, and Squibb Institute for Medical Research, all filed patents for INH as an anti-tuberculosis medication in 1951. However, the two doctoral students who had initially synthesised INH in Prague in 1912 were shown to have synthesised the structure first, nullifying any later claim from the three pharmaceutical companies.

Isoniazid was a significant leap forwards both in terms of efficacy in clearing Mycobacterial infections, and also in ease of administration, minimal side effects and low production costs. It was also very specific in targeting Mycobacteria, with a much higher potency than PAS or streptomycin, and capable of treating patients who had acquired or developed strains of TB which were resistant to these two drugs. Considering that a study of ambulatory patients in the early 1960s found that over 75% of *M. tuberculosis* strains isolated from these patients showed resistance to streptomycin, PAS or INH[206], using these drugs in combination would be helpful, but new medications were also needed.

Another drug which was tested in the 1950s against tuberculosis based on its chemical similarity to nicotinamide is pyrazinamide (PZA). PZA is uncommon in that it is ineffective *in vitro*, but active *in vivo* due to requiring an acidic pH to be active[207]. It was not commonly prescribed for TB patients until the 1970s due to its hepatic toxicity, however it was shown to be synergistic with rifampicin (RIF) and capable of shortening the treatment for TB from 12

months to 6 months if both PZA and RIF were used[205]. PZA is also highly effective at treating non-growing persister cells, which is unusual compared to most anti-Mycobacterials[205].

After PZA was shown to be effective against TB, there was a long wait until the next front-line anti-Mycobacterial was discovered. Ethambutol was immediately tested in an animal model for TB upon its discovery[208], and quickly replaced PAS in the standard treatment regimen due to its lower toxicity and high efficacy against replicating bacteria[209]. Shortly after, a new family of antibiotics was discovered by a group based in Milan. A researcher in the group returned from a holiday in France with a number of soil samples. One sample from the Cote d'Azure contained a novel Actinomycete which produced a family of antibiotics which showed efficacy against many bacteria, including *M. tuberculosis*[210]*.* The group named the antibiotic family "rifamycins", and worked to improve their *in vivo* activity as they were significantly more active *in vitro* than in an animal model[209]. The ultimate product was rifampicin, which was immediately included in the first-line treatment combination for TB patients and allowed for the standard treatment window to be significantly shortened[205].

Unfortunately, after this period no new anti-Mycobacterials were approved for use for decades. The impetus to find new treatments was, regrettably, from the immense pressure of the AIDS crisis[209] and the synergy of HIV and TB creating an increased disease burden[42]. Many antibiotics that had been approved for other infections were repurposed to treat TB, but the first new antibiotic specifically for Mycobacterial infections was bedaquiline (BDQ) which was approved for use in humans in 2012[211]. BDQ was also shown to be effective against non-replicating Mycobacteria in low oxygen environments[208], and has been shown to accumulate to biologically relevant concentrations in necrotic granulomas in mice[212]. Other drugs are mentioned in **Section 1.2.5**.

1.2.3 Granulomas, phagocytosis and Mycobacteria

A key aspect of Mycobacterial infections is the formation of structures within the host called granulomas (see **Figure 1.3 A, B** and **C**), which are utilised by the bacteria to create a closed, hospitable environment to evade the host immune system, divide, and eventually proliferate. Granulomas also provide a unique challenge in terms of treatment as they represent a physical barrier from the host immune system, are not supplied with blood or lymph which could contain soluble antibiotics, and the bacteria can be dormant or very difficult to diagnose using standard tests such as swabs or blood tests.

In humans, the invasive bacteria are engulfed by the host phagocytes and begin to replicate within them, instigating the migration of other immune cells (including macrophages) to the site of infection[213] which eventually leads to an accumulation of infected and non-infected cells[214]. This structure is called a granuloma, and initially allows the host to protect the surrounding tissue from infection (granulomas are not a tuberculosis-specific pathology). However the centre of the granuloma, filled with lipid-rich cell debris[213], can undergo necrosis[214] and eventually rupture, flooding the surrounding area with *M. tuberculosis* bacteria[213] and leaving a cavity in soft tissues like the lungs[215]. Granulomas are also the primary location of bacteria residing in a person with a latent, chronic TB infection.

The formation of granulomas which are comparable to those which form in humans is not a necessity in a model organism but is an extremely desirable trait. In terms of modelling granulomas, mice, rabbits and guinea pigs are capable of forming granulomas within their lungs. However, these are either morphologically different to human granulomas[215], don't show a true latency of infection[216], rupture far more easily than the human equivalent[215], causing death within a relatively short timeframe[217] or require *M. bovis* as an alternative species[217].

Zebrafish also require an alternative *Mycobacterium* to model infection, but exposure to *M. marinum* eventually produces lipid-rich and necrotic granulomas[218] in both adult and larval fish, despite the immature fish larvae having no adaptive immune system at that point in development[213].

Of course, it cannot be expected that simpler invertebrate models such as fruit flies and moth larvae will produce necrotising granulomas, yet some comparisons remain not only for the formation of granulomas but also in the host's general response to infection. A well-established and popular "basic" model is the nematode worm, *Caenorhabditis elegans.* However one group has reported that the worms are not susceptible to exposure to *M. fortuitum* and *M. marinum*[219], although they mention that they were unable to closely follow survival rates of the worms, so this result may not be accurate to the worm's true susceptibility. It has also been shown that infections with *M. avium* subspecies do not cause increased mortality in nematodes[220]. Other groups have used nematodes with *M. marinum* to assess the virulence of different Mycobacterial strains[221], and showed that *M. marinum* can colonise the intestinal tract and did increase morbidity and mortality in the worms.

Additionally the nematode immune system completely lacks mobile phagocytes or haemocytes[222] – considering this is a vital aspect of assessing how Mycobacteria respond in a model organism, *C. elegans* cannot be considered a viable model for these experiments and may be more valuable as a screening tool[223].

In contrast, *Drosophila melanogaster* (fruit fly) is becoming a well-established model for *M. marinum*[144]. Infected flies show evidence of "wasting"[224], a key indicator in human infections[225]. Fly phagocytes have been shown to engulf *M. marinum,* which replicate inside the phagocytes

and are then released and found extracellularly to the host cells[226] much in the same way that *M. tuberculosis* enters and overwhelms immune cells in humans.

Although *D. melanogaster* has the advantage of having a fully sequenced genome[227] and can easily have genes knocked in or knocked out, fruit flies are reared at 18 – 29 ^{o}C and cannot be easily inoculated with specific volumes of solution. It's preferable for a cell challenge or antibiotic dose to be precise and reproducible, and for the model to function at human physiological temperature (37 ^{o}C). Fruit flies also have the disadvantage in that researchers can only collect circulating haemocytes from fly larvae, as the haemocytes in adult flies become adhered to a variety of tissues within the fly and no longer circulate in the haemolymph[226,228], meaning short term effects of infection on circulating phagocytes can only be examined in larval flies and long term experiments can only be designed using adult flies[226].

A key aspect of the *G. mellonella* immune response is the mobilisation of haemocytes around invading bacteria or fungi, followed by production of melanin deposits and engulfment by plasmatocytes to form a nodule[229]. This is comparable to phagocytosis of foreign bodies by macrophages and neutrophils in mammals[230] and although the comparatively simplistic physiology of insects cannot provide a perfect equivalent of a granuloma, the ability of insects to form nodules may be described as analogous[231]. Foreign cells or particles are trapped by aggregated haemocytes which proceed to melanise[232]. Melanin provides a structural element[233,234] but also "scavenges" reactive oxygen species (ROS) to be used against the confined bacteria and protects the host from ROS and cytotoxic by-products of melanin production[233,234]. This primary aggregate is surrounded by specialised haemocytes (plasmatocytes) which are stimulated to attach to the cell cluster through chemotactic factors released by the haemocytes within the aggregate[232]. Ultimately, the haemocytes and foreign

cells in the melanised core of the nodule are necrotised, and surrounded by insect immune cells[232], much like the macrophages and invading microorganisms in human granulomas.

Researchers can also assess the response to an invading micro-organism by assessing the number of tagged cells taken up by haemocytes. This can provide a measure of how well the larvae respond to the immune challenge and whether the organism is capable of causing larval death despite being phagocytosed by the haemocytes.

The haemocytes of *G. mellonella* are discussed in more detail in **Section 1.3.3** and in **Chapter Five**.

1.2.4 Vaccines and preventative measures

Many infectious diseases have become very uncommon in the Western world and are slowly being eradicated from the planet through the use of vaccination. The basic premise of vaccination is to stimulate the production of antibodies without causing the full infection. This can be through the use of live-attenuated strains, inactivated strains, small doses of the toxin produced by the live bacteria, or exposure to subunits of the bacteria or virus such as proteins, sugars and other antigens.

There are huge benefits to be gained from vaccines – the eradication of smallpox is certainly due to effective and pragmatic use of vaccines, and the eradication of polio is within reach[235]. Vaccines are available for rabies, diphtheria, tetanus, meningitis, typhoid, and many more bacteria and viruses which were previously endemic and often disabling or fatal. However, there can be a myriad of difficulties with vaccination, from ineffective strains, reactivation of attenuated strains to cause an active infection, requiring multiple injections over

a lengthy period of time, complications around refrigerated storage and transport, and short shelf-lives.

The vaccine for *Mycobacterium* infections is not exempt from some of these issues. Protecting the individuals who are likely to be exposed to TB before they are infected ought to be feasible using an active and long-lasting vaccine – sadly, this does not describe the *M. bovis* BCG vaccine currently used to reduce TB transmission. Its efficacy has been strongly debated, with estimates of effectiveness differing immensely between publications[236] – some trials show extensive protection[237,238] while others show no benefit in vaccination whatsoever[239].

It is well established that the vaccine is most effective when given to young children, but has variable efficacy in adults – generally the vaccine is regarded as being ineffective when given to adult patients[240]. Intriguingly, there may be an association between geographic location and the efficacy of the BCG vaccine, with patients closest to the equator seeing a lower efficacy that those at higher latitudes[241].

Other techniques which can be applied to reduce tuberculosis incidence include basic controls such as the use of respirators and masks in settings such as hospitals and prisons, discouraging spitting in public spaces, and covering mouths when coughing and sneezing. Even regular cleaning, good ventilation, and letting lots of sunlight into living spaces can help prevent the spread of tuberculosis[242] (although Robert Koch suggested some rather charming tips for controlling exposure to tuberculosis, a favourite being "*in the case of death from pulmonary consumption, the walls of all rooms and apartments used by the deceased should be rubbed down with fresh baked bread, which is a sure method of removing the bacilli*"[243]).

In terms of other non-tuberculous Mycobacterial infections, disease prevention is mostly through identifying and managing environmental sources. Considering tap water is a source for

many Mycobacterial infections, using tap water to clean surgical or medical devices, or to dilute sterile solutions, should be discouraged[128]. Equally, biofilms are a recognised site for Mycobacterial growth, so regular disinfections of reservoirs such as hot tubs, footbaths, aquaria, and tubing in medical devices should be adhered to[128]. As mentioned previously, proper chlorination of swimming pools has been shown to have a drastic effect in reducing the likelihood of Mycobacterial infections[96,97].

1.2.5 Modern treatment pressures and drug discovery

Despite the huge strides made in disease prevention and treatment in a comparatively short space of time, there are some real issues with preventing and treating infections in the modern world. Fear of vaccination has been present since the introduction of the very first vaccine[244], and in some communities was negatively associated with the Western world – Pakistan and Afghanistan have had issues with vaccine providers being targeted in the belief that vaccines cause infertility and will cause a medical genocide[245]. More recently, the highly criticised work from Andrew Wakefield around the measles, mumps and rubella vaccine has triggered vaccine hesitancy in the Western world[246], allowing for the increased incidence of a number of diseases which were previously uncommon[247]. There does not seem to be much controversy in online anti-vaccination communities around the BCG vaccination, although this could be due to the vaccine not being compulsory in most European countries or in the USA, where anti-vaccination sentiments are most well established.

Simultaneously, issues with the use and abuse of antibiotics have also caused treatment pressures in clinical settings. The problems with antibiotic resistance were recognised very early in the 20th century – Alexander Fleming warned about misuse of antibiotics in his Nobel acceptance speech, and the strains of *Staphylococcus aureus* which were resistant to penicillin

were isolated as early as 1947[248], only a few years after mass production of penicillin began. Antibiotics are used to increase milk and egg production and muscle growth in animal crops but can be excreted from the kidneys at relatively high concentrations and leak into the soil and water in the environment[249]. In terms of humans, antibiotics are routinely over-prescribed and misused, and in many countries are readily available without a prescription[250]. Using the wrong type of antibiotics, or not completing a course of treatment until the infection has been fully cleared, can encourage the selection for resistant subgroups within a patient, and these can spread to other people, especially in a setting where many people are immune-compromised such as a hospital, prison, elderly care unit, drug rehabilitation centre, or nursery.

As mentioned in **Section 1.2.1**, *M. tuberculosis* is very capable of developing drug resistance, and some strains are now resistant to all readily available antibiotics[44–47]. The importance of using multiple antibiotics to treat TB was immediately apparent after the discovery of streptomycin by the Waksman group (see **Section 1.2.2**) as patients treated with streptomycin alone soon developed resistance to the drug, a mere handful of years after its discovery[251]. Although the standard treatment regime for TB includes drugs which are cheap, easily stored, and don't require intravenous or intramuscular injection, the length of treatment and the lack of obvious symptoms in patients means that they may stop taking their medication as soon as they feel better, rather than completing the full course[252].

Modern diagnosis pressures for tuberculosis often stem from a lack of familiarity with tuberculosis infections, particularly those which are non-pulmonary, and from little to no screening for infections. Misdiagnosis is also an issue for non-tuberculous Mycobacterial infections – most of the cases described in **Section 1.2.1.1** include a delay in diagnosis as the symptoms were unfamiliar or improper tests were performed to determine the infectious

agent. Historically, Western countries had widespread and efficient screening programmes to test large swathes of the population for tuberculosis. Mobile radiology units provided free, rapid, and simple x-rays of lungs to look for the signs of TB infections, and signage reminding to avoid spitting and coughing in public places were displayed in shops, public transport (see **Figure 1.4**), and alongside pavements. In recent times, transient radiology units have been used at airports and returned to high-risk inner-city communities. These units can also be taken to communities in developing nations to reach groups of people who would not be screened otherwise, such a rural farming communities, urban slums, construction sites[253], homeless camps and drug users[254]. Unfortunately, these communities are therefore also at risk of not taking their medication correctly.

In terms of the drug discovery efforts for novel Mycobacterial treatments, there are a number of drugs in the trial pipeline or newly licensed for use. Delamanid, which blocks the production of mycolic acids and creates an unstable cell wall, was released for medical use in 2014, and is considered a useful addition to treating MDR-TB. However, it has no statistically significant effect on treatment success compared to the placebo arm of the trial, where patients were given the "optimised background regimen" of existing drugs[255]. Equally, bedaquiline, which prevents Mycobacteria from producing ATP, has recently been permitted for medical use, and was also shown to have a small effect on curing patients over a background regimen[256]. Other treatments have reached phase II trials, but are either reiterations of existing compounds, or are unlikely to be studied further as they are toxic to the liver[257].

Bedaquiline and delamanid represent the first new anti-TB drugs to be approved for medical use for over 50 years, since rifampicin was first first marketed in the late 1960s (discussed in greater detail in **Section 1.2.2.3**). The discovery of new families of antibiotics has

dramatically decreased since the "golden era" of the 1950s and '60s, with only one new class of antibiotic discovered since the early 1990s[258]. The extent of the search for new antibiotics classes is discussed in **Section 4.1.2**.

Figure 1.4 – A sign used on Great Southern & Western Railways in Ireland to discourage spitting. Saliva contains TB bacteria in people with an active pulmonary infection, and spitting used to be very common, even in public places. Property of the Science Museum, London, and licenced under the Creative Commons Attribution (CC BY 4.0).

1.3 *Galleria mellonella* – the larvae of the Greater wax moth

1.3.1 What are *Galleria mellonella*?

Galleria mellonella larvae are recognised as a pest by apiarists across the world. They target the hives of honey bees, which are already in decline due to a combination of pressures including use of insecticides, habitat loss, and pathogens[259]. The larvae burrow into the honeycomb, damaging the structure of the comb and allowing honey to leak through the tunnels they have bored, but also eat honey, pollen, wax, and the larval bee brood. The larvae avoid being removed from the hive by consuming the contents of capped cells, where the bees cannot directly observe the larvae[260]. Other than the physical damage caused by the larvae tunnelling and consuming parts of the hive, the strands of silk the larvae leave in their tunnels physically entrap the bees, which then die from starvation. This can lead to the bees abandoning the hive, or colony collapse[259].

As the larvae grow and develop, they undergo a series of moulting stages before spinning a cocoon at the end of their final instar stage and undergoing metamorphosis into their moth form (see **Figure 1.5**). Moths will then go on to lay eggs in batches of 50 to 150 several times across 4 - 5 days, aiming to hide the eggs in cracks within the hive to reduce the risk of the bees locating and then removing the eggs. It can take as little as a month for an egg to become a moth, or as long as 6 months, depending on the temperature of the hive and the availability of food[261].

Under standard growth conditions, the moths take around six to eight weeks to complete their life cycle (see **Figure 1.5**). The larvae prefer to grow at 30 to 40 ^{o}C, but growth will halt completely at 10 ^{o}C[262]. They eat old wax, but will happily eat sawdust, plant matter, milk powder, honey[263] and have even been shown to consume polyethylene plastic[264] and the bottom of plastic 96-well plates[265]. When food is scarce, the larvae will turn to cannibalism[262]. It should

be noted that cannibalism was not observed at any point during the experiments reported in this thesis.

Galleria mellonella have been officially reported in at least 60 countries[259], although the likelihood that they are present in more countries is high, with reports beginning as early as the fourth century BC, where they were described by Aristotle in his *Historia Animalium*[262]. There is now evidence that they carry a number of pathogens[266], although there are anecdotal links to many more pathogens when consulting apiarist forums.

In terms of anatomy, the *G. mellonella* larval body looks to be divided into segments from the outside, but internally is essentially a continuous compartment[262]. The third of the larval body closest to the head contains the fat body and the heart, the aorta of which extends into the lower two thirds. The heart tissue is surrounded by pericardial cells. The middle third contains the alimentary canal, the main tracheal system, and the Malpighian tubules, which controls excretion and osmoregulation. The lowest third of the larvae is mostly fat body, which takes up a substantial portion of the full body cavity. The free spaces in the body contain blood, which passes through the heart and then freely circulates. The blood is pale yellow in colour, but quickly coagulates and turns black on contact with the air through melanisation. The larval fat body stores lipids provided by the larval diet, and also lipids synthesised from carbohydrates ingested by the larvae.

The larvae are freely available from a number of suppliers, with online shops making purchasing accessible through the postal service. Usually, these larvae are provided to use for fishing or to feed to exotic pets like snakes and lizards, but have been used by research groups in many countries to supply the larvae required for experimentation[267–269]. Hand raising a colony of larvae is also available but requires daily monitoring and care.

There are some issues with ordering larvae to be delivered through the post. Larvae have shown to be temperature sensitive[270] and to respond differently to infections after being vigorously shaken[271], so when parcels are transported in cold weather, left overnight in unheated warehouses, or disturbed in the postal vehicles, this may affect their response to infection and treatment. Also, the researcher has no control over the processes used to raise the larvae, such as the application of antibiotics or antifungals, variations in feeding, or the presence of parasites such as nematodes[272]. Starvation before use can affect the results of infection experiments[273], and the time since the last feed in uncertain. Also, the type of diet fed to the larvae seems to change the number of circulating immune cells in the haemolymph, and the response to infection[263]. Equally, many insect cultures are developed in the presence of antibiotics and these antibiotics have been shown to remain in the gut and affect digestive physiology and enzymes[274,275].

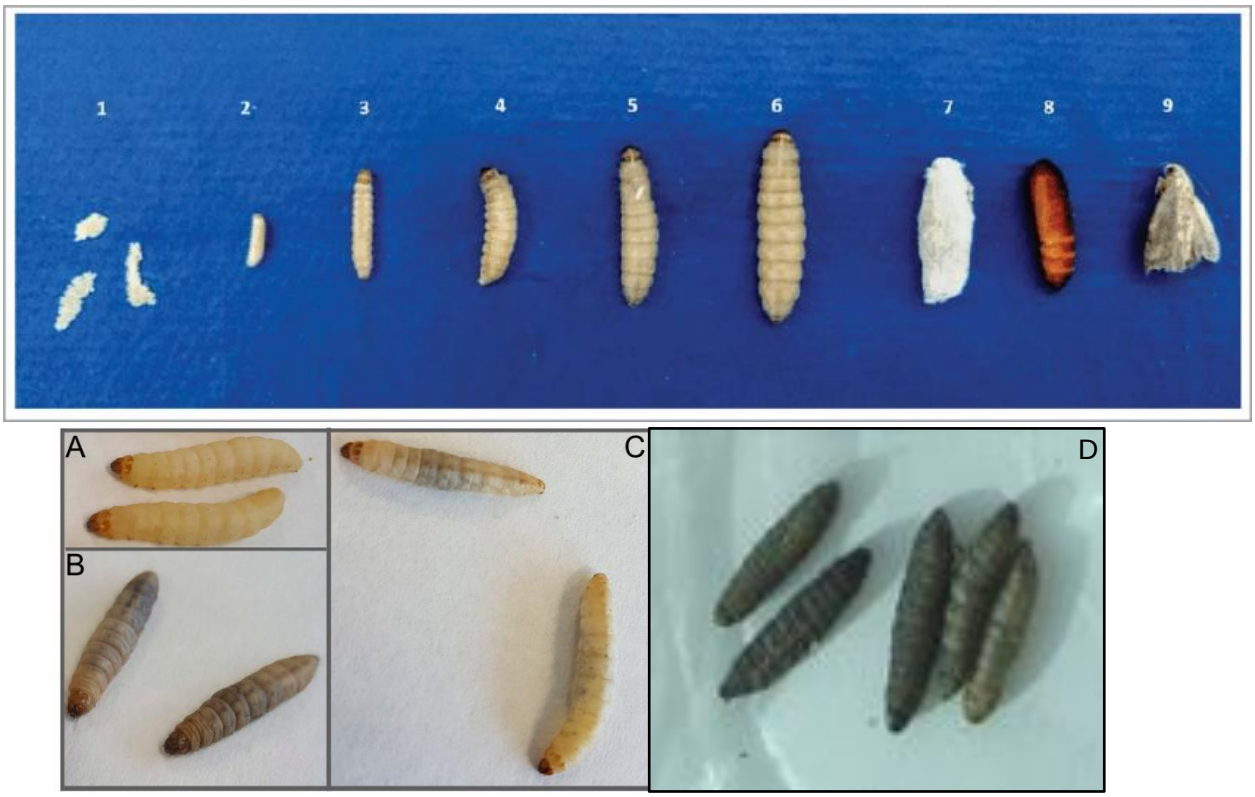

Figure 1.5 – The stages of the *Galleria mellonella* life cycle, from eggs (1) to moth (9) and in various stages of melanisation (A through D). Larvae of the appropriate weight for use in an experiment are generally in the final instar stage (6). Their ability to progress to the cocooning stage (8) is an indicator of health – larvae injected with saline can often form a cocoon over the observational period, but occasionally larvae which had been infected with bacteria and treated with an effective antibiotic dose would also be seen to cocoon. Portion of figure from Jorjão *et al.* (2018).

After an immune challenge, melanisations can be entirely absent (A) or only in some areas of the larval body (C). This often progresses to melanisation over the entire length of the body (B) and can eventually lead to heavy, black melanisation which is invariably associated with death (D). Stages A, B and C can be seen in larvae that are still alive and responsive to touch. Portion of figure from Kavanagh, K. & Sheehan, G (2018) and Tsai *et al.* (2015).

1.3.2 Moth larvae as a model organism

Insects have long been dissected and examined by natural philosophers. Robert Hooke's drawings of small insects such as fleas, gnats and flies, made possible by the novel invention of the microscope, brought the minute details of insect anatomy to a scale which both intrigued and shocked the world (Samuel Pepys wrote in his diaries that Hooke's volume of illustrations, *Micrographia,* was "*the most ingenious book that I ever read in my life*"[276]).

Tantalisingly, Gregor Mendel probably also worked on insects, and possibly even used them as a model organism for his studies on genetics. It is well documented that he wished to study genetics using mice, but the bishop in his Abbey was uncomfortable with a monk studying animal mating[277], so Mendel switched his studies to plants. It is also reported by multiple sources that he kept multiple bee colonies and hives[278,279], and no great leap of imagination to assume that he studied his bees as intently as he studied his pea plants. Considering that a major discovery in recent years was due to a researcher at Cambridge removing *G. mellonella* larvae from her beehives, keeping them in a plastic supermarket bag, then realising the larvae were eating the plastic bag[264], it seems that even casual contact with *G. mellonella* can lead to impromptu experiments.

However, the first modern use of insects as a model organism is in the late 1890s and early 1900s, with fruit flies (*Drosophila melanogaster*) used as models for genetic studies. Studies on the lifecycle of *G. mellonella* are recorded as early as 1737[280], although there will certainly have been a familiarity of this topic to any apiarist affected by the larvae. Papers on the anatomy[281], cell types[282,283], and phagocytic capabilities of haemocytes[282] in *G. mellonella* were published in end of the 19th century[262], yet the embrace of *G. mellonella* as a model organism occurred on a

similar timeline as *Drosophila,* with a respectable number of publications between 1900 and the 1940s.

However, there seems to have been a much lower rate of uptake compared to other model organisms, and other insect organisms such as *Drosophila*. Once *Drosophila* had an established record of high-profile publications, had been associated with Nobel prize winning research (as of 2019, eight Nobel prizes have been awarded for work using *Drosophila*), and become an established research tool in a large number of laboratories, *G. mellonella* was left behind as a research tool.

In tandem, models for infection and immunity were usually mice, rats, or other vertebrates. However, when the Laboratory Animal Welfare Act was passed in 1966 in the United States and the Animals (Scientific Procedures) Act passed in the United Kingdom in 1986, research laboratories turned to other model organisms to use in experiments as mammalian models became more expensive and logistically complicated to purchase and maintain. These pressures were compounded by the creation of the "3 Rs" in 1959[284] – replacement, reduction, and refinement – and the inclusions and implementation of these tenets into legislation, funding guidelines, and law.

Eventually, these compounding factors brought *G. mellonella* back into focus as a useful model organism for researchers wanting to find a useful bridge between *in vitro* and *in vivo* studies. Although *in vitro* and *in silico* studies are a key component of any drug discovery or development process, the leap between these models and experiments using a whole, multicellular organism can be surprisingly wide. The interplay between an immune response, different tissue types, and circulatory systems is practically impossible to recreate in a Petri dish (although growing complex tissues and "organs" from stem cells is a rapidly developing

research field), but researchers require reasonable confidence in a potential new compound or drug combination before testing them in a vertebrate model. *Galleria mellonella* can be a useful tool for testing these compounds in a whole organism quickly, cheaply, and with minimal specialised equipment. If the results suggest the compound is worth testing further, then the larval data can be used as important supporting evidence to advance to vertebrate models.

Hence, after a hiatus of several decades, *G. mellonella* underwent a revival in the 1980s[285] as a model for *Pseudomonas aeruginosa*[286], *Proteus mirabilis*[287], *Escherichia coli* and *Bacillius cereus*[288]. *Galleria mellonella* have been used as an infection model for bacteria (*Escherichia coli*[289], *Legionella pneumophilia*[290], *Klebsiella pneumonia*[291], *Micrococcus luteus*[292], *Acinetobacter baumannii*[293] and *Listeria monocytogenes*[294] for example), for fungi (including *Aspergillus fumigatus*[295], *Cryptococcus neoformans*[267], *Saccharomyces cerevisiae*[296] and a number of *Candida* species[285]) and even for a number of viruses (*Bovine herpes simplex virus*-1[297] and others[298]), indicating its ease-of-use and adaptability to suit a researcher's topic of interest. Importantly, the correlation between the results in a *G. mellonella* model and mammalian models is now well established for a number of micro-organisms[267–269,299–302], although there are some strains and species which are exceptions and do not give similar results[301,303].

1.3.3 Adaptive versus innate immunity

All jawed vertebrates, or gnathostomata, have an adaptive immune system[304], which allows for a host to create an "immunological memory" of an initial exposure to an antigen in order to respond more rapidly and effectively when re-exposed. The immune cells associated with the adaptive immune system, the B and T lymphocytes, are also able to recognise the antigens presented by the body's own cells to prevent an improper immune response to the "self"[305]. Exposure to non-self antigens creates a community of memory B and T cells, which retain an

immunological memory of the antigen so then, if recognised again in the future, these cells can rapidly differentiate into effector cells to respond to the infection.

The specific response to a foreign antigen varies depending on the target. Cytotoxic T cells release cytotoxins which target the foreign cell's plasma membrane, allowing an influx of water into the cells which then lyse, or causing apoptosis[305]. The remains of the lysed cells are then cleared by phagocytic cells such as macrophages. Alongside this, B cells produce antibodies which circulate in the lymph and blood plasma of the host and are specific to the antigens of the foreign cells. These antibodies can have a myriad of uses including binding multiple foreign cells into clump via agglutination, making foreign cells easier for phagocytic cells to recognise via opsonisation, or blocking the processes which these cells use to bind to surfaces like mucosal membranes that can be used as points of entry into the body[306].

Obviously, the adaptive immune system is found in animals which are higher in the evolutionary tree. The immune system used by plants, fungi and insects is the innate immune system, which differs from the adaptive immune in two major ways – the innate system is non-specific and responds in the same manner to all pathogens, and it does not create a "memory" of pathogens it has been exposed to previously to aid in responding promptly when exposed again. Yet the innate immune system is vital to all living organisms and is just as successful and intricate as the adaptive immune system. A key component of the innate immune system, the inflammatory response, is not only incredibly rapid but also highly effective[307]. Organisms can also recruit a variety of immune cells with a range of roles, and utilise a range of antimicrobial peptides[307].

In terms of *Galleria mellonella,* their immune response is usually divided into two sections – cellular and humoral responses. The immune cells in *G. mellonella* are called haemocytes,

which have roles in coagulation, nodulation, phagocytosis, encapsulation, and melanisation[308,309]. The humoral responses are antimicrobial peptides, opsonins, lytic enzymes, and a phenoloxidase enzyme which catalyses the formation of melanin[309]. Both components of the larval immune system will be described in **Chapter Five** but will also be reviewed here.

Although direct parallels may not be drawn between vertebrate white blood cells and invertebrate haemocytes, there are shared behaviours which are of interest to researchers. Both cell groups are important factors in wound repair[310,311], cell clustering around foreign bodies[312,313], innate immunity[313,314], phagocytosis[315] and production of reactive oxygen species (ROS) as a defence mechanism[314,316]. There are a variety of different types of haemocytes in invertebrates, with the most common being prohaemocytes, plasmatocytes, granular cells, spherule cells, and oenocytoids[317] (see **Figure 1.6**). The percentages of each type varying between species, within species, and even during the life cycle of a single individual[318] and these five haemocyte types have been recorded in all lepidopteran species[319].

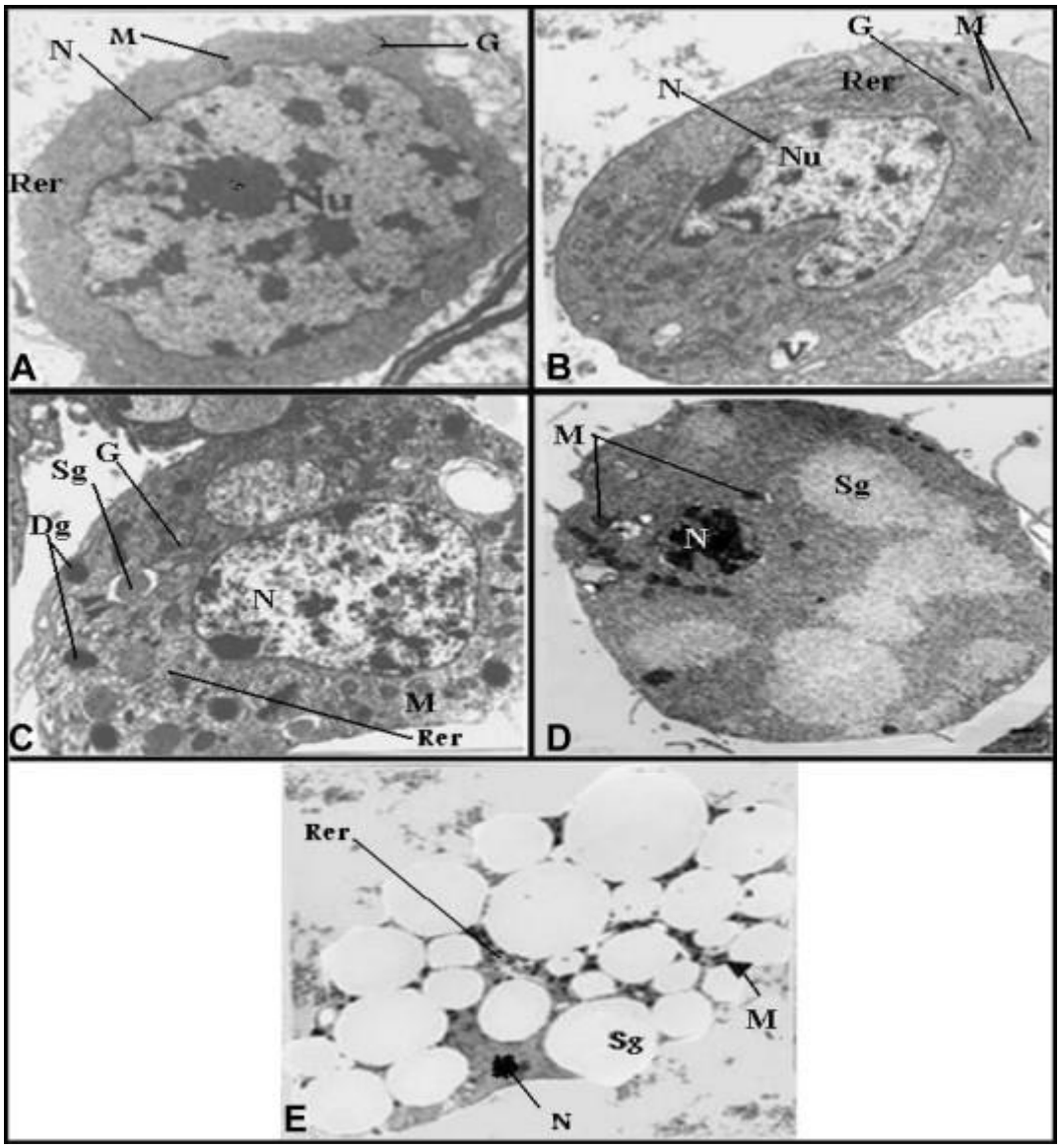

Figure 1.6 – The five different haemocyte types seen in final instar *Galleria mellonella* larvae. (TEM mag. = 12 Kx, bar: 2 nm). (A) prohaemocytes; (B) plasmatocytes; (C) granulocytes; (D) oenocytoids; (E) spherulocytes. N/Nu, nucleus; Rer, rough endoplasmic reticulum; M, mitochondria; G, Golgi body; Dg, dense granules; Sg, structured granules; V, vacuole. Figure from Salem *et al.* (2014).

Prohaemocytes (**Figure 1.6 A**) are analogous to haemopoietic stem cells, although the discussion on whether haemocytes primarily arise through mitosis or from haemopoietic tissues has been debated for many decades[318,319].

Plasmatocytes (**Figure 1.6** B) can have quite a variable morphology and degrees of granulation depending on the insect, and the percentage of haemocytes that are plasmatocytes varies even within insects of the same species[318]. Usually they represent 30 – 60% of the haemocyte population. They have a variety of roles within the insect – phagocytosis, encapsulation, wound repair, and nodule formation to name a few[318,319]. They can change their shape readily, and when binding to surfaces such as glass slides can become ameboid in appearance[319].

Granulocytes, or granular cells (**Figure 1.6 C**), are readily identified in *G. mellonella* as they are packed with granules and maintain a round or oval shape[319], unlike the plasmatocytes. They also represent 30 – 60% of the haemocyte population[318], and their primary role is in phagocytosis[320]. They are often the first type of cell to come into contact with a foreign microorganism or object inside the larvae, at which point they release their granular contents and assist in attracting plasmatocytes to the area[320] which shares some homology with the role of effector cells in vertebrate organisms. The cells then work in tandem to create a capsule or nodule around the foreign object. Hence, plasmatocytes and granulocytes in *G. mellonella* are generally regarded as the defensive component of the insect immune system[319].

Oenocytoids (**Figure 1.6 D**) are, in contrast, present in very small amounts in Lepidoptera[318], accounting for only 1 – 2% of the haemocyte population. They are large (up to 30 μm across) and identified by their erratic nucleus and fragile membrane. The oenocytoids isolated form *G. mellonella* can survive *in vitro*, but oenocytoids from other insects can lyse and

rupture minutes after extraction[320]. In *G. mellonella*, one of the phenoloxidases which synthesises melanin is found in these haemocytes. This enzyme is then released into the haemolymph, leading to spontaneous polymerization and formation of insoluble melanin[319]. Some groups have also shown oenocytoids to be capable of phagocytosis[321], however their low numbers mean that their efficacy in helping to clear an infection is likely minimal.

Surprisingly, the spherulocytes (**Figure 1.6 E**) are still poorly understood and their specific role in the immune system is unclear. They are present in reasonably low percentages, around 5%[318], and are filled with spherical inclusions that have very variable sizes within the cell. These inclusions have been observed to disappear when larvae begin to produce silk or start pupation[319].

In terms of the humoral aspects of the *G. mellonella* innate immune system, they can produce a very robust response, with production of at least 18 antimicrobial peptides[230,322], lytic enzymes and opsonins[309].

The antimicrobial peptides (AMPs) usually work by disrupting and destabilising the membranes of foreign cells, either by forming pores or by interfering with potentials across the membrane[323]. They can be very small proteins of only a handful of amino acids, or they can be much larger, more complicated proteins[323]. AMPs are mainly produced within the fat body and then released into the haemolymph[309], and act in synergy with lysozymes[324] against a broad spectrum of pathogens including fungi, bacteria, parasites and viruses[325]. For bacteria, this interaction allows lysozymes to damage bacterial cell wall by hydrolysing β-1,4 linkages[309].

This action is related to the production of opsonin proteins by *G. mellonella* post-infection – opsonins also recognise the foreign components of bacterial and fungal cells walls. Most opsonins recognise lipopolysaccharides (LPS), peptidoglycans, and β-1,3-glucan[309], which is a

similar action to pattern recognition receptors in mammals[326]. Once opsonins have bound to a bacterial or fungal cell, phagocytosis and encapsulation of the foreign cell occurs[326], and opsonins may increase the permeabilising activity of lysozymes[292].

In terms of pathogen recognition by haemocytes, pathogens recognition is made through calreticulin and apolipophorin[325]. Calreticulin is expressed on the surface of haemocytes, and shows a high degree of homology to mammalian calreticulin[327]. Apolipophorin is a lipid binding protein, allowing it to bind to LPS, and also has homologs in mammals[328]. It also enhances the activity of lysozymes [329].

1.3.4 A larval genome

The most commonly used insect model for genomic studies is *Drosophila melanogaster*, with at least eight Nobel prizes awarded for work associated with *D. melanogaster* as of 2019. More than 90% of their homobox genes have homology to a human equivalent[330], mutations in many genes associated with cancer can be studied in fruit flies[330], and around 75% of genes which cause disease in humans have a functional homolog in fruit flies[331]. Much of this work only became feasible once researchers had access to a sufficient portion of the fly genome, and *D. melanogaster* was the first complex organism to have its genome sequenced and published[227]. Sequencing the genomes of the nematode *Caenorhabditis elegans* and the Zebrafish *Danio rerio* also made these models more attractive as a model organism[331]. Now that a genome sequence for *G. mellonella* was published[332] in 2018, it will be interesting to see how this new resource will be applied in future studies, and if *G. mellonella* will eventually become as commonly used for genetic studies as fruit flies, nematodes, and Zebrafish. The paper reporting the *G. mellonella* genome has been cited 28 times as of the writing of this thesis.

1.3.5 Advantages and limitations of the model

As mentioned in several sections previously, the most immediate advantages of using *Galleria mellonella* as a model organism are their low cost, ease of use, simple maintenance and storage, rapidity of experimentation, and that they are readily accessible to anyone with a computer and a postal address. There are no governmental restrictions and no limitations on the number of larvae that can be used, unlike with higher organisms.

The larvae are large enough to be easily injected, dissected, or fixed for staining, but not so large that they require specialised housing, feeding or care. Unlike other popular invertebrate model organisms such as fruit flies or nematode worms, individual larvae can be examined for survival without the use of a microscope. *Drosophila* larvae are less than 10 mm in length, and *C. elegans* are about 1 mm long, so may not be assessed as easily.

Galleria mellonella larvae can survive at 37 ^{o}C for the length of a standard experiment, which is a key advantage over fruit flies[333] or nematode worms[334], who cannot survive extended periods at this temperature. Allowing experiments to be performed at 37 ^{o}C allows for temperature dependant virulence factors to be expressed[325,335]. This is especially important for microorganisms such as dimorphic fungi, which have different morphologies depending on temperature and in some cases (such as *Talaromyces marneffei*) are much more harmful to a host when at 37 ^{o}C[336]. However bacteria also have a variety of temperature dependant virulence factors[337], and *M. tuberculosis* has been shown to have a range of genes which are upregulated when at an increased temperature[338]. Hence, using a microorganism at a temperature close to human body temperature when possible is a key benefit to this model.

However, some aspects of this model are simultaneously an advantage and a drawback. As mentioned in **Section 1.3.1**, ordering the larvae through the post means that they are readily

available to researchers and the materials required to run an experiment can be easily accessed in a short period of time. However, the larvae are likely subjected to physical damage of an unknown duration and intensity, and there is no control over the temperatures that the larvae are kept at. On a related note, the selection of the larvae for experimentation is entirely the responsibility of the researcher – larvae are selected which are cream, free from melanisation, and are responsive to touch. However, if the larvae have been exposed to temperature fluctuations, they can be compromised without any physical evidence that they are unsuitable for injecting (personal observations, unpublished).

As shown in **Section 2.3.1.3**, larvae injected with *M. aurum* are totally unaffected by infection with this *Mycobacterium* – they show 100% survival for at least 144 hours post-infection, even when injected with the equivalent of 3,000,000 bacteria (see **Figure 2.7**). In tandem with this, the larvae also show no evidence of melanisation, reduction of movement, or any outward signs of immune response. This clearly shows that larvae which have been infected with bacteria, even a large number of bacteria, can look comparable to uninfected, uncompromised larvae. As described in **Section 7.2.2**, the larvae used throughout this work are raised without antibiotics, meaning that there is no guarantee that they are not incubating a bacterial (or even a viral) infection when sent out for delivery. If the larvae are compromised with a pre-existing infection, there may be no visible, recognisable response meaning that impaired larvae could be used for experimentation.

Another limitation of *G. mellonella* is the variability between stocks. There is no standard protocol for raising and feeding the larval broods, and the variations in diet[273] and environment[270,271] could easily affect the results of experiments. This also means that there is no guarantee that the selective agar plates used in this thesis will be effective for other

researchers, as their larval stocks are likely to have a different internal bacteria depending on the microbes they have been exposed to.

There have been efforts to create a more standardised system for raising larvae – a notable company in this area is BioSystems Technology who sell "research grade" larvae which can be ordered online and delivered via courier with "thermo packaging". These larvae are raised with no antibiotics or hormones, and are described as "decontaminated", which should mean that any control larvae injected with saline should remain alive during the experiment. However, the larvae are not described as sterile, with the assumption that they still have a microbial community in their gut, giving the same issues of using selective plates for certain experiments. Equally, the same issues with physical damage during shipping will still be applicable. Although the use of packaging which tries to moderate temperatures is an advantage over normal larvae suppliers, the effectiveness of this packaging when parcels are left over the weekend in an unheated warehouse until Monday is questionable, especially during the winter months when temperatures overnight are often below 0 ^{o}C.

In terms of the claims that "fish bait" larvae (such as the larvae used in this thesis) will always have at least one death in the mock-infected control groups, this statement is not aligned with the results in personal experiments. Occasionally there would be deaths in the control group, but this was usually traced to contaminated saline, equipment malfunction (a blockage in the syringe can lead to an injection of air into the larvae, which usually leads to death), or the entire order of larvae being unusable from improper shipping or exposure to low temperatures during shipping. All of these issues are equally possible with the larvae provided by BioSystems Technology. As of Autumn, 2019, an order of 100 larvae from BioSystems

Technology costs £132.00, whereas an order of 300 larvae (of which 100 or so will be suitable for experimentation) from a standard supplier is less than £10.

In terms of experimental design, there are largely two methods which are used to introduce microorganisms to the larvae – injections directly into the larval body, or force-feeding. Both techniques, when used correctly, allow a researcher to accurately, reliably and rapidly introduce a known number of cells, viral particles, or concentration of chemicals to every larvae, which is a key advantage when using this model. Injection is a more popular technique and can allow a large number of larvae to be injected more rapidly than force-feeding. However, improper injection can allow liquids to leak from the larval body, reducing the concentration of cells or chemicals inside the larvae. If sufficient larval haemolymph leaks from the larval body, the larvae will die. Equally, every injection will cause a degree of physical trauma to the larvae, regardless of how well the researcher performs the injections.

In contrast, force-feeding is less common, but should reduce the physical trauma to the larvae. They are held to a blunt-ended syringe, aiming between the mandibles, and a fixed volume of liquid is slowly forced through the syringe. Depending on how accurate the researcher can be in targeting the oral cavity there should not be any leakage, allowing for the same accurate and reliable introduction of a set concentration of cells or chemicals into the larvae as seen with injection. However physical trauma can still be introduced by holding the larvae too firmly, or mis-locating the mandibles and causing trauma to the head of the larvae.

A key difference between force-feeding and injecting the larvae is beautifully demonstrated by Coates *et al*[339], who performed both techniques with 20 μL of 0.4% trypan blue (see **Figure 1.7**). For the injected larvae, blue dye is spreads within the entire body cavity within a few seconds, and the entire larvae is blue after a few minutes. The fed larvae clearly

contain blue dye, but the blue is entirely isolated to the intestinal tract, primarily the midgut. Hence, it may seem logical that larvae which are injected with a microorganism may have a lower survival rate than those which are force-fed, as the researcher is essentially mimicking a systemic, whole-body infection. However, the larval gut has been shown to readily leak solutions into the body cavity[340], and larvae which have been force-fed bacteria have been shown to have viable bacteria in their body cavity 24 hours post-infection[341]. Studies where bacteria were injected with okadaic acid, which causes larval death, showed no difference in survival between larvae that were injected and those that were force-fed[339]. Therefore, the differences in survival depending on the method of infection are unclear.

In addition to these points, researchers should be aware of the role that the larval microbiome may play in affecting experimental outcomes. Researchers have shown that the bacteria present in the gut of *G. mellonella* can suppress the growth of other bacteria which are consumed in the food provided to the larvae[342]. The suppression is non-specific and was shown to effectively eliminate Gram-positive and Gram-negative bacteria from the digestive tract. Obviously, this suggests that injection of the bacteria into the larvae may be more suitable, as it contravenes any issues with the larval microbiome reducing the bacterial burden.

There have been a number of publications where an alternative method of infection has been used – including microorganisms in the food provided to the larvae, and allowing them to consume the contaminated food at will[343,344]. This allows an infection to be introduced into the model with no physical trauma to the larvae, and as with the force-feeding method[341], bacteria given orally are later found in the larval body cavity[343]. It is also more akin to how larvae would be infected in the natural environment, and control groups can be fed a diet which is bacteria free to determine whether changes between the experimental and control groups are due to

the contaminated food. However, there is no method to control the consumption of the food and, by extension, the number of microorganisms ingested by the larvae. Although the researcher can increase the concentration of microorganisms in the food, which would lead to larvae consuming larger numbers of bacteria than those provided with food containing a lower concentration of microorganisms.

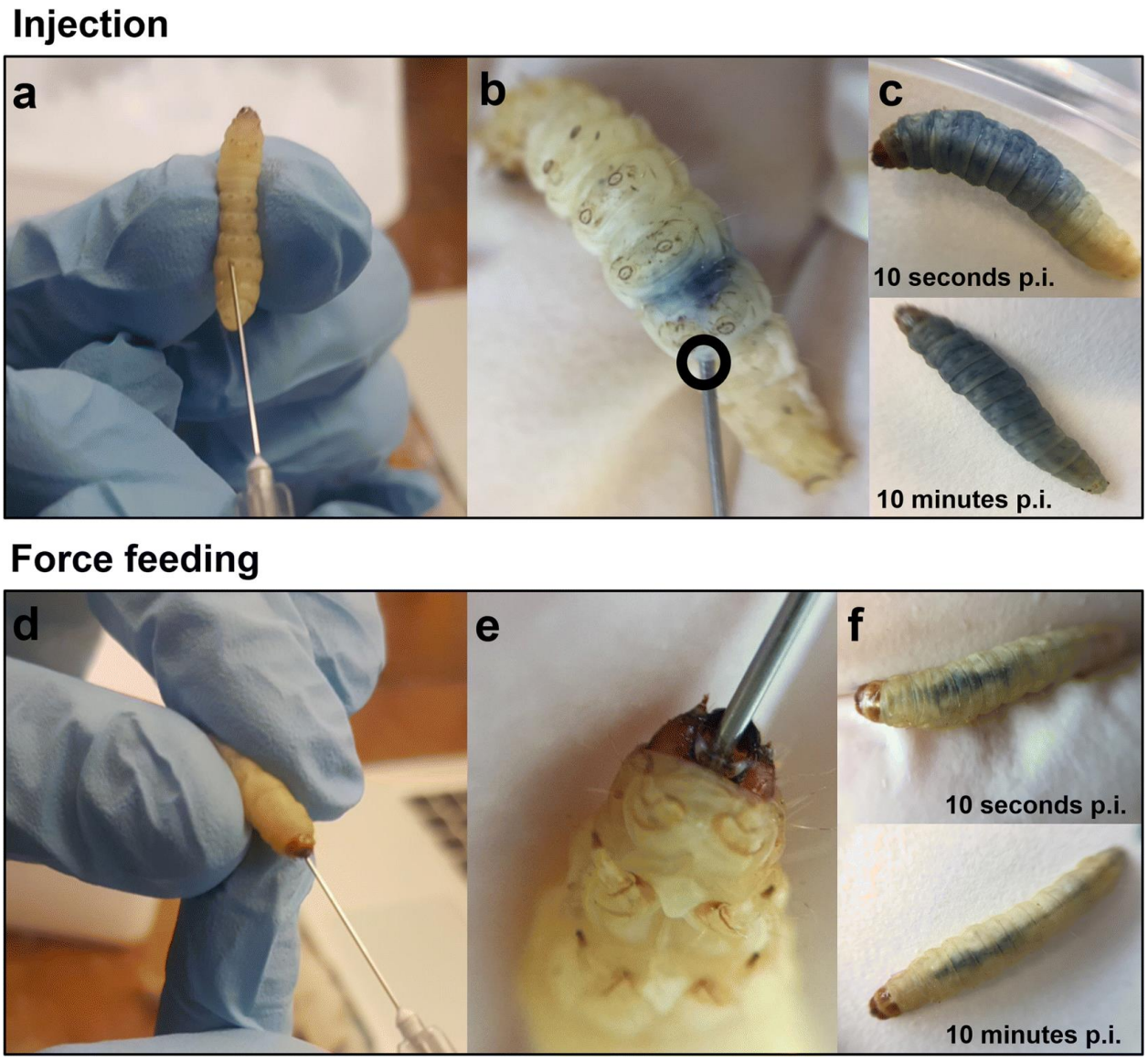

Figure 1.7 – Spreading of blue colour through larval body depending on method of exposure. Larvae were injected (panels a and b) or force-fed (panels d and e) with 20 µL of 0.4% (w/v) Trypan blue and assessed 10 seconds and 10 minutes post-inoculation (p.i, panels c and f). The blue dye is clearly restricted to the gut of the larvae after force feeding, whereas it has diffused throughout the entire larval body after injection. Figure from Coates *et al.* (2019).

1.4 Existing models for Mycobacterial infections

The use of models, whether they are *in silico, in vitro,* or *in vivo*, to understand and develop treatments for bacterial, fungal, and viral infections has become a hallmark of modern science and medicine, starting from Gregor Mendel's first forays into genetic studies with pea plants.

1.4.1 *In silico* models

In the 21st century, the computer has become an indispensable tool in most aspects of professional research and investigation, not simply for sharing data and papers with international collaborators and journals, but also for running computer-based experiments and models. Obviously, high-power analysis and modelling only became a viable option once computers became capable of holding and manipulating large quantities of inputted data, but computer models have become a valuable means for epidemiological and drug discovery studies.

Mycobacteria are an interesting component to asses in epidemiological models as many people can be asymptomatic, or have latent infections, for long periods (months or years, rather than hours or days) before becoming "active" infections or capable of spreading the bacteria to others. This adds many layers of complexity to predictive epidemiological studies[345], and the balance between latent and active carriers, and the triggers that can increase the percentage of active cases.

Equally, modelling the interactions of the bacteria and a host immune system and tissues can be very informative for Mycobacterial infections. Mycobacteria are able to isolate themselves in complex granuloma structures (see **Section 1.2.3**) which isolates them from circulating immune cells and can also grow very slowly. Both of these factors can be modelled

in silico, in terms of how this affects host-pathogen interactions, how granulomas form, which agents can penetrate their structure, and clearance patterns[346,347]. Computer models can also be used to run virtual clinical trials, identify drug targets, and optimise antibiotic treatment protocols[348].

1.4.2 *In vitro* models

In vitro models allow researchers to isolate and investigate fundamental pathways, processes, or interactions in an environment where multiple parameters, components, or conditions can be manipulated with great control by the researcher.

Some of these experiments may be logically simple. For example, Lawrence Wayne noted in the mid-1970s that *M. tuberculosis* cultures would not grow when settled in a flask, as the concentration of dissolved O_2 was decreased, but growth could be restarted if the flasks were shaken to increase the dO_2 concentration[349]. This was a very simple *in vitro* model for the hypoxia *M. tuberculosis* bacteria experience when in a granuloma and was the experiment which showed that *M. tuberculosis* grows in response to an oxygen gradient. Wayne went on to develop "the Wayne model", which showed that when oxygen saturation is at 1% the bacteria no longer divide but are still actively producing ATP in high levels and repairing DNA. The bacteria remain viable, even at 0.06% oxygen saturation[350]. Interestingly, this paper also showed that some antibiotics lost all efficacy at killing *M. tuberculosis* bacteria in these low oxygen conditions, but other antibiotics which were ineffective at high oxygen levels only became bactericidal only at 0.06% dO_2. This model has since been adapted for high-throughput screening assay to test the MIC of compounds at decreasing oxygen concentrations, and for analysis of the changes in gene regulation in these different conditions[351].

Other *in vitro* models take advantage of the ability to grow cells in culture, such as human macrophages. These models can be used to assess the rates of phagocytosis, production of reactive oxygen species, secretion of cytokines[352] , apoptosis, and necroptosis[353]. Other cell cultures of interest are alveolar pneumocyte epithelial cells[354] (which line the air sacs in lungs), macrophages isolated from blood[354,355], macrophages differentiated from monocytes[355] (white blood cells which can also become dendritic cells), and T cells[355]. These models are widely used, popular, and have been used in thousands of research papers.

More complicated tissue models such as 3D lung epithelial models have also been used to study Mycobacteria *in vitro*[356]. A new tool called "organoids" is also being applied to TB infections, which make use of microfluidics, 3D matrix scaffolding[357], and even 3D printing[358]. Organoids are miniature, simplified organs produced from embryonic or induced pluripotent stem cells which can develop complex, multi-levelled tissues, and acquire the cellular organisation and function of the tissue they were isolated from[359]. They have been used to assess the initial interactions between *M. tuberculosis* bacteria and lung epithelial cells, and one day researchers may be able to grow organoids which can represent the organs of a person with HIV, or with other compounding factors such as diabetes, smoking, or malnutrition[357] to assess how they affect the disease phenotype or treatment protocols. However, because they rely on stem cells, organoids have a very low rate of reproducibility, and the experiments cannot be effectively scaled up[360]. Constructing 3D organs is still an expensive and time-consuming protocol, and unfortunately organoids are unlikely to become feasible in standard research laboratories for many years.

1.4.3 Vertebrate models

As mentioned in **Section 1.2.3**, a number of vertebrate models have been used to model Mycobacterial infections, including mice, rabbits, guinea pigs and primates. Obviously, as these model organisms have similar anatomy and immune responses to humans, the represent an advantage over models which lack homologs to lungs or other organs, blood, lymph, or soft tissues like skin. However, other vertebrates which lack some of these traits, such as Zebrafish, can still be invaluable. A Zebrafish model for granulomas using *M. marinum* as an alternate *Mycobacterium* to *M. tuberculosis* is an important tool for researchers, and has many advantages over other traditional vertebrate models[213,218,361].

However, the use of mice still dominates the literature[357], and many research institutes have the facilities to work with mice but not fish. This is despite mice not being a natural host for *M. tuberculosis*, and there being marked differences in disease progression and pathologies depending on the strain of mouse used[362,363]. The route of infection and the microbiome of the mouse have also been suggested to impact on the experimental results[357]. Mouse granulomas show very little homology with those seen in humans, and a general inability to model chronic infections limits the scope of experiments[215].

Guinea pigs have a well-established use as a model for Mycobacterial infections, and were used by Robert Koch to determine his Four Postulates[364]. They are very susceptible to infections with *M. tuberculosis*, especially in aerosol[365], where as few as five bacterial cells can cause death[364]. Guinea pigs have been used for a variety of experimental purposes, including vaccine development, drug development, characterisation of TB, and virulence studies[364]. They also form well-organised granulomas which progress to necrotic stages in a manner which is similar to humans[215]. However, there are severe limitations to the number of experimental protocols

that can be applied to the model[215] and there is a far smaller range of genetic varieties to work with[357].

The third most popular vertebrate model for TB infections[357] are rabbits which, like mice, can exhibit different results depending on the rabbit genotype used[366]. However, they can show very similar disease phenotypes to humans, showing development of granulomas and necrotic or caseous granulomas, and give useful results when used with *M. bovis*[367], which is a lower risk pathogen for researchers to use. Yet, as with guinea pigs, the low number of experimental protocols available constrains the data which researchers can collect from this model.

Of course, the animals which show the strongest homology with humans in terms of anatomy, immune response, and general disease development are monkeys and non-human primates. There are many parallels in granuloma and lung cavity formation, development of non-pulmonary infections, and in co-infection with simian immunodeficiency virus, an equivalent to HIV[215,366]. Importantly, these animals are the only true models for latent TB[368]. Yet experiments which should lead to the death of the subjects are far more expensive and impractical with monkeys and primates than with other vertebrates as the costs of housing, maintenance and care are so high. These experiments will also attract the attention of animal-welfare groups and the media, often in a negative context. Hence, papers on TB research utilising primates represent a mere 1% of publications[357].

Equally, Zebrafish are also used in 1% of TB research publications[357], although considering they are far more amenable to larger scale experiments and are subject to far fewer restrictions than primates so are likely to increase in popularity in the future. As *M. marinum* is a bacterium which primarily infects fish and amphibians in the natural environment[369], there is no need to create mutant or inbred strains of Zebrafish to allow for an infection – they are naturally

susceptible already, and will fall to predictable patterns of morbidity and mortality. Hence, they will produce necrotising granulomas when infected with *M. marinum*[370], which is a very desirable trait. Being able to use Zebrafish embryos also allows easy use of fluorescent reagents, as the embryos are transparent and fluorescence can be assessed with ease[215].

1.4.4 Invertebrate models

As mentioned in **Section 1.2.3**, fruit flies can be a useful tool in assessing phagocytosis and intracellular survival of *M. marinum*[226], which is a key mechanism that Mycobacteria use to evade the immune system of their host and establish a latent infection. Fruit flies also have the advantage of a wealth of genetic tools and protocols to use, and they are easy to raise and maintain[371]. However, as mentioned in **Section 1.2.3**, there are limitations to the model, and the number of publications using *D. melanogaster* as a model for Mycobacterial infections is surprisingly low, especially considering the popularity of this model elsewhere.

Equally, nematode worms are not a widely used model for Mycobacteria, although there some groups who are using them with *M. marinum*[221] and *M. avium*[220,223]. There is some intriguing work in using them as a diagnostic tool to detect volatile compounds indicative of a TB infection from patient sputum samples[372], yet if nematodes are to become a more valuable tool for modelling Mycobacterial infections then further investigations are required – much as is the case with *G. mellonella*.

Another resource which can be used to model phagocytosis of Mycobacteria is the single celled slime mould amoeba *Dictyostelium discoideum.* Often described as a "professional phagocyte", these cells have many molecular features which are homologous with mammalian macrophages[373], a fully sequenced genome, and a variety of genetic transformation methods[374]. Although the use of this model in combination with Mycobacteria has been relatively recent, in

2002 *D. discoiduem* was shown to phagocytose *M. avium,* which were then capable of intracellular growth within their host cells and eventually triggering cell lysis[375], and these results were soon repeated with *M. marinum*[376], which showed similar patterns of phagocytosis, growth and lysis. Intriguingly, this model also showed that, when clumps of *M. marinum* are phagocytosed, these clumped Mycobacteria cannot replicate within the amoeba, and remain dormant[376] . Although slime moulds cannot replicate a granuloma, dormant Mycobacteria than are undigested and not eradicated from a host phagocytic cells are akin to the behaviour of Mycobacteria within a granuloma.

1.5 *Galleria mellonella* as a model for Mycobacteria

Previous experiments which have used *G. mellonella* as a host for Mycobacterial infections are described in more detail in **Section 2.1** – briefly, there are two researchers who used this host-bacterium combination, one in the early 1900s and the other in the 1930s. There has also been an investigation into the effects of lipidolytic enzymes harvested from *G. mellonella* on *M. tuberculosis* bacteria[377]. Hence, this thesis represents the first thorough investigation into the use of *G. mellonella* as a host and model for *Mycobacterium* infections in the modern era.

Since commencement, a group at Imperial College in London have also begun using *G. mellonella* as a model for Mycobacterial infections, although making use of *M. bovis* in place of *M. tuberculosis*[378,379], funded by the National Centre for Replacement, Refinement and Reduction of Animals in Research. Larvae were infected with increasing concentrations of *M. bovis* BCG bacteria and survival was assessed over a 96 hour observational window, unlike the 144 hours utilised in this thesis, although larvae were also incubated at 37 ^{o}C. Lower concentrations of bacteria (1 x 10^5 and 1 x 10^6 colony forming units (c.f.u) per mL) did not cause any larvae death, but 2 x 10^7 c.f.u/mL gave 100% mortality at 96 hours. This group also performed experiments

assessing the phagocytosis of *M. bovis* by larval haemocytes, and formation of granuloma-like structures. These results will be discussed in more detail in the opening of **Chapter Five**, as similar experiments were undertaken as part of this thesis**.**

Another group based in Israel have also used *G. mellonella* in combination with Mycobacteria – in this case, *M. abscessus*[380,381]*.* They showed that the type strain for *M. abscessus* and a clinical strain can both cause larval mortality when infected with 1 x 10^3 c.f.u/mL bacteria, although larvae were observed for 15 days.

1.6 Testing toxicity, novel compounds and new drug combinations using *G. mellonella*

Due to the ease with which experiments can be designed and performed with *G. mellonella,* testing an unknown but potentially useful compound or drug combination requires minimal effort or preparation to a researcher.

An interesting application of the larvae is to assess the toxicity of compounds unrelated to medical treatments or infections, such as food additives[382–384], biocides, pesticides, cosmetic additives[385], and other chemicals[339,386]. They have even been used to assess the safety of Gram-negative bacteria associated with traditional French cheeses[387]. Larvae often produce melanin when exhibiting an immune response, even if a compound or pathogen does not cause larval death. It may be valuable to measure the amount of melanisation seen in the larvae as an indicator of the level of an immune response. There are a variety of techniques which can be utilised to assess melanisation, including simple image analysis[388], a more complicated high-throughput image analysis[389], a "health index score"[390] or similar scoring system[391], or analysis of the larval haemolymph[339] and fixed sections[392]. If a compound of interest, especially one

which will be ingested or injected, triggers melanisation in *G. mellonella*, this could be useful information for further studies using higher organisms.

In a similar context, *G. mellonella* have been shown to be a viable model for pharmacokinetic and pharmacodynamic studies[393–395], which would also be a valuable tool for informing future experiments examining new compounds or treatment combinations, or providing results which can be used to obtain funding and permissions for animal experiments. Clinical trials could benefit greatly from including *G. mellonella* studies as a simple, rapid stage between testing treatments *in vitro* and using multi-cellular, whole organism models. This would also be in alignment with the 3Rs – reduction, replacement and refinement of model organisms[385,396].

As discussed in **Sections 1.2.1, 1.2.5, 3.2.2** and **4.1**, antibiotic resistance from a range of Mycobacteria is an ever-present burden on healthcare providers and represents a great risk as antibiotic resistance increases while the development of new antibiotic classes stagnates. Although we will never return to the position of having no usable antibiotics whatsoever for all common infections, it is clear that efforts to assess compound libraries (**Section 1.2.2.3**) and other molecules of interest, including antimicrobial peptides and plant phytochemicals (**Section 4.1.2**), will be an important part of finding new antibiotic families. Experiments utilising *Galleria mellonella* have low running cost, can be used for high speed assessments of pharmacokinetics and pharmacodynamics for novel compounds, and could be an invaluable resource for testing potential drugs or combination treatments. This application will be discussed further in **Chapter Four**.

1.7 Aims and objectives

The initial, and most important, aim of this thesis was to demonstrate that *Galleria mellonella* are susceptible to infections with non-tuberculous Mycobacteria in a dose-dependent manner (**Chapter Two**). Larval death would be required, if not at all bacterial concentrations then certainly at the highest concentrations, to allow for survival to be "rescued" when appropriate doses of effective antibiotics are administered.

Initially, "standard" antibiotics for treatment of Mycobacterial infections were used to improve larval survival (**Chapter Three**) both in single and combination doses. The effect of these antibiotics on the internal bacterial burden within the larvae was also assessed. Once these antibiotics had been investigated a range of non-standard compounds including efflux pump inhibitors, β lactam/β lactamase inhibitor combinations, and anti-psychotics were assessed both alone and in combination with standard anti-Mycobacterials (**Chapter Four**).

Finally, as the response of the host's immune cells to foreign *Mycobacterium* bacilli is a key component for models which use Mycobacteria, the responses of larval haemocytes were examined (**Chapter Five**). The changes in circulating haemocyte numbers, the formation and enumeration of nodules and melanised haemolymph, and the rates of phagocytosis were assessed, both with and without antibiotic treatment. The effects of dexamethasone, a corticosteroid used as an immunosuppressant in animal and *in vitro* models[397], were also examined, both in terms of the changes in larval survival and also for changes in the haemocyte responses to a Mycobacterial challenge.

Chapter Two

2.0 Establishing *Galleria mellonella* as a model for Mycobacteria infections

The aim of the experiments in this chapter was to demonstrate that *G. mellonella* are susceptible to infection with various Mycobacterial species, that their survival decreases across a 144-hour observation period, and that survival is negatively correlated to an increase in the number of bacterial cells injected.

2.1 Previous experiments using *Galleria mellonella* and Mycobacteria

Before commencement of this project, there were no modern research papers describing *G. mellonella* as a model organism for Mycobacterial infections. The lone paper in English that uses *G. mellonella* as a host for Mycobacteria was published in 1934 by Gordon Roy Cameron – "*Inflammation in the Caterpillars of Lepidoptera*"[262]. Cameron uses *M. tuberculosis* and *M. smegmatis* and assesses the phagocytosis of these bacteria by the insect immune cells.

The paper is very detailed and informative and contains key results such as post-infection changes in the numbers and types of haemocyte, interactions with the pericardial and fat body cells, formation of nodules, and persistence of these nodules in the pericardial cells and occasionally the fat cells. These nodules contain acid-fast bacteria in their centres, do not seem to be discharged from the larvae, and persist even through metamorphosis of the larvae into a moth, where they can be re-isolated and can produce tuberculosis if injected into guinea pigs.

Cameron refers to a French language paper – "*Infection microbienne et l'immunité chez la mite des abeilles Galleria mellonella*"[398] authored by Sergiĕĭ Ivanovich Metal'nikov. Sadly, a copy of this manuscript could not be located, but a review of the monograph in Nature is available[399]. It describes that "*the [larval] defence mechanism is shown to be similar to that of man and the*

higher animals, except that in the larvae the process is much more rapidly brought about, being a matter of hours only". In 1906, Metal'nikov also observed bacteriolysis of *Mycobacterium tuberculosis* in the haemolymph of Galleria[400].

These papers are not readily available due to their age, and in this sense, this topic could be approached as a blank slate, as it had not been studied in the 21st century or the late 20th century. As discussed in **Chapter One**, other non-mammalian models have been used to model Mycobacterial infections, although invertebrate models have not been utilised to the degree that is seen with other microorganisms, such as *Candida* yeasts or nosocomial bacteria. Yet the use of invertebrate models in a more generalised sense seems to be a natural progression in the study of Mycobacteria – including, but not limited to, the use of *G. mellonella.*

2.2 Experimental design when using Mycobacteria with *G. mellonella*

2.2.1 Mycobacterial growth at 37 °C and in a host

In **Chapter One**, the advantages and disadvantages of experimental design when using *G. mellonella* were discussed in a generalised overview. These points are of key consideration when using the model, but further considerations must be addressed for the use of Mycobacteria specifically.

Mycobacterium fortuitum is more than capable of growing at 37 °C and grows rapidly in comparison to other Mycobacteria. These growth characteristics mean *M. fortuitum* infections in immunocompromised patients can be very serious and potentially systemic. However, this is also beneficial to the design of experiments using *M. fortuitum. Galleria mellonella* can be kept incubated at 37 °C with no detrimental effects on survival, meaning that the behaviours of *M. fortuitum* can be studied at human body temperature. Mycobacteria do not show vast changes

in morphology based on temperature as can be seen in, for example, dimorphic fungi such as *Candida albicans.* However, there is some evidence that the ratios of different mycolic acids in the cell walls of *M. tuberculosis* can change based on temperature, and synthesis of mycolic acids is decreased overall at lower temperatures, meaning that experiments should ideally be performed at 37 ^{o}C to reduce the risk of these changes interfering with results.

Mycobacterium marinum is more complicated, as its preferred growth temperature is between 25 ^{o}C and 35 ^{o}C. This means that *M. marinum* infections are most commonly seen in cold-blooded animals, such as fish and amphibians. Consequently, the term for *M. marinum* infections in most patients is a "fish tank granuloma", as infections are often seen in people who keep aquaria or work in pet shops. Infections in humans are usually of the skin and are rarely systemic unless the patient is heavily immunocompromised, although well-developed infections can often affect deeper structures such as tendons, joints and bones.

In these experiments, *M. marinum* was grown in liquid culture at 27 ^{o}C and maintained on agar plates incubated at 27 ^{o}C, yet once the bacteria were injected into *G. mellonella* the larvae were kept at 37 ^{o}C. Initially, it was not clear if this would result in larval death, especially as a common treatment for mild fish tank granulomas is for a hot compress to be held on the point of infection to take advantage of the bacteria's heat sensitivity [100]. However, as this chapter will show, the larvae are susceptible to *M. marinum* despite being maintained at 37 ^{o}C, so experiments were continued at this temperature to allow results to be comparable.

Mycobacterium aurum was included in initial experiments due to its use in several drug-screening protocols, and suggested use as a suitable substitute for *M. tuberculosis* as it can also survive intracellularly and grows much more rapidly than *M. tuberculosis*[147]*.* It can grow in culture and on agar at 37 ^{o}C and is distinctive in its production of bright orange pigments. As *M.*

aurum can be easily identified when grown on plates, this means determination of contamination can be easily assessed but also that samples taken from larvae (for example, during a burden experiment) can be read easily without concerns about background growth from other bacteria. The use of a selective agar should prevent the growth of non-Mycobacterial bacteria, but easy identification is always an advantage.

However, *M. aurum* has been shown to be incapable of survival in low oxygen conditions, when deprived of nutrients, or when exposed to acidic pH[151]. These are important factors to consider when assessing a potential surrogate for *M. tuberculosis,* as *M. tuberculosis* persists under these conditions when sequestered to granulomas. In terms of *M. aurum* and its capacity to grow within a human host, there are three reports of *M. aurum* infections in patients, and all three were extremely immunocompromised[149,150]. The compatibility of *M. aurum* with a larval host was deemed unsuccessful and will be discussed further in this chapter (see **Section 2.5**).

2.2.2 Selective growth of Mycobacteria in culture and on plates

In terms of ensuring experiments using the larvae were not compromised by the internal bacterial community naturally present in the larvae, it was important to recognise the limitations of the agar used to grow Mycobacteria. Mycobacteria will successfully grow on a range of different types of media, but none of these are specifically selective for Mycobacteria. Penicillins and nalidixic acid can be added to media to reduce the likelihood of Gram-negative and Gram-positive bacteria, but the standard growth media used in these experiments do not contain these additions. M7H9 base media supplemented with bovine albumin fraction V, dextrose, and catalase (ADC) was used throughout, with some agar plates containing piperacillin (a broad-spectrum β-lactam antibiotic) to prevent the growth of bacteria from the gut of the larvae.

When trying to develop selective plates to use in burden experiments a variety of different antibiotics from a range of families were assessed, with β-lactams being the most reliable and successful at preventing growth of bacteria isolated from unmanipulated larvae (i.e. bacteria present in their digestive tract) but allowing growth of the Mycobacteria used in these experiments. Unmanipulated larvae from several deliveries were assessed in case there were variations in their microbiomes across batches. Piperacillin was selected from the β-lactams tested because it is a broad-spectrum β-lactam and is often combined with the β-lactamase tazobactam, which would be used in future experiments (see **Chapter 3**).

The vast majority of researchers working with *G. mellonella* order their larvae from suppliers that raise larvae for a variety of uses – fishing bait, food for exotic pets, or bird food. Hence, these larvae are not sterile and the larvae have a digestive tract that is colonised by a variety of bacteria (see **Section 1.3.1**). They also ingest bacteria when consuming their preferred food of bees' wax. The only species of bacteria that is regularly found in wax moth larvae, throughout all stages of development and across multiple generations, is *Enterococcus faecalis* (known as *Streptococcus faecalis* before 1984)[342,401].

2.2.3 Length of experiments

The vast majority of experiments using *G. mellonella* use larvae in their final instar stage and observe the survival of the larvae for several days post-infection or post-injection. Considering that *M. marinum* strains, and Mycobacteria in general, can grow quite slowly, it seemed unlikely that experiments would produce meaningful data if larvae were only assessed for a few days post-infections as it would take time for the bacteria to establish an infection, interact with the immune system, and begin dividing, before death could be observed. Hence, it was decided that larvae should be assessed for 144 hours (6 days) after infection to allow

sufficient time for larval death to occur. However, this is probably the maximum length that meaningful experiments can be performed with *G. mellonella* – often control plates will contain larvae that started to cocoon after only a few days (see **Section 1.3** for lifecycle) and inevitably, the larvae will eventually become moths.

The length of the larval survival experiments (144 hours) worked well for this combination of bacteria and host organism. Other types of experiment, such as those where haemocyte numbers or nodulation were assessed, were generally much shorter (usually to a maximum of 48 hours), as the initial responses of these systems to bacteria and/or antibiotics would be observed almost immediately after injection, and results seen at 48 hours would likely be the same as those seen at 144 hours.

2.2.4 Trauma from delivery and injections

A key requirement of working with *G. mellonella* is to reduce the amount of trauma inflicted on them. Evidence has shown that the physical trauma of shaking the larvae in cupped hands causes them to respond differently to infections compared to larvae that have not been shaken[271], so minimising this type of action is important to producing comparable experiments. Most *G. mellonella* are delivered from breeders through the postal system, which will unavoidably cause some physical trauma to the larvae, alongside variations in temperature depending on the time of year. Larvae that are kept at a very high or low temperature before commencing experiments have been shown to have an increased resistance to infection than larvae kept at room temperature[270].

The supplier who provided the larvae used in these experiments kindly assisted by packing all larvae in boxes with plenty of wood shavings and packing material, which minimises jostling during transportation, insulates the parcel from minor fluctuations in temperature, and

also allows the larvae to have ready access to food (wood shavings) for the duration of delivery. Larvae that had been denied access to food before infection showed an increased sensitivity to infection[273] so ensuring the larvae are not nutrient deprived before beginning experiments is a key factor to control. Once experiments began, all larvae were kept in plastic Petri dishes without a food source. There have been some reports of larvae being able to chew through plastic dishes[265] and other types of plastic[264,402], however no evidence was seen that the larvae damaged or consumed the plastic Petri dishes used throughout this work.

Considering the known effects of temperature fluctuation on the response to infection, all larvae were transferred to a large, sealed box with air holes in the lid to allow air to circulate in the box and moisture produced by the larvae to diffuse away, as the build-up of condensation in the box rots the wood shavings the larvae are stored in. The box was kept in a dark cupboard in a part of the lab that does not receive any sunlight, allowing the larvae to be kept at a very stable temperature. Larvae that are exposed to light will pupate faster than larva kept in the dark[403] so the larvae were only brought out when needed for experimentation. Larvae were used within a week of receipt, as larvae kept for longer seemed to be less resilient to experimentation and developed generalised melanisation (personal observations, unpublished).

In terms of injecting the larvae, the standard syringe used by most researchers is a Hamilton syringe as they allow for accurate injection of small volumes, and the needles are easy to keep sharp and clean. The larvae have delicate thin skin, and a bent needle can easily rip large wounds, which lead to larval death within a few hours. The syringe was also kept clean, as the high fat content in the larval body can cause an accumulation of fat in the shaft and barrel of the

syringe. Dirt and haemolymph on the user's gloves can also collect on the plunger and block the syringe from the end of the barrel.

To confirm that injections were being performed correctly and the saline or water used to dilute bacterial cultures or antibiotics was not contaminated, every experiment included a control plate of larvae injected only with saline or water at the same time as bacteria, antibiotics, or other reagents. If these larvae died over the course of the experiment, this suggests injections were performed incorrectly, the saline or water was contaminated, or the larvae had been mishandled before the experiment. The experiment was discarded and restarted.

In terms of minimising the trauma from injections, some researchers have expressed concerns about holding larvae between fingers when injecting and have suggested alternatives such as bracing larvae against a pipette tip taped to the bench, or trapping larvae in a sponge[404]. However, these techniques can be very slow (3-4 larvae injected per minute, as opposed to 6-9 when holding larvae between fingers) and although the risk of a stick injury to the researcher is reduced, the larvae can still make unexpected movements and seriously wound themselves in the process of injection.

2.2.5 Heat killed bacterial controls

In the same necessity of demonstrating that any saline or water used in larval injections is free from contaminations, it is also important to show that larval death is due to exposure to living bacteria. This can be achieved by heat killing a volume of liquid bacterial culture in a 100 ^{o}C water bath for 10 minutes and tracking larval survival when the dead bacteria are injected. The bacteria can also be plated onto agar plates to confirm that the water bath killed all bacteria.

Larvae should survive when injected with dead bacteria, although it is not uncommon to see some mild melanisation in response to injection, as some bacteria trigger a melanisation response even when the bacteria are non-viable. In these experiments, this was seen especially with *M. fortuitum* NCTC 8573, which produces mild to heavy melanisation in larvae within a few minutes of viable bacterial injection. Although the non-viable bacteria are incapable of causing harm to the larvae, the physical presence of a foreign particle within the cuticle will still stimulate an immune response[308,318,405–407].

For the experiments described in this chapter, heat killed bacteria were included as controls in almost all cases. However, the heat-killed bacteria were only plated on agar in the first instance to confirm that 10 minutes in a 100 ^{o}C water bath was sufficient to kill the bacteria.

2.3 Results

2.3.1 *G. mellonella* are susceptible to infection with viable *M. fortuitum* and *M. marinum,* but not *M. aurum*

2.3.1.1 *M. fortuitum* NCTC 10394, *M. fortuitum* NCTC 8573, and *M. fortuitum* R365371Q

The impact of *M. fortuitum* infections on the global population is described in **Chapter One**, as are the origins of these strains and the reasoning for their inclusion in this work.

Initially, these bacteria were assessed by diluting liquid cultures according to their optical density at 600 nm in a standard UV-Vis spectrophotometer to optical densities of 0.5, 1.0, and 1.5, then injecting these dilutions into 15 larvae in duplicate (n = 30). Once it was clear that larvae were dying from bacteria at these densities (data not shown), plating assays (see **Section 7.3.3**) were used to determine the number of colony forming units (c.f.u) in cultures of optical densities 0.5, 1.0, and 1.5 (see **Table 2.1**). Further injection experiments were then performed using bacterial cultures at specific c.f.u/mL values, and larval survival was assessed every 24 hours for 144 hours post-infection.

With all *M. fortuitum* strains used, larval survival decreased as inoculum size increased (see **Figures 2.1, 2.2** and **2.3**). Heat killed bacteria had no effect on larval survival, although *M. fortuitum* NCTC 8573 did produce melanisation when heat killed and viable bacteria were injected. The response was rapid, with melanisation seen within 10 – 30 minutes of injection and generalised, full body melanisations that was maintained for the duration of the observation period.

When viable bacteria were used, melanisation developed through the 144-hour observation for many of the larvae, although melanisation was not necessarily correlated with death or survival. For *M. fortuitum* NCTC 10394 and *M. fortuitum* R365371Q, many dead larvae were observed that still maintained the cream, uniform cuticle colour of the healthy larvae selected for experiments. Larvae which had generalised, full body melanisation but were still responsive to touch and therefore alive were also observed. Occasionally the level of melanisation was so extreme that the larvae would have been judged to be dead if survival was based on observation alone. This highlights the necessity to assess larval survival through touch response and not just visual inspection.

Table 2.1 – The relationship between optical density (OD) of bacterial liquid cultures at 600 nm and the number of viable colony forming units in a millilitre.

Species	Strain	c.f.u/mL		
		OD 0.5	OD 1.0	OD 1.5
M. fortuitum	NCTC 10394	2.60×10^8	4.24×10^8	1.07×10^9
	NTCT 8573	2.51×10^8	6.38×10^8	1.10×10^9
	R365371Q	5.93×10^8	1.10×10^9	1.98×10^9
M. marinum	Strain M	1.27×10^7	2.99×10^7	3.39×10^7
	NCTC 2275	5.10×10^5	1.39×10^6	4.53×10^6
	R356933F	3.23×10^6	14.90×10^7	1.90×10^7
M. aurum	NTCT 10437	6.40×10^7	2.11×10^8	3.32×10^8

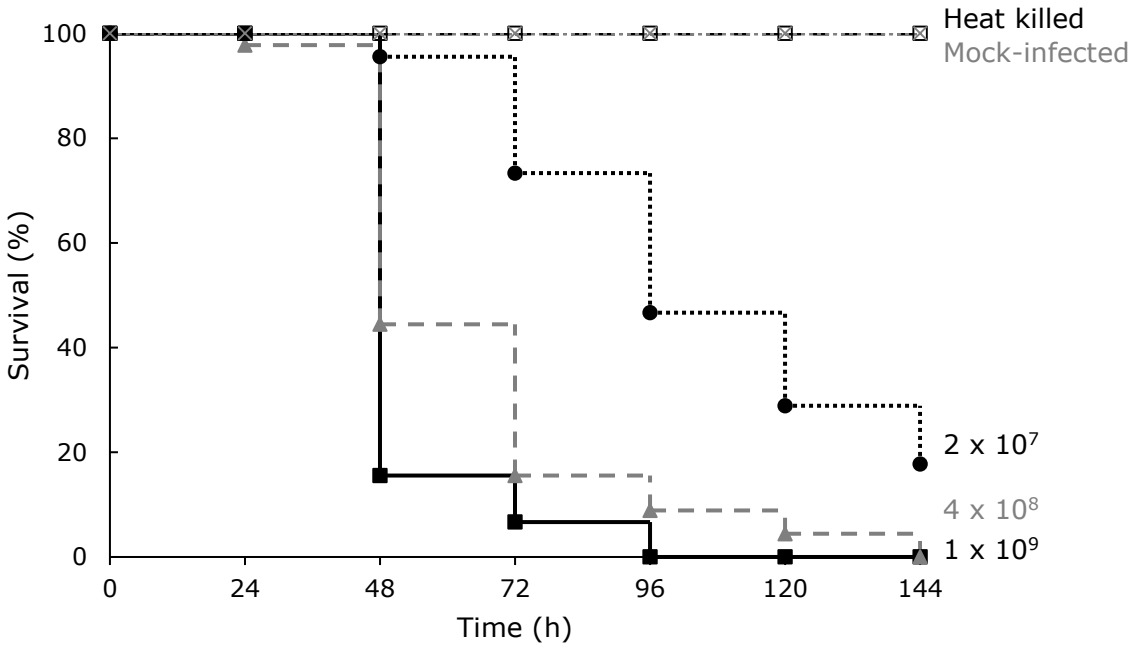

Figure 2.1 – Kaplan-Meir curve indicating the survival of larvae across a 144-hour observation window after infection with *M. fortuitum* NCTC 10394. Larvae were injected with 10 µL of bacteria at the c.f.u/mL value shown to the right of the chart. Highest bacterial concentrations, black filled squares; middle concentration, grey filled triangles; lowest concentration, black filled circles. Controls of larvae mock-infected with PBS (grey crosses) and injected with heat-killed bacteria at an equivalent concentration to the highest c.f.u/mL used (heat-killed, black open squares) are also shown. *n* = 30.

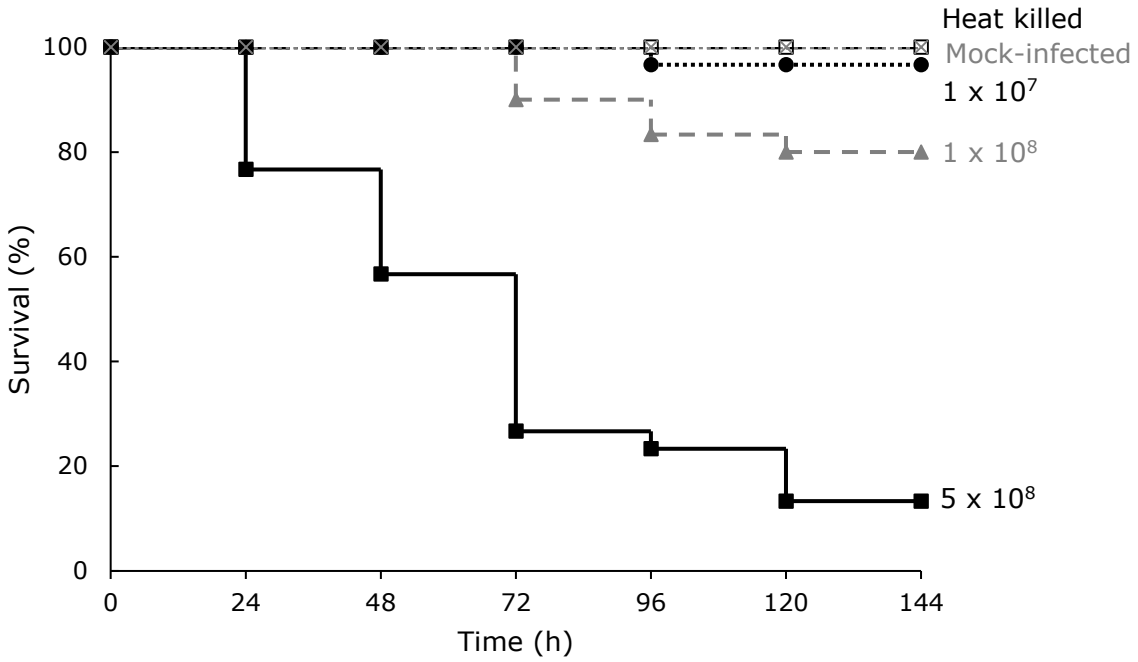

Figure 2.2 – Kaplan-Meir curve indicating the survival of larvae across a 144-hour observation window after infection with *M. fortuitum* NCTC 8573. Larvae were injected with 10 µL of bacteria at the c.f.u/mL value shown to the right of the chart. Highest bacterial concentrations, black filled squares; middle concentration, grey filled triangles; lowest concentration, black filled circles. Controls of larvae mock-infected with PBS (grey crosses) and injected with heat-killed bacteria at an equivalent concentration to the highest c.f.u/mL used (heat-killed, black open squares) are also shown. *n* = 30.

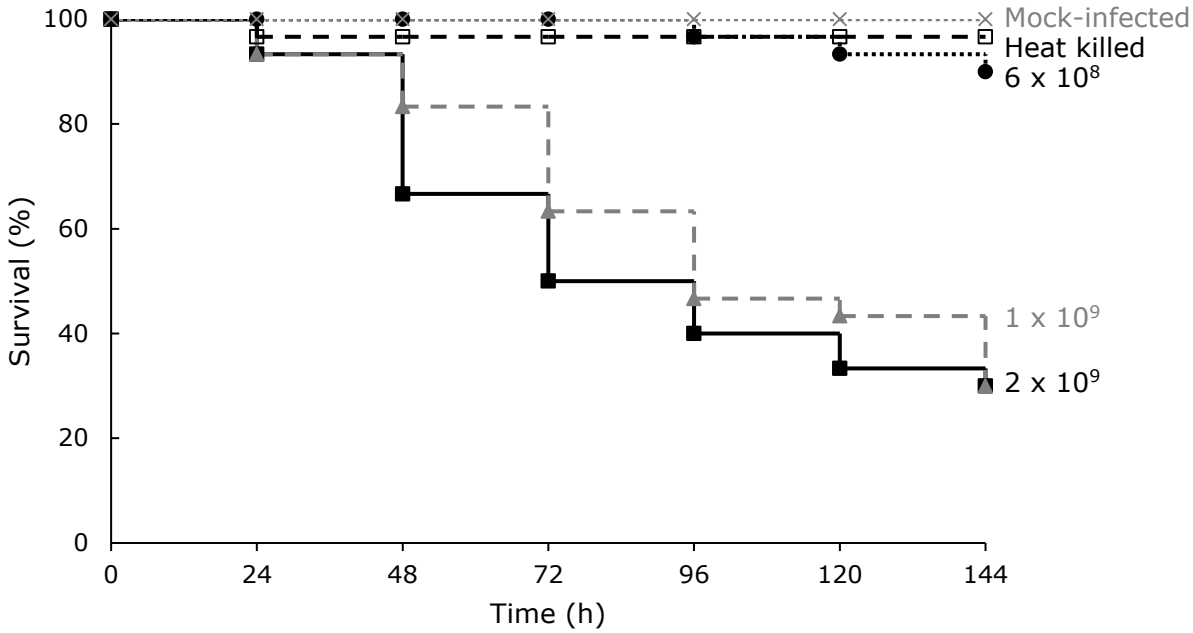

Figure 2.3 – Kaplan-Meir curve indicating the survival of larvae across a 144-hour observation window after infection with *M. fortuitum* R365371Q. Larvae were injected with 10 µL of bacteria at the c.f.u/mL value shown to the right of the chart. Highest bacterial concentrations, black filled squares; middle concentration, grey filled triangles; lowest concentration, black filled circles. Controls of larvae mock-infected with PBS (grey crosses) and injected with heat-killed bacteria at an equivalent concentration to the highest c.f.u/mL used (heat-killed, black open squares) are also shown. *n* = 30.

2.3.1.2 *M. marinum* Strain M, *M. marinum* NCTC 2275, and *M. marinum* R356933F

The impact of *M. marinum* infections on the global population is also described in **Chapter One**, as are the origins of these strains and the reasoning for their inclusion in this work.

As described in **Section 2.3.1** with *M. fortuitum*, the bacteria were assessed by diluting liquid cultures according to their optical density at 600 nm in a standard UV-Vis spectrophotometer to optical densities of 0.5, 1.0, and 1.5, then injecting these dilutions into 15 larvae in duplicate (n = 30). Once these densities were shown to cause larval death, the bacteria were plated to determine accurate c.f.u values (see **Table 2.1**).

As with the *M. fortuitum* strains, all *M. marinum* strains all produced a decrease in larval survival as inoculum size increased (see **Figures 2.4, 2.5** and **2.6**), and heat killed bacteria had no effect on survival.

In comparison to the *M. fortuitum* experiments, the *M. marinum* bacteria were much slower to produce larval death, with no death seen in the first few days even at the highest inoculum concentration. This is especially clear with *M. marinum* NCTC 2275 (**Figure 2.6**). There was also a distinct lack of melanisation for the first 48 hours, although melanisation would develop in many of the larvae after this point.

There were also variations in virulence between the *M. fortuitum* and *M. marinum* strains. Far fewer bacteria were required to reduce larval survival substantially, with *M. fortuitum* strains requiring up to 30 times as many larvae to have a similar survival curve compared to *M. marinum.*

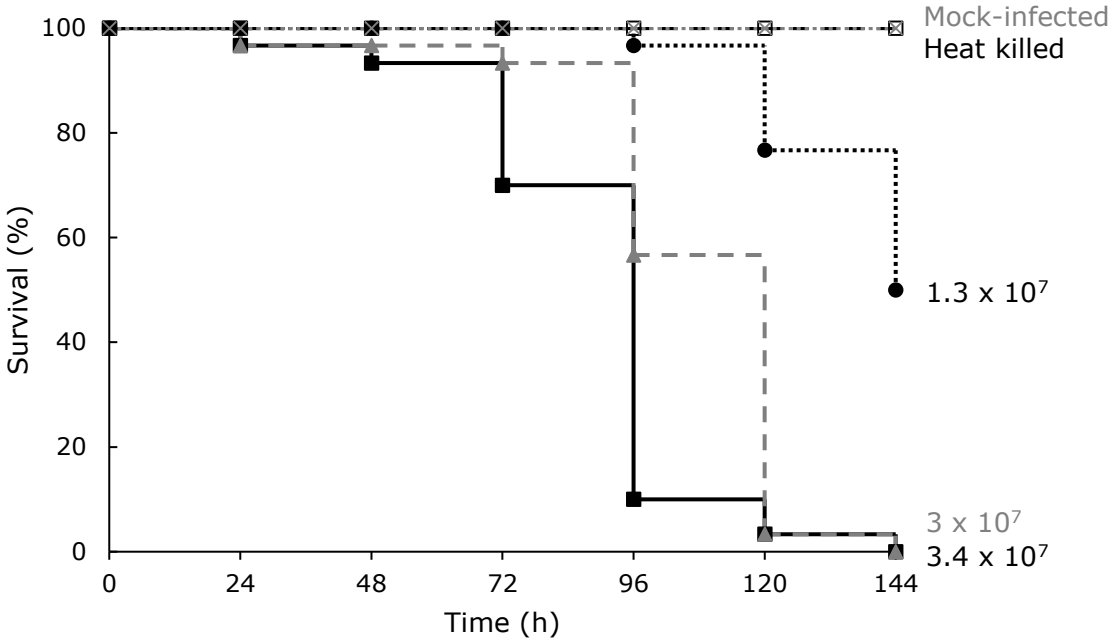

Figure 2.4 – Kaplan-Meir curve indicating the survival of larvae across a 144-hour observation window after infection with *M. marinum* Strain M. Larvae were injected with 10 µL of bacteria at the c.f.u/mL value shown to the right of the chart. Highest bacterial concentrations, black filled squares; middle concentration, grey filled triangles; lowest concentration, black filled circles. Controls of larvae mock-infected with PBS (grey crosses) and injected with heat-killed bacteria at an equivalent concentration to the highest c.f.u/mL used (heat-killed, black open squares) are also shown. *n* = 30.

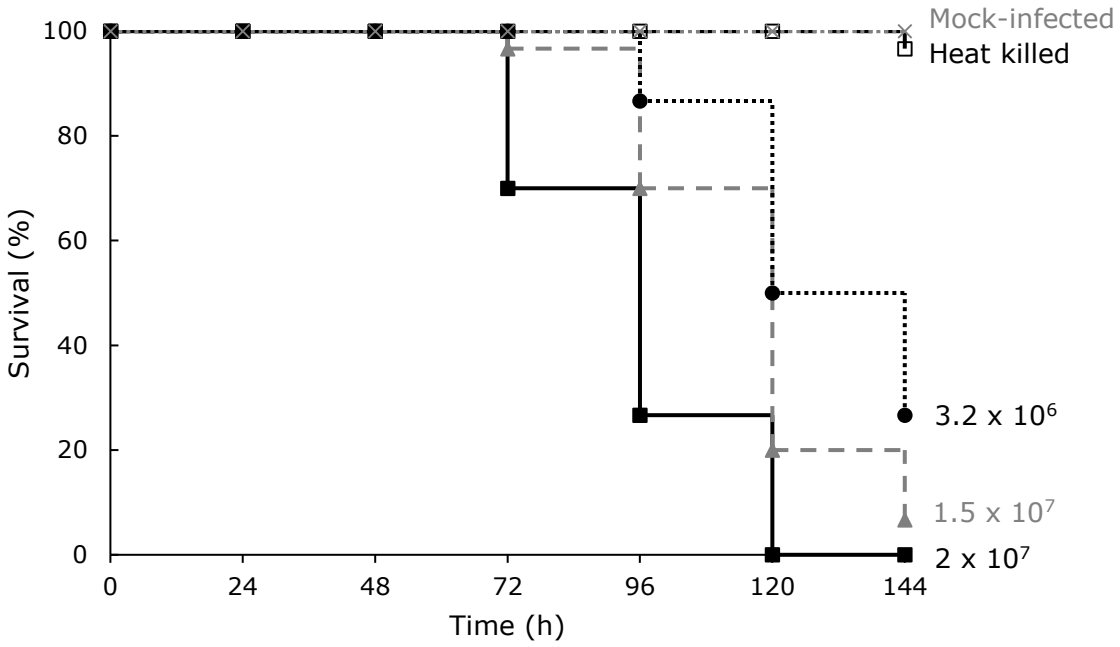

Figure 2.5 – Kaplan-Meir curve indicating the survival of larvae across a 144-hour observation window after infection with *M. marinum* R356933F. Larvae were injected with 10 µL of bacteria at the c.f.u/mL value shown to the right of the chart. Highest bacterial concentrations, black filled squares; middle concentration, grey filled triangles; lowest concentration, black filled circles. Controls of larvae mock-infected with PBS (grey crosses) and injected with heat-killed bacteria at an equivalent concentration to the highest c.f.u/mL used (heat-killed, black open squares) are also shown. *n* = 30.

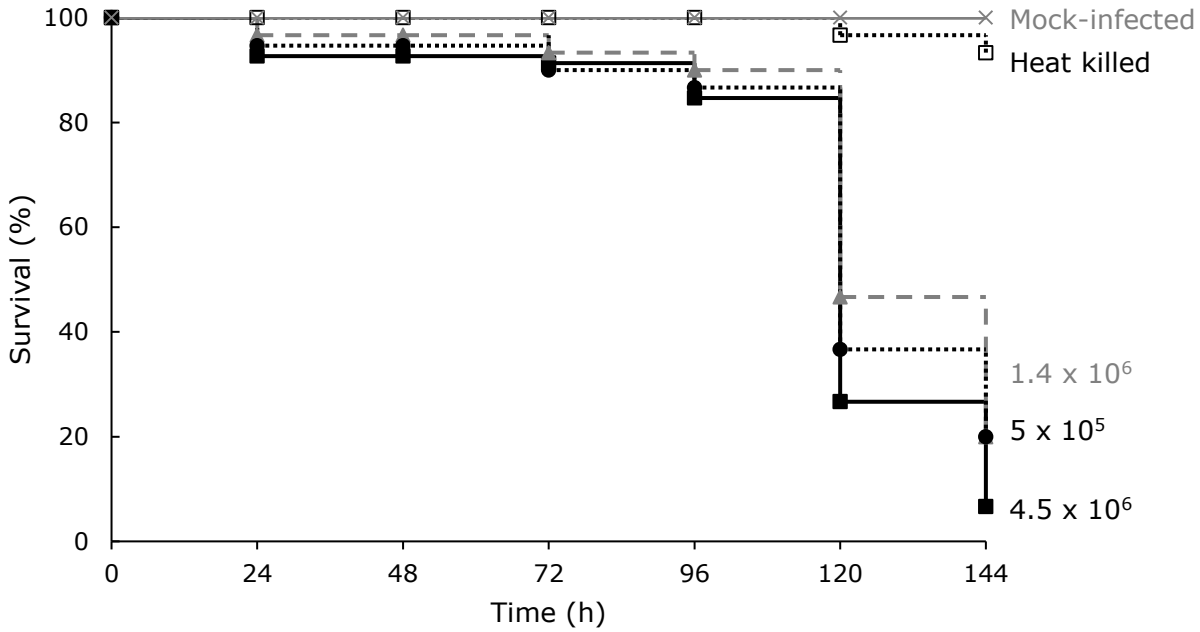

Figure 2.6 – Kaplan-Meir curve indicating the survival of larvae across a 144-hour observation window after infection with *M. marinum* NCTC 2275. Larvae were injected with 10 µL of bacteria at the c.f.u/mL value shown to the right of the chart. Highest bacterial concentrations, black filled squares; middle concentration, grey filled triangles; lowest concentration, black filled circles. Controls of larvae mock-infected with PBS (grey crosses) and injected with heat-killed bacteria at an equivalent concentration to the highest c.f.u/mL used (heat-killed, black open squares) are also shown. *n* = 30.

2.3.1.3 *M. aurum* NCTC 10437

Unlike the *M. marinum* and *M. fortuitum* strains, initial experiments with *M. aurum* using bacteria diluted according to optical density were not helpful in determining the number of colony forming units required to cause larval death. Even at the highest density (1.5 at 600 nm, the equivalent of 3,320,000 bacterial cells) larval survival remained at 100% for the duration of the experiment (see **Figure 2.7**).

As mentioned in **Section 2.2.1** and **Chapter One**, *M. aurum* have been used in a number of other experimental designs as a substitute for *M. tuberculosis.* However, they are clearly unsuitable in the context of a survival assay. Much in the same way that they cannot cause an infection in humans[149,150], the larvae seem completely unaffected by injections of *M. aurum.* Interestingly, they also showed a complete lack of melanisation. Considering melanisation is triggered even by non-viable bacteria (**Section 2.2.5**), the complete lack of melanisation is surprising.

Although *M. aurum* cannot cause larval death, this species can still be useful for further experiments as a positive control for viable bacterial injections.

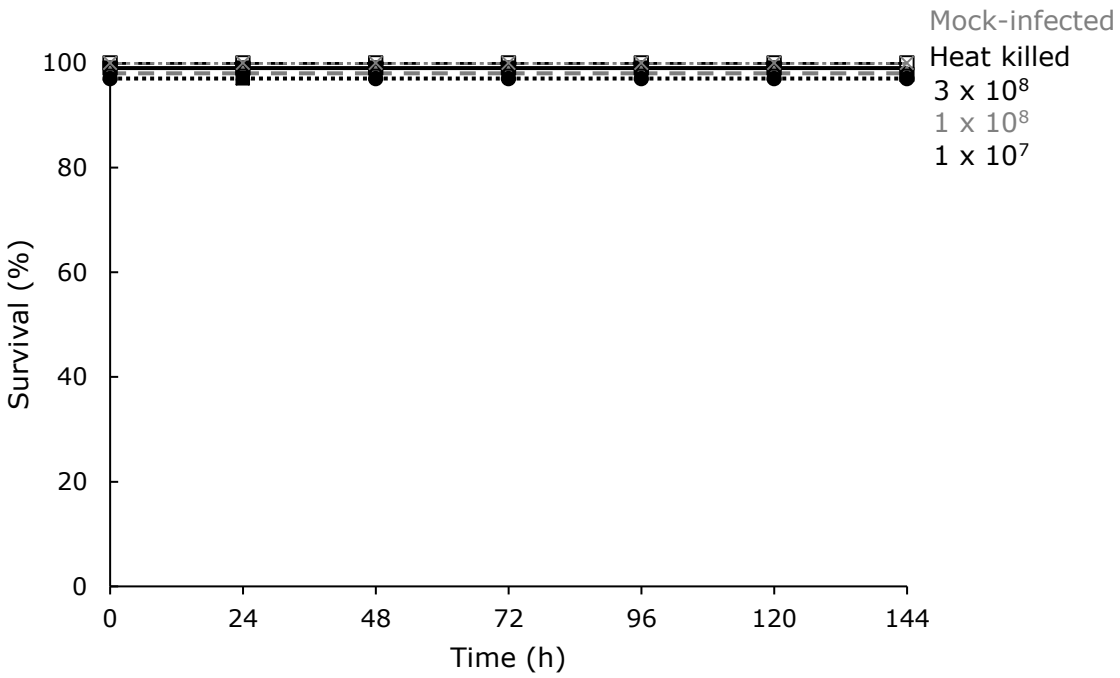

Figure 2.7 – Kaplan-Meir curve indicating the survival of larvae across a 144-hour observation window after infection with *M. aurum* NCTC 10437. Larvae were injected with 10 µL of bacteria at the c.f.u/mL value shown to the right of the chart. Survival values were 100% throughout for all inoculations but have been spaced out to indicate that all five experimental conditions are represented at 100%. Highest bacterial concentrations, black filled squares; middle concentration, grey filled triangles; lowest concentration, black filled circles. Controls of larvae mock-infected with PBS (grey crosses) and injected with heat-killed bacteria at an equivalent concentration to the highest c.f.u/mL used (heat-killed, black open squares) are also shown. *n* = 30.

2.4 Summary

The strains of *M. fortuitum* and *M. marinum* used in these experiments were capable of reducing larval survival across a 144-hour observation period. Larger inoculums of bacteria decreased survival or caused 100% larval death earlier in the observation period, however heat killed bacteria were incapable of causing larval death in any experiments.

M. aurum NCTC 10437 was unable to cause larval death. Other strains were not assessed but considering *M. aurum* is widely reported to be incapable of causing active infections in an immune-competent host, testing further strains would likely produce a similar result.

Melanisation is a common post-infection response, although is not an indicator of survival. Non-melanised dead larvae and heavily melanised living larvae were observed in a number of experiments in this chapter, and in subsequent experiments.

2.5 Discussion

At the time that these experiments were performed, these initial findings were the first modern usage of *G. mellonella* as a model for Mycobacteria. Considering that Cameron's working in 1934[262] indicated that *M. tuberculosis* is not capable of causing larval death, it was uncertain whether other species of *Mycobacterium* would cause larval death. Although there is a plethora of useful tests that can be used in tandem with a *Galleria* model without larval death being required (many of the experiments in **Chapter Five** are designed to minimise larval death), the most popular experimental format with *Galleria* are Kaplan-Meir curves, of which larval death is a necessity. Equally, assessing the effectiveness of existing anti-Mycobacterial drugs, novel treatments, and repurposed drugs is undeniably evidenced when larval survival is improved.

Mycobacterium aurum was not shown to cause larval death in these experiments, which is not unexpected considering the inability of this bacteria to cause infections in immunocompetent hosts (as discussed in **Sections 2.2.1** and **2.3.3**). As mentioned in **Section 2.1**, Cameron's work using *Galleria* and *M. tuberculosis* indicates that the tuberculosis bacteria can subsist inside of the larvae for the full duration of their development and can be re-isolated from adult moths. It is also unlikely that other strains of *M. aurum* would be capable of causing larval death – although the strain used here was isolated from soil rather than a patient, it is the type strain for this species.

Mycobacterium fortuitum and *M. marinum* were both capable of causing larval death, although there is a clear difference in the way that these bacteria cause harm to the larvae and how larval survival changes across 144 hours.

Larval death is consistently seen later in the 144 hours of observation when infected with *M. marinum,* with as many as three full days (72 hours) before any larval death is observed. Larval death also occurs with fewer bacteria. *Mycobacterium marinum* Strain M and *M. marinum* R356933F both reached 100% mortality at 120 or 144 hours after infection with 3×10^7 and 2×10^7 c.f.u/mL respectively, whereas *M. fortuitum* NCTC 10394 required 4×10^8 to reach 100% mortality at 144 hours, and *M. fortuitum* NCTC 8573 never reached 100% mortality despite injections of 5×10^8 c.f.u/mL. This difference is likely linked to the ability of the larval immune system to respond effectively to the bacterial challenge (nodulation, internal bacterial burdens, and haemocyte responses to bacteria will be discussed in **Chapter Five**), but also to how quickly these bacteria grow. As mentioned in **Section 7.3.2**, liquid cultures of *M. fortuitum* are ready for use after incubation overnight, whereas *M. marinum* need approximately 36 hours to be dense enough for use. Although burden experiments (**Sections 3.5.2** and **3.5.4**) indicate that there is a rapid response to reducing the number of internal *M. fortuitum* bacteria within the

first 24 hours of infection, this response is matched to the speed at which the bacteria kill the larvae.

As with the importance in relating larval death to infection with viable Mycobacteria, these results have also shown that the larval death is dose-responsive – injections with larger numbers of bacteria leads to a higher percentage of larvae dying, with death occurring earlier in the 144-hour observation period.

For most of the bacteria used here, even the lowest c.f.u/mL of bacteria will eventually cause some larval death, as the larvae are not capable of clearing the bacteria to the point where 100% of the larvae can survive. The exceptions are *M. fortuitum* NCTC 8573, where 1 x 10^7 c.f.u/mL (an injection of 100,000 bacteria, **Figure 2.2**) only caused death in 3% of larvae, and *M. fortuitum* R365371Q, where 6 x 10^8 c.f.u/mL (an injection of 6,000,000 bacteria, **Figure 2.3**) caused death in 10% of larvae. This shows that, even when the bacterial challenge isn't particularly small, larval death is not an inevitability. There are many experiments in the upcoming chapters where larval death was to be avoided, and hence injections with 1 x 10^7 c.f.u/mL were selected as a bacterial challenge sufficiently low to prevent mortality.

Showing that these bacteria are capable of causing larval death was integral to the further investigations in this thesis. Once it had been demonstrated that the larval have a dose dependent survival response to infection, it was then possible to assess the capability of existing anti-Mycobacterials to improve survival. The results of this chapter represent the first modern assessment of *Galleria mellonella* when challenged with a *Mycobacterium* infection at the time of execution. These results were published, along with results from **Chapters Three, Four** and **Five** in the spring of 2018[408].

Chapter Three

3.0 Assessing the effects of common anti-Mycobacterials on the survival of *Galleria mellonella* infected with *M. fortuitum* and *M. marinum*

The aim of the experiments in this chapter was to treat *G. mellonella* with several common anti-Mycobacterial drugs after infection with *M. fortuitum* and *M. marinum* strains. Survival was then assessed, as was the internal bacterial burden across 144 hours.

These drugs were then used in combination treatments, and survival and internal bacterial burden assessed using a selection of these combinations.

3.1 Anti-Mycobacterials

The drug discovery history and timelines are described in **Sections 1.2.2** and **1.2.4**, however a brief overview of each antibiotic used in this chapter is discussed here.

3.1.1 Anti-Mycobacterials in this thesis

There are a huge range of antibiotics which could have been selected for examination in this thesis. Four key drugs are used in the WHO recommended treatment regimen, isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA). Streptomycin is also classed as a first-line anti-TB drug[409]. INH, RIF and EMB were all selected for use.

Second-line anti-TB drugs include different groups of drugs. Several fluoroquinolones including ofloxacin (OFX), levofloxacin (LEV), moxifloxacin (MOX) and ciprofloxacin (CIP) have been used. Kanamycin (KAN), amikacin (AMK) and capreomycin (CAP), which are all aminoglycosides, are also available as second-line drugs. Other drugs are listed within this group, including *para*-aminosalicylic acid (PAS), but are not as common or not as effective[409].

From this group of antibiotics, CIP and AMK were selected for use. AMK is especially effective against Mycobacteria[410,411], and CIP is used for *M. fortuitum*[412–414] and *M. marinum*[415] infections.

3.1.1.1 Amikacin (AMK)

Amikacin is an aminoglycoside antibiotic, which can act as a bactericidal or bacteriostatic agent depending on the concentration used and inhibits bacterial function by irreversibly binding to 16S rRNA and therefore blocking the operation of the 30S ribosomal subunit. This prevents the bacteria from being able to produce proteins, as mRNA cannot bind effectively to the ribosome, the ribosome cannot read the mRNA codons effectively, and tRNA cannot attach to binder sites. Amikacin is chemically derived from kanamycin A and became commercially available in the mid-1970s. Administration is usually via intravenous injections, although intramuscular injections and nebulised preparations are also effective. Amikacin is not absorbed from the gastrointestinal tract, but can cause nephrotoxicity and ototoxicity, with effects on the auditory nerve and cochlea being reported by around 25% of patients[416].

When combined with penicillins, some microorganisms will become more susceptible to amikacin. This is also seen with Gram-positive bacteria when combined with carbapenems, which, like penicillins, are based on a central β-lactam ring. Some combinations, including clindamycin, chloramphenicol, and tetracycline, will inactivate amikacin[417].

3.1.1.2 Ciprofloxacin (CIP)

Ciprofloxacin is a broad-spectrum fluoroquinolone, which is active against Gram-positive and Gram-negative bacteria but is generally more effective against Gram-negative bacteria. As with all fluoroquinolones, the mechanism of action is to inhibit DNA gyrase and topoisomerases leading to an inability to unwind and duplicate the bacterial DNA, preventing cell duplication

and bacterial division. Hence, it is generally bactericidal, although has been shown to be bacteriostatic under certain conditions. It has been used clinically since the mid-1980s.

Ciprofloxacin is commonly prescribed as tablets, eye and ear drops, or as intravenous solutions. After the patent for ciprofloxacin expired, generic equivalents have become very cheap and it is now one of the most commonly prescribed generic drugs worldwide. Ciprofloxacin interacts with a number of metals, including calcium, iron, and zinc, to form insoluble salts which cannot be readily absorbed, and also inhibits drug metabolising enzymes. This reduces the clearance of other compounds and can lead to massively increased serum concentrations of co-administered drugs.

3.1.1.3 Ethambutol (EMB)

Ethambutol is one of the "four pillars" of tuberculosis treatment and has been part of the standard treatment regimen for tuberculosis for over half a century, since its first use in the early 1960s. Its mechanism of action is different to amikacin and ciprofloxacin as it targets the formation of the cell wall, meaning it is generally regarded as bacteriostatic. The drug inhibits arabinosyl transferase, which polymerises arabinogalactan, a major structural component of the cell wall of Mycobacteria. The bacteria cannot grow or divide, and their cell wall becomes increasingly permeable, however the specifics of how ethambutol specifically interacts with bacterial arabinosyl transferase are not yet known.

Ethambutol is taken as a tablet and is readily absorbed from the GI tract.

3.1.1.4 Isoniazid (INH)

Isoniazid is also one of the oldest treatments for tuberculosis, having been in use since the 1950s, and is another of the "four pillars" of treatment. Like ethambutol, it targets the bacterial

cell wall, but as a prodrug it requires a mycobacterial catalase to convert to an active oxyferrous enzyme complex. This complex then inhibits the synthesis of mycolic acids by blocking the action of the bacterial fatty acid synthase. Mycolic acids are intrinsic to the structure of the mycobacterial cell wall, although slow growing Mycobacteria will simply stop growth in the presence of isoniazid (bacteriostatic), but it is bactericidal for dividing bacteria[418].

Isoniazid is most commonly prescribed as a tablet but is also available as intramuscular and intravenous injections. Unusually, the human population is divided into those who metabolise isoniazid quite slowly and those who metabolise it much more rapidly. However, isoniazid seems to be equally effective between the two groups. Yet isoniazid can affect the metabolism and clearance of several key drugs, most notably acetaminophen (paracetamol).

3.1.1.5 Rifampicin (RIF)

Rifampicin is the third of the "four pillars" used in this thesis and was first used in a clinical setting in the mid-1960s. Originally isolated from a soil bacterium, rifampicin is a polyketide produced as a secondary metabolite by the bacterium. Its mechanism of action is to prevent the elongation of RNA by sterically blocking the bacterial RNA polymerase active site. It can be bacteriostatic or bactericidal depending on the target bacteria but is considered bactericidal to Mycobacteria.

Rifampicin is usually prescribed as a tablet as it is easily absorbed from the GI tract but is also used intravenously. It is distinctive due to its bright red/orange colour, which remains when in solution and can cause urine, sweat, and tears to become red. In conjunction to its physically observable side effects, rifampicin is an incredibly potent inducer of cytochrome P450 enzymes in the liver. This enzyme family accounts for around 75% of total drug metabolism in humans, both bioactivating drugs into their active states and deactivating drugs

before excretion. Hence, rifampicin can both increase and decrease concentrations and activities of a plethora of medications and compounds. Birth control pills, antiretrovirals, antibiotics, antifungals, even antidepressants, are all susceptible to changes in efficacy and absorption.

3.2 Treatments for *Mycobacterium* infections

3.2.1 Regimen choices

Infections with Mycobacteria are usually treated with a minimum of two agents to reduce the risk of developing resistance. However, Mycobacteria usually produce persistent and extended infections, especially if associated with medical implants or devices, or in patients who are immunosuppressed. Therefore, it is common to use more than two drugs in combination.

In terms of *M. tuberculosis,* the WHO recommended treatment protocol includes a combination of four antibiotics are taken for the first two months, usually pyrazinamide, ethambutol, isoniazid and rifampicin, with isoniazid and rifampicin continuing to be taken for a further four months. Other antibiotics are added for patients with drug resistant strains, which also extends the treatment period to as long as 24 months.

One of the most problematic aspects of the treatment protocol for *M. tuberculosis* is that therapy lasts for many months. The same concern applies to *M. fortuitum* and *M. marinum* infections – treatment also takes several months, and for some patients treatment can take well over a year[101].

Usually, combination treatments are applied to an infection where a broad antibacterial spectrum is required (especially if the causative agent has not been isolated), when multiple

microbes need to be treated (for example, when bacteria from a microbe-rich area, such as the gut, leak into a sterile area like the peritoneal cavity), when two or more drugs are needed to act synergistically (such as a β-lactam with a β-lactamase inhibitor), or when an emergence of resistance seems likely[419]. As the treatments for Mycobacterial infections need to be effective for many months, using a combination treatment helps reduce the risk of antibiotic resistance emerging[420].

3.2.2 Antibiotic resistance

When considering the development of antibiotic resistance during an infection, a key factor to consider is the ability of bacteria to communicate genetic information through a bacterial community and even between species[421]. Once resistance arises, resistance genes on plasmids can be distributed through conjugation and create drug resistant communities of bacteria[422] through horizontal gene transfer. Hence, it is important to target not only the "standard" bacteria in an infectious population, but also those which may have pre-existing resistance to a drug and could share these genes. There is also discussion on the effects of antibiotics on conjugation, and if exposure to antibiotics makes the sharing of resistance genes on plasmids more likely[423].

Mycobacteria, particularly *M. tuberculosis* and *M. leprae*, are unusual in this regard, in that they are generally considered as lacking natural plasmids or performing horizontal gene transfer[424]. In terms of Mycobacteria in the laboratory, transformation protocols have been developed[425,426], but often with far more stages, equipment, and reagents than a conventional transformation protocol to address the very low Mycobacterial membrane permeability, and therefore transformation efficiency. In the environment, a handful of plasmids have been found in *M. fortuitum*[427], *M. ulcerans*[428], and *M. marinum*[424], and multiple plasmids from *M. avium*[429,430].

The natural movement of plasmids between Mycobacterial species has also been observed[431], however considering that the movement of resistance genes between Mycobacteria and Mycobacterial species is not clinically recognised, this is not considered when deciding a treatment protocol for Mycobacteria.

Rather than acquiring resistance from external mobile genetic elements, the evolution of drug resistant strains of *M. tuberculosis* is partly attributed to patients taking their medications irregularly, unpredictable drug availability, low-quality drugs, poor implementation of infection control measures, and patient non-adherence[432]. However, resistant bacterial strains have also been isolated from communities where patients adhered to strict treatment protocols using reliable and high-quality medications[433,434], meaning that drug resistance is influenced by factors other than patient conduct and drug quality.

A notable trait of Mycobacterial infections is that infected areas (or lesions) are only populated with Mycobacteria[420]. Polymicrobial diseases are surprisingly common and diverse[435,436], and present varied challenges for both diagnosis and treatment. Prescribing antimicrobials for only one microbe in a mixed infection inevitably means that other pathogens will grow to replace these missing community members, even if combination treatments were used against the target microbe.

Although Mycobacterial lesions are not populated with other bacteria, multiple strains of *M. tuberculosis* can infect a patient[420,437,438], and patients can be infected with new strains, including drug-resistant strains, while undergoing treatment for existing *M. tuberculosis* infections[439,440]. Examples of this have not been shown for patients infected with *M. fortuitum* or *M. marinum,* but patients with advanced HIV infections can be infected with multiple *M. avium* strains[441]. The possibility of a patient being infected with multiple strains of the same

Mycobacteria, which may have different responses to antibiotics, should be considered when developing a treatment. The WHO guidelines on tuberculosis treatment suggest monitoring the cultures present in patient samples throughout the treatment period[442] to determine any resistance as early as possible.

In terms of how Mycobacteria can develop resistance to treatment, rifampicin and isoniazid resistance are usually the first types of resistance to develop. Multi-drug resistant (MDR) TB is defined as a strain which is resistant to at least INH and RIF, and 161,000 new MDR-TB cases were reported by the WHO in 2017[443]. Extremely drug resistant (XDR) strains are resistant to INH, RIF plus any fluoroquinolone (such as ciprofloxacin, ofloxacin, levofloxacin and moxifloxacin) and at least one of amikacin, kanamycin, or capreomycin[444].

3.2.2.1 Isoniazid resistance

INH is most effective in the period just after the medication is taken, clearing almost all Mycobacteria, and then other drugs, particularly RIF, clear the small number of remaining bacteria[418,445]. Resistance to isoniazid has been associated with over twenty different mutations[418,446]. Some mutations lead to a greater phenotypic resistance – mutations in *katG*, which encodes a catalase-peroxidase that activates the INH in its prodrug form into its active form, lead to much higher drug resistance than mutations in *inhA*[418], which codes for a NADH-dependent enoyl-[ACP] reductase. Mutations in this reductase decrease the affinity of InhA for the isoniazid-NAD adduct[447]. One, mutation in *katG, a* Ser315Thr substitution, is present in 50 – 90% of all INH-resistant strains[448].

Reducing the incidence of isoniazid resistance is most easily managed by detecting which patients have INH-resistant strains of TB, then working to prevent their transmission.

Preventing the resistance developing in the first instance can be aided by ensuring adherence to treatment and avoiding breaks in the treatment protocol.

3.2.2.2 Rifampicin resistance

As rifampicin is effective against actively metabolising and dormant bacteria, it is seen as one of the most effective anti-TB drugs[449]. As rifampicin functions by sterically blocking the bacterial RNA polymerase active site of the bacteria, preventing the elongation of RNA, resistance to rifampicin comes from changes to the polymerase structure. Almost all *M. tuberculosis* strains which show resistance to rifampicin have mutations in a 81 base-pair region of the *rpoB* gene[449,450] – mutations in codons 516, 526 and 531 are the most commonly observed. This gene codes for the β subunit of RNA polymerase, which is where rifampicin usually binds, hence reducing the affinity for rifampicin to the bacterial RNA polymerase[451].

Interestingly, monoresistance to isoniazid is not uncommon, but strains which are susceptible to isoniazid and resistant to rifampicin are very rare[452]. More than 90% of strains which have RIF resistance are also resistant to INH[453], but strains that are resistant to RIF and susceptible to INH seem to arise in cases where patients are non-compliant with their TB medication regimen, or have an inability to absorb RIF[454]. They have been recorded in communities with HIV/AIDs[454,455] but also in the wider patient community. The loss of rifampicin as part of the treatment protocol for patients with MDR-TB is a crucial concern to healthcare providers, as it is key component in eradicating *M. tuberculosis* cells in the quiescent, dormant phenotype.

3.2.2.3 Ethambutol resistance

Ethambutol inhibits arabinosyl transferase, which polymerises arabinogalactan, a major structural component of the Mycobacterial cell wall. Hence, resistance to ethambutol is associated with mutation in the gene which codes for this enzyme – *embB.* Mutations to codon 306 are the most commonly observed[456], but these mutations are often associated with resistance to other antibiotics, not simply a monoresistance to ethambutol[457]. However, some strains which show a resistance to EMB have no mutations to the *embB* gene, so another mechanism of resistance must be accessible to the bacteria[449,457,458].

3.2.2.4 Amikacin resistance

Amikacin prevents protein synthesis by disrupting the function of the bacterial ribosomes, so mutations to the 16S rRNA binding site (*rrs*) are the most common types of mutation in amikacin resistant bacteria. This can confer resistance to other antibiotics, but is not guaranteed[449,458].

3.2.2.5 Ciprofloxacin resistance

Ciprofloxacin targets bacterial DNA gyrase and topoisomerases. DNA gyrases in *M. tuberculosis* are encoded by *gyrA* and *gyrB*, and mutations associated with resistance in these genes is so common that regions within both genes are called "quinolone resistance-determining regions"[457]. Changes in the amino acids in *gyrA* at positions 90, 91 and 94 are the most frequently associated with fluoroquinolone resistance, but codons 74 and 88 have also been linked to resistance[457]. These mutations alter the binding affinity of fluroquinolones to the DNA gyrase enzyme, allowing for DNA replication and DNA supercoiling which would have

otherwise been inhibited by the antibiotic. However, mutations in *gyrB* associated with fluoroquinolone resistance are uncommon.

Resistance to fluoroquinolones can arise when patients are inadvertently given monotherapy treatment, as their doctor mistakenly prescribes the medication to treat a lower respiratory tract infection which is actually pulmonary tuberculosis[459].

3.3 Reducing bacterial burdens using single antibiotic therapy and combination treatments

There are several ways that the number of bacteria or fungi can be tracked during an infection in a human patient. For tuberculosis, a sputum smear can be used to enumerate the number of bacteria in a patients lungs[460]. Other tests can use the polymerase chain reaction (PCR) to test for the presence of a particular microorganisms genetic material[461,462]. Enzyme-linked immunosorbent assays (ELISAs) can also be used to look for specific antigens belonging to a microorganism[462–464]. Flow cytometry can also be used to assess microorganisms in a liquid, and can assess samples quickly and with great specificity[462].

Assessing the internal load of an infectious agent inside *G. mellonella* has been used with a variety of bacteria[269,465–467] and fungi[267,468,469], and is a useful tool to determine the pathogenicity of a microorganism[468], but also the efficacy for antimicrobials to reduce the growth of a pathogen or eradicate an infection.

Histology can be used[470,471], but the procedure requires a more complicated protocol and investment of time, and although it can show where microorganisms are found within the larvae, it is difficult to see exactly how many microorganisms are in the total larval body.

Equally, there have been some novel protocols which have used fluorescent bacteria and tracked the development of an infection internally within the larvae by assessing the levels of fluorescence either in collected haemolymph[380] or in whole, live larvae[381]. However, this procedure requires specialised equipment, and must be carefully calibrated to give an accurate correlation between fluorescence and the number of bacteria in the larvae or haemolymph. Strains of Mycobacterium are available which constitutively express fluorescent protein, which would be useful for this protocol[379,380].

Instead, homogenising the whole larval body and plating the contents onto agar plates means that all internal, viable microorganisms can be visualised and enumerated. Injecting the larvae with microorganisms and then using standard or non-standard treatments to assess if the microorganisms are still capable of surviving or dividing after these treatments is easily tested with this method.

3.4 Additive efficacy versus synergy

Using combinations of drugs to treat an illness is the foundation of many treatment protocols, including HIV[472], cancer[473], and sepsis[474]. As to whether a combination is synergistic or additive; additive combinations mean the medications used have the same effect proportionately when combined as each separate component would have separately (such as taking aspirin with paracetamol), whereas synergistic combinations mean that the medications taken together have a greater overall effect that the sum of their individual efficacies (such as an insecticide with a fertiliser).

Finding new treatment combinations that may provide synergistic interactions when used with *M. tuberculosis* has been tried[475–477], with some promising *in vitro* and *in vivo* results. In terms of experiments into synergy between the more "standard" anti-TB drugs, surprisingly

few studies have been performed, with a handful testing double combinations[478,479] and even fewer testing triple combinations[479–482]. However, synergy has been shown when using EMB + INH + RIF in *M. tuberculosis* strains with INH resistance, although synergy was not seen when this combination was used with drug susceptible strains[479]. Synergy was also seen with ofloxacin (a fluoroquinolone, similar to ciprofloxacin) combined with RIF and EMB, for both drug resistant and drug susceptible strains. However, evidence of synergy for antibiotic combinations used in this chapter could not be found as synergy experiments are assessed *in vitro*, usually through the use of the checkerboard method, multiple-combination bactericidal antimicrobial testing (MCBT), Etests, and time-kill curve assays[483].

3.5 Results

3.5.1 Antibiotics will improve larval survival after infection with Mycobacteria, and their efficacy is mostly correlated with their minimum inhibitory concentration (MIC) values

The reasoning for the panel of antibiotics used in this section is described in **Section 3.1.1**. The MIC values were found using MIC strips as described in **Section 7.4.1**, in triplicate, and the values are shown in **Table 3.1**. These MIC values were used to guide which doses of antibiotic should be used for larval injections, but if the highest dose of antibiotic did not improve larval survival compared to an untreated control then the dose would generally be increased up to 100 mg/mL. Equally, if the lowest antibiotic dose conferred a large improvement in larval survival, then a lower dose would generally also be tested.

Table 3.1 – MICs of five antibiotics for *M. fortuitum* NCTC 10394, *M. fortuitum* NCTC 8573, *M. marinum* Strain M and *M. marinum* R356933F using three independent biological replicates

mg/L

	M. fortuitum		*M. marinum*	
	NCTC 10394	NCTC 8573	Strain M	R356933F
AMK	0.12 - 0.25	0.12 - 0.25	1.0	2.0
CIP	0.008	0.05	0.5	0.25 - 0.5
EMB	2.0	>256	0.125 - 0.19	0.19 - 0.25
INH	1.0 - 2.0	>256	4.0 - 6.0	2.0 - 3.0
RIF	12.0	<0.016	6.0 - 8.0	0.38 - 0.5

AMK, amikacin; CIP, ciprofloxacin; EMB, ethambutol; INH, isoniazid; RIF, rifampicin.

3.5.1.1 *M. fortuitum* NCTC 10394 – larval survival with single dose antibiotics

The *M. fortuitum* NCTC 10394 bacteria were used at 4 x 10^8 c.f.u/mL, and antibiotics were injected 2 hours later. The Kaplan-Meir curves are shown in **Figure 3.1**. Larvae injected with PBS alone (un-infected larvae) had 100% survival throughout.

Amikacin, ciprofloxacin, and isoniazid are all effective antibiotics, significantly improving survival. Amikacin was especially successful, with very high rates of survival even at relatively low doses, and despite only receiving a single injection of antibiotic.

However, ethambutol and rifampicin had no effect on larval survival. Occasionally the larval survival would be higher than the untreated group at a single time-point, but the final survival was consistently low.

The MIC values (see **Table 3.1**) show some correlation with these results – the MIC values for AMK and CIP are both very low, as is INH. However, the MIC for INH and EMB are comparable, yet EMB was unable to increase larval survival. The MIC for RIF is a mediocre 12 μg/mL, which is not unreasonably high but far higher than the MIC for AMK or INH.

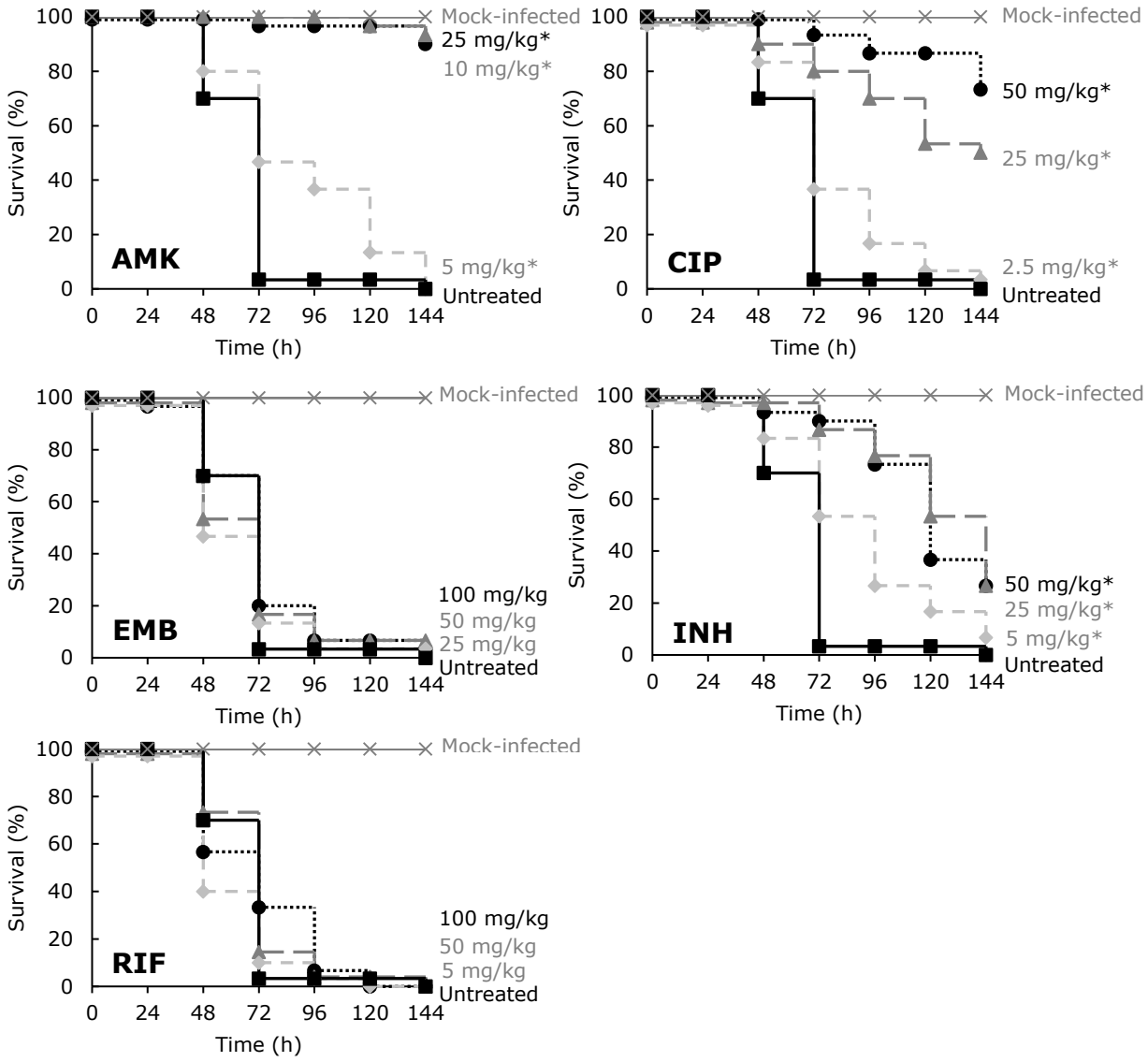

Figure 3.1 – Kaplan-Meir curves indicating the survival of larvae across a 144-hour observation window after infection with *M. fortuitum* NCTC 10394 and treatment with a single antibiotic dose. Larvae were injected with 10 µL of bacteria at 4 x 10^8 c.f.u/mL, then with a single dose of antibiotic two hours later. The different mg/kg doses of antibiotic used are shown to the right of each curve. The antibiotic used is shown in bold in the left corner of each curve. Infected and untreated larvae, black filled squares; highest antibiotic dose, black filled circles; medium antibiotic dose, grey filled triangles; lowest antibiotic dose, light grey filled diamonds. Controls of larvae mock-infected with PBS (grey crosses) are also shown. When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant antibiotic dose. n = 30. AMK, amikacin; CIP, ciprofloxacin; EMB, ethambutol; INH, isoniazid; RIF, rifampicin.

3.5.1.2 *M. fortuitum* NCTC 8573 – larval survival with single dose antibiotics

The *M. fortuitum* NCTC 8573 bacteria were used at 5 x 10^8 c.f.u/mL and antibiotics were injected 2 hours later. The Kaplan-Meir curves are shown in **Figure 3.2**. Larvae injected with PBS alone (un-infected larvae) had 100% survival throughout.

Amikacin, ciprofloxacin, and rifampicin were effective at improving larval survival compared to untreated infected larvae. Amikacin was not as effective as it had been with *M. fortuitum* NCTC 10394 – for NCTC 10394, 25 mg/kg raised survival to almost 100%, but for *M. fortuitum* NCTC 8573 100 mg/kg amikacin had a final survival of 57%. Rifampicin is more effective – 100 mg/kg gave very high survival throughout.

Ethambutol and isoniazid were both ineffective at increasing larval survival. Isoniazid gave a very slight increase in survival at most time points, but this increase was not statistically significant and the final survival at 144 hours was comparable to the untreated larvae.

The MIC values (see **Table 3.1**) for *M. fortuitum* NCTC 8573 are very closely aligned with the survival data. The values for AMK, CIP and RIF are all very low (below 0.25 μg/mL) whereas the values for EMB and INH were higher than the maximum MIC on the test strips (above 256 μg/mL).

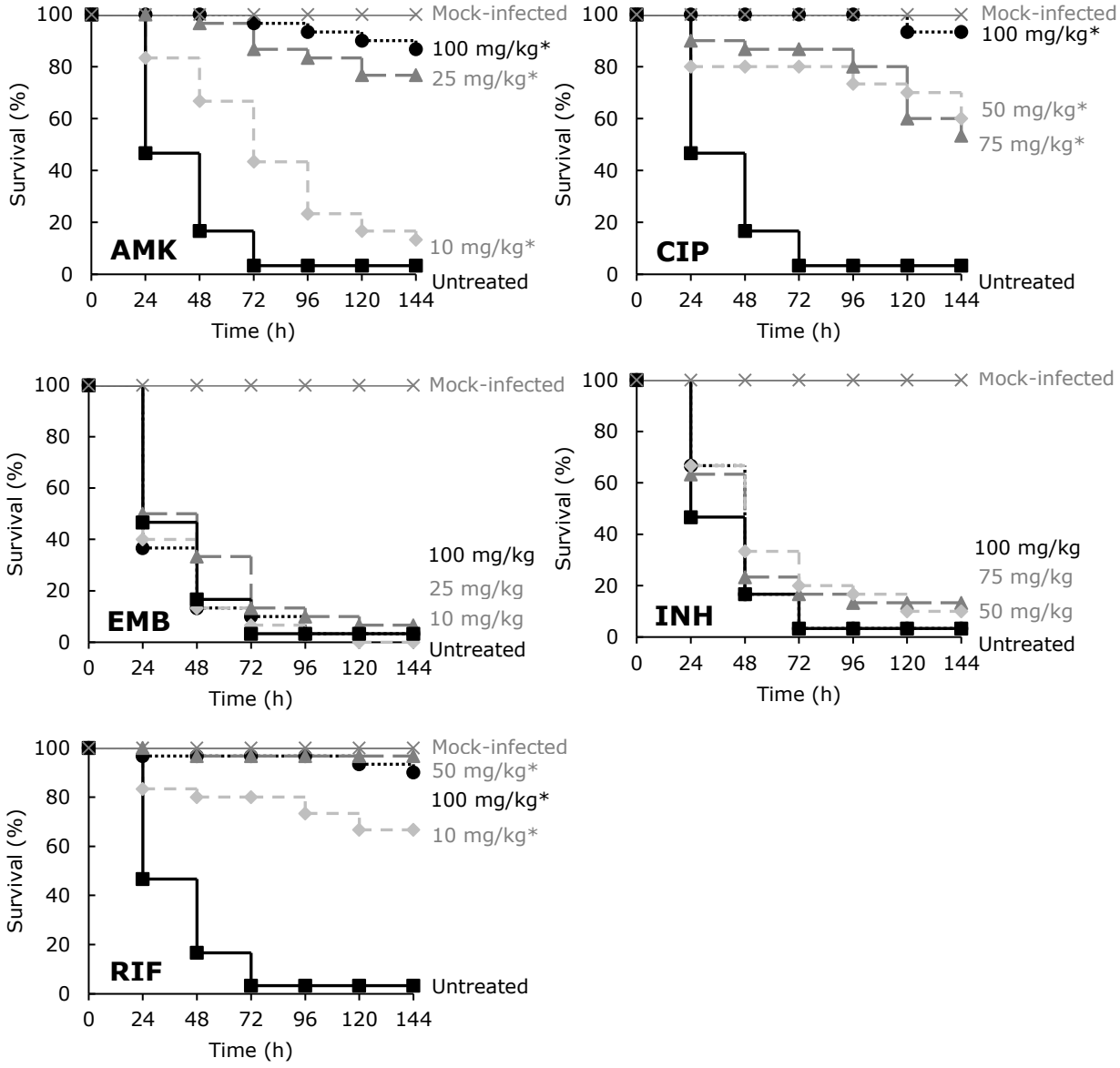

Figure 3.2 – Kaplan-Meir curves indicating the survival of larvae across a 144-hour observation window after infection with *M. fortuitum* NCTC 8573 and treatment with a single antibiotic dose. Larvae were injected with 10 µL of bacteria at 5 x 10^8 c.f.u/mL, then with a single dose of antibiotic two hours later. The different mg/kg doses of antibiotic used are shown to the right of each curve. The antibiotic used is shown in bold in the left corner of each curve. Infected and untreated larvae, black filled squares; highest antibiotic dose, black filled circles; medium antibiotic dose, grey filled triangles; lowest antibiotic dose, light grey filled diamonds. Controls of larvae mock-infected with PBS (grey crosses) are also shown. When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant antibiotic dose. n = 30. AMK, amikacin; CIP, ciprofloxacin; EMB, ethambutol; INH, isoniazid; RIF, rifampicin.

3.5.1.3 *M. marinum* Strain M – larval survival with single dose antibiotics

The *M. marinum* Strain M bacteria were used at 3 x 10^7 c.f.u/mL and antibiotics were injected 2 hours later. The Kaplan-Meir curves are shown in **Figure 3.3**. Larvae injected with PBS alone (un-infected larvae) had 100% survival throughout.

Larval survival was increased significantly when larvae were treated with amikacin, isoniazid, and rifampicin. For all three of these antibiotics, the highest dose used gave a very high larval survival of close to 100% across the 144 hours. However, lower doses (for example, amikacin at 0.25 mg/kg) gave survival curves which were similar to untreated larvae.

However, ciprofloxacin and ethambutol had a minimal effect on improving larval survival. Ciprofloxacin had been effective at improving larval survival for *M. fortuitum* NCTC 10394 and NCTC 8573, but ethambutol had been ineffective for both *M. marinum* strains as well.

These survival curves have very little correlation to the MIC values (see **Table 3.1**). The lowest MIC is for EMB, which has no effect on larval survival, with the next lowest MIC being for CIP, which also has no effect on improving larval survival. The highest MIC is for RIF, which was very effective at treating the larvae, INH also has a high MIC value which does not match its efficacy in the survival assays.

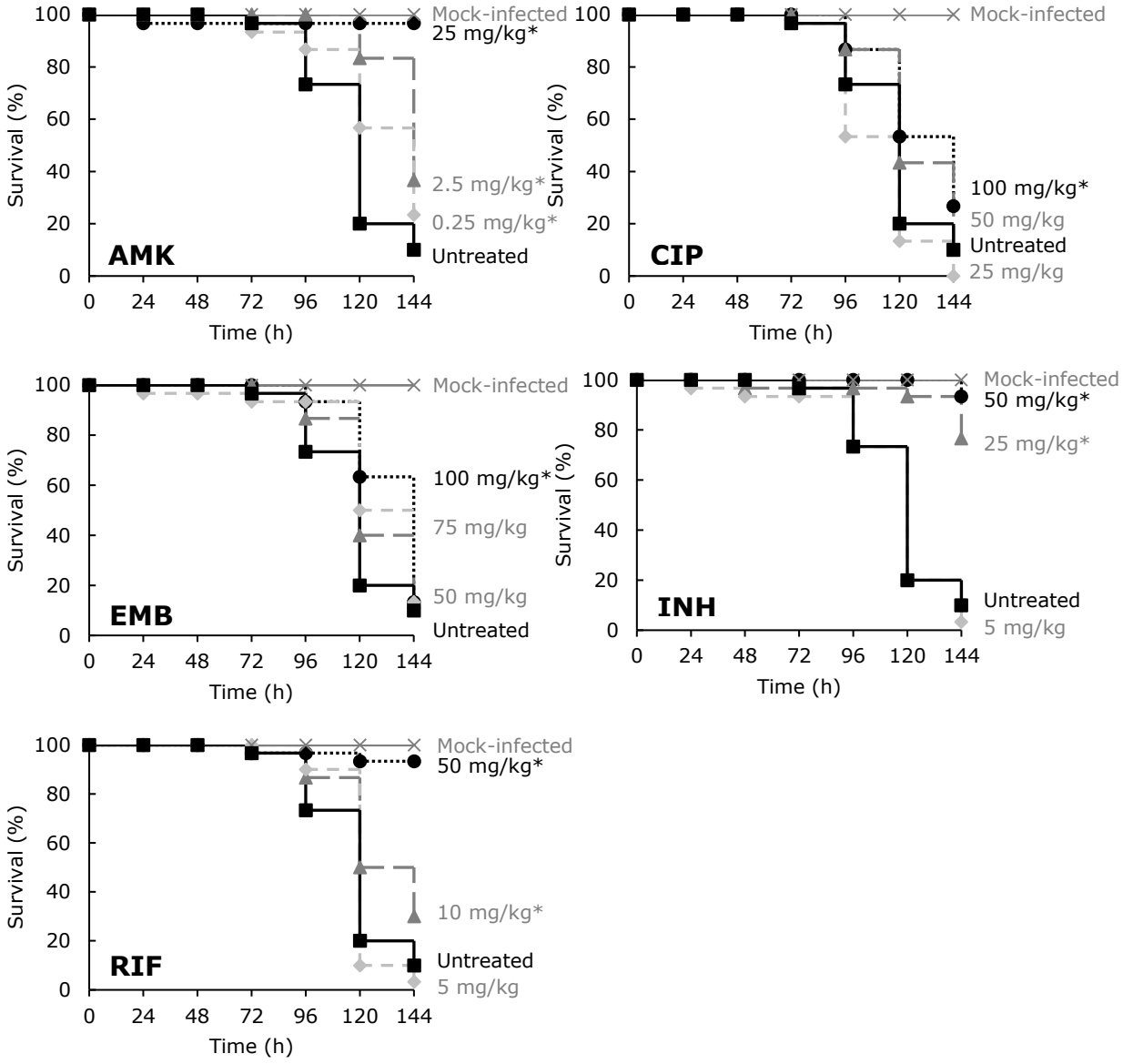

Figure 3.3 – Kaplan-Meir curves indicating the survival of larvae across a 144-hour observation window after infection with *M. marinum* Strain M and treatment with a single antibiotic dose. Larvae were injected with 10 µL of bacteria at 3 x 10^7 c.f.u/mL, then with a single dose of antibiotic two hours later. The different mg/kg doses of antibiotic used are shown to the right of each curve. The antibiotic used is shown in bold in the left corner of each curve. Infected and untreated larvae, black filled squares; highest antibiotic dose, black filled circles; medium antibiotic dose, grey filled triangles; lowest antibiotic dose, light grey filled diamonds. Controls of larvae mock-infected with PBS (grey crosses) are also shown. When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant antibiotic dose. $n = 30$. AMK, amikacin; CIP, ciprofloxacin; EMB, ethambutol; INH, isoniazid; RIF, rifampicin.

3.5.1.4 *M. marinum* R356933F – larval survival with single dose antibiotics

The *M. marinum* R356933F bacteria were used at 9 x 10^8 c.f.u/mL and antibiotics were injected 2 hours later. The Kaplan-Meir curves are shown in **Figure 3.4**. Larvae injected with PBS alone (un-infected larvae) had 100% survival throughout.

Amikacin, isoniazid, and rifampicin were all effective at improving larval survival, with amikacin being especially effective. Even at very low doses (0.25 mg/kg) larval survival was significantly improved, and injections of 25 mg/kg gave almost 100% survival at 144 hours.

As with *M. marinum* Strain M, ciprofloxacin and ethambutol were ineffective at increasing larval survival. This means that both bacterial species used here were unaffected by ethambutol – it did not give improved survival at any dose, even at 100 mg/kg. The Kaplan-Meir curves for ciprofloxacin were very similar between *M. marinum* Strain M and R356933F. There were some time points were larval survival was slightly higher for the CIP treated larvae, but overall there were no significant difference between the treated and untreated larvae.

There are some inconsistencies between the survival assays for *M. marinum* R356933F, although not as stark as those with *M. marinum* Strain M. CIP and EMB both have very low MIC values but were not effective treatments to improve larval survival. The antibiotics with the highest MICs were AMK and INH, which were both able to improve larval survival. AMK was especially effective, even at low doses.

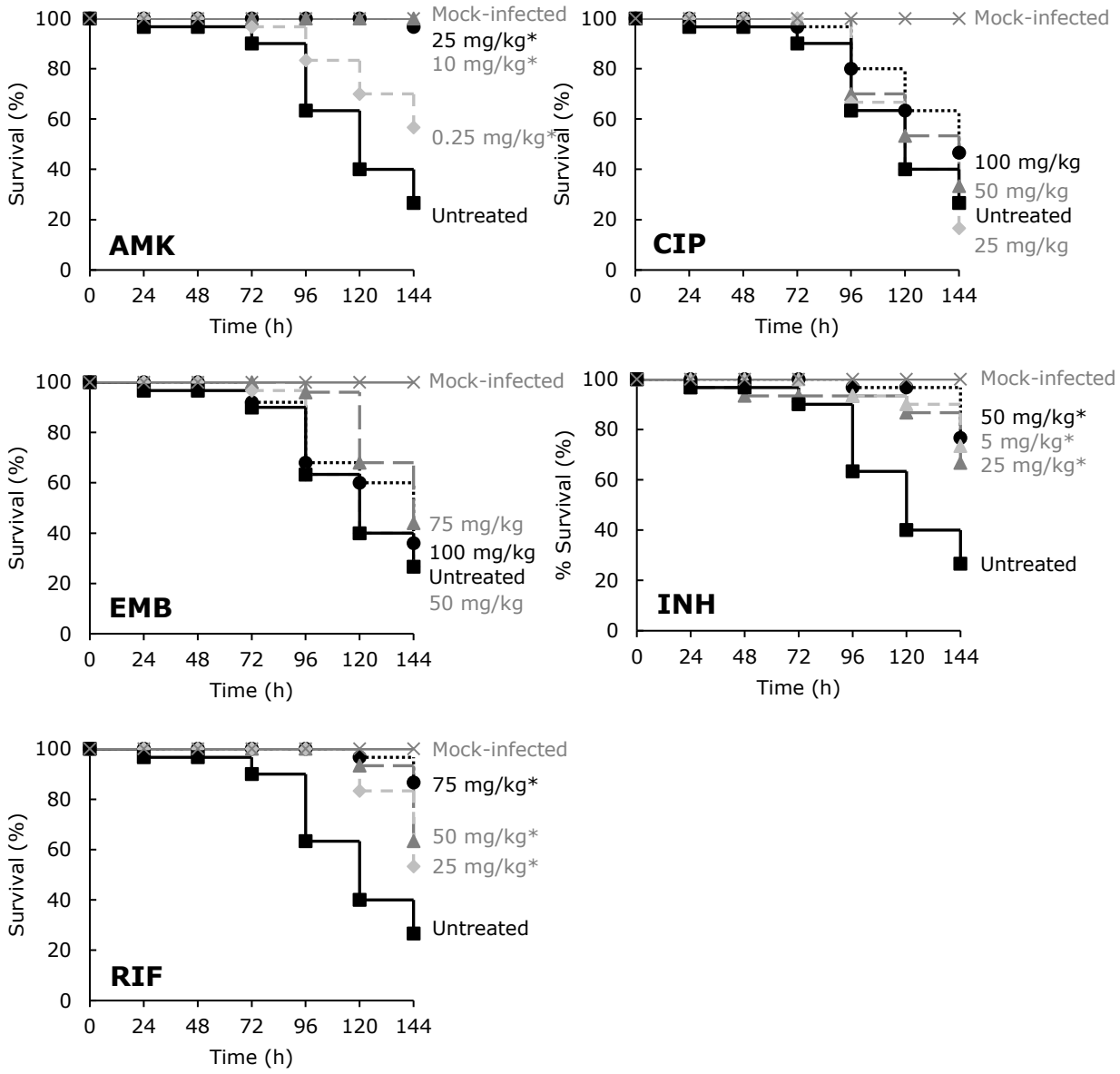

Figure 3.4 – Kaplan-Meir curves indicating the survival of larvae across a 144-hour observation window after infection with *M. marinum* R356933F and treatment with a single antibiotic dose. Larvae were injected with 10 µL of bacteria at 9 x 10^8 c.f.u/mL, then with a single dose of antibiotic two hours later. The different mg/kg doses of antibiotic used are shown to the right of each curve. The antibiotic used is shown in bold in the left corner of each curve. Infected and untreated larvae, black filled squares; highest antibiotic dose, black filled circles; medium antibiotic dose, grey filled triangles; lowest antibiotic dose, light grey filled diamonds. Controls of larvae mock-infected with PBS (grey crosses) are also shown. When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant antibiotic dose. n = 30. AMK, amikacin; CIP, ciprofloxacin; EMB, ethambutol; INH, isoniazid; RIF, rifampicin.

3.5.2 Antibiotics can help to reduce the bacterial burden of larvae infected with Mycobacteria

Due to the size and expense of burden experiments, only *M. fortuitum* NCTC 10394 and *M. marinum* Strain M were selected to assess with three antibiotics as they are the type strains for each species. Both species showed little response to treatment with EMB, and a high sensitivity to AMK, so both species were examined in combination with these antibiotics. *Mycobacterium fortuitum* NCTC 10394 was also tested with CIP, and *M. marinum* Strain M with INH, as they showed sensitivity to each of these.

Initially, these experiments were run using standard M7H9 + ADC agar plates, but it became clear that there was unexpected growth of bacteria other than the Mycobacteria. Due to *M. marinum* being photochromic, meaning the colour of the bacteria changes when exposed to light, the identity of the bacteria on the plate can easily be assessed by leaving them in sunlight for a day. If the colonies turn yellow, they are almost certainly *M. marinum*, yet the plates had a number of white colonies which did not change colour. As one of the stages in the burden protocol (see **Section 7.6.1**) is to surface sterilise the larvae with ethanol, this meant the bacterial colonies originated from bacteria inside the larvae.

To stop the growth of these unwanted internal bacteria, an antibiotic needed to be found to stop their growth but leave the Mycobacteria to grow unaffected. A panel of antibiotics were assessed as described in **Section 2.2.2**, and various β-lactams were effective at preventing growth of the contaminating bacteria but permitting the growth of *M. fortuitum* NCTC 10394, *M. fortuitum* NCTC 8573, *M. marinum* Strain M, and *M. marinum* R356933F. Piperacillin was selected, and used in plates at a concentration of 256 μg/mL.

3.5.2.1 *M. fortuitum* NCTC 10394 – burden results

The number of bacteria isolated from the larvae across the 144 hours is shown in **Figure 3.5**. The number of bacteria isolated 5 hours post-infection was far lower than the number injected. For the untreated group the number of internal, viable bacteria slowly increases across the observation period, For the larvae treated with ciprofloxacin, there was an increase in the number of internal bacteria in the larvae, although the increase in comparatively small compared to the untreated larvae. The larvae treated with AMK exhibited a decrease in the number of internal bacteria, down to as low as 32,100 c.f.u/mL at 96 hours, which is less than 0.01% of the bacteria injected into the larvae. The final collection at 144 hours shows an increased number of bacteria per mL, but still below the value for the untreated larvae.

Although there was a reasonable amount of variation in the number of internal bacteria in each larvae, the standard error of the mean for each point is usually very small and the overall trend in bacterial numbers are clear.

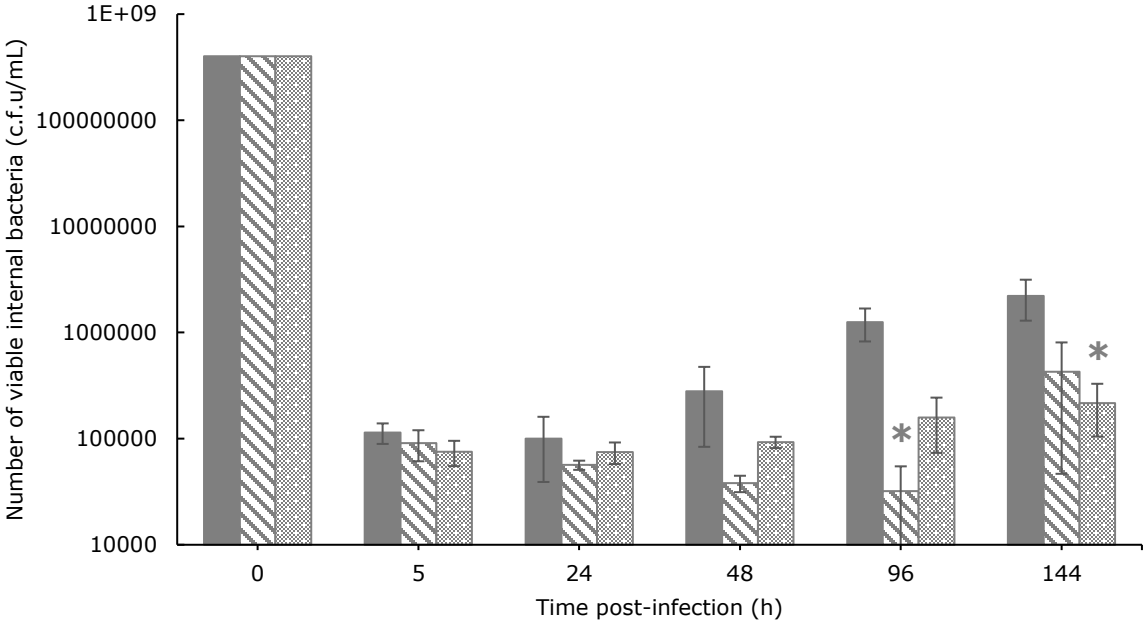

Figure 3.5 – The number of internal viable *M. fortuitum* bacteria is reduced in larvae treated with amikacin or ciprofloxacin. Larvae were injected with 10 µL of bacteria at 4 x 10^8 c.f.u/mL, then with a single dose of antibiotic two hours later; either 10 mg/kg of amikacin, or 50 mg/kg of ciprofloxacin. Infected and untreated larvae, grey filled bars; larvae treated with amikacin, diagonal grey stripes; larvae treated with ciprofloxacin, dotted grey bars. Each bar is the averaged value from five larvae and the SEM of each point is also shown. When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, Mann-Whitney *U* test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown above the relevant antibiotic bar.

3.5.2.2 *M. marinum* Strain M – burden results

The number of bacteria isolated from the larvae across the 144 hours is shown in **Figure 3.6**. As with *M. fortuitum* NCTC 10394, there is an initial decrease in the number of viable bacteria in the period after injection, although the decrease is not as pronounced. In contrast to the *M. fortuitum* assay, the untreated larvae show a decreasing number of internal bacteria for the duration of the experiment, as do the larvae treated with amikacin. The larvae treated with isoniazid have consistently fewer internal bacteria than the untreated larvae, except at the 144-hour collection point, where the number of bacteria is statistically similar to the untreated larvae.

As with the *M. fortuitum* assay, there is variation between each larvae from the treatment and control groups at each timepoint, but the standard error bars are small and the patterns of bacterial growth are well-defined.

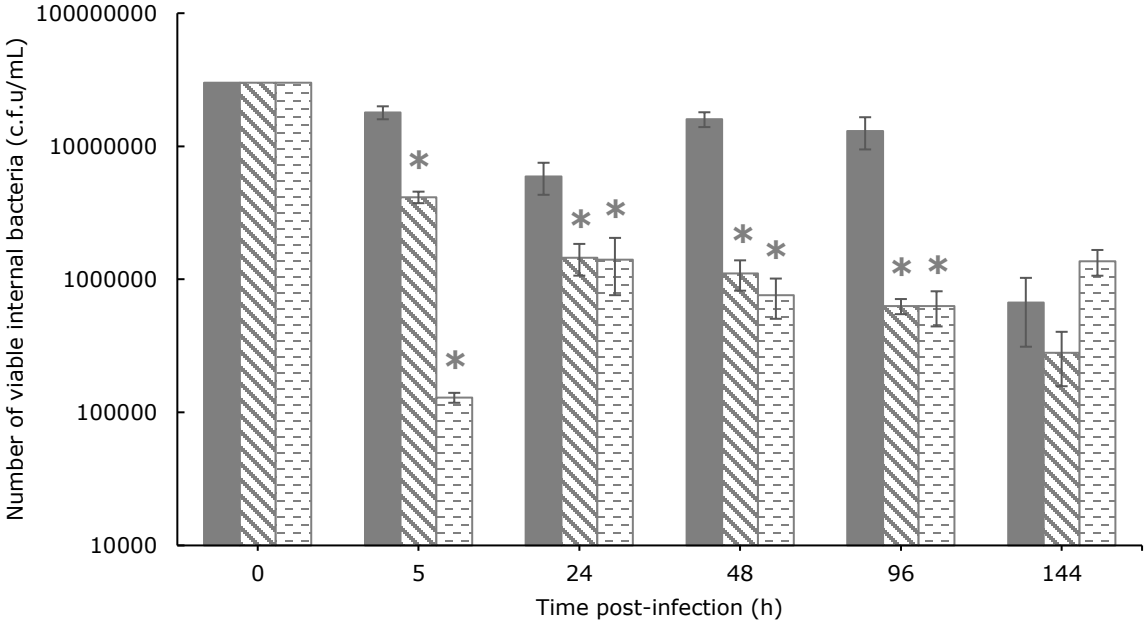

Figure 3.6 – The number of internal viable *M. marinum* bacteria is reduced in larvae treated with amikacin or isoniazid. Larvae were injected with 10 µL of bacteria at 3 x 10^7 c.f.u/mL, then with a single dose of antibiotic two hours later; either 25 mg/kg of amikacin, or 50 mg/kg of isoniazid. Infected and untreated larvae, grey solid bars; larvae treated with amikacin, diagonal grey lines; larvae treated with isoniazid, horizontal grey dashes. Each bar is the averaged value from five larvae and the SEM of each point is also shown. When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, Mann-Whitney *U* test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown above the relevant antibiotic bar.

3.5.3 Combining antibiotics can further improve larval survival compared to single antibiotic treatment

As seen in **Sections 3.5.1.1** through **3.5.1.4**, a single dose of antibiotic can significantly improve larvae survival, although not all antibiotics are effective and not every dose will increase survival.

Antibiotics doses in this section were selected because they gave survival curves that were statistically similar to untreated larvae. Therefore, any enhanced efficacy of combinations compared with the ineffective monotherapies would be readily apparent. For example, ethambutol was ineffective at improving survival even at 100 mg/kg, so was used at this dose in the combined treatments. However, for *M. fortuitum* NCTC 10394 and ciprofloxacin, 25 mg/kg still gave 50% survival at 144-hours, but 2.5 mg/kg gave almost no survival, so 2.5 mg/kg was used.

3.5.3.1 *M. fortuitum* NCTC 10394 – larval survival with combined antibiotic treatments

Larvae were injected with combined injections of AMK, CIP, EMB, INH and RIF, along with single antibiotic injections of each drug at the dose used in the combined injection, and an untreated control. The results are shown in **Figures 3.7, 3.8** and **3.9**.

Combinations which included AMK (**Figures 3.7 and 3.8**) did especially well in comparison to single-dose antibiotics. In the group injected with AMK + EMB + INH + RIF, survival at 144 hours was 96.7%, meaning that only one larva from the experiment died. Equally, larvae injected with AMK + INH + RIF had 93.3% survival at 144 hours, meaning only two larvae died.

Combinations including CIP were not as effective as those with AMK (see **Figure 3.7 and 3.9**). The survival curves still indicate a higher survival rate than the untreated larvae, which is to be expected, but the larvae did not survive any better than larvae that had single doses of CIP.

In contrast, the combination of EMB + INH + RIF improved larval survival to 26.7%, which is not as impressive as the AMK combination results but is much higher than the survival of the untreated larvae and also the single dose injections of each of these antibiotics alone.

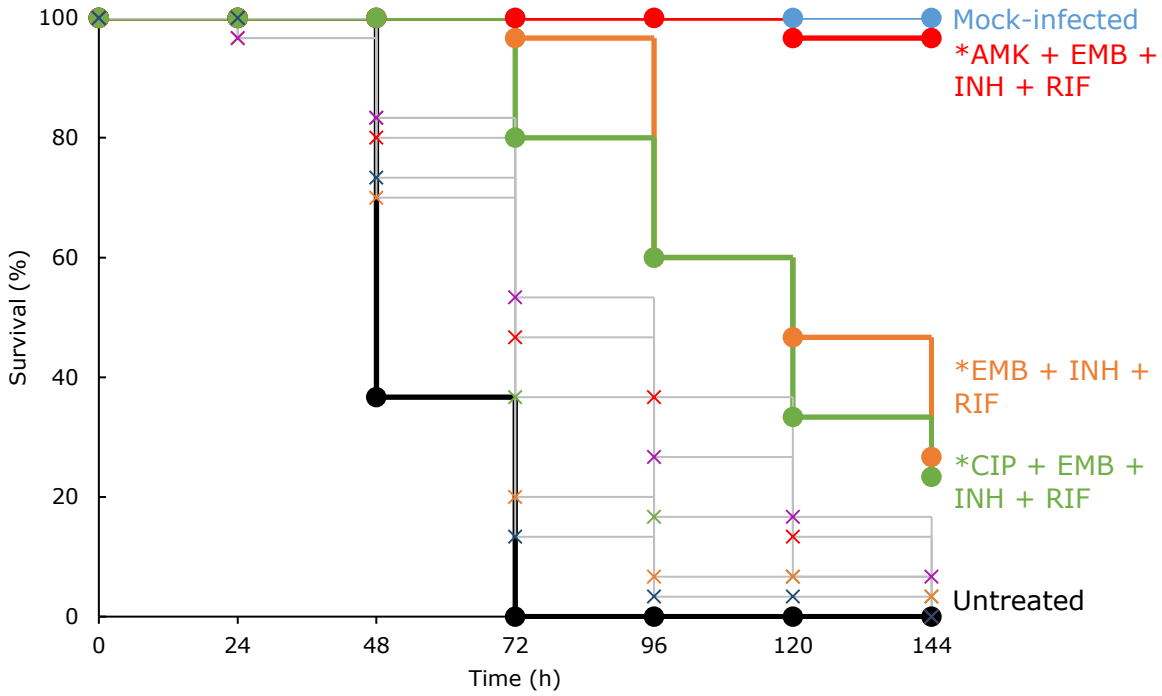

Figure 3.7 – Kaplan-Meir curves indicating the increased survival of larvae across a 144-hour observation window after infection with *M. fortuitum* NCTC 10394 and treatment with a combined antibiotic dose. Larvae were injected with 10 µL of bacteria at 4 x 10^8 c.f.u/mL, then with a combined dose of antibiotic two hours later. The different mg/kg doses of antibiotic were 5 mg/kg of amikacin, 2.5 mg/kg of ciprofloxacin, 100 mg/kg of ethambutol, 5 mg/kg of isoniazid, or 30 mg/kg of rifampicin. The antibiotic used in each combination are shown to the right of each curve. Infected and untreated larvae, black filled circles; AMK + EMB + INH + RIF, red filled circles; CIP + EMB + INH + RIF, green filled circles; EMB + INH + RIF, orange filled circles.

Controls of larvae mock-infected with PBS (blue filled circles) are also shown, as are the curves for the antibiotics when used on their own (shown with light grey lines, and coloured crosses rather than filled circles). When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant combination curve. For this data, the combination treatments were all significantly improved over the monotherapies ($p < 0.05$, not highlighted on figure). $n = 30$. AMK (red), amikacin; CIP (green), ciprofloxacin; EMB (orange), ethambutol; INH (purple), isoniazid; RIF (blue), rifampicin.

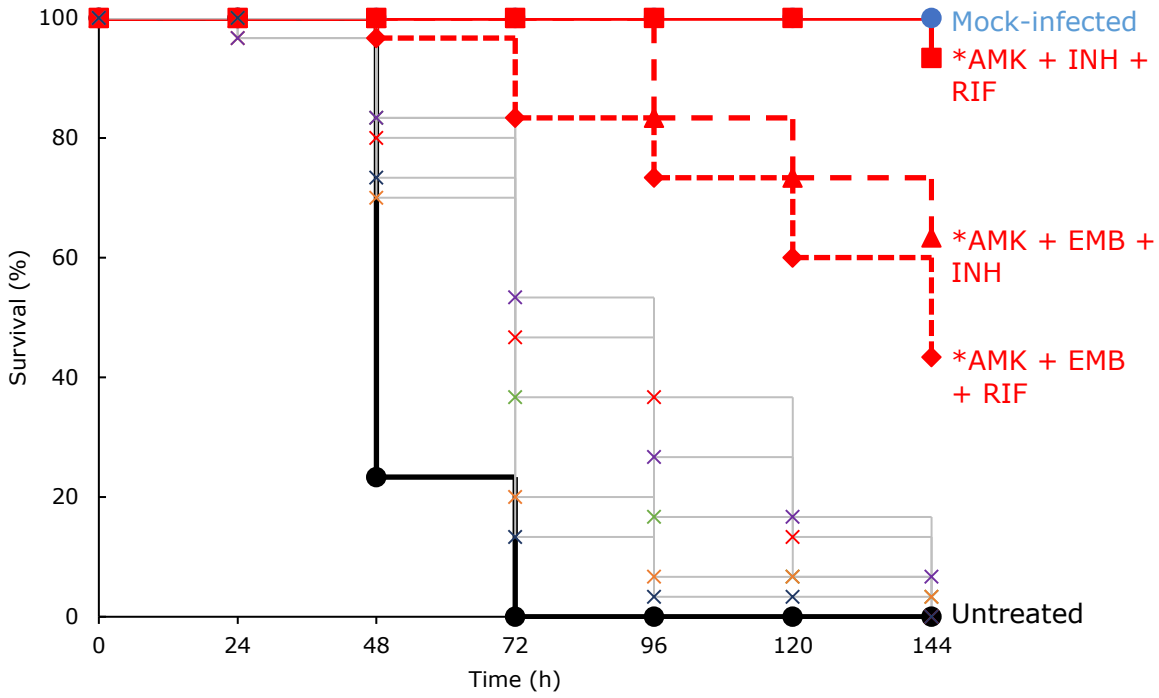

Figure 3.8 – Kaplan-Meir curves indicating the increased survival of larvae across a 144-hour observation window after infection with *M. fortuitum* NCTC 10394 and treatment with a combined antibiotic dose. Larvae were injected with 10 µL of bacteria at 4 x 10^8 c.f.u/mL, then with a combined dose of antibiotic two hours later. The different mg/kg doses of antibiotic were 5 mg/kg of amikacin, 100 mg/kg of ethambutol, 5 mg/kg of isoniazid, or 30 mg/kg of rifampicin. The antibiotic used in each combination are shown to the right of each curve. Infected and untreated larvae, black filled circles; AMK + INH + RIF, red filled squares; AMK + EMB + INH, red filled triangles; AMK + EMB + RIF, red filled diamonds.

Controls of larvae mock-infected with PBS (blue filled circles) are also shown, as are the curves for the antibiotics when used on their own (shown with light grey lines, and coloured crosses rather than filled circles). When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant combination curve. For this data, the combination treatments were all significantly improved over the monotherapies ($p < 0.05$, not highlighted on figure). $n = 30$. AMK (red), amikacin; EMB (orange), ethambutol; INH (purple), isoniazid; RIF (blue), rifampicin.

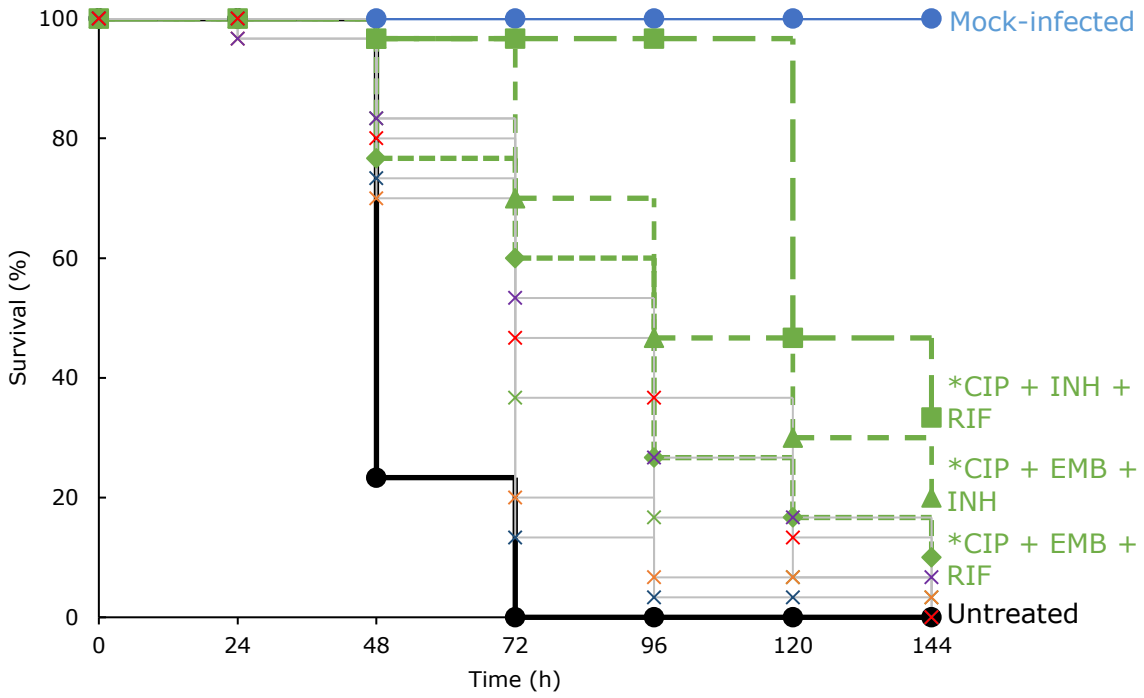

Figure 3.9 – Kaplan-Meir curves indicating the increased survival of larvae across a 144-hour observation window after infection with *M. fortuitum* NCTC 10394 and treatment with a combined antibiotic dose. Larvae were injected with 10 µL of bacteria at 4 x 10^8 c.f.u/mL, then with a combined dose of antibiotic two hours later. The different mg/kg doses of antibiotic were 2.5 mg/kg of ciprofloxacin, 100 mg/kg of ethambutol, 5 mg/kg of isoniazid, or 30 mg/kg of rifampicin. The antibiotic used in each combination are shown to the right of each curve. Infected and untreated larvae, black filled circles; CIP + INH + RIF, green filled squares; CIP + EMB + INH, green filled triangles; CIP + EMB + RIF, green filled diamonds.

Controls of larvae mock-infected with PBS (blue filled circles) are also shown, as are the curves for the antibiotics when used on their own (shown with light grey lines, and coloured crosses rather than filled circles). When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant combination curve. For CIP + INH + RIF, survival significantly improved over the monotherapies ($p < 0.05$, not highlighted on figure). CIP + EMB + RIF had significantly higher survival than the EMB monotherapy but no other monotherapies. CIP + EMB + INH had significantly higher survival over all monotherapies except for INH. $n = 30$. CIP (green), ciprofloxacin; EMB (orange), ethambutol; INH (purple), isoniazid; RIF (blue), rifampicin.

3.5.3.2 *M. marinum* Strain M – larval survival with combined antibiotic treatments

Larvae were injected with combined injections of AMK, CIP, EMB, INH and RIF, along with single antibiotic injections of each drug at the dose used in the combined injection, and an untreated control. The results are shown in **Figures 3.10, 3.11** and **3.12**.

As with *M. fortuitum* NCTC 10394, the combination which improved larval survival the most effectively was the group injected with AMK + EMB + INH + RIF, with only one larvae dying during the 144 hours of observation (**Figure 3.10**). However, the EMB + INH + RIF and CIP + EMB + INH + RIF groups also have a significantly higher survival than untreated larvae.

The efficacy of triple combination treatments including AMK also gave significantly higher rates of survival than untreated larvae (**Figure 3.11**), and these combinations also gave improved survival over single antibiotic injections at an equivalent dose. However, triple antibiotic combinations including CIP did not improve survival above single antibiotic injections (**Figure 3.12**). The survival across the full 144 hours of observation may be significant compared to untreated larvae ($p > 0.05$), but the final survival is still low.

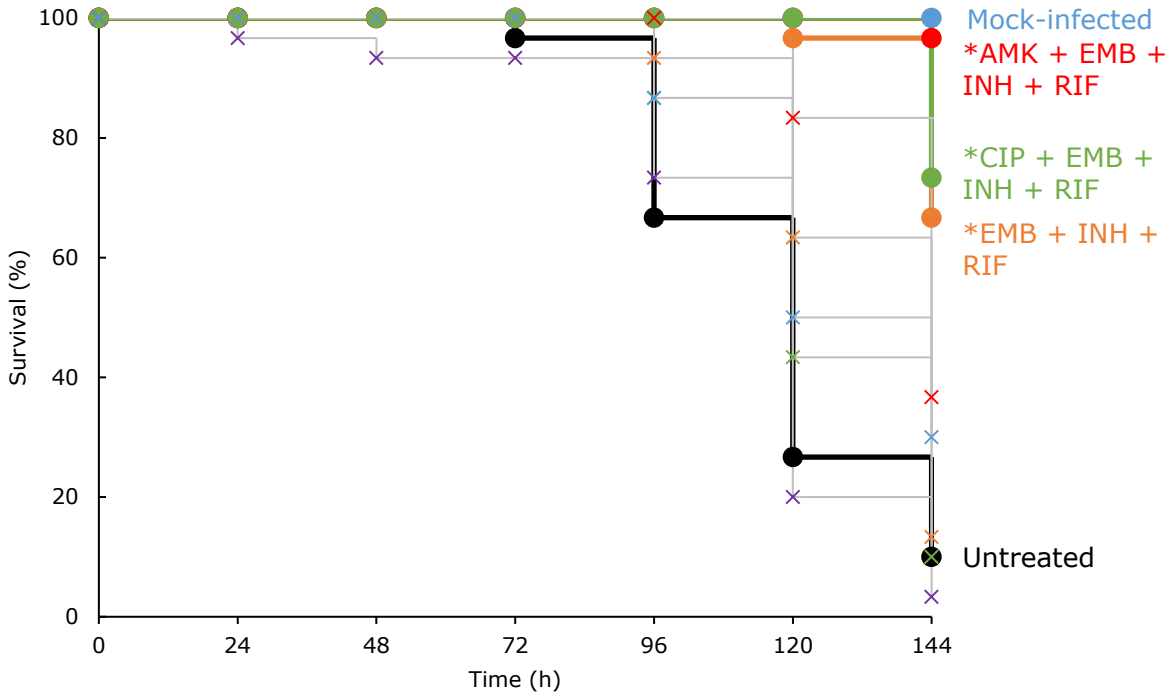

Figure 3.10 – Kaplan-Meir curves indicating the increased survival of larvae across a 144-hour observation window after infection with *M. marinum* Strain M and treatment with a combined antibiotic dose. Larvae were injected with 10 µL of bacteria at 3 x 10^7 c.f.u/mL, then with a combined dose of antibiotic two hours later. The different mg/kg doses of antibiotic were 2.5 mg/kg of amikacin, 50 mg/kg of ciprofloxacin, 100 mg/kg of ethambutol, 5 mg/kg of isoniazid, or 10 mg/kg of rifampicin. The antibiotic used in each combination are shown to the right of each curve. Infected and untreated larvae, black filled circles; AMK + EMB + INH + RIF, red filled circles; CIP + EMB + INH + RIF, green filled circles; EMB + INH + RIF, orange filled circles.

Controls of larvae mock-infected with PBS (blue filled circles) are also shown, as are the curves for the antibiotics when used on their own (shown with light grey lines, and coloured crosses rather than filled circles). When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant combination curve. For this data, the combination treatments were all significantly improved over the monotherapies ($p < 0.05$, not highlighted on figure). $n = 30$. AMK (red), amikacin; CIP (green), ciprofloxacin; EMB (orange), ethambutol; INH (purple), isoniazid; RIF (blue), rifampicin.

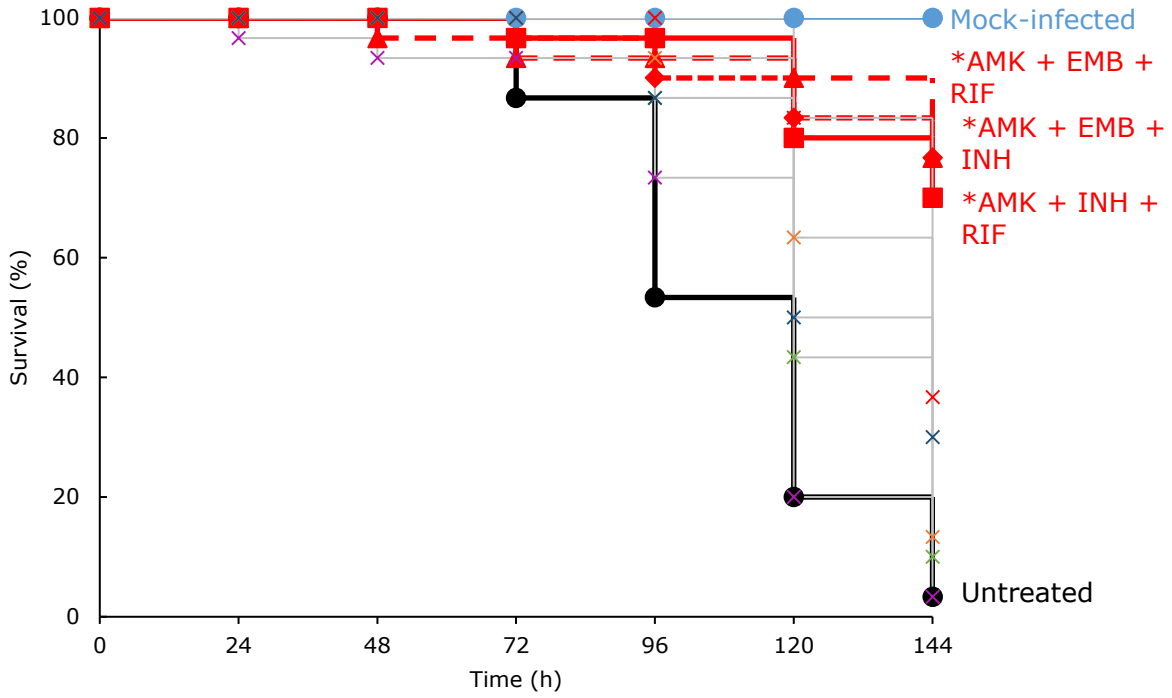

Figure 3.11 – Kaplan-Meier curves indicating the increased survival of larvae across a 144-hour observation window after infection with *M. marinum* Strain M and treatment with a combined antibiotic dose. Larvae were injected with 10 µL of bacteria at 3 x 10^7 c.f.u/mL, then with a combined dose of antibiotic two hours later. The different mg/kg doses of antibiotic were 2.5 mg/kg of amikacin, 100 mg/kg of ethambutol, 5 mg/kg of isoniazid, or 10 mg/kg of rifampicin. The antibiotic used in each combination are shown to the right of each curve. Infected and untreated larvae, black filled circles; AMK + INH + RIF, red filled squares; AMK + EMB + INH, red filled triangles; AMK + EMB + RIF, red filled diamonds.

Controls of larvae mock-infected with PBS (blue filled circles) are also shown, as are the curves for the antibiotics when used on their own (shown with light grey lines, and coloured crosses rather than filled circles). When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant combination curve. For this data, the combination treatments were all significantly improved over the monotherapies ($p < 0.05$, not highlighted on figure). $n = 30$. AMK (red), amikacin; EMB (orange), ethambutol; INH (purple), isoniazid; RIF (blue), rifampicin.

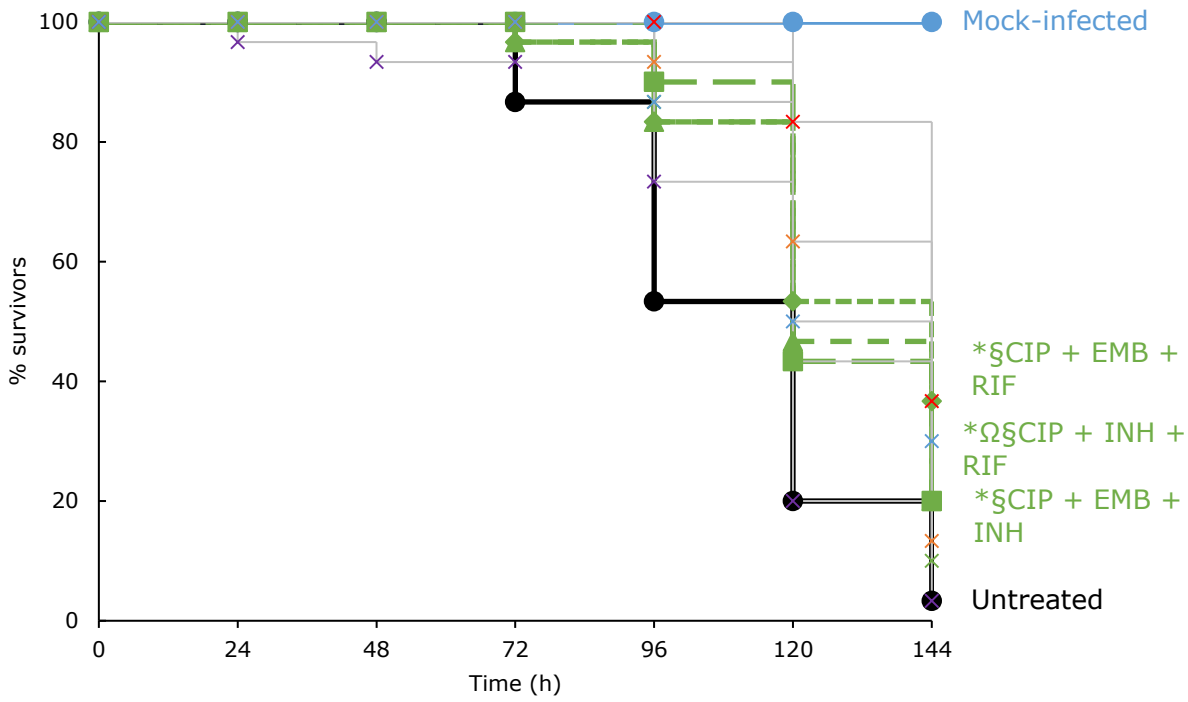

Figure 3.12 – Kaplan-Meir curves indicating the increased survival of larvae across a 144-hour observation window after infection with *M. marinum* Strain M and treatment with a combined antibiotic dose. Larvae were injected with 10 µL of bacteria at 3 x 10^7 c.f.u/mL, then with a combined dose of antibiotic two hours later. The different mg/kg doses of antibiotic were 50 mg/kg of ciprofloxacin, 100 mg/kg of ethambutol, 5 mg/kg of isoniazid, or 10 mg/kg of rifampicin. The antibiotic used in each combination are shown to the right of each curve. Infected and untreated larvae, black filled circles; CIP + INH + RIF, green filled squares; CIP + EMB + INH, green filled triangles; CIP + EMB + RIF, green filled diamonds.

Controls of larvae mock-infected with PBS (blue filled circles) are also shown, as are the curves for the antibiotics when used on their own (shown with light grey lines, and coloured crosses rather than filled circles). When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant combination curve. When the combination treatments were significantly ($p > 0.05$) improved over AMK monotherapy a Ω symbol is shown beside the curve. When the combination treatments were significantly ($p > 0.05$) improved over INH monotherapy a § symbol is shown beside the curve. $n = 30$. CIP (green), ciprofloxacin; EMB (orange), ethambutol; INH (purple), isoniazid; RIF (blue), rifampicin.

3.5.4 Combinations of antibiotics can decrease the internal bacterial burden inside larvae more effectively than when single antibiotic treatments are used

3.5.4.1 *M. fortuitum* NCTC 10394 – combined antibiotics burden results

The number of bacteria isolated from the larvae across the 144 hours is shown in **Figure 3.13**. The data from the larvae mock-treated with PBS is also shown. The antibiotics used in the combinations are the same as those in **Figures 3.7, 3.8** and **3.9**, and were selected because they did not improve larval survival when injected as single doses (see **Figure 3.1**).

The larvae treated with EMB + INH + RIF or CIP + EMB + INH + RIF have bacterial burden curves that are quite similar to those for larvae treated with a single antibiotic dose (**Figure 3.5**). The number of internal viable bacteria sharply drops from the number that were injected, but then the number of bacteria remains between 10,000 and 100,000 c.f.u/mL. The bacteria are not cleared from the larvae, but the number of bacteria stays reasonably constant for the duration of the experiment.

However, the larvae treated with AMK + EMB + INH + RIF show a very distinct decrease in internal bacteria. By 144 hours, the average number of bacteria per mL in these larvae was 1,400, a very small number compared to two million bacteria per mL in the mock-treated larvae or the 426,600 bacteria per mL in the larvae treated with AMK alone (**Figure 3.5**).

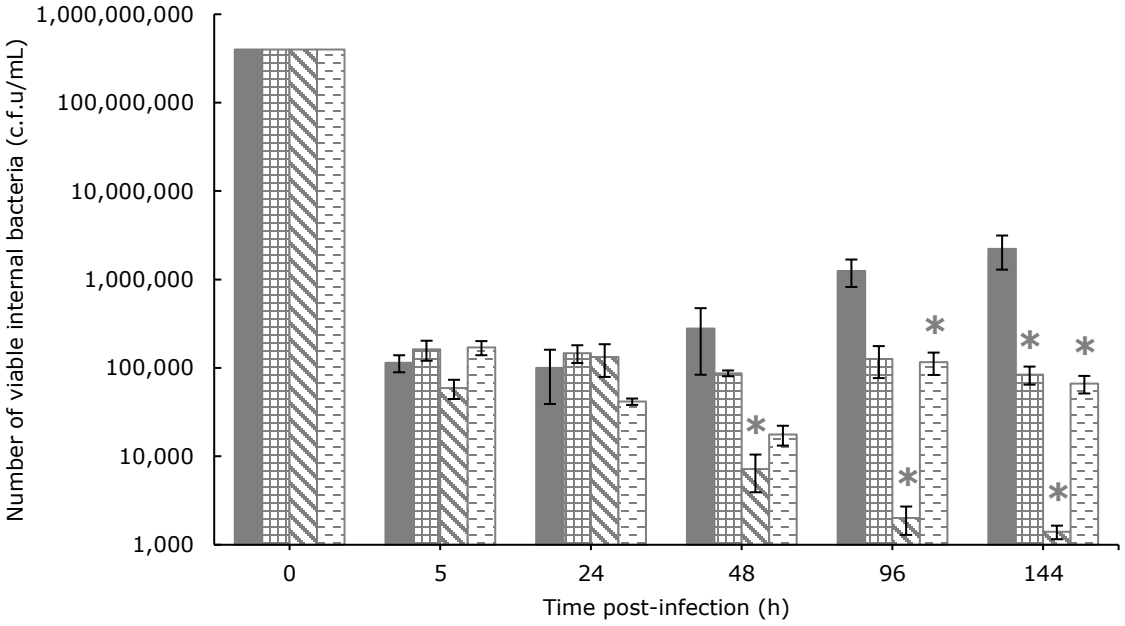

Figure 3.13 – The number of internal viable *M. fortuitum* bacteria is reduced in larvae treated with injections of combined antibiotics. Larvae were injected with 10 µL of bacteria at 4 x 10^8 c.f.u/mL, then with a single injection of a combined dose of antibiotics two hours later. The combinations of antibiotics are shown to the right of each curve. Infected and untreated larvae, solid grey bars; larvae treated with EMB + INH + RIF, grey grid-pattern; larvae treated with AMK + EMB + INH + RIF, diagonal grey bars; CIP + EMB + INH + RIF, horizontal grey dashes. Each bar is the averaged value from five larvae and the SEM of each point is also shown. When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, Mann-Whitney *U* test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown above the relevant antibiotic bar. AMK, amikacin at 5 mg/kg; CIP, ciprofloxacin at 2.5 mg/kg; EMB, ethambutol at 100 mg/kg; INH, isoniazid at 5 mg/kg; RIF, rifampicin at 30 mg/kg.

3.6 Summary

In these experiments, AMK, CIP, INH and RIF have been shown to be successful at improving larval survival when administered 2 hours post-infection, although some antibiotics were more effective than others and some were only effective against *M. fortuitum* or *M. marinum* infections, but not both. For example, RIF was effective at treating *M. marinum* R356933F infections, but ineffective for infections with *M. fortuitum* NCTC 10394.

EMB was essentially ineffective at improving larval survival in all cases, with survival at 144 hours being reliably similar to untreated larvae. These results were dose-dependent, with higher doses conferring increased larval survival over lower doses.

When doses of antibiotics that did not significantly improve larval survival in single dose injections were combined into triple and quadruple injections, larval survival was improved over untreated larvae in all cases, occasionally to very high survival percentages. This was seen most clearly in the quadruple injections of AMK + EMB + INH + RIF and CIP + EMB + INH + RIF. However, survival was not always increased in comparison to the larvae treated with the antibiotic in a single dose injection.

Burden assays also showed that single doses of bacteria could reduce the internal bacterial burden within larvae. Although the results for *M. fortuitum* NCTC 10394 were not statistically significant for the majority of datapoints, the overall decrease in internal burden was still clear. A similar result was seen with *M. marinum* Strain M, although with more support from the Mann-Whitney *U* analysis.

When combinations of antibiotics are used, the internal bacterial burden of *M. fortuitum* is reduced even further. These experiments used antibiotics at doses which were poor at

improving larval survival when injected as single doses, and the burden combinations were the same as those used in the survival curves.

3.7 Discussion

Ethambutol, isoniazid and rifampicin were used in these experiments for their inclusion in standard TB treatment regimens[484] and amikacin and ciprofloxacin for their use as second-line treatments for MDR-TB[485]. These anti-Mycobacterials have been used with *M. fortuitum*[486–491] and *M. marinum*[101,139,143] both *in vitro* and *in vivo,* although *in vivo* experiments use combination treatments in the vast majority of cases*.* When used in patients, amikacin can be used at 15 mg/kg for *M. fortuitum* infections[486] or 400 mg daily for *M. marinum*[492]. Ciprofloxacin has been used at 8 mg/kg for *M. fortuitum* infections[493] and 500 mg twice daily for *M. marinum*[494]*.* Daily doses of ethambutol at 25 mg/kg and rifampicin at 600 mg is a well-established treatment for *M. marinum* infections[495] and isoniazid at 10 mg/kg has been shown to be an effective treatment for *M. marinum* in Zebrafish[496]. However, ethambutol, isoniazid and rifampicin are not commonly prescribed for *M. fortuitum* infections.

The results from the survival experiments using these antibiotics showed a degree of correlation between the data for patients – in particular, amikacin was effective even at quite low doses, and ciprofloxacin was useful for treating *M. marinum* infections. Isoniazid was also very effective for *M. marinum* treatment.

The results for *M. marinum* Strain M (**Figure 3.3**) and *M. marinum* R356933F (**Figure 3.4**) were also quite comparable – AMK, INH and RIF were all very effective, but CIP and EMB has very little to no effect on increasing survival. However, rifampicin improved survival for larvae infected with *M. fortuitum* NCTC 8573 (**Figure 3.2**), but not for larvae infected with *M. fortuitum* NCTC 10394 (**Figure 3.1**). Equally, larvae infected with *M. fortuitum* NCTC 10394 were

responsive to INH, but larvae infected with *M. fortuitum* NCTC 8573 saw no benefit from INH treatment, even when used at 100 mg/kg.

When compared to the MIC values (**Table 3.1**) these results correlate quite well – the MIC for INH with *M. fortuitum* NCTC 10394 was less than 2 mg/L, but over 256 mg/L for *M. fortuitum* NCTC 8573. The same correlation is seen to some degree with RIF (<0.016 mg/L for *M. fortuitum* NCTC 8573, but 12 mg/L for *M. fortuitum* NCTC 10394), but not to the same extremes. Yet this does not explain why there is such a significant difference is results between two strains of the same species. *Mycobacterium fortuitum* NCTC 10394 is the type strain for *M. fortuitum,* although both strains were isolated from human patients. There have been studies which show significant variation in the antibiotic susceptibility profiles of different *M. fortuitum* strains[126,497], and there is also a wide degree of variation in the biochemical and antigenic properties between strains[498]. Hence, as the MIC and survival data for INH and RIF correlate well, the strain variation is likely to be due to the well-reported variance between *M. fortuitum* strains.

It is also clear that the antibiotics that were effective in improving larval survival showed a dose-dependent response. This meant that survival over the 144 hours of observation was correlated with the amount of antibiotic injected, so a low dose of antibiotic could reliably lead to a low larval survival that would be comparable to an untreated group of larvae. Hence, these ineffective doses of antibiotic could be combined and if an improvement in survival were observed, it would due to the combined dose of antibiotics, as they had been ineffective when used alone.

The results from combined antibiotic injections (**Section 3.5.3**) indicate that combined antibiotic injects are effective at improving larval survival over single doses, however it is not

obvious if the improvement is due to an additive effect or synergy without *in vitro* experiments. However, the inclusion of AMK in any combination seems to improve survival to a high degree – this is particularly clear in **Figure 3.8** when *M. fortuitum* NCTC 10394 infections are treated with AMK + INH + RIF, and in **Figure 3.7** when larvae infected with *M. fortuitum* NCTC 10394 are treated with AMK + EMB + INH + RIF. Considering AMK has been shown to act synergistically *in vitro* with other antibiotics with non-tuberculous Mycobacteria previously[499–502], these results suggest that synergy could also be improving larval survival in these conditions. The efficacy of the AMK + EMB + INH + RIF is also clear in **Figure 3.13**, where the internal bacterial burden of *M. fortuitum* NCTC 10394 is reduced to a remarkable degree.

Considering that researchers have known for decades that Mycobacterial infections need to be treated with combinations of antibiotics[201], these results support the reasoning that combination treatments can be far more useful than single-dose treatments. Although experiments with *G. mellonella* are too short to show much evidence for the development of drug-resistance, the increase in larval survival and the decrease in internal bacterial burdens align with the existing knowledge on treating Mycobacterial infections. Hence, this model may be able to indicate the worth of novel compounds in combinations with other novel drugs, or with existing antibiotics and anti-Mycobacterials. These experiments will be described in **Chapter Four**.

Chapter Four

4.0 Combining antibiotic drugs with alternative and repurposed compounds

The aim of the experiments in this chapter was to assess how the survival of *G. mellonella* larvae is affected when traditional antibiotic drugs are administered alongside less conventional compounds, or β lactams are given with β lactamase inhibitors. Survival was assessed every 24 hours for a 144 hour period.

4.1 Novel compounds and combating antibiotic resistance

4.1.1 Why is it important to find new drugs to treat infections?

The risks that bacteria posed to the human species were once felt to a much higher degree than we recognise in the modern Western world. A rotten tooth, a scratch from an animal, a cough caught while walking in the rain – any opportunity for bacteria to enter the body could lead to septicaemia or pneumonia, not to mention the dangers associated with any kind of surgical, dental, or obstetric procedure.

Unfortunately, despite antibiotics being described as one of humankind's greatest discoveries, bacteria which showed resistance to penicillin were first reported in 1940, even before penicillin had been used on a human for the first time. The period between the 1950s and 1970s has been described as the golden age of antibiotic discovery – around half of the antibiotics in use today were discovered in the 1950s alone. Yet only one new class of antibiotic has been discovered since the early 1990s[258], and resistance to antibiotics is commonly observed. This is unsurprising, considering that we are exposed to antibiotics from a huge

variety of environmental sources (food, water, and soil[503]), there are many countries where antibiotics can be purchased without a prescription, antibiotics are used in many countries to increase the yield of animal crops (both meat and dairy products), and there is a plethora of issues with patient non-adherence to antibiotic treatment protocols.

This means that many researchers feel we are approaching a "post-antibiotic era", where bacterial infections will once again become a ever-present threat. Numerous bacteria are becoming resistant to commonly used antibiotics, with some becoming resistant to all current drug treatments. Of particular concern are families of bacteria that frequently infect patients in hospital or clinical settings (nosocomial bacteria). These can infect surgical wounds, medical implants, devices, or be transmitted through air, water, or fomites. As patients are usually on one or more antibiotics, this creates a strong selective pressure for bacteria to develop resistance. Infections acquired by people who are already ill and immunosuppressed are more likely to cause long-term damage to organs and tissues, take longer to clear, and can result in patient death.

4.1.2 The search for new antibiotics

Historically, the most abundant source of antibiotics has been soil bacteria, which naturally produce antibiotics to supress the growth of competitors. Many groups are now looking at other resources to find antimicrobial compounds, such as marine bacteria, ocean sediment, bacteria isolated from deep cave and mine systems, and animal and insect sources including alligators, pandas, frogs and cockroaches. Many micro and macro organisms produce antibiotics which can be extracted and adapted to use in clinical cases.

Attention is also turning to alternative antibiotic structures, such as peptides produced by prokaryotes and eukaryotes as part of the innate immune response. They can be highly

potent and effective against a variety of microbial targets. However, only a small range of antimicrobial peptides are available for prescriptions (such as bacitracin)[504], and the peptides themselves have very short half-lives due to their high clearance rates[504].

Other alternative sources are phytochemicals isolated from plants, including cumin, coriander, green tea, ginger, garlic, and cinnamon. Plants produce an enormous variety of primary and secondary metabolites, many of which have been used as treatments in traditional medicine for many centuries. The most well-known example is probably salicin, which can be consumed in willow bark tea to treat pain and inflammation and is the natural equivalent of aspirin (acetylsalicylic acid). Many phytochemicals have been shown to improve the growth and health of livestock[505], and have been reported to have anti-obesity, anti-diabetes, anti-cancer, and anti-aging properties, alongside protective actions against cardiovascular disease, inflammatory bowel disease, and Alzheimer's disease[506]. They have also been shown to have a variety of antibacterial activities against human pathogens[507], although the minimum inhibitory concentrations are many times higher than those for "traditional" antibiotics. Hence, phytochemicals combined with antibiotics can be used to decrease the dose of antibiotic and increase their activity[508,509].

This approach, of combining a secondary compound with a primary antibiotic or antibiotics, is also a key research interest. For example, many bacteria utilise efflux pumps to actively transport antibiotics out of the bacterial cell. Efflux pumps play a myriad of roles within a bacteria ranging from metabolic processes and transport of hydrogen and sodium ions, to expulsion of toxins and antibiotics[510]. Hence, the role of efflux pumps in pathogenicity is clear, but it is likely that they are also involved in virulence and quorum sensing[511]. Efflux pumps can be used to reduce the internal concentration of antibiotics, which can bring them below the

minimum inhibitory concentration – this means they are no longer effective at killing the bacteria, but the presence of a low concentration of antibiotics also means it can be more likely for resistance to develop[510]. These pumps can be specific to a single substrate, or can eliminate entire families of antibiotics.

However, the use of an inhibitor for these pumps can ensure that antibiotics remain inside the target bacteria at a sufficient concentration for an appropriate amount of time to allow for activity. Antibiotics which had been discarded as ineffective due to their efflux from the target microbe could become valuable treatments when combined with an efflux pump inhibitor (EPI). Increasing the range of antibiotics which can be prescribed for an infection by combining the treatment with an EPI could be a valuable asset to healthcare providers, as it reduces the risk of resistance developing, compared to using a small range of antibiotics repeatedly for an infection. As it stands, there have only been a small number of EPIs which have reached clinical trials, as there are issues with potency, toxicity, mediocre pharmacokinetic properties, or low *in vivo* efficacy[510]. A lead compound was tested as an aerosol to be used in tandem with ciprofloxacin for patients with cystic fibrosis or ventilator-associated pneumonia, but did not continue past phase I trials due to issues with tolerability[510].

Similar to efflux pumps, many bacteria also utilise enzymes to reduce the efficacy of an antibiotic by altering the structure of the drug. The most well-known example of this is the β lactamase family, which is a group of enzymes produced by a variety of bacteria to disrupt β lactam antibiotics. These enzymes have been produced by bacteria for millions of years and are produced when β lactam-producing microorganisms try to eradicate competitors in a polymicrobial community. To prevent their elimination from the community, bacteria began to produce hydrolases which could be secreted into the environment and essentially deactivate

the antibiotic. In a healthcare setting, the prescription of β lactam antibiotics to bacteria which have β lactamase activity has created strains which are totally unaffected by these types of antibiotics. Instead of using β lactam antibiotics, these types of infection were more susceptible to carbapenem classes, which also use a central β lactam ring structure but have a broader spectrum of activity and are less susceptible to β lactamase activity. However, the increase in bacteria which produce a subgroup of β lactamase called carbapenemases, especially in the Enterobacteriaceae family, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, is of great concern. Bacteria that showed resistance to carbapenems used to be very uncommon, and were almost unheard of before the millennium, but are reported at an increasing rate every year[512].

4.1.3 β lactams and Mycobacteria

Mycobacteria owe much of their antibiotic resistance to their impermeable cell envelope and their inaccessibility to antibiotics while sequestered in macrophages and granulomas[513], but their resistance to β lactam antibiotics is also due to β lactamase activity. The BlaC β lactamase which has been isolated from *M. tuberculosis* is an Ambler class A β lactamase and shows a broad substrate profile for both penicillins and cephalosporins. Class A β lactamases have also been isolated from *M. fortuitum*[514], *M. smegmatis*[515], *M. avium*[516] and *M. abscessus*[517]. This class of β lactamase are generally unable to hydrolyse carbapenems[513], and combinations of a β lactamase inhibitor and a carbapenem have shown to be effective against *M. tuberculosis*, including extremely drug resistant strains[518,519].

The Mycobacteria mentioned above (*M. fortuitum, M. smegmatis, M. avium* and *M. abscessus*) have been assess *in vitro* with various combinations of β lactam and β lactamase inhibitor[515]. There have been trials examining β lactams/carbapenems with β lactamase inhibitor drugs in patients with tuberculosis[520–522], with evidence that they can treat the slow

growing bacteria in a hypoxic environment such as a granuloma. As they are already readily available, cheap, and have a low toxicity to the liver, adding the combination of β lactams and β lactamase inhibitors to a regimen for multi drug resistant or extremely drug resistant strains of *M. tuberculosis* would be a very practical solution.

In terms of other Mycobacteria, *G. mellonella* have been used as an *in vivo* model for effectively treating *M. abscessus* with a β lactamase inhibitor (avibactam) and a β lactam antibiotic (piperacillin)[381], which is also discussed in **Section 4.2.1**. A Zebrafish model has also been used to test avibactam with cefoxitin and imipenem[523]. There have also been *in vitro* tests of other β lactam/lactamase inhibitors with *M. abscesses*[524]*.* There have been *in vitro* assessments of *M. fortuitum*[525–529] and *M. marinum*[527] with β lactam/lactamase inhibitor combinations, but no evidence could be found in the literature for any *in vivo* experiments.

4.1.4 Efflux pump inhibitors and Mycobacteria

As discussed in **Section 4.1.2**, the development of effective efflux pump inhibitors (EPIs) to prevent bacteria from expelling antibiotics is a key research interest, particularly in relation to bacteria which are resistant to standard treatment regimens.

Mycobacterium tuberculosis contains four transporters which are involved in virulence control, and other resistance, nodulation, and cell division (RND) proteins involved with polyketide synthesis, lipid transport, and certainly drug resistance[530]. Mycobacteria also make use of a wide array of efflux mechanisms, including several ATP-binding cassette (ABC) transporters and a major facilitator superfamily[531,532]. These efflux pumps are reviewed in detail in de Rossi *et al.*[532]*,* reporting a number of well-characterised pumps*.*

The effects of EPIs on *M. tuberculosis* in combination with antibiotics have been assessed *in vitro*[533–536] and in mouse models[534,537]. The efflux pump inhibitor timcodar was shown to have synergy with rifampicin, moxifloxacin, and bedaquiline, and to substantially reduce the pulmonary bacterial burden in mice when combined with rifampicin or isoniazid[537]. Verapamil was also shown to reduce pulmonary burdens in mice when combined with isoniazid, rifampicin, or pyrazinamide[534].

However, as Mycobacteria have the ability to "hide" from the host immune system inside tissues, macrophages and granulomas, EPIs can also have a role in allowing anti-Mycobacterial treatments reach bacteria which would otherwise be protected by the efflux pumps in the cells of the host.

When *M. tuberculosis* is carried around the body in the bloodstream, it is most likely to colonise parts of the body with high levels of oxygen, such as the brain[538]. Tuberculosis meningitis has the highest mortality rate of all *M. tuberculosis* infections, resulting in 30% and 65% mortality for non-HIV positive and HIV positive patients respectively, and causing long-term neurological disabilities in 50% of survivors[539]. Hence, TB medications to treat TB meningitis must be capable of crossing the blood-brain barrier and not being pumped out. A number of EPIs have been assessed in human and rodent brains[540], however there are issues in finding concentrations of EPI that can be safely administered to humans[541].

More generally, as Mycobacteria can reside in host macrophages to avoid detection by other immune cells, enabling anti-Mycobacterial drugs to cross the macrophage membrane and remain internalised within the macrophage is also clinically important[542]. EPIs have been shown to allow bioaccumulation of antibiotics intracellularly in the macrophage[538]. Macrophages which have been modified to overexpress certain efflux pumps can also have their

antibiotic susceptibility returned to that of a wild-type macrophage by depriving them of ATP, which is required for active transport of antibiotics out of the macrophage[543]. However, the lack of knowledge on this topic, and the wider role of EPIs in Mycobacterial infections, means that there is no clear result expected from combining EPIs with non-tuberculous Mycobacteria. A range of EPIs have been assessed with *M. avium* strains[533], although not with any of the EPIs used here. *Mycobacterium avium* has also been examined with EPIs but without an antibiotic[544]. There has also been a study using *M. smegmatis, M, phlei, M. avium, M. flavescence* and *M. fortuitum*[545], although *M. fortuitum* was not used in combination with any of the EPIs used in this work. Otherwise, there do not seem to be other examples in the literature of non-tuberculous Mycobacteria being treated with EPIs and antibiotics.

4.2 Assessing novel combinations using *G. mellonella*

4.2.1 β lactams and *G. mellonella*

As mentioned in many previous sections, *G. mellonella* are easy to use, allow rapid experiments, and have low purchasing and housing costs. This means they are very well-suited to simple, *in vivo* assessments of drug combinations that have promising *in vitro* data or would be expected to have a measurable effect on an experimental group compared to a control group.

A good example of this application is the use of *G. mellonella* to assess combinations of β lactam antibiotics with β lactamase inhibitors. As resistance to β lactam antibiotics is an increasingly important clinical issue, identifying and assessing potential compounds which can restore the efficacy of β lactam antibiotics is a key concern.

A paper which clearly demonstrates the use of *G. mellonella* for these types of investigations used a newly identified inhibitor of class B metallo-β-lactamases, ML302F, in

combination with meropenem against 31 carbapenem resistant Gram-negative clinical isolates[546]. These included strains of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa, Escherichia coli* and *Providencia stuartii,* and 30 of these were resistant to meropenem as a monotherapy. When ML302F and meropenem were used in combination, larval survival was significantly improved for selected *P. aeruginosa*, *E. coli* and *K. pneumoniae* strains over 96 hours of observation. Until this paper, ML302F had not been assessed in any *in vivo* models indicating the usefulness of *G. mellonella* in this context.

The research group in Israel mentioned in **Section 1.5** have also assessed a β lactamase inhibitor, avibactam, in combination with piperacillin against infections with *M. abscessus* in a *G. mellonella* infection model[381]. When injected as monotherapies, piperacillin and avibactam alone had no effect on improving larval survival or reducing the number of internal bacteria compared to an untreated control. However, combining piperacillin and avibactam significantly reduced the internal bacterial burden and improved survival. These results were comparable to larvae treated with meropenem, which is a carbapenem. This combination would be a useful treatment alternative to patients who are co-infected with *M. abscessus* and bacteria susceptible to carbapenems such as *P. aeruginosa*, as this will prevent the development of carbapenem resistant strains. This is particularly relevant to patients with cystic fibrosis, as co-infection with *M. abscessus* and *P. aeruginosa* are often observed in this group[547].

Galleria mellonella were also used to assess a conjugate of tobramycin, an aminoglycoside antibiotic, and cyclam, an organic compound which acts as a chelator, against infections with *P. aeruginosa*[548]*.* When combined with aztreonam or meropenem, the tobramycin-cyclam conjugate significantly improved survival over monotherapies. Importantly, when the tobramycin-cyclam conjugate was used in a triple combination with a β lactam (cefrazidime)

and β lactamase inhibitor (avibactam), survival was improved over the double combination of ceftazidime/avibactam.

There have also been studies which look at combinations of β lactam antibiotics without a β lactamase inhibitor[465,549], as testing novel combinations of existing drugs *in vivo* is a valuable application of *G. mellonella.*

4.2.2 Efflux pump inhibitors and *G. mellonella*

Although EPIs have been assessed using a *G. mellonella* model by multiple research groups, the number of publications on this topic are surprisingly restricted. As mentioned in **Section 4.1.2**, plants have been a particularly rich source of EPIs[550], including reserpine from Indian snakeroot[551], piperine from the peppercorn plant[552], and compounds from rosemary[553], curry plant[554], thyme[555] and turmeric[556]. Many of the publications which combine EPIs and antibiotics in a *G. mellonella* model utilise EPIs sourced from plants, which indicates their usefulness in testing "uncommon" compounds. A selection of these papers, including ones which use EPIs from a non-plant sources, are discussed below.

Curcumin, isolated from turmeric root, has been shown to enhance the therapeutic effect of levofloxacin and piperacillin when used in combination, compared to each drug as a monotherapy, in the *G. mellonella* model with a multi-drug resistant (MDR) strain of *Pseudomonas aeruginosa*[557]. This expands on other publications, which have indicated curcumin can reduce *P. aeruginosa* pathogenicity in nematode worms and *Arabidopsis thaliana* infection models[558], although the effects of curcumin on *P. aeruginosa* on a higher organism model do not seem to have been investigated.

Pseudomonas aeruginosa has also been shown to respond to treatment with extracts from *Holarrhena antidysenterica*, a small flowering plant which is a popular treatment for gastric

upset in East Asia[559]. One alkaloid in particular, conessine, significantly improved larval survival when combined with levofloxacin, and also reduced the bacterial burden within the larvae. This was also seen for larvae infected with MDR strains of *P. aeruginosa.*

The polyphenol that gives cranberries their deep red colour and is suggested to help treat urinary tract infections[560], cranberry proanthocyanidin, has been tested in a *G. mellonella* model combined with the antibiotic sulfamethoxazole to treat *P. aeruginosa* infections[561]. Larval survival was significantly improved when cranberry proanthocyanidin and sulfamethoxazole were injected 3 hours post-infection, whereas survival was not improved when each compound was injected as a monotherapy. This group also demonstrated that cranberry proanthocyanidin is an effective efflux pump inhibitor.

Other groups have also looked at a quintessential EPI, Phe-Arg-β-naphthylamide (PAβN), with *P. aeruginosa* in a *G. mellonella* model[511]. PAβN is a dipeptide amide which was first synthesised in 1999[562]. It is often combined with fluoroquinolone antibiotics, and although it inhibits efflux pumps in the resistance/nodulation/division (RND) superfamily[563], it has also been shown to permeabilise the outer membrane of Gram-negative bacteria[564]. This group looked to assess wild-type and mutant *P. aeruginosa* strains in *G. mellonella*, and demonstrated that a efflux pump triple-deletion mutant of *P. aeruginosa,* which lacked all RND efflux pumps, had a significantly reduced virulence in the larval model[511]. They also showed that PAβN protected *G. mellonella* from infections with wild-type and clinical strains of *P. aeruginosa*. PAβN was not combined with antibiotics in this study.

The EPIs trimethoprim (which is also an antibiotic) and sertraline (which is more commonly known by the antidepressant trade name “Zoloft”) have also been assessed in a *G. mellonella* model with wild-type *P. aeruginosa,* strains which overexpress three RND efflux

pumps, and MDR strains[565]. Trimethoprim and sertraline both increased larval survival when combined with levofloxacin over monotherapies for both wild-type and MDR *P. aeruginosa*.

A conjugate of rifampicin and tobramycin which was demonstrated to act as an efflux pump inhibitor for *P. aeruginosa* was assessed using the larval *in vivo* model[566], indicating the use of *G. mellonella* in preliminary assessments of newly synthesised compounds. When combined with doxycycline or chloramphenicol, the conjugate compound of rifampicin and tobramycin significantly improved larval survival over monotherapies. This study used an extensively drug resistant strain of *P. aeruginosa* to demonstrate the potential of their new compound, which was shown to have a low toxicity in *G. mellonella* and human kidney (HEK293) and liver (HepG2) cells *in vitro.*

Although there will certainly be other publications on EPIs used in a *G. mellonella* model, it is clear that *G. mellonella* is not commonly used for these types of experiment, despite positive results in all of the publications discussed above.

4.2.3 Compounds selected to investigate with *G. mellonella*

The compounds selected to use in this thesis were selected based on a lack of *in vivo* data, but literatures suggesting that they could be potentially useful treatments. For ease of use, compounds which are soluble in water and were readily available for purchase from suppliers were prioritised.

A paper from 2016 reported a range of β lactams in combinations with β lactamase inhibitors against various MDR-TB strains[567]. Nine different β lactams were assessed, of which amoxicillin, cephalothin and cefaclor were selected to test *in vivo* with *G. mellonella*. The group also assessed three β lactamtase inhibitors – clavulanate, tazobactam, and sulbactam. Tazobactam was chosen to apply here.

Several papers were consulted to select the efflux pump inhibitors used in this thesis[568–570]. Machado *et al.* focussed on verapamil, thioridazine, chlorpromazine, flupenthixol, and haloperidol, and indicated that all compounds exhibited synergistic inhibitory activities when combined with isoniazid and rifampicin[568]. They also killed *M. tuberculosis* bacteria by blocking ion channels and interfering with energy metabolism and were also shown to decrease the intracellular load of Mycobacteria via phagosome acidification. All of the compounds used in this paper were selected for use with *G. mellonella*, however haloperidol is not readily soluble in water so was not chosen.

De Knegt *et al.* also assessed verapamil, but in combination with moxifloxacin and linezolid[569]. These antibiotics were selected because they had been shown to be helpful in treating XDR-TB patients in Argentina when combined with the efflux pump inhibitor thioridazine[571]. This data further supported in inclusion of verapamil and thioridazine in this thesis.

Vesenbeckh *et al.* examined a number of phenothiazine antipsychotics, which are used to reduce hallucinations and delusions in patients with schizophrenia and other illnesses associated with psychosis[570]. They also reference the promising results of thioridazine use in Argentina[571], and the use of thioridazine to treat XDR-TB patients in India[572], but also examine trifluoperazine and triflupromazine. Vesenbeckh *et al.* showed that these compounds accumulated up to 100-fold in macrophages containing *M. tuberculosis in vitro*, which allowed these bacteria to be killed but was not toxic to the macrophages[570]. Trifluoperazine was selected for examination with *G. mellonella*, but triflupromazine could not be found at a reasonable price so was not included.

4.3 Results

4.3.1 β lactams combined with a β lactamase inhibitor can initially improve larval survival, but survival at 144 hours is still low

The larvae were injected with bacteria at an appropriate concentration to cause sufficient larval death over 144 hours, and then treated two hours later with a β lactam antibiotic combined with a β lactamase inhibitor. Both of these were administered at 50 mg/kg, and controls where each drug was given as a monotherapy were also included. These could be compared to assess if the combination was superior to the monotherapy, and both were compared to an untreated control that was mock-treated with saline two hours post-infection.

Tazobactam was selected due to its use as a common β lactamase inhibitor, especially in combination with piperacillin, which was also utilised in these experiments. A dosing ratio of 1:1 was examined here, although β lactams and lactamase inhibitors are prescribed in a variety of different dosing ratios[573,574].

4.3.1.1 *M. fortuitum* NCTC 10394 – larval survival with β lactams and a β lactamase inhibitor

Larvae were injected with *M. fortuitum* NCTC 10394, then with either PBS, a β lactam alone, or a β lactam with a β lactamase inhibitor, injected two hours post-infection. Larval survival was assessed every 24 hours for the next 144 hours. The results of this experiment are shown in **Figure 4.1**. For all combinations of β lactam and β lactamase inhibitor, the combined β lactam and β lactamase inhibitor gave a higher survival rate than the β lactamase inhibitor as a monotherapy (data not shown).

Amoxicillin alone improves larval survival at 50 mg/kg, but larval survival is improved further when AMX is combined with tazobactam, a β lactamase inhibitor. This improved survival is significantly higher than AMX alone, and considerably higher than the survival for un-treated larvae. However, the final survival is still low – only 2 larvae are still alive at 144 hours.

Cefaclor gives similar results – CEC alone at 50 mg/kg improves larval survival (although only very slightly in this case), and CEC combined with TZO gives a significantly higher rate of survival compared to un-treated larvae. Larvae treated with CEC + TZO also had a statistically higher rate of survival compared to larvae injected with TZO alone. Yet the final result for the CEC + TZO larvae at 144 hours is 0% survival.

When cephalothin (CEF) is used with TZO, the larvae do better than the un-treated larvae in terms of log rank testing, but not better than larvae injected with CEF alone. Although the CEF + TZO have a higher rate of survival than un-treated larvae, in reality the survival curves for the two groups look practically identical, and essentially the larvae are all dead by 96 hours.

This is also true for the larvae injected with piperacillin and TZO. The log rank test indicates that the survival curves are statistically different, but the curves are very similar and the larvae are dead by 96 hours. The larvae injected with PIP + TZO do a little better than larvae injected with TZO alone, but do not have a statistically higher survival rate compared to larvae injected with PIP alone.

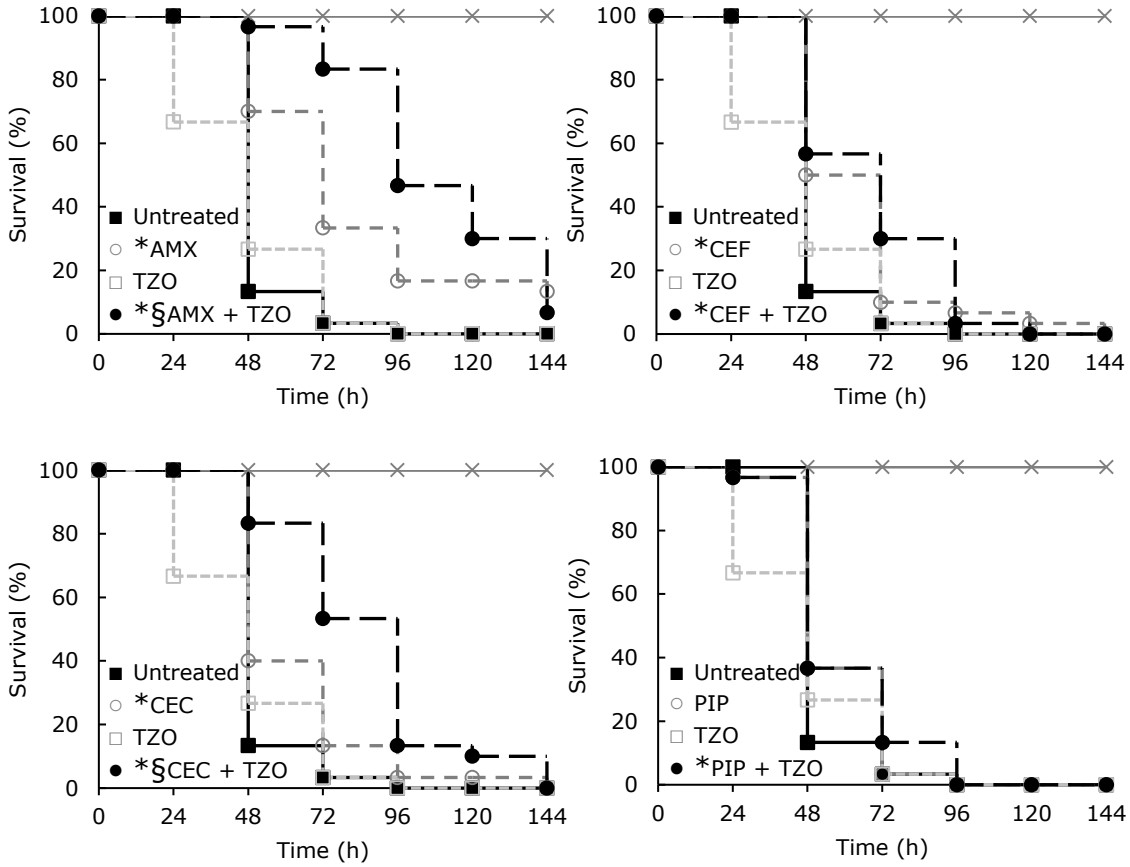

Figure 4.1 – Larvae infected with *M. fortuitum* NCTC 10394 can have a higher survival rate when treated with a β lactam combined with a β lactamase inhibitor than with either drug alone. Larvae were injected with 10 µL of bacteria at 4 x 10^8 c.f.u/mL, then with a single dose of drug two hours later. The β lactam and β lactamase inhibitor were both used at 50 mg/kg, both alone and when in combination. The combinations used are shown on the key to the left of each curve. Infected and untreated larvae, black filled squares; β lactam alone, grey open circles; β lactamase inhibitor alone, light grey open squares; combined β lactam and β lactamase inhibitor, black filled circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant experimental group on the key. When the change in survival compared to the β lactam monotherapy is statistically significant, a section symbol (§) is shown beside the relevant group. $n = 30$. AMX, amoxicillin; CEF, cephalothin; CEC, cefaclor; PIP, piperacillin; TZO, tazobactam.

4.3.1.2 *M. fortuitum* NCTC 8573 – larval survival with β lactams and a β lactamase inhibitor

Larvae were injected with *M. fortuitum* NCTC 8573, then with either PBS, a β lactam alone, or a β lactam with a β lactamase inhibitor, injected two hours post-infection. Larval survival was assessed every 24 hours for the next 144 hours. The results of this experiment are shown in **Figure 4.2**.

Unlike when amoxicillin (AMK) was used alone on larvae injected with *M. fortuitum* NTCT 10394 (**Figure 4.1**), AMX did not improve the survival of larvae when used on its own to treat larvae injected with *M. fortuitum* NCTC 8573. TZO did improve larval survival when used on its own, which was not observed with *M. fortuitum* NCTC 10394. However, when combined with TZO, survival in increased even further, with over 80% of the larvae surviving for 144 hours. This is in contrast to the results when AMX + TZO were used in combination for larvae infected with *M. fortuitum* NCTC 10394, where larval survival was significantly improved with AMX +TZO but survival at 144 hours was only 13%.

Larval survival was not improved over the untreated controls when cephalothin (CEF) was used alone. When CEF and TZO were used in combination, survival was slightly improved over TZO as a monotherapy, however the increase in survival was not significant. The combination treatment of CEF + TZO was more effective at improving survival compared to CEF as a monotherapy and compared to the untreated larvae. However, the survival of larvae treated with CEF + TZO at 144 hours was 47%, so much lower than the final survival of larvae treated with AMX + TZO.

When cefaclor (CEC) was used as a monotherapy to treat larvae, larval survival was improved over untreated larvae, but the survival at 144 hours was only 16% - only slightly

higher than the untreated larvae. Yet, as with CEF + TZO, when CEC was combined with TZO the survival of the larvae was higher than CEC or TZO alone, but the final survival was only 47%.

Piperacillin (PIP) also increased larvae survival when used as a monotherapy, with the survival curve for a PIP monotherapy sharing a similar curve to a TZO monotherapy. When PIP and TZO were used in a combination therapy, survival was higher than the monotherapies, but the final survival at 144 hours was 57%. PIP + TZO improved survival significantly over TZO as a monotherapy. The improvement in survival over PIP alone was significant, however the final survival values for the PIP monotherapy (33%) and the PIP + TZO combination (56%) were still reasonably low.

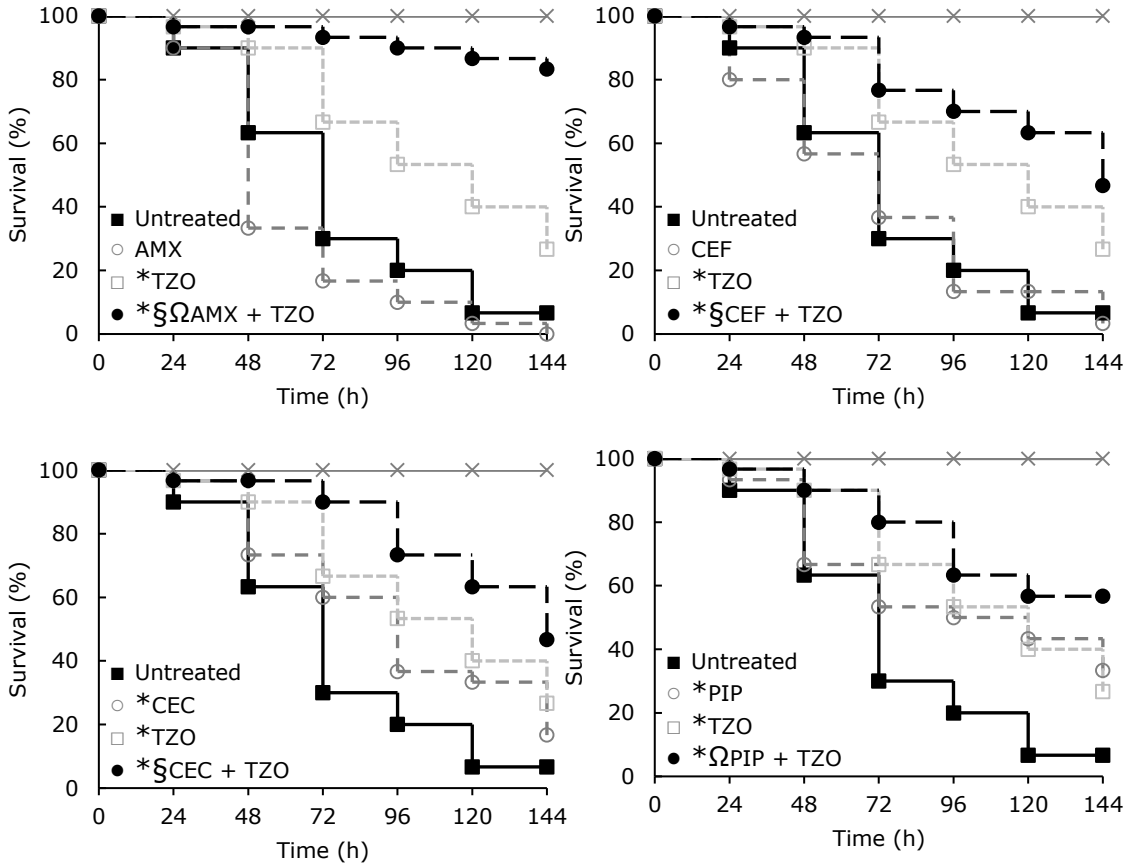

Figure 4.2 – Larvae infected with *M. fortuitum* NCTC 8573 can have a higher survival rate when treated with a β lactam combined with a β lactamase inhibitor than with either drug alone. Larvae were injected with 10 µL of bacteria at 6 x 10^8 c.f.u/mL, then with a single dose of drug two hours later. The β lactam and β lactamase inhibitor were both used at 50 mg/kg, both alone and when in combination. The combinations used are shown on the key to the left of each curve. Infected and untreated larvae, black filled squares; β lactam alone, grey open circles; β lactamase inhibitor alone, light grey open squares; combined β lactam and β lactamase inhibitor, black filled circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant experimental group on the key. When the change in survival compared to the β lactam monotherapy is statistically significant, a section symbol (§) is shown beside the relevant group. When the change in survival compared to the β lactamase inhibitor (TZO) monotherapy is statistically significant, an omega symbol (Ω) is shown beside the relevant group. $n = 30$. AMX, amoxicillin; CEF, cephalothin; CEC, cefaclor; PIP, piperacillin; TZO, tazobactam.

4.3.1.3 *M. marinum* Strain M – larval survival with β lactams and a β lactamase inhibitor

Although there are data points on the curves where the larvae injected with a β lactam or a β lactam combined with a β lactamase inhibitor had improved survival over untreated larvae, at the 144 hour timepoint the survival of these larvae was indistinguishable from untreated larvae (see **Figure 4.3**). Unlike the results seen for both *M. fortuitum* strains used, where at least one β lactam/β lactamase inhibitor combination improved survival significantly from monotherapies or saline mock-treatment, there are no combinations that improved survival above 16% at 144 hours.

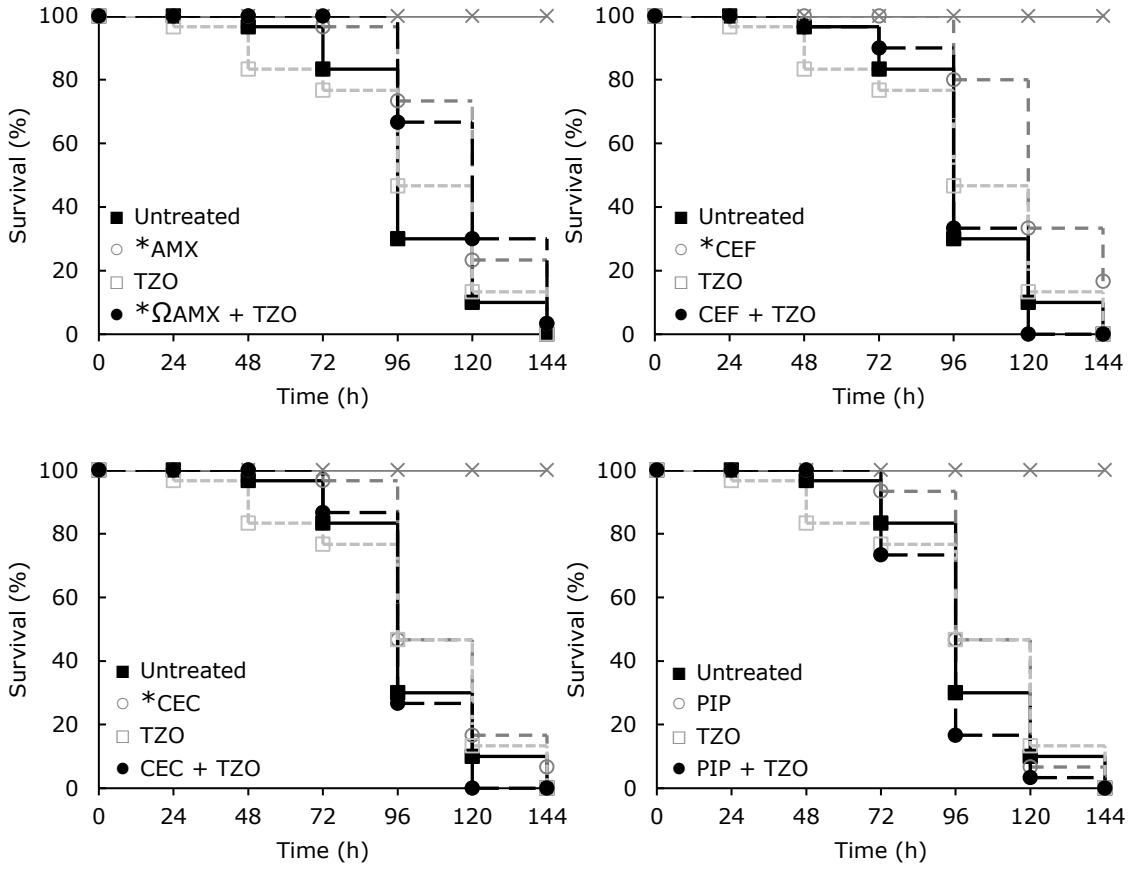

Figure 4.3 – Larvae infected with *M. marinum* Strain M do not have an improvement in survival when treated with a β lactam combined with a β lactamase inhibitor. Larvae were injected with 10 µL of bacteria at 3 x 10^7 c.f.u/mL, then with a single dose of drug two hours later. The β lactam and β lactamase inhibitor were both used at 50 mg/kg, both alone and when in combination. The combinations used are shown on the key to the left of each curve. Infected and untreated larvae, black filled squares; β lactam alone, grey open circles; β lactamase inhibitor alone, light grey open squares; combined β lactam and β lactamase inhibitor, black filled circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant experimental group on the key. When the change in survival compared to the β lactamase inhibitor (TZO) monotherapy is statistically significant, an omega symbol (Ω) is shown beside the relevant group. n = 30. AMX, amoxicillin; CEF, cephalothin; CEC, cefaclor; PIP, piperacillin; TZO, tazobactam.

4.3.1.4 *M. marinum* R356933F – larval survival with β lactams and a β lactamase inhibitor

As seen with *M. fortuitum* NCTC 8573, TZO as a monotherapy was able to improve larval survival above that of the untreated controls (**Figure 4.4**). However, when looking that the survival curves, the curve for TZO is practically identical to that of the untreated control. This also applies to the PIP monotherapy curve.

Of the combination treatments, PIP + TZO and CEF + TZO were not significantly different from the untreated controls. AMX + TZO and CEC + TZO do show an improvement, but the final survival at 144 hours was 40% and 33% respectively. This may seem high, but as larvae infected with *M. marinum* (Strain M and R356933F) die later in the experimental window than those infected with *M. fortuitum,* these values should be far higher if the treatment combination is truly effective.

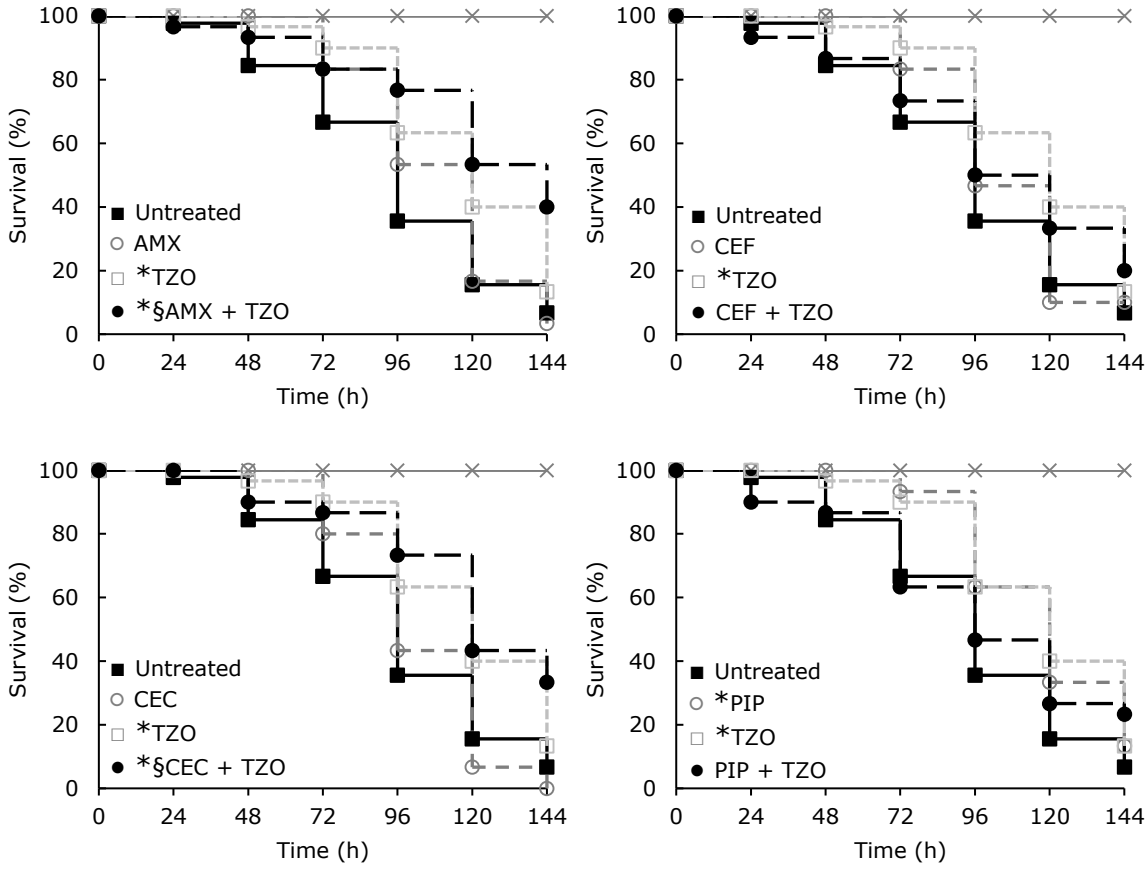

Figure 4.4 – Larvae infected with *M. marinum* R356933F have a minimal increase in survival when treated with a β lactam combined with a β lactamase inhibitor. Larvae were injected with 10 µL of bacteria at 9 x 10^8 c.f.u/mL, then with a single dose of drug two hours later. The β lactam and β lactamase inhibitor were both used at 50 mg/kg, both alone and when in combination. The combinations used are shown on the key to the left of each curve. Infected and untreated larvae, black filled squares; β lactam alone, grey open circles; β lactamase inhibitor alone, light grey open squares; combined β lactam and β lactamase inhibitor, black filled circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant experimental group on the key. When the change in survival compared to the β lactam monotherapy is statistically significant, a section symbol (§) is shown beside the relevant group. $n = 30$. AMX, amoxicillin; CEF, cephalothin; CEC, cefaclor; PIP, piperacillin; TZO, tazobactam.

4.3.2 Efflux pump inhibitors can improve larval survival when combined with a sub-active dose of antibiotic, although the majority of combinations tested here were ineffective

Larvae were injected with bacteria, then with antibiotics and an efflux pump inhibitor two hours later. Larval survival was assessed every 24 hours for the next 144 hours. Efflux pump inhibitors were tested at three different dose – 10 mg/kg, 1 mg/kg, and 0.1 mg/kg. As so many different combinations of efflux pump inhibitor and antibiotic were being assessed, it was not feasible to test each combination with the usual protocol of 30 larvae, so small tests were initially performed using n = 5, 10 or 15 larvae. Combinations which significantly improved larval survival were then tested with n = 30 larvae.

The efflux pump inhibitors used here (VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine) were selected as they have been using with *M. tuberculosis in vitro*[568–570] but have either only has brief analysis *in vivo*, or have not been assessed *in vivo*. The doses used were based on the concentrations utilised in these studies.

4.3.2.1 *M. fortuitum* NCTC 10394 – larval survival with efflux pump inhibitors and low antibiotic doses

Initial tests of these drug combinations were initially tested on smaller groups of larvae than the standard experimental design used elsewhere in this thesis. The results of these experiments are shown in **Figures 4.5, 4.6, 4.7, 4.8** and **4.9**. Generally, when combined with an ineffective dose of a standard anti-TB drug the lower doses of efflux pump inhibitor (1 mg/kg and 0.1 mg/kg) did not improve larval survival in comparison to the untreated larvae. Usually,

the final number of surviving larvae was very low, even if survival was statistically higher than the untreated larvae. However, some combinations did improve survival sufficiently to give a 144 hour survival which was much higher than the untreated larvae. These combinations were then tested with the standard number of larvae (n = 30) to determine the validity of these results.

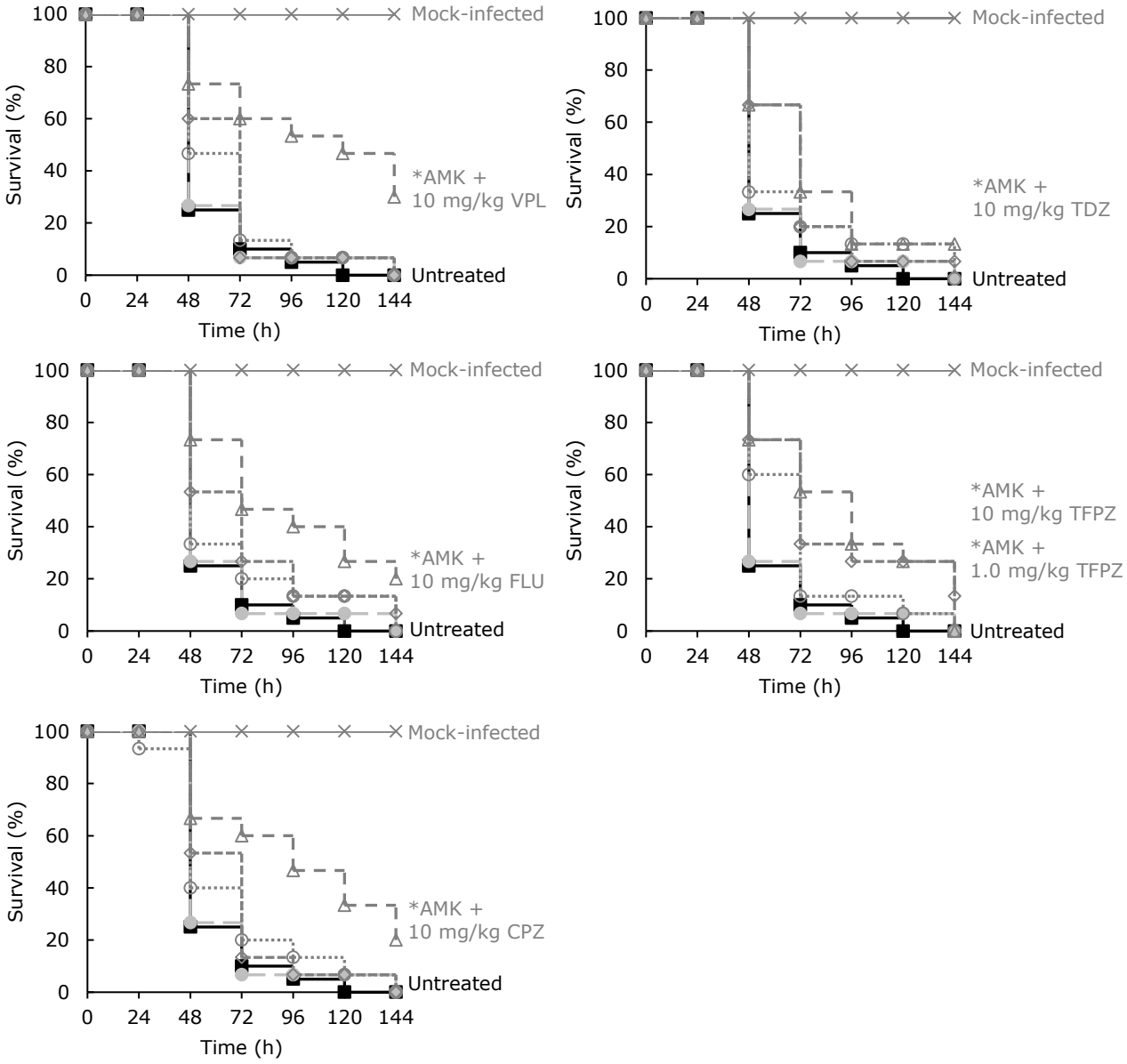

Figure 4.5 – Larvae infected with *M. fortuitum* NCTC 10394 can have a marginally improved survival rate when treated with amikacin combined with an efflux pump inhibitor. Larvae were injected with 10 µL of bacteria at 4 x 10^8 c.f.u/mL, then with a single dose of drug two hours later. Amikacin was used at 2.5 mg/kg, both alone and when in combination. The efflux pump inhibitors were used at 10, 1.0 and 0.1 mg/kg. The combinations used are shown on the right of each curve – only the highest combination of AMK and EPI is labelled unless the results were significant. Infected and untreated larvae, black filled squares; AMK alone, pale grey filled circles; AMK + 10 mg/kg EPI, grey open diamonds; AMK + 1.0 mg/kg EPI, grey open triangles; AMK + 0.1 mg/kg EPI, grey open circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant experimental group on the key. n = 15. AMK, amikacin; VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine; EPI, efflux pump inhibitor.

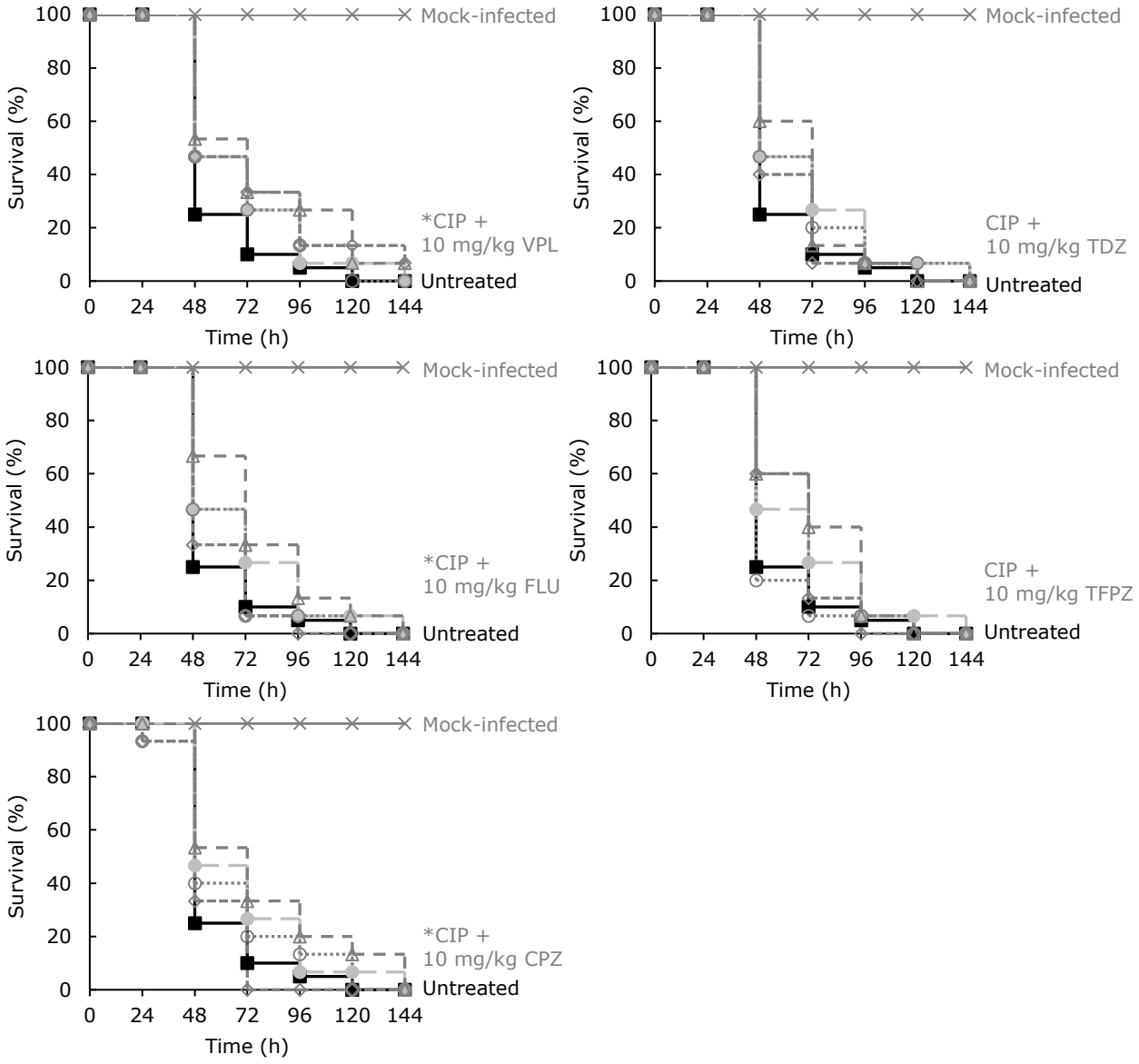

Figure 4.6 – Larvae infected with *M. fortuitum* NCTC 10394 rarely have marginally improved survival rate when treated with ciprofloxacin combined with an efflux pump inhibitor. Larvae were injected with 10 µL of bacteria at 4 x 10^8 c.f.u/mL, then with a single dose of drug two hours later. Ciprofloxacin was used at 2.5 mg/kg, both alone and when in combination. The efflux pump inhibitors were used at 10, 1.0 and 0.1 mg/kg. The combinations used are shown on the right of each curve – only the highest combination of CIP and EPI is labelled unless the results were significant. Infected and untreated larvae, black filled squares; AMK alone, pale grey filled circles; CIP + 10 mg/kg EPI, grey open diamonds; CIP + 1.0 mg/kg EPI, grey open triangles; CIP + 0.1 mg/kg EPI, grey open circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. When the change in survival compared to the untreated larvae was statistically significant (p < 0.05, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant experimental group on the key. n = 15. CIP, ciprofloxacin; VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine; EPI, efflux pump inhibitor.

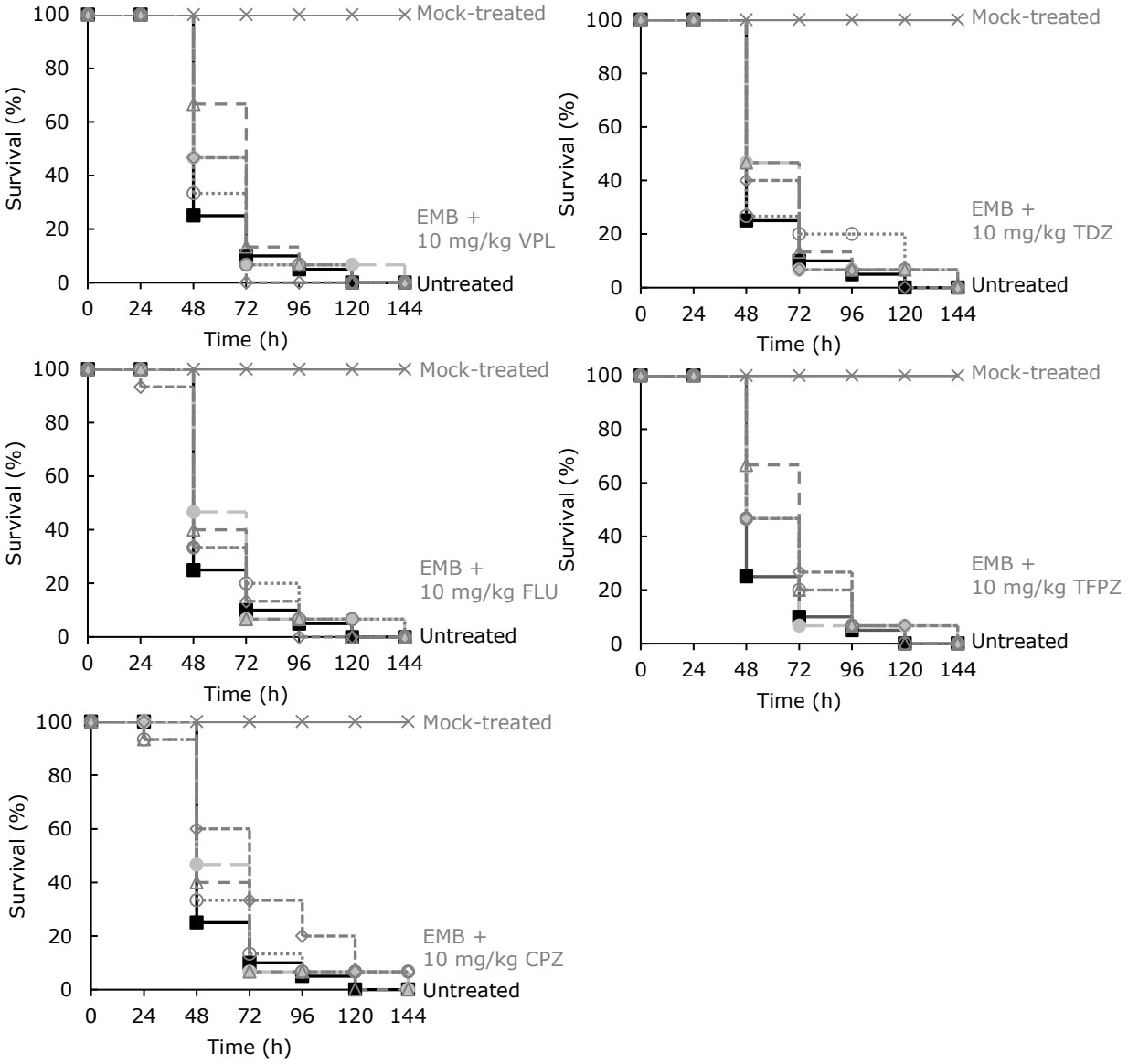

Figure 4.7 – Larvae infected with *M. fortuitum* NCTC 10394 show no improvement in survival when treated with ethambutol combined with an efflux pump inhibitor. Larvae were injected with 10 µL of bacteria at 4 x 10^8 c.f.u/mL, then with a single dose of drug two hours later. Ethambutol was used at 100 mg/kg, both alone and when in combination. The efflux pump inhibitors were used at 10, 1.0 and 0.1 mg/kg. The combinations used are shown on the right of each curve, although only the highest combination of EMB and EPI is labelled. Infected and untreated larvae, black filled squares; EMB alone, pale grey filled circles; EMB + 10 mg/kg EPI, grey open diamonds; EMB + 1.0 mg/kg EPI, grey open triangles; EMB + 0.1 mg/kg EPI, grey open circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. *n* = 15. EMB, ethambutol; VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine; EPI, efflux pump inhibitor.

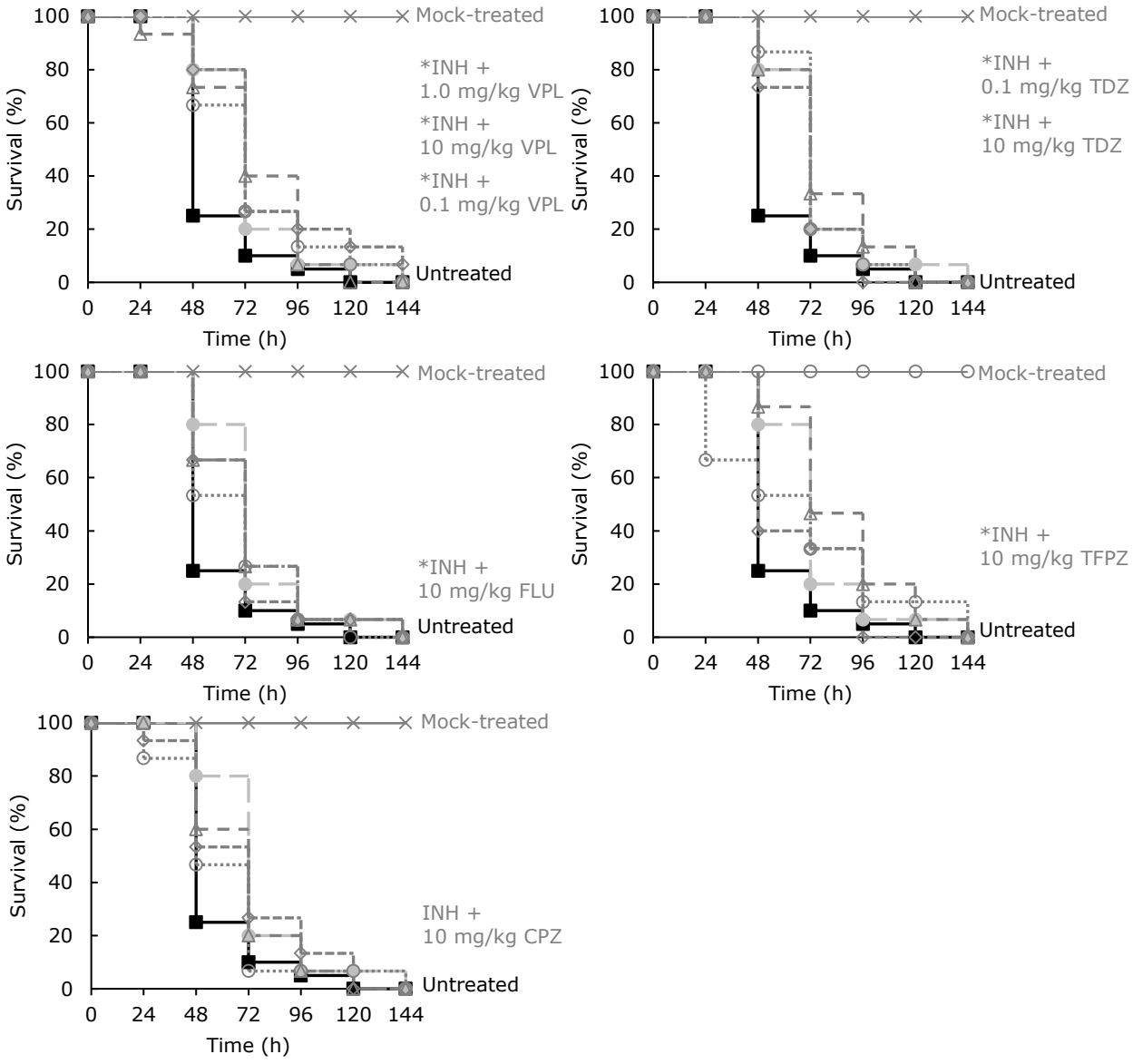

Figure 4.8 – Larvae infected with *M. fortuitum* NCTC 10394 can have a marginally improved survival rate when treated with isoniazid combined with an efflux pump inhibitor. Larvae were injected with 10 µL of bacteria at 4 x 10^8 c.f.u/mL, then with a single dose of drug two hours later. Isoniazid was used at 2.5 mg/kg, both alone and when in combination. The efflux pump inhibitors were used at 10, 1.0 and 0.1 mg/kg. The combinations used are shown on the right of each curve – only the highest combination of INH and EPI is labelled unless the results were significant. Infected and untreated larvae, black filled squares; INH alone, pale grey filled circles; INH + 10 mg/kg EPI, grey open diamonds; INH + 1.0 mg/kg EPI, grey open triangles; INH + 0.1 mg/kg EPI, grey open circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant experimental group on the key. n = 15. INH, isoniazid; VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine; EPI, efflux pump inhibitor.

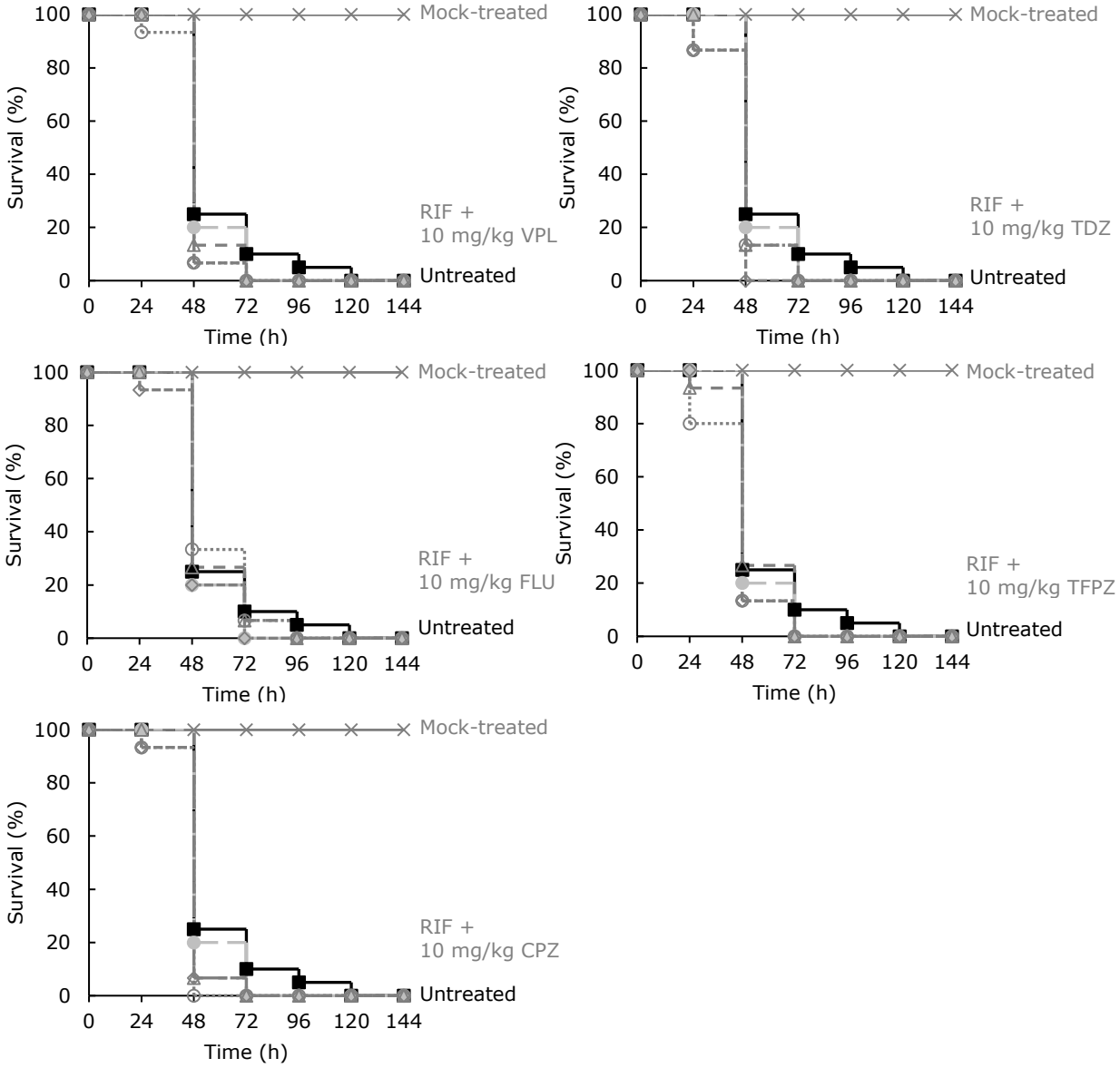

Figure 4.9 – Larvae infected with *M. fortuitum* NCTC 10394 show no improvement in survival when treated with rifampicin combined with an efflux pump inhibitor. Larvae were injected with 10 µL of bacteria at 4 x 10^8 c.f.u/mL, then with a single dose of drug two hours later. Rifampicin was used at 30 mg/kg, both alone and when in combination. The efflux pump inhibitors were used at 10, 1.0 and 0.1 mg/kg. The combinations used are shown on the right of each curve, although only the highest combination of RIF and EPI is labelled. Infected and untreated larvae, black filled squares; RIF alone, pale grey filled circles; RIF + 10 mg/kg EPI, grey open diamonds; RIF + 1.0 mg/kg EPI, grey open triangles; RIF + 0.1 mg/kg EPI, grey open circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. *n* = 15. RIF, rifampicin; VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine; EPI, efflux pump inhibitor.

Amikacin at 2.5 mg/kg is an ineffective dose of antibiotic when used to treat larvae infected with *M. fortuitum* NCTC 10394 (p = 0.696, see **Figure 4.5**). When combined with verapamil, there was some evidence that 10 mg/kg VPL improved larval survival (p = 0.003, 30% survival at 144 hours compared to 0% for untreated larvae).

Combining AMK with thioridazine (TDZ) showed some improvement in the first few days of assessment (see **Figure 4.5**). When AMK was combined with 10 mg/kg or 1 mg/kg TDZ, 67% of larvae were alive at 48 hours compared to 25% of un-treated larvae. However, the later time points have very similar survival curves between the treated and un-treated groups, and the final survival at 144 hours is very low for all groups.

AMK combined with flupenthixol has a similarly low survival at 144 hours (**Figure 4.5**), but survival of larvae injected with AMK + FLU (10 mg/kg FLU) is higher than un-treated larvae and larvae injected with AMK alone (p = 0.00103). This can also be seen for larvae injected with AMK and trifluoperazine (TFPZ). Larvae injected with AMK and chlorpromazine (CPZ, 10 mg/kg) have a higher rate of survival throughout the majority of the 144 hour experiment, although the final time-point has only 13.3% survival.

Because of the statistically increased survival of some of these combinations, VPL, FLU, TFPZ and CPZ were selected to be tested again at 10 mg/kg with 2.5 mg/kg AMK using a larger group of larvae (n = 30). The results of these experiments are shown in **Figure 4.10**. Although the AMK + 10 mg/kg TDZ combination in the smaller sample size (see **Figure 4.5**) was shown to have a higher survival than untreated larvae, the final survival was very low and the time points which showed the biggest increase in survival over the untreated larvae were in the earlier portion of the experiment. Hence, this combination was not assessed, however 10 mg/kg of VPL, FLU, TFPZ and CPZ was assessed. These results were comparable to those in **Figure 4.5**

– survival was clearly improved over untreated larvae, and also over larvae treated with a sub-optimal dose of amikacin alone. However, the final number of surviving larvae at 144 hours post-infection is still fairly low.

For all other combinations of antibiotics (CIP, EMB, INH, and RIF) and efflux pump inhibitors, there were no groups which gave increased larval survival for the full length of the experiment (**Figure 4.6, 4.7, 4.8** and **4.9**). There were occasional combinations, such as CIP + FLU (FLU at 10 mg/kg, **Figure 4.6**), where larval survival at 48 and/or 72 hours was higher than the survival of the un-treated larvae. Occasionally this meant the log-rank tests indicated a *p*-value below 0.05, but the rest of the survival curve was very close to the un-treated data, and the final survival was low.

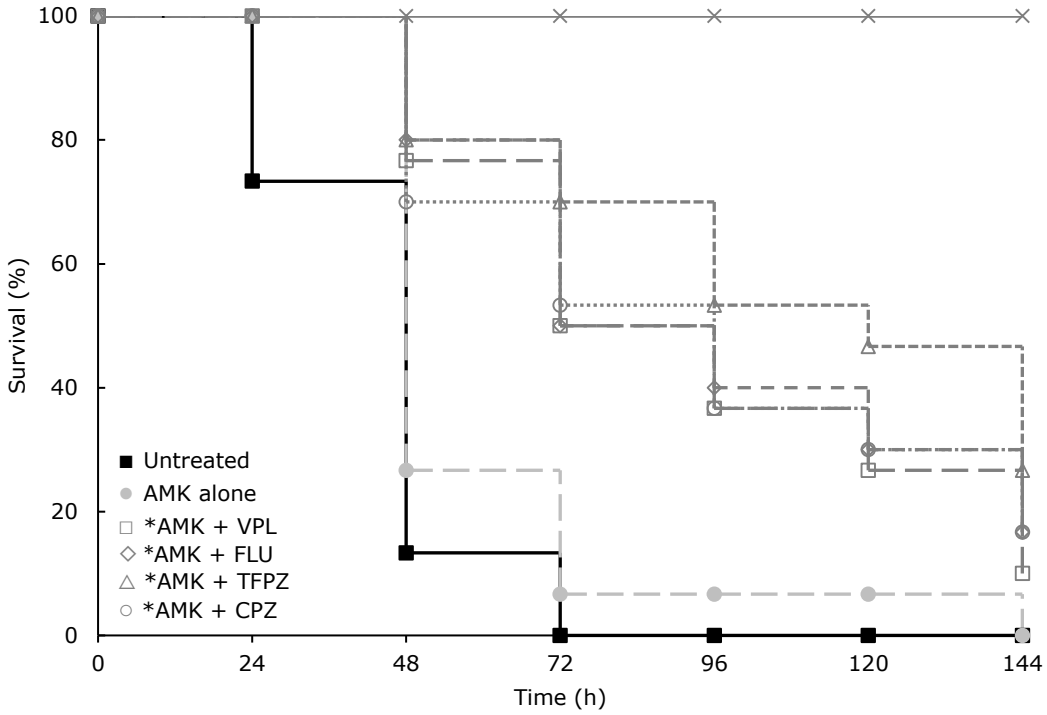

Figure 4.10 – The improvement in survival when larvae infected with *M. fortuitum* NCTC 10394 are treated with a sub-optimal dose of amikacin combined with an efflux pump inhibitor remains when the combinations are tested in a larger experimental group. Larvae were injected with 10 µL of bacteria at 4 x 10^8 c.f.u/mL, then with a single dose of drug two hours later. Amikacin was used at 2.5 mg/kg, both alone and when in combination. The efflux pump inhibitors were used at 10 mg/kg. The combinations used are shown to the left of the curve. Infected and untreated larvae, black filled squares; AMK alone, pale grey filled circles; AMK + 10 mg/kg VPL, grey open squares; AMK + 10 mg/kg FLU, grey open diamonds; AMK + 10 mg/kg TFPZ, grey open triangles; AMK + 10 mg/kg CPZ, grey open circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant experimental group on the key. $n = 30$. AMK, amikacin; VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine; EPI, efflux pump inhibitor.

4.3.2.2 *M. fortuitum* NCTC 8573 – larval survival with efflux pump inhibitors and low antibiotic doses

In testing combinations of antibiotics and efflux pump inhibitors against infections with *M. fortuitum* NCTC 8573, the issues of performing log-rank tests using data from small sample groups is compounded against the slow rate of death in un-treated larvae. Larvae injected with *M. fortuitum* NCTC 8573 take a reasonably long time to die (see **Figures 2.2, 4.11, 4.12, 4.13, 4.14** and **4.15**), especially compared to *M. fortuitum* NCTC 10394. Log-rank tests on small data sets, or data sets where the changes in larval survival are very small between each time point, will not show a statistically significant difference between groups.

A good example of this is AMK + VPL with VPL at 10 mg/kg (see **Figure 4.11**). Larvae treated with this combination have a consistently higher survival than untreated larvae or larvae treated with AMK alone, and the survival at 144 hours is 40%, yet the log-rank gives a *p* value which is far from significant. Equally, larvae treated with AMK + FLU with FLU at 10 mg/kg, the survival was 40% at 144 hours and larvae survival was greater than for the un-treated group throughout, but the log-rank *p* value was still short of significance – $p = 0.0596$.

In general, EPIs did very little to improve larval survival when combined with an antibiotic, and survival was comparable to infected but untreated larvae. This was particularly seen in larvae treated with an EPI + EMB (see **Figure 4.13**) and EPI + INH (**Figure 4.14**). For other combinations, survival could be improved up to 70% (RIF + 10 mg/kg TFPZ in **Figure 4.15**). As mentioned, AMK + VPL and AMK + FLU improved larval survival but not above 60%, and these combinations were not statistically significant. The combinations of RIF and all five EPIs used in this work were more successful, yet only one of these combinations gave a statistically significant increase in survival (RIF + 10 mg/kg TFPZ).

Figure 4.12 shows that CIP combined with several of the EPIs gives survival which is significantly higher than the untreated controls. However, when these results are compared to the larvae that received CIP as a monotherapy, significance was lost and survival was statistically comparable to the CIP monotherapy group. The difference in survival between the CIP monotherapy group and the untreated group was not significant.

As the survival curves indicated so few combinations of antibiotic and EPI which could marginally improve larval survival, let alone to a significant degree, experiments which used a larger number of larvae to reassess these combinations were deemed unnecessary.

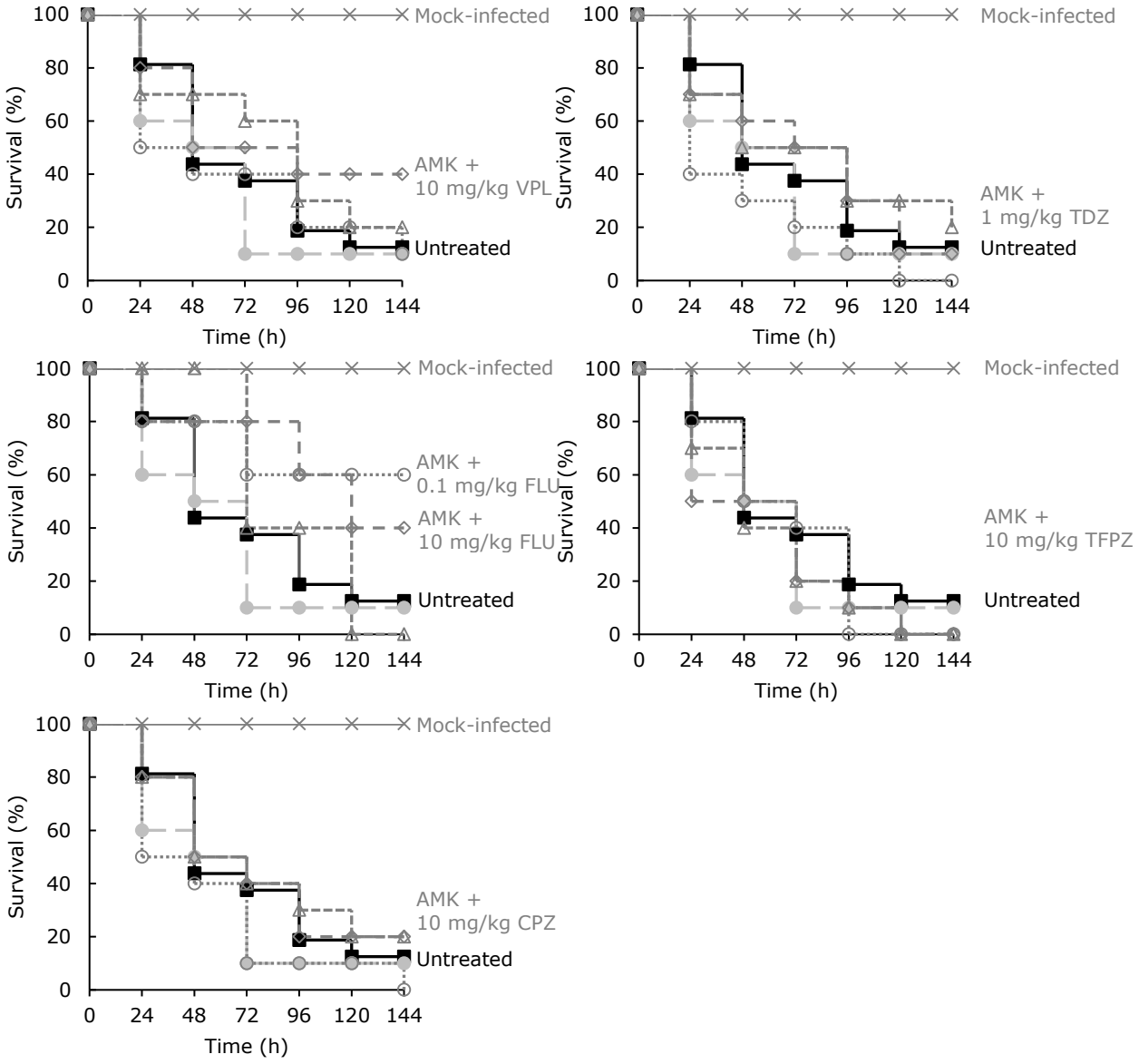

Figure 4.11 – Larvae infected with *M. fortuitum* NCTC 8573 generally show no improvement in survival when treated with amikacin combined with an efflux pump inhibitor. Larvae were injected with 10 µL of bacteria at 6 x 10^8 c.f.u/mL, then with a single dose of drug two hours later. Amikacin was used at 1.0 mg/kg, both alone and when in combination. The efflux pump inhibitors were used at 10, 1.0 and 0.1 mg/kg. The combinations used are shown on the right of each curve – only the highest combination of AMK and EPI is labelled unless the results were significant. Infected and untreated larvae, black filled squares; AMK alone, pale grey filled circles; AMK + 10 mg/kg EPI, grey open diamonds; AMK + 1.0 mg/kg EPI, grey open triangles; AMK + 0.1 mg/kg EPI, grey open circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. *n* = 10. AMK, amikacin; VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine; EPI, efflux pump inhibitor.

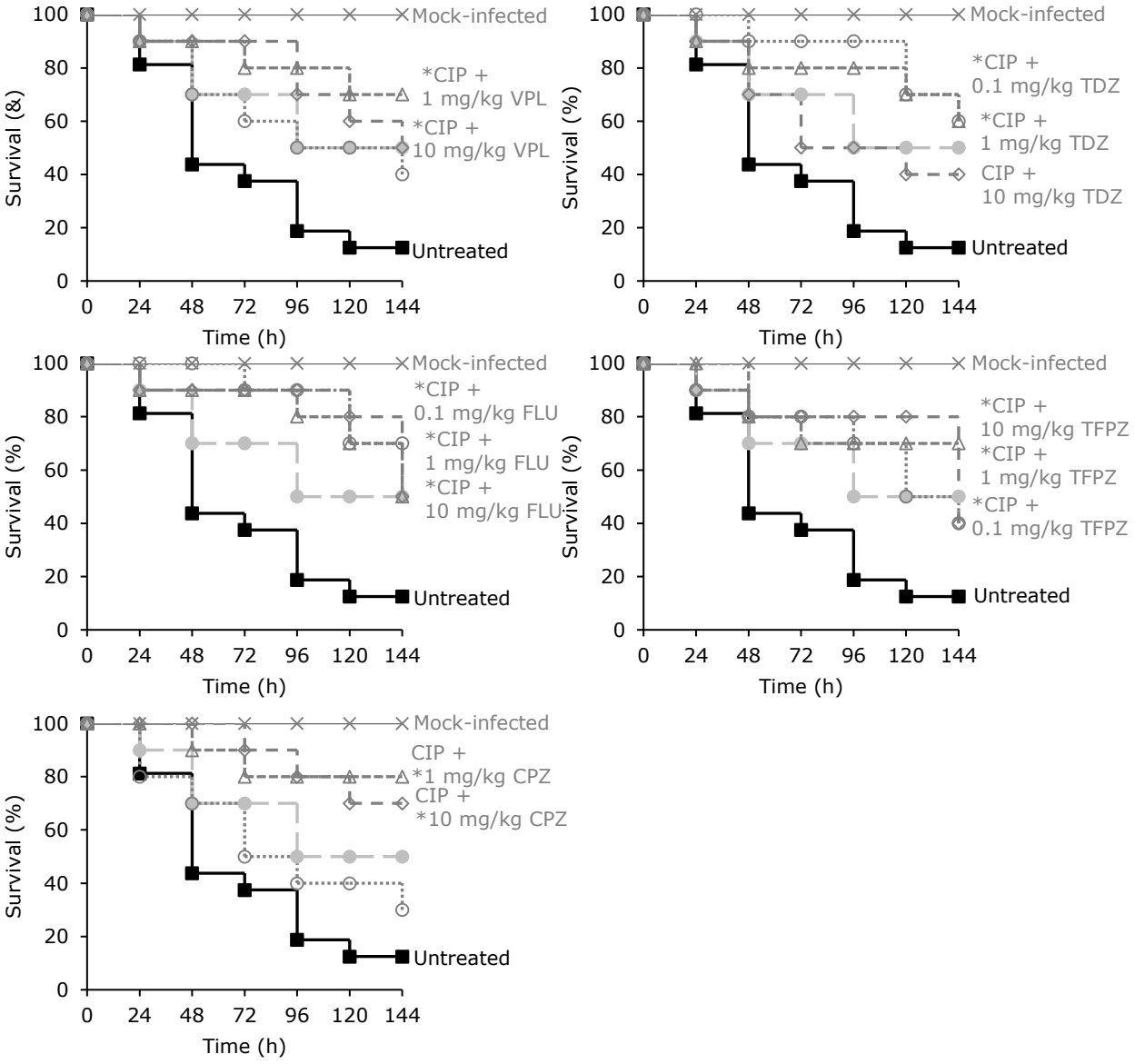

Figure 4.12 – Larvae infected with *M. fortuitum* NCTC 8573 generally show no improvement over a CIP monotherapy in survival when treated with ciprofloxacin combined with an efflux pump inhibitor. Larvae were injected with 10 µL of bacteria at 6 x 10^8 c.f.u/mL, then with a single dose of drug two hours later. Amikacin was used at 1.0 mg/kg, both alone and when in combination. The efflux pump inhibitors were used at 10, 1.0 and 0.1 mg/kg. The combinations used are shown on the right of each curve – only the highest combination of CIP and EPI is labelled unless the results were significant. Infected and untreated larvae, black filled squares; CIP alone, pale grey filled circles; CIP + 10 mg/kg EPI, grey open diamonds; CIP + 1.0 mg/kg EPI, grey open triangles; CIP + 0.1 mg/kg EPI, grey open circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant experimental group on the key. $n = 10$. CIP, ciprofloxacin; VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine; EPI, efflux pump inhibitor.

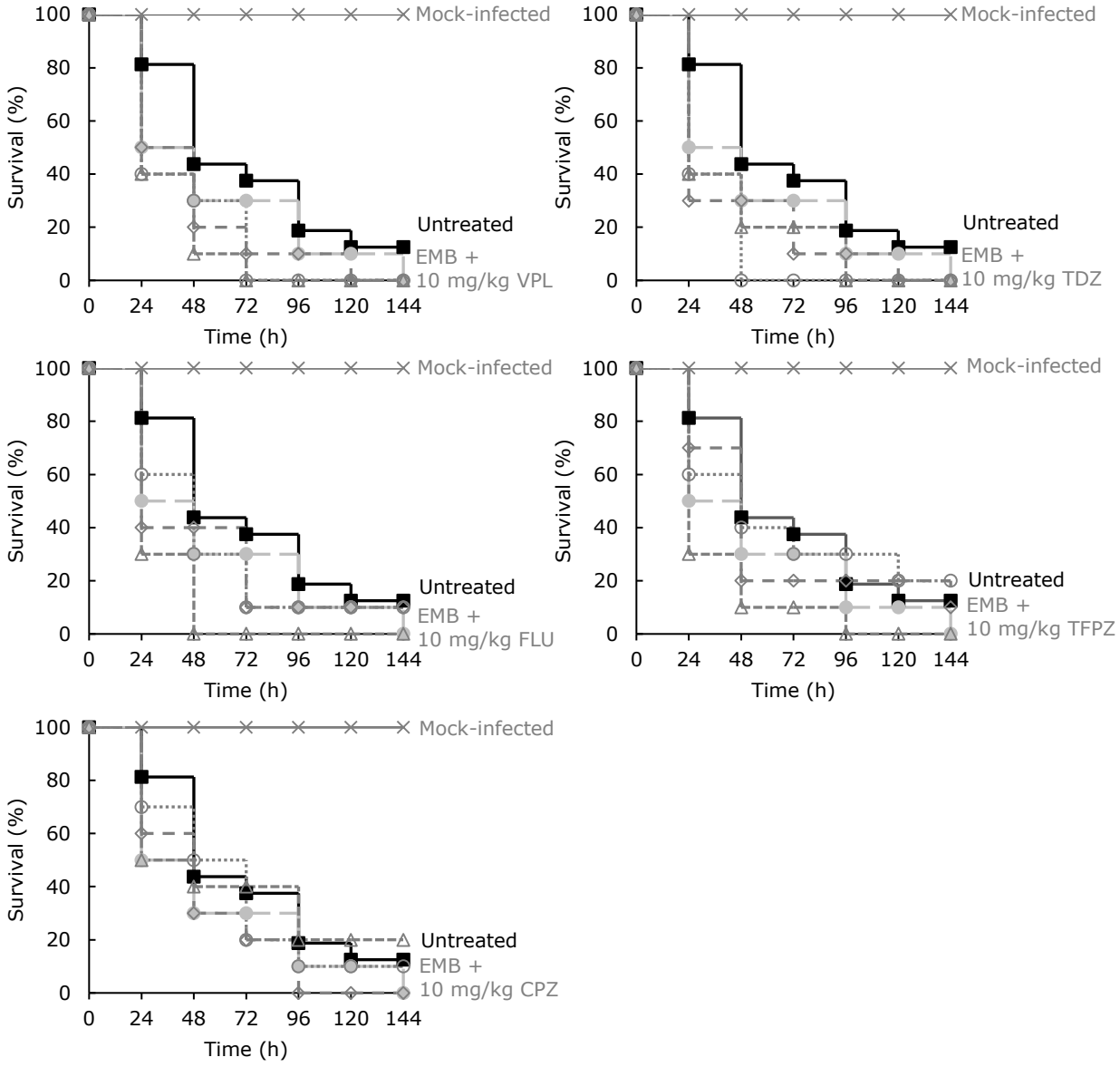

Figure 4.13 – Larvae infected with *M. fortuitum* NCTC 8573 show no improvement in survival when treated with ethambutol combined with an efflux pump inhibitor. Larvae were injected with 10 µL of bacteria at 6 x 10^8 c.f.u/mL, then with a single dose of drug two hours later. Ethambutol was used at 100 mg/kg, both alone and when in combination. The efflux pump inhibitors were used at 10, 1.0 and 0.1 mg/kg. The combinations used are shown on the right of each curve, although only the highest combination of EMB and EPI is labelled. Infected and untreated larvae, black filled squares; EMB alone, pale grey filled circles; EMB + 10 mg/kg EPI, grey open diamonds; EMB + 1.0 mg/kg EPI, grey open triangles; EMB + 0.1 mg/kg EPI, grey open circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. *n* = 10. EMB, ethambutol; VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine; EPI, efflux pump inhibitor.

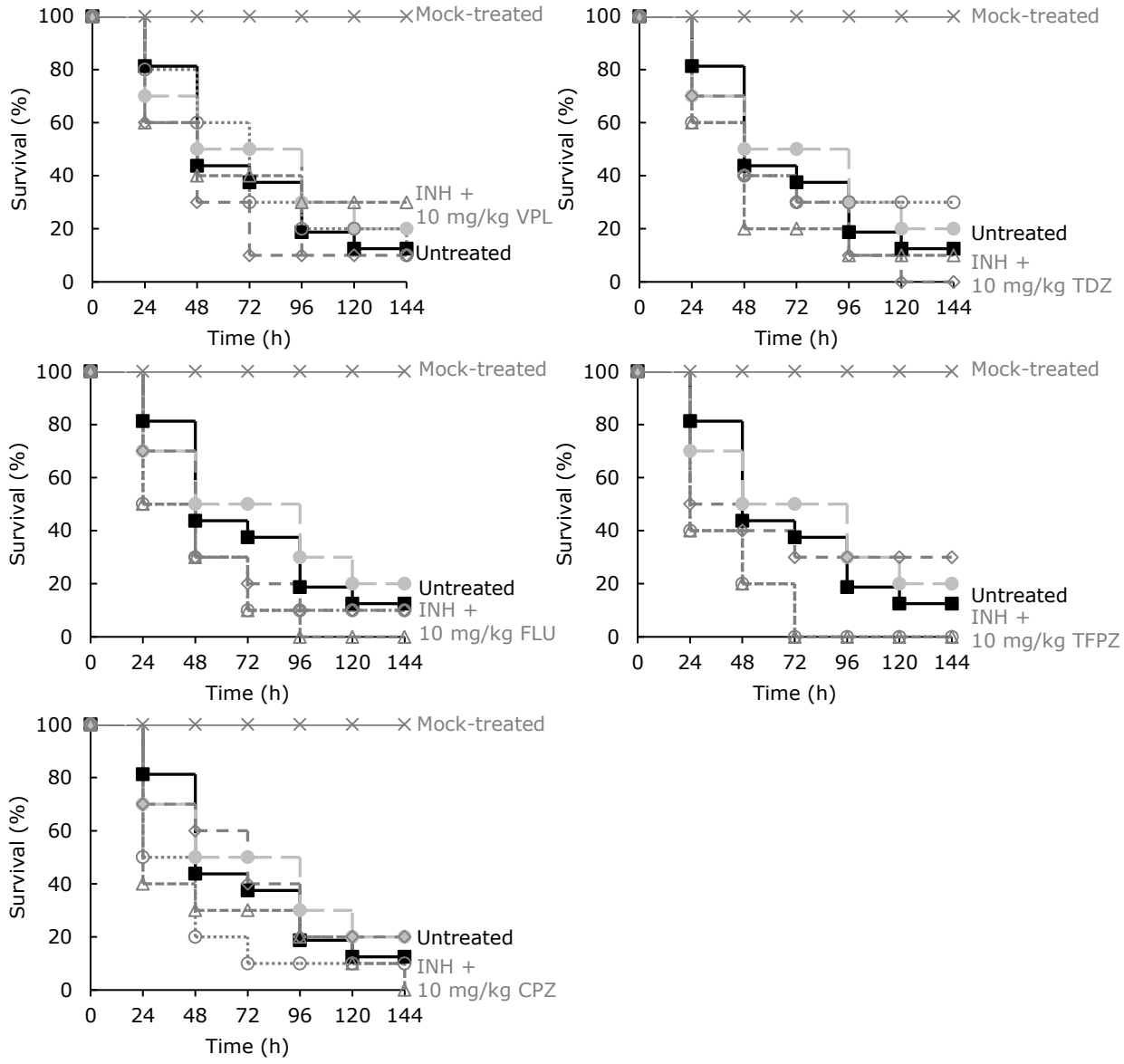

Figure 4.14 – Larvae infected with *M. fortuitum* NCTC 8573 show no significant improvement in survival rate when treated with isoniazid combined with an efflux pump inhibitor. Larvae were injected with 10 µL of bacteria at 6 x 10^8 c.f.u/mL, then with a single dose of drug two hours later. Isoniazid was used at 50 mg/kg, both alone and when in combination. The efflux pump inhibitors were used at 10, 1.0 and 0.1 mg/kg. The combinations used are shown on the right of each curve – only the highest combination of INH and EPI is labelled. Infected and untreated larvae, black filled squares; INH alone, pale grey filled circles; INH + 10 mg/kg EPI, grey open diamonds; INH + 1.0 mg/kg EPI, grey open triangles; INH + 0.1 mg/kg EPI, grey open circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. *n* = 10. INH, isoniazid; VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine; EPI, efflux pump inhibitor.

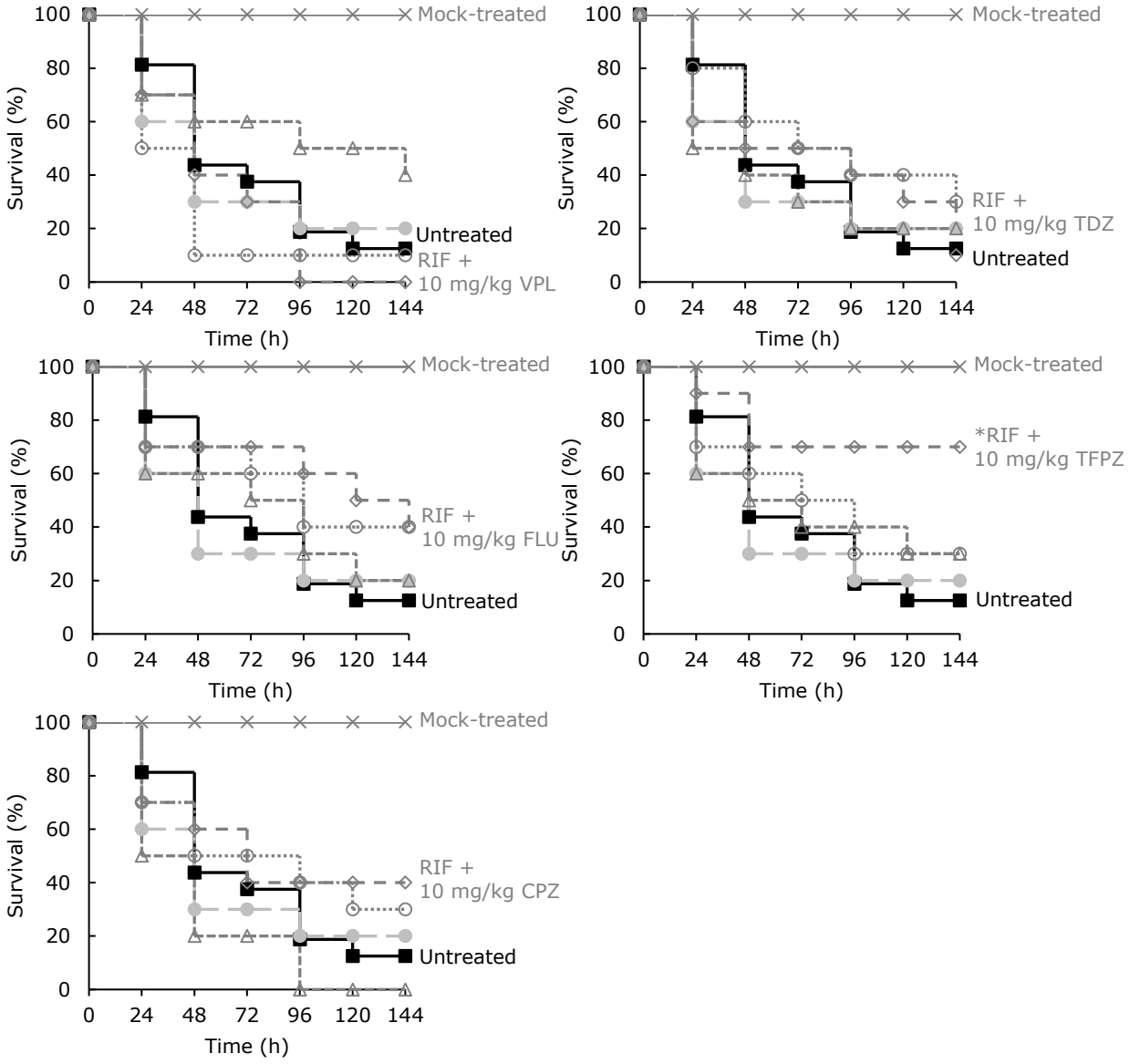

Figure 4.15 – Larvae infected with *M. fortuitum* NCTC 8573 can show an improvement in survival when treated with rifampicin combined with an efflux pump inhibitor. Larvae were injected with 10 µL of bacteria at 6 x 10^8 c.f.u/mL, then with a single dose of drug two hours later. Rifampicin was used at 0.5 mg/kg, both alone and when in combination. The efflux pump inhibitors were used at 10, 1.0 and 0.1 mg/kg. The combinations used are shown on the right of each curve, although only the highest combination of RIF and EPI is labelled. Infected and untreated larvae, black filled squares; RIF alone, pale grey filled circles; RIF + 10 mg/kg EPI, grey open diamonds; RIF + 1.0 mg/kg EPI, grey open triangles; RIF + 0.1 mg/kg EPI, grey open circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant experimental group on the key. n = 10. RIF, rifampicin; VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine; EPI, efflux pump inhibitor.

4.3.2.3 *M. marinum* Strain M – larval survival with efflux pump inhibitors and low antibiotic doses

The results for efflux pump inhibitors combined with antibiotics indicate that these combinations are unable to treat larvae infected with *M. marinum* Strain M (see **Figures 4.16, 4.17, 4.18, 4.19** and **4.20**). The larvae have consistently low survival rates at 144 hours, and generally the progression of the survival curves is indistinguishable from the untreated controls. This applies to the monotherapies of antibiotics and EPIs as well as the combined treatments.

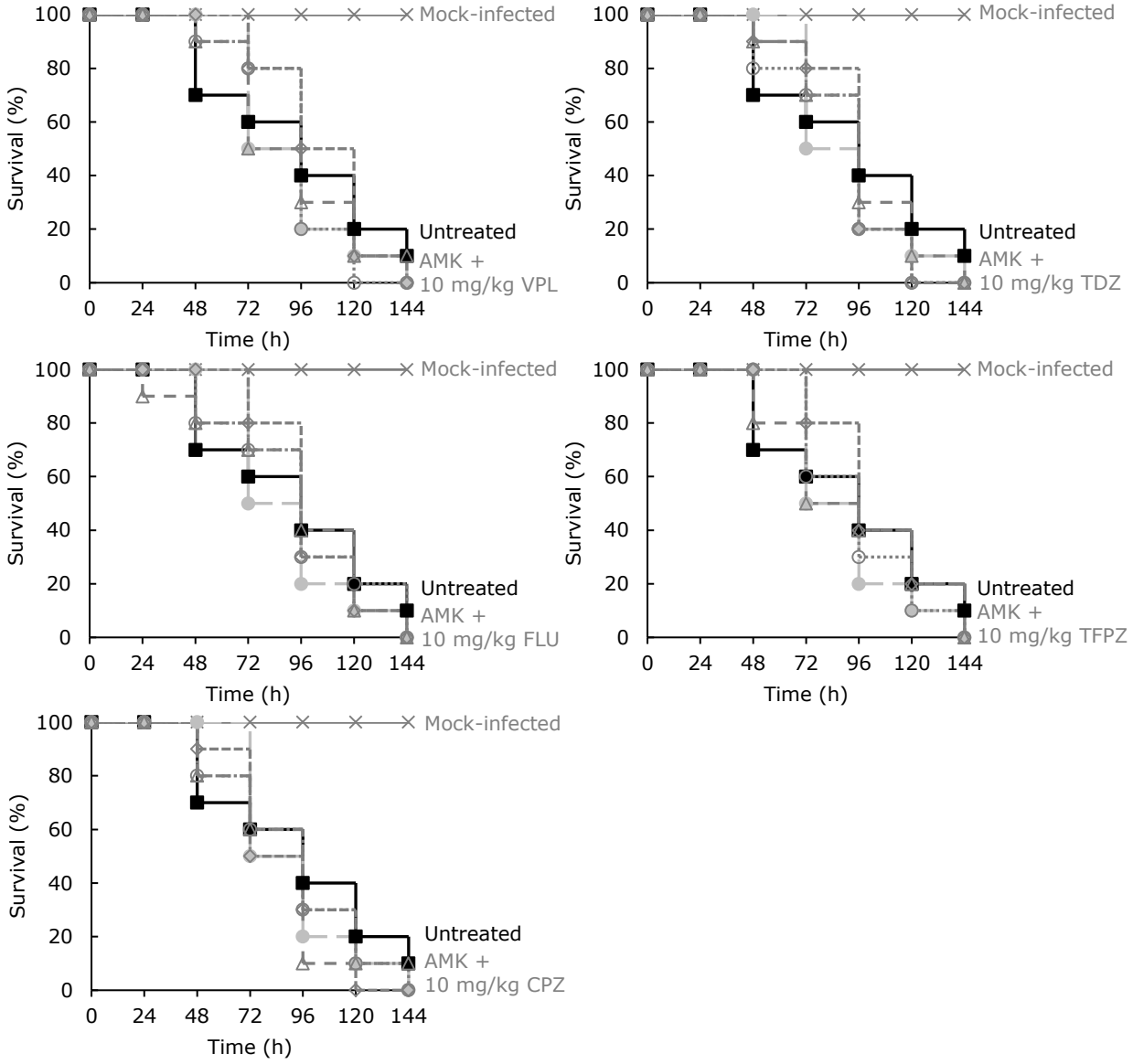

Figure 4.16 – Larvae infected with *M. marinum* Strain M show no improvement in survival when treated with amikacin combined with an efflux pump inhibitor. Larvae were injected with 10 µL of bacteria at 3 x 10^7 c.f.u/mL, then with a single dose of drug two hours later. Amikacin was used at 0.1 mg/kg, both alone and when in combination. The efflux pump inhibitors were used at 10, 1.0 and 0.1 mg/kg. The combinations used are shown on the right of each curve, although only the highest combination of AMK and EPI is labelled. Infected and untreated larvae, black filled squares; AMK alone, pale grey filled circles; AMK + 10 mg/kg EPI, grey open diamonds; AMK + 1.0 mg/kg EPI, grey open triangles; AMK + 0.1 mg/kg EPI, grey open circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. *n* = 10. AMK, amikacin; VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine; EPI, efflux pump inhibitor.

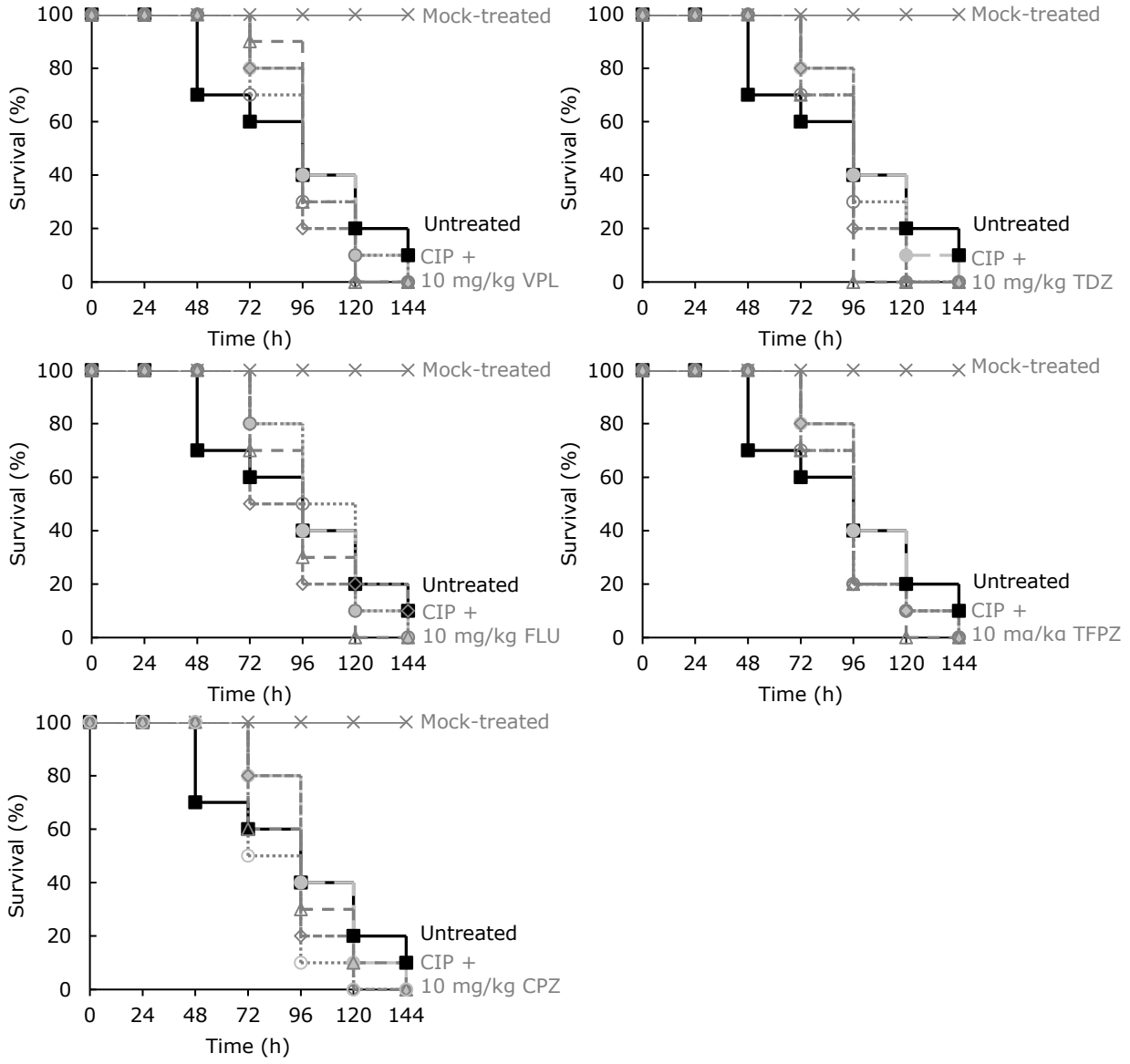

Figure 4.17 – Larvae infected with *M. marinum* Strain M show no improvement in survival when treated with ciprofloxacin combined with an efflux pump inhibitor. Larvae were injected with 10 µL of bacteria at 3 x 10^7 c.f.u/mL, then with a single dose of drug two hours later. Ciprofloxacin was used at 25 mg/kg, both alone and when in combination. The efflux pump inhibitors were used at 10, 1.0 and 0.1 mg/kg. The combinations used are shown on the right of each curve, although only the highest combination of CIP and EPI is labelled. Infected and untreated larvae, black filled squares; CIP alone, pale grey filled circles; CIP + 10 mg/kg EPI, grey open diamonds; CIP + 1.0 mg/kg EPI, grey open triangles; CIP + 0.1 mg/kg EPI, grey open circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. *n* = 10. CIP, ciprofloxacin; VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine; EPI, efflux pump inhibitor.

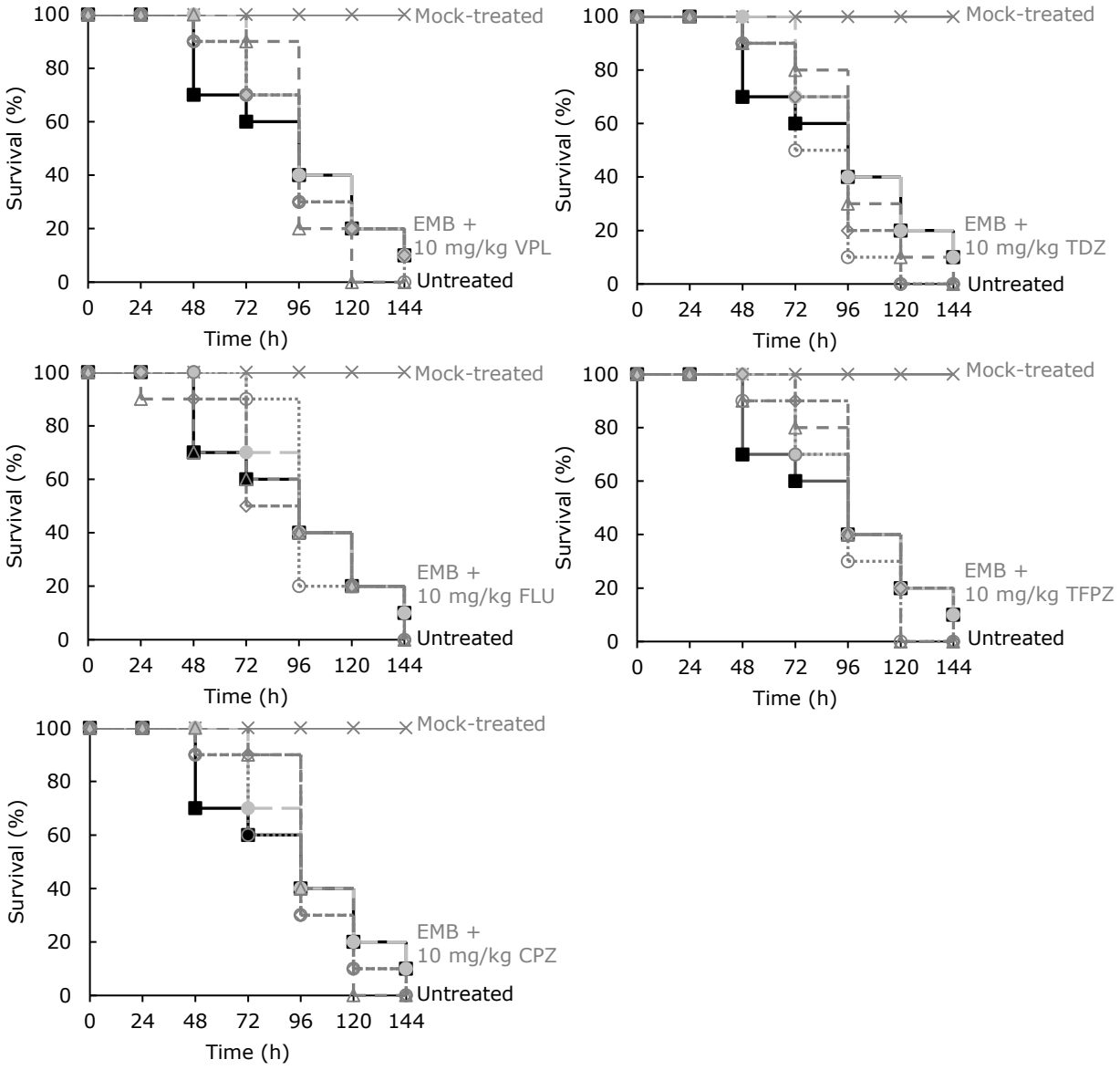

Figure 4.18 – Larvae infected with *M. marinum* Strain M show no improvement in survival when treated with ethambutol combined with an efflux pump inhibitor. Larvae were injected with 10 µL of bacteria at 3 x 10^7 c.f.u/mL, then with a single dose of drug two hours later. Ethambutol was used at 50 mg/kg, both alone and when in combination. The efflux pump inhibitors were used at 10, 1.0 and 0.1 mg/kg. The combinations used are shown on the right of each curve, although only the highest combination of EMB and EPI is labelled. Infected and untreated larvae, black filled squares; EMB alone, pale grey filled circles; EMB + 10 mg/kg EPI, grey open diamonds; EMB + 1.0 mg/kg EPI, grey open triangles; EMB + 0.1 mg/kg EPI, grey open circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. *n* = 10. EMB, ethambutol; VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine; EPI, efflux pump inhibitor.

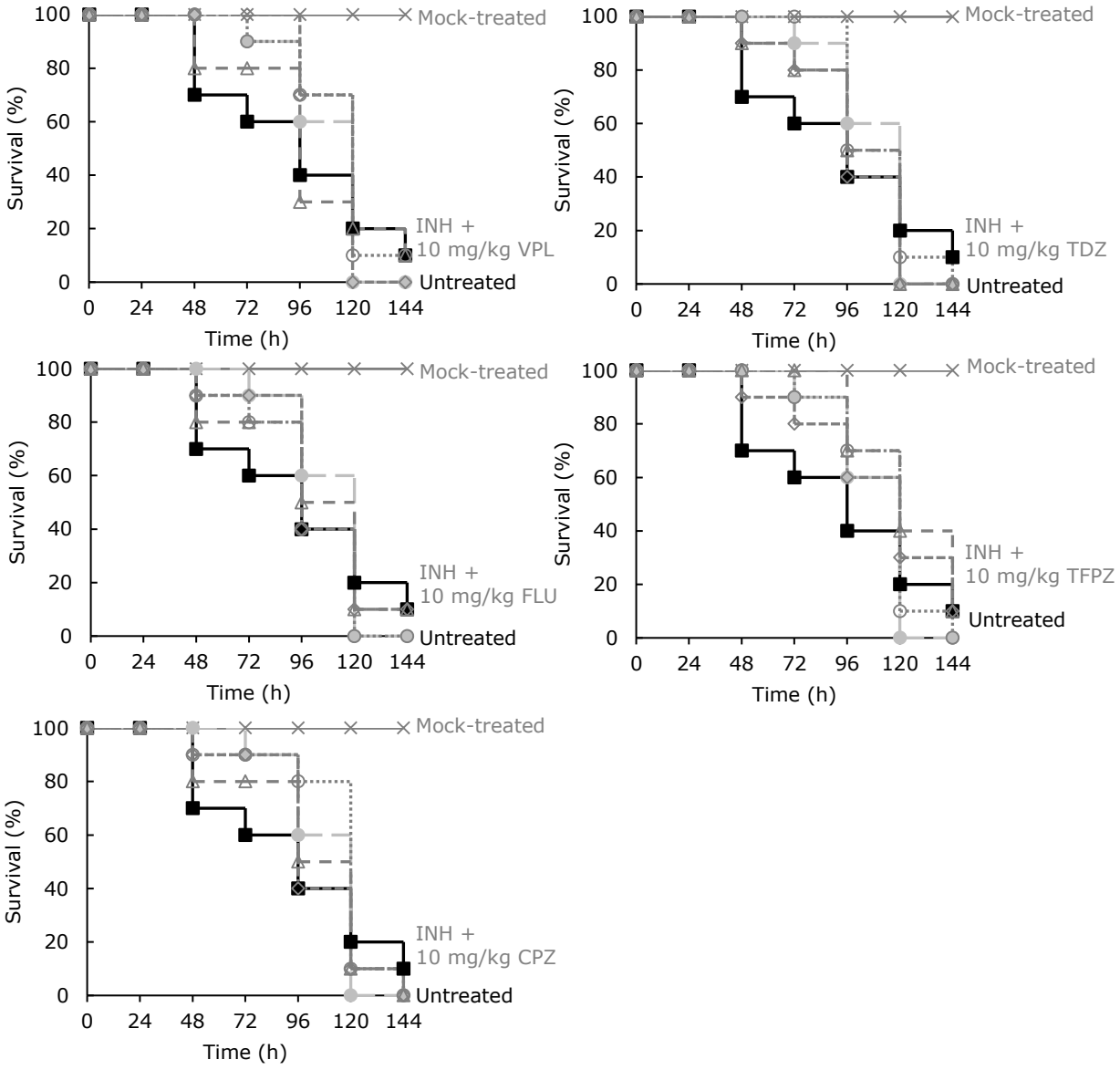

Figure 4.19 – Larvae infected with *M. marinum* Strain M show some improvement in survival when treated with isoniazid combined with an efflux pump inhibitor, but the overall effect on survival rate is minimal. Larvae were injected with 10 µL of bacteria at 3 x 10^7 c.f.u/mL, then with a single dose of drug two hours later. Isoniazid was used at 5.0 mg/kg, both alone and when in combination. The efflux pump inhibitors were used at 10, 1.0 and 0.1 mg/kg. The combinations used are shown on the right of each curve, although only the highest combination of INH and EPI is labelled. Infected and untreated larvae, black filled squares; INH alone, pale grey filled circles; INH + 10 mg/kg EPI, grey open diamonds; INH + 1.0 mg/kg EPI, grey open triangles; INH + 0.1 mg/kg EPI, grey open circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. *n* = 10. INH, isoniazid; VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine; EPI, efflux pump inhibitor.

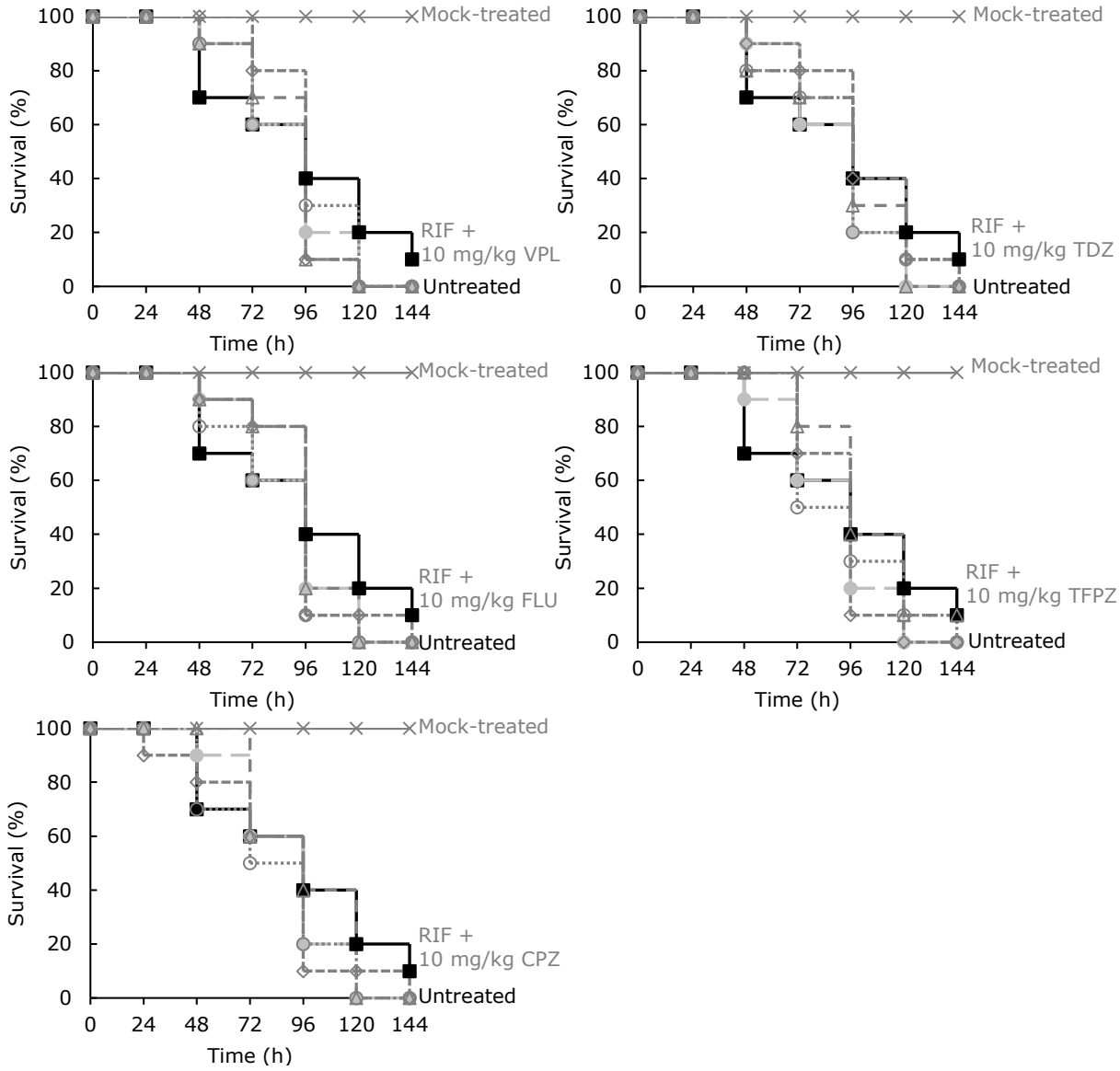

Figure 4.20 – Larvae infected with *M. marinum* Strain M show no improvement in survival when treated with rifampicin combined with an efflux pump inhibitor. Larvae were injected with 10 µL of bacteria at 3 x 10^7 c.f.u/mL, then with a single dose of drug two hours later. Rifampicin was used at 5.0 mg/kg, both alone and when in combination. The efflux pump inhibitors were used at 10, 1.0 and 0.1 mg/kg. The combinations used are shown on the right of each curve, although only the highest combination of RIF and EPI is labelled. Infected and untreated larvae, black filled squares; RIF alone, pale grey filled circles; RIF + 10 mg/kg EPI, grey open diamonds; RIF + 1.0 mg/kg EPI, grey open triangles; RIF + 0.1 mg/kg EPI, grey open circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. *n* = 10. RIF, rifampicin; VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine; EPI, efflux pump inhibitor.

4.3.2.4 *M. marinum* R356933F – larval survival with efflux pump inhibitors and low antibiotic doses

The results for efflux pump inhibitors combined with antibiotics indicate that these combinations are generally unable to treat larvae infected with *M. marinum* R356933F (see **Figures 4.21, 4.22, 4.23, 4.24** and **4.25**). The survival curves for the untreated controls and controls injected with antibiotics as a monotherapy are mostly indistinguishable from the larvae injected with antibiotics combined with EPIs.

There are occasional combinations which improve larval survival above the controls in **Figures 4.21, 4.22, 4.23** and **4.25**, However, the survival for these larvae never remains higher than 40% by the end of the 144 hour observation period. Equally, a similar issue to the results for *M. fortuitum* NCTC 8573 (**Section 4.3.2.2**) is seen – as the larvae die much later in the 144 hour observation period, increases in survival can look significant but log-rank testing shows that the result is not significant ($p > 0.05$).

Yet the results shown in **Figure 4.24** indicate that INH combined with all five of the EPIs in this study improves larval survival above that of of the untreated controls, and the controls injected with INH alone. Survival in these preliminary experiments was even as high as 100% for some combinations.

Because of the statistically increased survival of some of these combinations, VPL, TDZ, FLU, TFPZ and CPZ were selected to be tested again at 10 mg/kg with 0.5 mg/kg INH using a larger group of larvae ($n = 30$). The results of these experiments are shown in **Figure 4.26**.

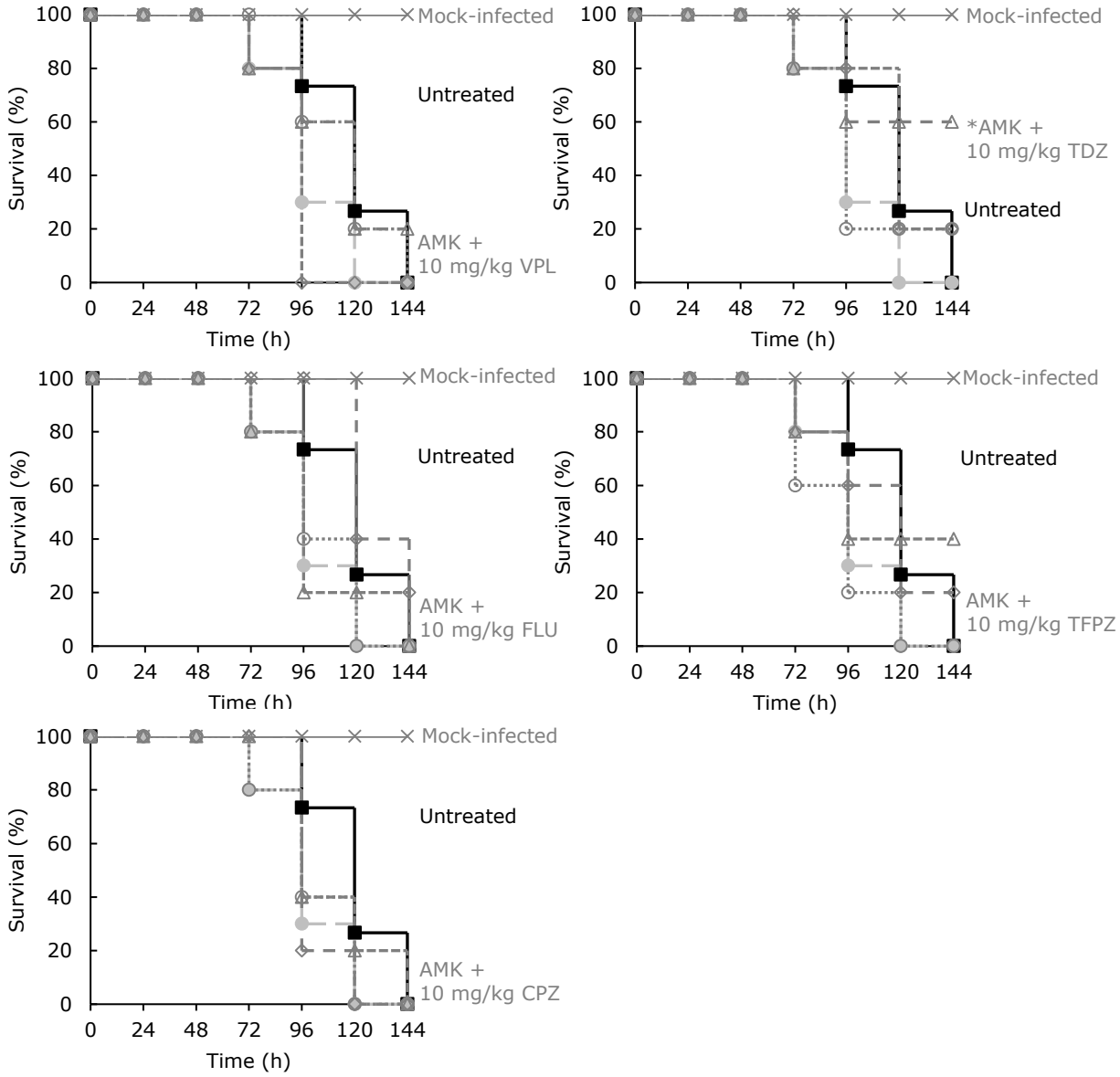

Figure 4.21 – Larvae infected with *M. marinum* R356933F show minimal improvement in survival when treated with amikacin combined with an efflux pump inhibitor. Larvae were injected with 10 µL of bacteria at 9 x 10^8 c.f.u/mL, then with a single dose of drug two hours later. Amikacin was used at 0.1 mg/kg, both alone and when in combination. The efflux pump inhibitors were used at 10, 1.0 and 0.1 mg/kg. The combinations used are shown on the right of each curve; the highest combination of AMK and EPI is labelled on all curves, and combinations where the final survival were higher than the untreated controls are also shown. Infected and untreated larvae, black filled squares; AMK alone, pale grey filled circles; AMK + 10 mg/kg EPI, grey open diamonds; AMK + 1.0 mg/kg EPI, grey open triangles; AMK + 0.1 mg/kg EPI, grey open circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant experimental group on the key. $n = 30$ for the untreated controls, $n = 10$ for the antibiotic monotherapy controls, $n = 5$ for the antibiotic and EPI combinations. AMK, amikacin; VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine; EPI, efflux pump inhibitor.

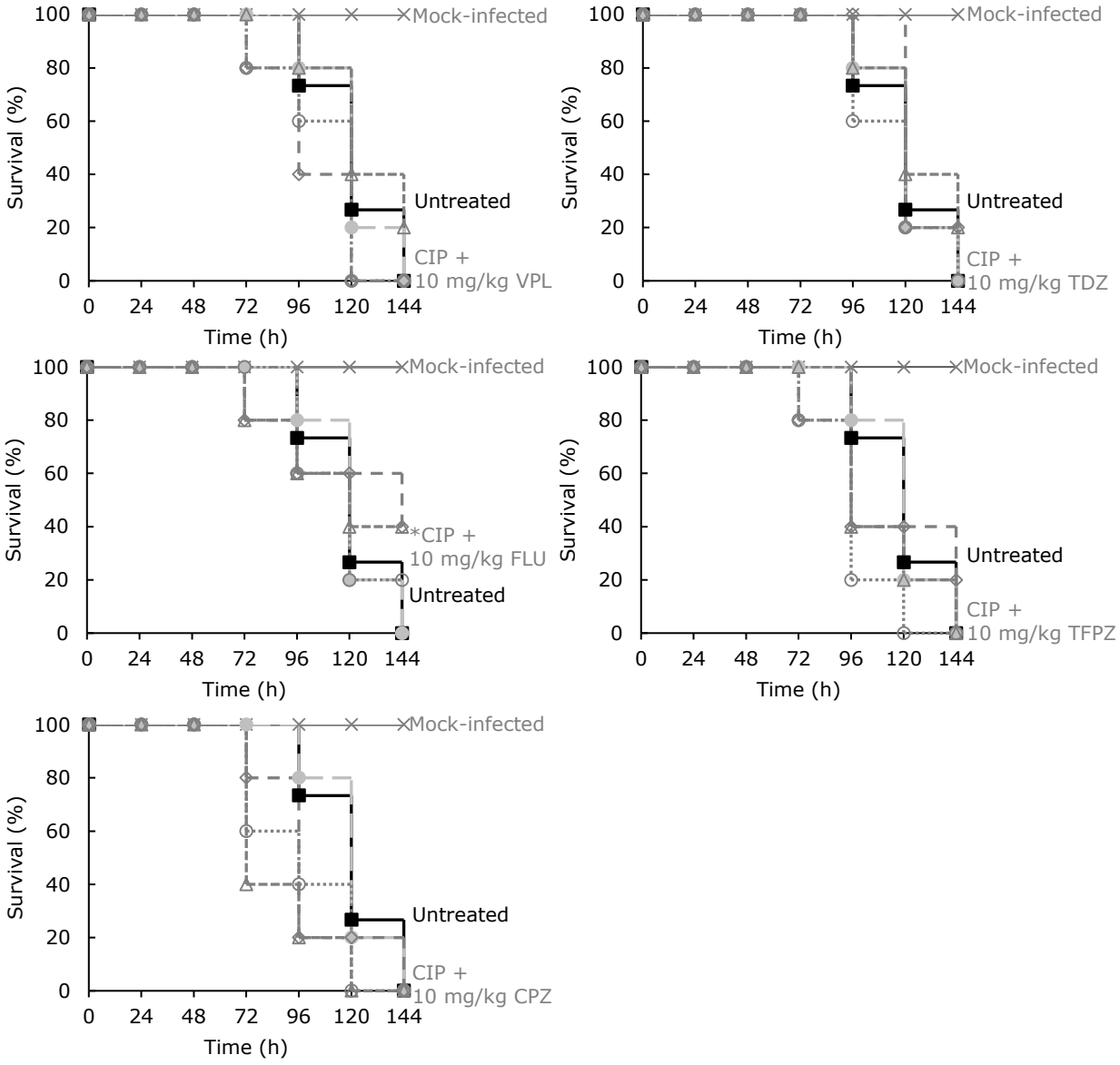

Figure 4.22 – Larvae infected with *M. marinum* R356933F show minimal improvement in survival when treated with ciprofloxacin combined with an efflux pump inhibitor. Larvae were injected with 10 µL of bacteria at 9 x 10^8 c.f.u/mL, then with a single dose of drug two hours later. Ciprofloxacin was used at 2.5 mg/kg, both alone and when in combination. The efflux pump inhibitors were used at 10, 1.0 and 0.1 mg/kg. The combinations used are shown on the right of each curve, although only the highest combination of CIP and EPI is labelled. Infected and untreated larvae, black filled squares; CIP alone, pale grey filled circles; CIP + 10 mg/kg EPI, grey open diamonds; CIP + 1.0 mg/kg EPI, grey open triangles; CIP + 0.1 mg/kg EPI, grey open circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant experimental group on the key. n = 30 for the untreated controls, n = 10 for the antibiotic monotherapy controls, n = 5 for the antibiotic and EPI combinations. CIP, ciprofloxacin; VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine; EPI, efflux pump inhibitor.

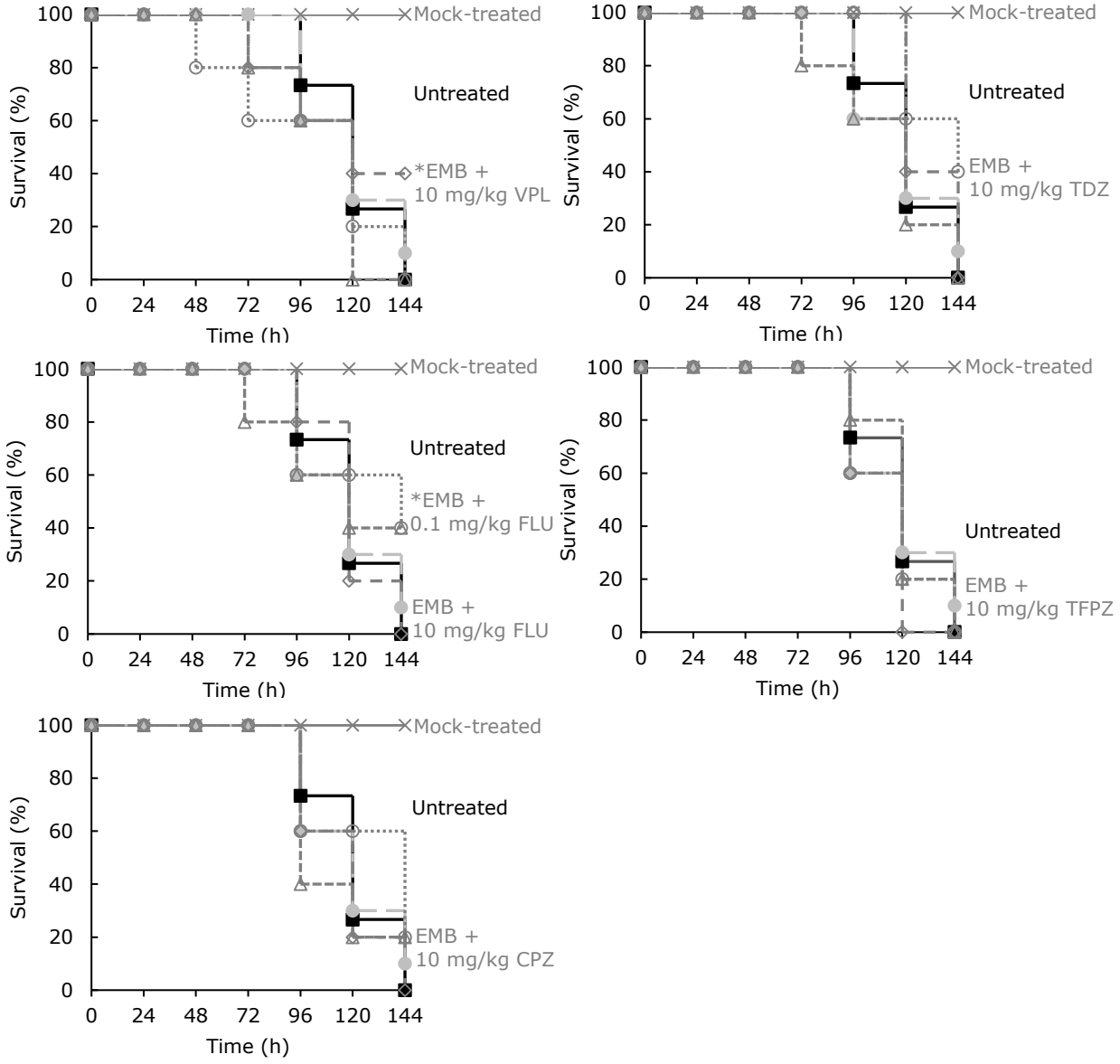

Figure 4.23 – Larvae infected with *M. marinum* R356933F generally show no improvement in survival when treated with ethambutol combined with an efflux pump inhibitor. Larvae were injected with 10 µL of bacteria at 9 x 10^8 c.f.u/mL, then with a single dose of drug two hours later. Ethambutol was used at 50 mg/kg, both alone and when in combination. The efflux pump inhibitors were used at 10, 1.0 and 0.1 mg/kg. The combinations used are shown on the right of each curve, although only the highest combination of EMB and EPI is labelled unless another dataset is significant. Infected and untreated larvae, black filled squares; EMB alone, pale grey filled circles; EMB + 10 mg/kg EPI, grey open diamonds; EMB + 1.0 mg/kg EPI, grey open triangles; EMB + 0.1 mg/kg EPI, grey open circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant experimental group on the key. $n = 30$ for the untreated controls, $n = 10$ for the antibiotic monotherapy controls, $n = 5$ for the antibiotic and EPI combinations. EMB, ethambutol; VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine; EPI, efflux pump inhibitor.

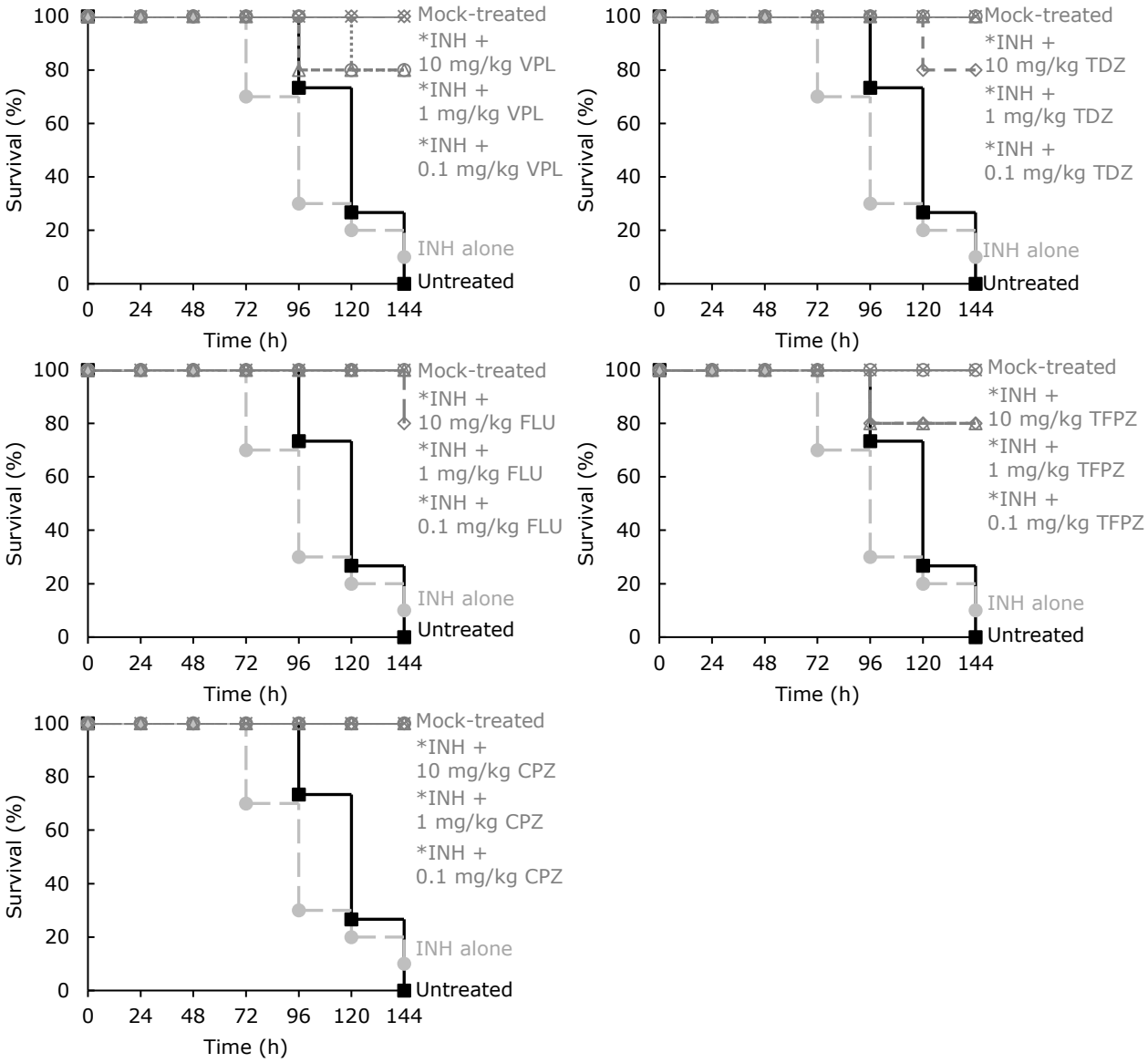

Figure 4.24 – Larvae infected with *M. marinum* R356933F have increases survival rates when treated with isoniazid combined with an efflux pump inhibitor. Larvae were injected with 10 µL of bacteria at 9 x 10^8 c.f.u/mL, then with a single dose of drug two hours later. Isoniazid was used at 0.5 mg/kg, both alone and when in combination. The efflux pump inhibitors were used at 10, 1.0 and 0.1 mg/kg. The combinations used are shown on the right of each curve – as the survival of some combination is 100%, the results may be overlaid with other datapoints. Infected and untreated larvae, black filled squares; INH alone, pale grey filled circles; INH + 10 mg/kg EPI, grey open diamonds; INH + 1.0 mg/kg EPI, grey open triangles; INH + 0.1 mg/kg EPI, grey open circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant experimental group on the key. n = 30 for the untreated controls, n = 10 for the antibiotic monotherapy controls, n = 5 for the antibiotic and EPI combinations. INH, isoniazid; VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine; EPI, efflux pump inhibitor.

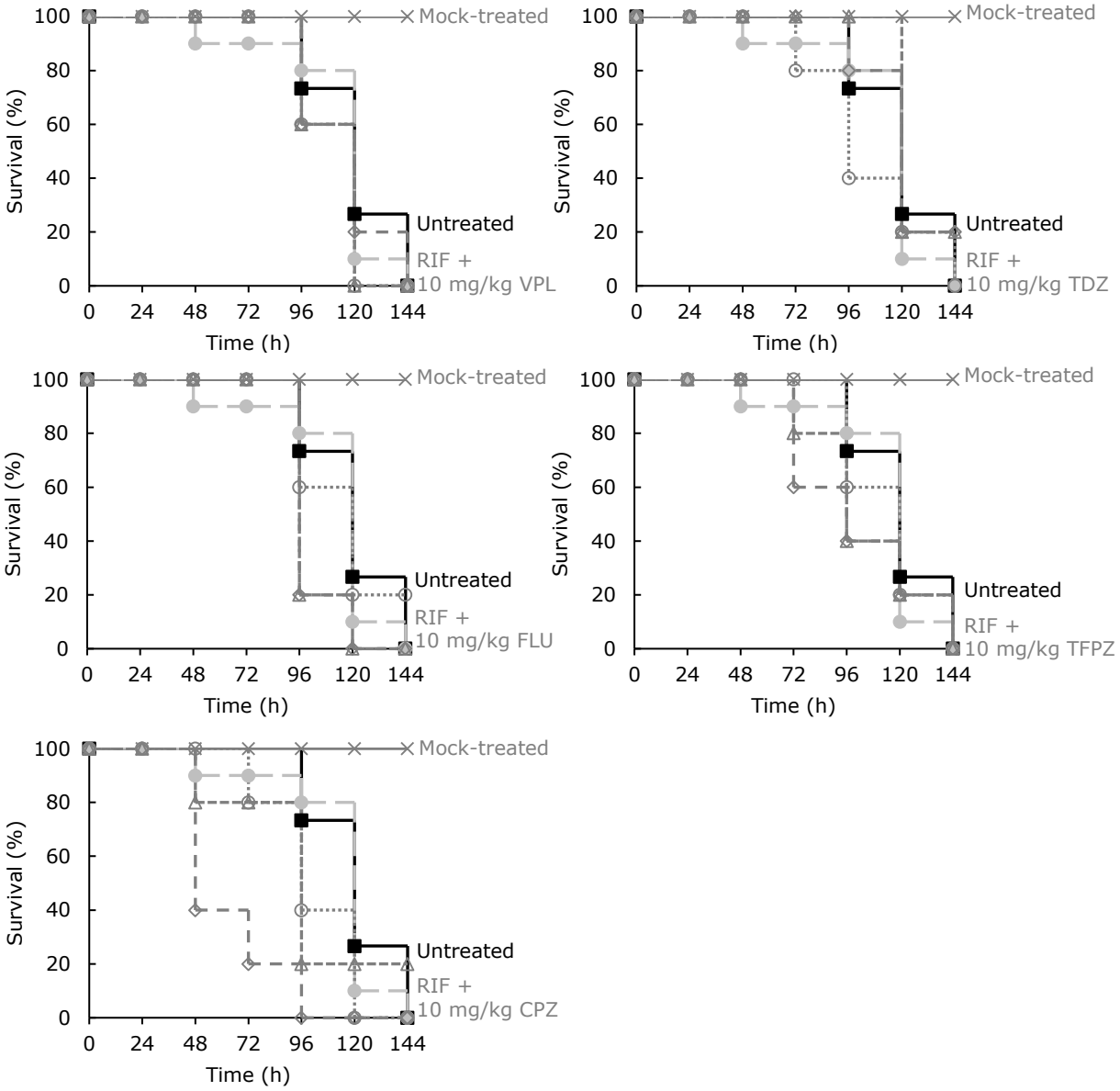

Figure 4.25 – Larvae infected with *M. marinum* R356933F show no improvement in survival when treated with rifampicin combined with an efflux pump inhibitor. Larvae were injected with 10 µL of bacteria at 9 x 10^8 c.f.u/mL, then with a single dose of drug two hours later. Rifampicin was used at 5.0 mg/kg, both alone and when in combination. The efflux pump inhibitors were used at 10, 1.0 and 0.1 mg/kg. The combinations used are shown on the right of each curve, although only the highest combination of RIF and EPI is labelled. Infected and untreated larvae, black filled squares; RIF alone, pale grey filled circles; RIF + 10 mg/kg EPI, grey open diamonds; RIF + 1.0 mg/kg EPI, grey open triangles; RIF + 0.1 mg/kg EPI, grey open circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. *n* = 30 for the untreated controls, *n* = 10 for the antibiotic monotherapy controls, *n* = 5 for the antibiotic and EPI combinations. RIF, rifampicin; VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine; EPI, efflux pump inhibitor.

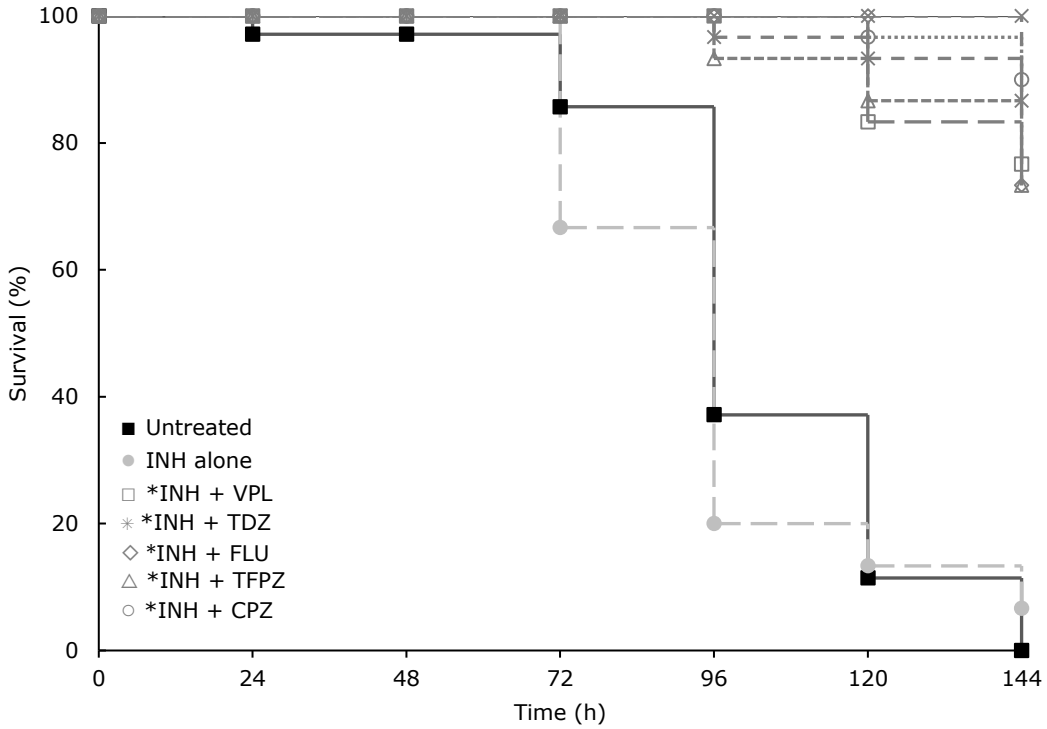

Figure 4.26 – The improvement in survival when larvae infected with *M. marinum* R356933F are treated with a sub-optimal dose of isoniazid combined with an efflux pump inhibitor remains when the combinations are tested in a larger experimental group. Larvae were injected with 10 µL of bacteria at 9 x 10^8 c.f.u/mL, then with a single dose of drug two hours later. Isoniazid was used at 0.5 mg/kg, both alone and when in combination. The efflux pump inhibitors were used at 10 mg/kg. The combinations used are shown to the left of the curve. Infected and untreated larvae, black filled squares; INH alone, pale grey filled circles; INH + 10 mg/kg VPL, grey open squares; INH + 10 mg/kg TDZ, grey eight-pointed star; INH + 10 mg/kg FLU, grey open diamonds; INH+ 10 mg/kg TFPZ, grey open triangles; INH + 10 mg/kg CPZ, grey open circles. Controls of larvae mock-infected with PBS (grey crosses) are also shown. When the change in survival compared to the untreated larvae was statistically significant ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons) an asterisk (*) is shown beside the relevant experimental group on the key. $n = 30$. INH, amikacin; VPL, verapamil; TDZ, thioridazine; FLU, flupenthixol; TFPZ, trifluoperazine; CPZ, chlorpromazine; EPI, efflux pump inhibitor.

4.4 Summary

There is encouraging evidence that β lactam antibiotics combined with a β lactamase inhibitor could be a promising combination for improving larval survival after infections with Mycobacteria. However, the final survival rates at 144 hours were almost uniformly low, and in some cases the β lactam and β lactamase inhibitor performed no better than each component as a monotherapy. The largest improvements in survival were made with *M. fortuitum* NCTC 8573 (**Figure 4.2**), with amoxicillin giving the most impressive increase in survival.

For the experiments combining common anti-Mycobacterials with efflux pump inhibitors (EPIs), the vast majority of antibiotic and EPI combination performed no better than each as a monotherapy, and many died at the same rate as the untreated larvae who had been mock-treated with saline.

However, there were a small number of combinations which were effective at improving larvae survival. For *M. fortuitum* NCTC 10394, these combinations were amikacin combined with verapamil, flupenthixol, trifluoperazine and chlorpromazine (see **Figure 4.10**). With *M. marinum* R356933F, amikacin combined with thioridazine, then isoniazid combined with verapamil, thioridazine, flupenthixol, trifluoperazine and chlorpromazine (see **Figure 4.26**). For the *M. fortuitum* NCTC 10394, survival was still disappointingly low at the end of the experiment. But for *M. marinum* R356933F, the survival at 144 hours for larvae treated with EPIs with isoniazid was very high by 144 hours.

4.5 Discussion

Although it is disappointing that there are very few successful combinations in this chapter, these experiments represent some of the greatest advantages of experiments using *Galleria mellonella.* The experiments in this chapter looked at five antibiotics, five efflux pump

inhibitors, four β lactams combined with a β lactamase inhibitor, with associated monotherapy controls and tested against four Mycobacterial strains. This represents over 150 different experimental conditions, not including replicate experiments with larger groups of larvae. This size and scope of experiment would be entirely unfeasible with a higher order model organism, and would require huge costs in terms of animals, reagents, labour, and time. Considering the "exploratory" nature of these tests, where there were no expectations of whether the combinations would rescue larval survival, these experiments would not have been an attractive investment for funding bodies or ethics boards.

4.5.1 β lactams combined with a β lactamase inhibitor

In terms of the results assessing β lactams with a β lactamase inhibitor, the combination of tazobactam and piperacillin is a well-established β lactam/lactamase inhibitor treatment for Gram-negative and Gram-positive bacterial infections[575]. This combination was anticipated to have some use within this model, although PIP + TZO has had very little testing against Mycobacteria in the literature. The combination was assessed by a group in the 1990s against growing *Mycobacterium avium, M. chelonei, M. haemophilum, M. microti, M. scrofulaceum* and *M. simiae* in liquid cultures, although was not the most active combination tested and was not effective for all Mycobacteria in this study[576]. There are a handful of clinical case studies where PIP + TZO has been assessed[577–580], usually with the *Mycobacterium* in question showing *in* vitro resistance to this combination[581,582]. Often PIP + TZO would be combined with existing anti-Mycobacterial antibiotics in a combined treatment protocol, which would certainly be a worthwhile investigation using *G. mellonella* in the future.

For *M. fortuitum* NCTC 10394, the most successful combination was tazobactam with amoxicillin (**Figure 4.1**). AMX + TZO is not a common drug combination – TZO is almost

ubiquitously combined with piperacillin. AMX + TZO has been examined *in vitro* with *Heliobacteria pylori*, where it was shown to be ineffective at killing these bacteria over a 6 hour experiment but did increase rates of cell lysis[583]. A group in China have also assessed AMX + TZO with 128 clinically isolated bacteria, including drug-resistant strains[574]. A 2:1 ratio of AMX to TZO was found to be the most effective dose and indicated antibacterial activity against multi-drug resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*, methicillin-susceptible *Staphylococcus aureus*, and *Escherichia coli.* However, this paper is not available in English so could not be assessed further.

The use of AMX + TZO in a 2:1 ratio also indicates an interesting criticism of these results. PIP + TZO is routinely administered in an 8:1 ratio[573], and adjusting the ratios of β lactam to β lactamase inhibitor can have direct effects on the susceptibility of bacteria to these treatments[584]. In this work, all combinations were assessed at a 1:1 ratio (50 mg/kg of β lactam antibiotics and of tazobactam), so combinations which showed no effect over the β lactam and tazobactam monotherapies may be more effective at a different ratio. Similarly, combinations which showed some efficacy may have improved results if the ratios were adjusted. Despite AMX + TZO, and also CEC + TZO, clearly improving larval survival after infection with *M. fortuitum* NCTC 10394 (**Figure 4.1**), survival at 144 hours was still very low, so the beneficial effects of these antibiotics could not be maintained for the duration of the experiment.

AMX + TZO was also an effective combination for larvae infected with *M. fortuitum* NCTC 8573 (**Figure 4.2**), much more so than with *M. fortuitum* NCTC 10394. Survival at 144 hours was over 83% with the former, compared to less than 7% with the latter. However, TZO as a monotherapy was also effective at improving larval survival as a monotherapy. AMX and PIP were both effective at improving larval survival significantly above the TZO monotherapy

(indicated with an Ω symbol on the key of the relevant survival curve), but CEC and CEF did not increase survival significantly above the TZO monotherapy.

TZO is not routinely tested as a monotherapy in combination studies. Usually, a panel of β lactams are assessed alone, and then in combination with β lactamase inhibitors such as sulbactam and tazobactam, but not with β lactamase inhibitors as monotherapies (a good example of this style of experiment with *M. tuberculosis* strains is shown in reference[567]). TZO is a heavily modified β lactam – essentially a modified sulphone derivative of penicillin[585] – although whether this would allow for TZO to have residual antibiotic properties is not clear. Hence, the antimicrobial activity of TZO is uncertain but likely to be absent[586]. This is in contrast to other β lactamase inhibitors – clavulanic acid[587] and sulbactam[588] – which have been shown to have antibiotic activities as monotherapies[586].

TZO was also seen to have to have an effect on improving survival of larvae infected with *M. marinum* R356933F (**Figure 4.4**), although not to the same degree as with *M. fortuitum* NCTC 8573. This could be further evidence that TZO can improve larval survival, but survival at 144 hours is still low (almost 7%). Looking at the results for *M. marinum* R356933F as a whole, the β lactam antibiotics and β lactamase inhibitors are generally ineffective. CEF + TZO and PIP + TZO are statistically indistinguishable from the untreated infected control. Although AMX + TZO and CEC + TZO are more effective at improving survival than the β lactam monotherapy, the final survival is not particularly high and neither combination improves survival over the larvae treated with TZO alone. As mentioned earlier, perhaps these results could be improved by adjusting the dosing ratios between the β lactam and β lactamase inhibitor.

Lastly, the results for *M. marinum* Strain M (**Figure 4.3**) also indicate that β lactams and a β lactamase inhibitor are ineffective at improving larval survival in this experimental design.

AMX, CEF and CEC are all slightly able to improve survival significantly above the untreated control, as is AMX + TZO, but in reality, these survival curves are practically identical to the untreated controls.

Other than adjusting ratios of β lactam and β lactamase inhibitor, other modifications to this experiment could include assessment of other β lactamase inhibitors – clavulanic acid, sulbactam or avibactam – or including other β lactam antibiotics. Injecting the drugs at several timepoints will also indicate if there is an issue with either drug being cleared too rapidly from the larval body and may improve survival as seen in **Section 3.5.5**. *Galleria mellonella* has been used as a tool for pharmacokinetic studies previously[393–395], which may be a useful application here.

4.5.2 Efflux pump inhibitors combined with low dose anti-Mycobacterials

As mentioned at the beginning of this discussion, it is disappointing to see so few effective combinations of efflux pump inhibitors and anti-Mycobacterial combinations, but these experiments represent the value of the *G. mellonella* system. A large number of *in vivo* tests could be assessed with relative ease and speed, especially when compared to the difficulty a group would have in analysing this number of combinations in murine or other vertebrate models.

As discussed in **Section 4.1.2**, only a small number of EPIs have been assessed in clinical trials, but none have been approved for clinical use and there have been persistent issues with potency, toxicity, mediocre pharmacokinetic properties, or low *in vivo* efficacy[510]. EPIs have been assessed with antibiotics and non-tuberculous Mycobacteria, but only in a small number of papers[545,589]. As some of the EPIs used in this study had been used elsewhere with

Mycobacteria *in vito*[533–535], there was some anticipation that positive results would be seen in a subset of the experiments performed here. However, the vast majority of EPIs and antibiotics were ineffective at improving larval survival above the level of the untreated controls. The small number which clearly improved larval survival generally didn't lead to especially high larval survival at 144 hours for *M. fortuitum* infections, although INH and EPIs improved the larval survival for one *M. marinum* strain, but not the other.

Considering that microbial multidrug efflux was only reported in 1980[590], a lack of publications combining EPIs and antibiotics for non-tuberculous Mycobacterial infections is not surprising, but does mean that it is not immediately clear why so many of these EPI and antibiotic combinations were ineffective.

In terms of *M. fortuitum* NCTC 10394, the most effective combinations all contained amikacin. The DrugBank database suggests that amikacin is capable of reducing the excretion rate of efflux pump inhibitors including verapamil and chlorpromazine, leading to increased serum levels. However, no published literature could be found to support these claims and it is not clear which excretion processes are affected (excretion from human cells, or excretion by the kidneys?), and therefore how these results could be related to excretion processes in *G. mellonella*. Additionally, DrugBank lists similar data for ethambutol, rifampicin and isoniazid, although reports INH as decreasing the metabolism of chlorpromazine. DrugBank also lists that ciprofloxacin can decrease the metabolism of EPIs.

EPIs used with AMK, INH and RIF against strains of *M. tuberculosis* have shown that verapamil, thioridazine, chlorpromazine and flupentixol can all reduce the MICs of these antibiotics[568]. If these EPIs reduce the MIC for INH and RIF as well as AMK, why are there no effective combinations in the INH and RIF experiments? This extends to the other experiments

with *M. fortuitum* NCTC 8573, and *M. marinum* Strain M and R356933F, all of which exhibited very low numbers of effective combinations. The exception to this is *M. marinum* R356933F with INH and the EPIs used in this thesis. The reasoning for this is uncertain, especially as survival was increased so dramatically but these combinations were ineffective for *M. marinum* Strain M.

Unlike the experiments with β lactams and β lactamase inhibitors, as no EPIs have been approved for clinical use, there is very little guidance on biologically effective ratios of EPIs and antibiotics. It may be that the doses of EPI selected were improper, although 0.1 mg/kg, 1.0 mg/kg and 10 mg/kg were expected to cover sufficient range to assess sensitivity to each EPI. Increased doses of EPI may give higher survival, mirroring the issues in potency, toxicity, mediocre pharmacokinetic properties, and poor *in vivo* efficacy seen by others[510].

Repeated injections of EPIs and/or antibiotics might also improve survival. Other EPIs could also be assessed; however, a reasonable range of EPIs were examined here, and using them with anti-Mycobacterial antibiotics would be the rational combination.

Chapter Five

5.0 The larval haemocyte and its response to infection and treatment

This chapter examines the immune cells, or haemocytes, of the larvae and the changes in their numbers, nodulation, melanisation, and microscopic interactions with bacteria post-infection.

5.1 The role of the haemocyte

As discussed in **Section 1.3.3**, the immune response of *G. mellonella* is divided into two sections – cellular and humoral responses. This Chapter primarily focusses on haemocytes, but the humoural response is discussed in **Section 1.3.3**.

Haemocytes are essentially the immune cells of invertebrates. As insects and other invertebrates lack an adaptive immune system, these cells are key to protecting the invertebrate from invasive bacteria, fungi, parasites and viruses.

5.1.1 Innate vs. adaptive cellular immunity

Unlike in higher organisms, invertebrates and insects have generally been regarded to have no ability to generate an immunological "memory" of pathogens which they have encountered previously. Hence, each new immune challenge has an equally likely chance of causing an immune response regardless of whether that individual was challenged with that stimulus previously. The ability of insects to overcome an infection is dependent on their innate immune system, which responds rapidly and effectively[307] to a stimulus but is generally non-specific in these responses.

However, there have been some publications that evidence that invertebrates may have some ability to recognise a previous antigen, indicating that the scientific community's

understanding of innate immune systems may require further investigation. Reviewed in Vigneron *et al.*[591], examples of immunological memory, or immune priming, have been investigated in fruit flies[592], mosquitos[593], mealworms[594], flour beetles[595], brine shrimp[596] and even *Galleria mellonella*[597,598]. It is worth noting that the vast majority of these papers have been published within the last 4 years, indicating that this topic is breaking new ground in this field.

The cells which primarily interact with invasive pathogens – haemocytes – share some homology with the immune cells utilised by vertebrates (see **Section 1.3.3**). As with the white blood cells used by vertebrates in the adaptive immune system, haemocytes have a variety of classifications and specialisations to align with their roles in the immune response. There is little evidence that haemocytes play a role in immune priming[591], although fruit fly haemocytes have been shown to take up double-stranded RNA produced by other haemocytes as a template to produce *de nova* RNA more rapidly, allowing for inhibition of viral expression via RNAi[591,592].

In vertebrates, when a pathogen which has been previously encountered by the immune system is recognised again at a later time, the generation, activation and objective of the various white blood cells is directed by memory cells which retain an immunological memory of the foreign antigen. This response is absent in insects, but a key benefit of the innate immune system is that, as it is generalised, the speed with which insects can respond to a pathogen is very rapid. Yet there is increasing evidence that epigenetics – the addition of methyl groups to DNA – could affect the ability of invertebrates to respond to immune challenges both within and across generations[594–596,598,599]. Considering that epigenetics is a very young field of research, with contemporary definitions only being described in the 1990s, it is likely that the previous understanding of invertebrate immunology will adapt as research into this area evolves and progresses.

5.1.2 Types of haemocyte

As discussed in **Section 1.3.3,** the haemocytes observed in all lepidopteran species are the prohaemocytes, plasmatocytes, granular cells, spherule cells, and oenocytoids[317]. These are colourless cells, most of which are freely moving within the haemolymph which fills the larval body, but they can attach to organs within the larvae.

Surprisingly, there is still debate over the classification, nomenclature, origins, and roles of the various haemocytes in *G. mellonella* and invertebrates more generally. Although prohaemocytes are generally described as insect "stem-type" cells[318], even in the modern era researchers struggle to identify them and to clarify whether prohaemocytes are generated through mitosis[600–602]. However, prohaemocytes lack differentiation and lack a role in actual immune interactions such as phagocytosis, nodulation, or adherence in *G. mellonella*[312].

In contrast, plasmatocytes have a variety of roles within the direct immune responses of *G. mellonella*, including phagocytosis, encapsulation, wound repair, and nodule formation[318,319]. They contain large numbers of microtubules to aid in encapsulation of foreign pathogens and in nodulation[312], and may be classified further into more specialised groups such as vermiform cells and podocytes[318,603]. Their specific roles in phagocytosis varies between insects[312,604], however most researchers describe them as phagocytic in *G. mellonella*[318,603,605].

Granulocytes are the most commonly isolated haemocytes in *G. mellonella*[312] and represent around half of the haemocyte population[318]. They arise from the plasmatocyte population[318], and release their granular contents onto foreign particles in the haemolymph and attract plasmatocytes to the area[320,603]. This is analogous to the immune cells in vertebrates, which also cluster around foreign bodies and attract other immune cells through the use of chemokines[606]. The granulocytes closest to the foreign object then flatten and forms layers to

create a capsule around the object, utilising gap-junctions between the layers to allow passage of nutrients, hormones, and cytokines for intercellular signalling[603]. Hence, the combination of phagocytosis by plasmatocyte cells, and capsule formation by granulocytes, means that these cells types are the predominant haemocytes utilised by *G. mellonella* to isolate and degrade invasive pathogens (see **Figure 5.1**).

Other haemocytes in *G. mellonella* include oenocytoids and spherulocytes, Oenocytoids mostly participate in melanisation, wound healing and encapsulation. There is some evidence that oenocytoids rupture very shortly after immune stimulation, and release a number of extracellular nucleic acids which stimulate a number of humoral and cellular responses[607]. This may include inducing the degranulation of the granulocytes, which is supported by observations that oenocytoids vanish from the haemolymph during infection[312]. Spherulocytes have also been observed to disappear from the haemolymph of infected larvae[312] but have been linked to silk production and pupation[319], and are suggested to transport cuticular components[607]. An accurate summation of their function is not known[320].

5.1.3 Changes in numbers and populations

As mentioned, oenocytoids and spherulocytes have been observed to disappear from haemolymph during infections, and in other areas of the larval lifecycle. Changes in the haemocyte populations can also be triggered by non-infective wounding[608], exposure to non-infective stimuli such as acetic acid vapour[318], or pre-incubation at 15 ^{o}C for long periods of time[609]. Injections with chemicals such as potassium nitrate[383], toxins[339], non-steroidal anti-inflammatories[340], antifungals[610,611], antibiotics[549], sterile broth[612] and sugars[613] can also affect the number of haemocytes. This is also seen with nematode worms[614], fungi[468,615], and

bacteria[230,290,291,315,616] after infection. Any trigger, even those which are not caused by exposure to an infectious agent, can alter the number of circulating haemocytes – usually an increase, but occasionally a decrease. An increase in free haemocyte numbers seems to be from the mobilisation of reservoirs of fixed haemocytes[318], and plasmatocytes can become absent from haemolymph during infection[315,612] but not in all cases. However, there are examples of *G. mellonella* being infected and the number of circulating haemocytes not changing[617].

Clearly, there can be a variety of responses from larval haemocytes after the immune system is stimulated, regardless of whether stimulation is chemical or pathogenic. Although there are a sizeable number of publications which look at the changes in haemocyte numbers and populations, there are no clear patterns to predicting whether a specific reagent, pathogen, or procedure is going to cause an increase or decrease in these values. Perhaps, if a greater volume of studies in this area can be produced and reviewed, patterns and correlations may be exposed.

5.2 Interactions between haemocytes and bacteria

The complex array of interactions between a host immune system and invasive pathogen are just as multifaceted and convoluted for an insect as they are for a human. Untangling the responses of the larval immune system to infection can give valuable information of the usefulness of the *G. mellonella* model for infections with Mycobacteria. However, as mentioned in **Sections 1.5** and **2.1**, only a handful of modern papers have been published on the use of Mycobacterial species with *G. mellonella.*

The first English-language paper on this host-pathogen combination was published in 1934[262] – the author describes the interactions between *M. tuberculosis* and the haemocytes of *G. mellonella* in remarkable detail. Within 30 minutes of injection, the Mycobacterial cells have started to be phagocytosed by plasmatocytes and the number of haemocytes begins to rise. In the first hour, phagocytosis of the bacteria is still active, although there are still free bacteria in the haemolymph, and the number of haemocytes continues to increase. Four hours post-infection, clumps of bacteria are surrounded by granulocytes and the boundaries between these cells becomes unclear, but no bacteria can be seen in oenocytoids or spherulocytes. Twelve hours post-infection, granulocytes can represent up to 90% of the non-adhered haemocytes in the haemolymph, but the number of plasmatocytes has decreased. A full day post-infection, nodules have surrounded the bacteria in layers large enough to mean the nodules are visible to the naked eye and there are almost no free-floating bacteria in the haemolymph. The number of granulocytes is still elevated above normal. For the rest of the experiment, nodules containing acid-fast, viable bacteria can be isolated from the larvae. Even after metamorphosis, these nodules and bacteria can be isolated from adult moths. The author states he found similar results with *M. smegmatis*.

Interestingly, the author also states that *M. tuberculosis*, *M. bovis* and *M. avium* cannot cause larval death. Modern research efforts using *M. bovis* BCG have shown these bacteria are more than capable of causing larval death[378,379], and the results described in the earlier Chapters of this thesis indicate that *M. fortuitum* and *M. marinum* are also pathogenic to *G. mellonella.* Hence, the author of the 1934 paper may have used an insufficient number of *M. tuberculosis* bacteria to cause death. Until *M. tuberculosis* is used in a modern publication, this question will remain unanswered.

5.2.1 Clustering of cells and nodulation

Cell clustering is a common technique used by vertebrate and invertebrate immune cells to isolate a foreign body while sending signals to other immune cells to come to the point of reaction[618,619]. For vertebrates, the primary response to isolate pathogens from the host is phagocytosis and drawing other immune cells to the vicinity through chemotaxis. Invertebrates also utilise nodule formation alongside phagocytosis to isolate pathogens, but the evidence for chemotaxis is minimal. Plasmatocytes seem to be drawn towards bacterial/granulocyte complexes, and larval haemocytes have been shown to be attracted to *Aspergillus flavus* conidia *in vitro*[318].

However, the use of nodulation and phagocytosis to isolate pathogens has been well studied in *G. mellonella*. Phagocytosis will be discussed in **Section 5.2.3**. Nodulation is, as described in **Section 1.2.3**, analogous with granuloma formation in higher organisms – a central core of melanised, necrotic haemocytes and associated pathogens surrounded by morphologically altered blood cells[318,620] (see **Figure 5.1**). Much as is seen with human granulomas[621], nodules in *G. mellonella* can be due to an infection with a variety of pathogens

including bacteria, fungi and protozoa, but also non-pathogenic triggers such as ink particles and silica or latex beads[318,406,407].

The nodulation response is incredibly rapid, and is mostly due to the action of granulocytes and their entrapment of foreign objects in granular discharge, rather than the phagocytic activities of plasmatocytes[312]. However, plasmatocytes seem to be attracted to the newly-forming nodule through chemotactic factors released by the granulocytes[312,318,620].

Despite a thick layer of immune cells encasing the pathogen within the nodule, bacteria have been shown to survive inside nodules[262] and even to escape the nodules and multiply in the haemolymph[622]. More contemporary examples include the survival of *Aspergillus* in melanised nodules[623], and the destruction of nodules after infection with *Pseudomonas aeruginosa*[624].

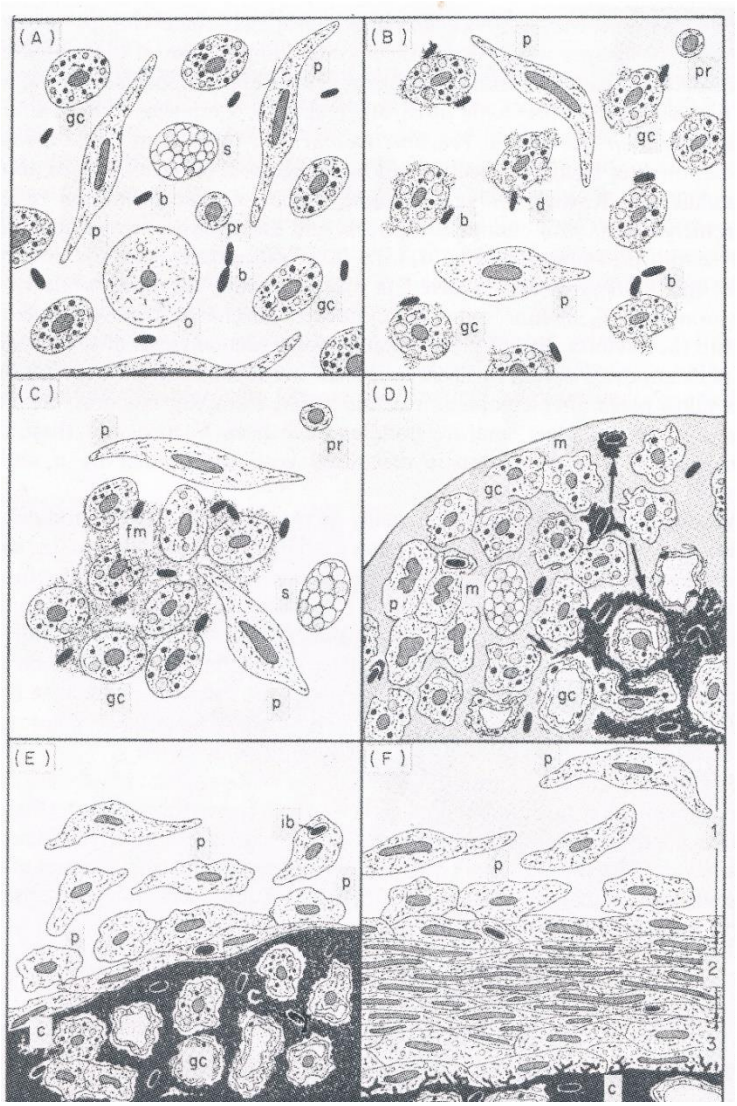

Figure 5.1 – Diagram representation of the stages of nodule formation in *G. mellonella*. (A) Free-floating haemocytes in the haemolymph at the point of infection. (B) Once the granulocytes make contact with the bacteria, their granular contents are discharged to trap the bacteria. (C) Within a minute of infection, granulocytes and their contents have collectively surrounded and coated the bacteria and themselves with granular material. (D) in the 5-30 minutes post-infection, the clumps have compacted and melanisation has started (unlabelled arrows), especially in the areas where the bacteria are present. (E) The second stage of nodulation begins as the plasmatocytes begin to attach to the nodule in large numbers. Some contain phagocytosed intracellular bacteria. The inner core of the nodule is now highly melanised. (F) By 12-24 hours post-infection, a mature nodule has formed. New cells are attaching to the outer layer (1) and the middle region (2) contains extremely flattened cells, those of which that are closest to the melanised core may contain melanised inclusions. gc, granular cells; b, bacteria; p, plasmatocytes; pr, prohaemocytes; s, spherulocytes; o, oenocytoids; d, discharge; fm, flocculant material; m, matrix; ib, intracellular bacteria; c, melanised core.

Figure adapted from Ratcliffe and Rowley (1981), originally in Ratcliffe and Gagen (1977)

5.2.2 Melanisation

Melanin is produced by organisms for a variety of purposes. In humans, melanin is usually associated with the colour of our skin, and is associated with exposure to UV light from the sun[625]. Other uses of melanin include the ink produced by cephalopods[626], the colouring of coats in mammals[627], and the colour of feathers in birds[628].

The production of melanin is a key component of the nodulation process (**Section 5.2.1** and **Figure 5.1**). As mentioned in **Section 1.2.3**, melanin formation in *G. mellonella* is catalysed by phenoloxidases and is activated by pattern recognition receptors binding to microbial surface proteins such as lipopolysaccharide[326]. The melanin "scavenges" reactive oxygen species (ROS) to be used against the confined bacteria and protects the host from ROS and cytotoxic by-products of melanin production[233,234]. Hence, levels of melanisation can occasionally be correlated with the strength of the larval response to a stimulus – a selection of experimental protocols to assess melanisation are described in **Section 1.6**. However, as shown by the results with *M. aurum* in **Chapter Two**, and from the results of other researchers[302], melanisation does not necessarily occur after infection. Melanisation can also be triggered by non-pathogenic stimuli, similarly to the complement system in higher organisms,

5.2.3 Phagocytosis

The activity of phagocytic cells is the major component of the host defence mechanism in vertebrates. Their role in dynamically isolating and then killing the engulfed organisms protects the host from a wide range of pathogens[629]. Although many aspects of the immune system in *G. mellonella* are not associated with phagocytosis[318], the phagocytic capabilities of insect haemocytes remains an important factor of their immune function. These cells also play a role in clearing cell debris during metamorphosis[630].

5.2.3.1 Efficacy of phagocytosis

The phagocytic cells in *G. mellonella* are primarily plasmatocytes, but oenocytoids[321] and granulocytes[320] have been shown to be capable of phagocytosis. Some studies have indicated that the phagocytic ability of plasmatocytes is only observed when granulocytes are also available – haemocytes which have been separated into monocellular populations are unable to perform phagocytosis, but their activity is recovered if plasmatocytes and granulocytes are recombined[631]. This supports the theory that plasmatocytes are drawn to the location of granulocytes in the early stages of nodulation[312,318,620].

As the innate immune system predominantly responds to stimuli with a reasonably small arsenal of responses, the efficacy of phagocytosis by haemocytes could be assumed to be reasonably non-variable. However, rates of phagocytosis can be altered by a range of compounds, both increasing[632] and decreasing[633] phagocytosis. Experiments which look at the changes in phagocytosis when an infection is combined with treatment are not common,

5.3 Labelling bacteria to assess phagocytosis

Assessing the progression of phagocytosis by labelling the pathogen and following the infection using microscopy of haemocytes is a popular use for *G. mellonella*.

5.3.1 FITC fluorescent labelling

The use of fluorescein isothiocyanate (FITC) to label proteins, antibodies and cells is very common for experiments in flow cytometry, ELISA assays, Western blotting and other detection experiments. Although it will eventually photobleach if overexposed, FITC is very stable, highly absorbent, water soluble and fluoresces brightly. In this context, FITC binds covalently to membrane proteins[634], and FITC staining has been shown to work with Mycobacteria[635,636].

The disadvantage of the protocol used in this thesis is that the bacteria are heat-killed during the staining protocol. Experiments have been performed using viable FITC stained Mycobacteria[636], but this paper also showed that the rates of phagocytosis for murine macrophages were very similar between viable and non-viable Mycobacteria.

5.3.2 Kinyoun staining

Kinyoun is a modified version of the more common Ziehl-Neelsen stain and is more commonly used with fixed and sectioned samples. Fixing and staining sections of *G. mellonella* is a protocol which has been used by a number of groups[290,391,616], and Ziehl-Neelsen stains have been used by a group assessing *M. abscessus* infections in *G. mellonella*[380] and a group using *M. bovis* in *G. mellonella*[378].

Staining of fixed sections can be a very helpful protocol to observe the interaction between Mycobacteria and haemocytes. Staining of haemocytes in the haemolymph is less common, but has been shown in early papers[262].

5.3.3 Fluorescein diacetate and propidium iodide staining

There are several variations of fluorescein diacetate and propidium iodide staining used by researchers as a live/dead assay. These include propidium iodide in combination with acridine orange[624], with Hoechst 33342[637], with Syto 9[638], and with FITC-Annexin V[639], all of which have been used with *G. mellonella.* However, the experiments need to be designed carefully to ensure that the researcher is either examining the viability of haemocytes, or the viability of the pathogens phagocytosed by the bacteria. Live/dead assays can be designed using target-specific antibodies or bioluminescent pathogens, but these may not always be economically or experimentally feasible.

5.4 Dexamethasone

5.4.1 What is dexamethasone?

Dexamethasone (DEX) is a corticosteroid which can be ingested, injected intramuscularly, or administered intravenously. It has a wide variety of applications in healthcare and veterinary science and is considered particularly long lasting after dosing. It has uses as an anti-inflammatory and immunosuppressant, so is often used to treat inflammatory and autoimmune diseases such as arthritis[640], cancers[641], and cerebral and pulmonary oedema associated with high-altitude mountain climbing[642].

DEX is a synthetic glucocorticoid, meaning it has a higher affinity, greater bioavailability, and less effective metabolism than endogenous corticosteroids such as cortisol[397]. This allows DEX to remain in circulation for several days, unlike endogenous corticosteroids which are released in circadian patterns and do not remain at a steady concentration for long periods of time[397].

Human models which utilise synthetic steroids to assess inflammation and immunosuppression have historically used prednisolone and methylprednisolone[397] although hydrocortisone has also been used[643]. DEX is more commonly used in animal studies and *in vitro*, however DEX and prednisolone should not be considered interchangeable – multiple reports have shown that *in vitro* and *in vivo* responses to DEX and prednisolone can vary[644–646].

5.4.2 Use as an immunosuppressant in humans and animal models

Human patients who require DEX as an immunosuppressant usually have a pre-existing condition, such as cancer or arthritis, which require modulation of their immune response in tangent with their treatment for the disease. For example, patients with brain cancer have been given corticosteroids to prevent cerebral oedema since the early 1950s[647] which was replaced

with DEX after its synthesis in 1958[648,649]. DEX is superior to other corticosteroids in this purpose due to its reduced rates of sodium and water retention[648].

Corticosteroids, and DEX in particular, are also key medications in preventing immune-related adverse events (irAEs) that could occur during immunotherapy[649], including during hyper- or-hypothyroidism, gastrointestinal irAEs[650], and hepatotoxicity related to immunotherapy[651].

In terms of DEX and animal models, DEX is a common corticoid steroid used with models including mice[649], rats[652], rabbits[653,654] and monkeys[654,655]. It has a well-recognised use in veterinary medicine and is regularly prescribed to pets and larger animals such as horses[656]. DEX has been shown to decrease survival rates in mice infected with *M. fortuitum,* and potentially allows for dormant bacteria to become an active infection[127]*.* It has also been used to create a model for latent *M. tuberculosis* infections in rabbits[657].

5.4.3 Dexamethasone and *Galleria mellonella*

Dexamethasone is well established as an immune suppressant in human and animals. It has been shown to inhibit phagocytosis by *G. mellonella* haemocytes *in vitro*, and haemocyte spreading[229] with similar results for a wide range of other insects[658], including a reduced clearance rate of bacteria and increased mortality.

Dexamethasone acts as a phospholipase A_2 inhibitor - phospholipase A_2 catalyses the hydrolysis of membrane glycerophospholipid fatty acids to release arachidonic acid, which is a precursor to eicosanoids[659]. Eicosanoids have been linked to the function and regulation of the insect immune system for a variety of stimuli, including bacteria, fungi, parasites and viruses[660]. Injecting DEX with arachidonic acid also produces survival responses which are comparable to non-immunosuppressed larvae[229,660].

As the immune system of *G. mellonella* is suppressed when exposed to DEX, it is unsurprising that larval survival is reduced when challenged by a pathogen[229,660–662]. Previous work from our lab has indicated that DEX can be injected in a water-soluble form at 200 μg per larvae with no effects on melanisation or lethality[662]. Other groups have used DEX dissolved in ethanol[229]. DEX was also shown to reduce the phagocytic rate of haemocytes exposed to FITC labelled *E. coli,* increase the ability for *E. coli* and *K. pneumoniae* bacteria to cause larval death, and reduced the efficacy of otherwise effective antibiotic doses[662]. However, there is little else information on *G. mellonella* and DEX used in combination.

5.5 Results

5.5.1 The number of circulating haemocytes changes when the larvae are injected with antibiotics or bacteria

5.5.1.1 Saline does not increase the number of circulating haemocytes, but antibiotics do

Two controls were used in these experiments – larvae which were not injected at all ("unmanipulated" on **Figure 5.2**) and larvae which were injected with phosphate buffered saline ("PBS" on **Figure 5.2**). **Figure 5.2** shows the results in comparison to the PBS controls.

There was no statistically significant difference between the haemocyte numbers in the unmanipulated and PBS controls. However, injections of antibiotics did increase the number of circulating haemocytes above a background level.

AMK was selected for experimentation as it was generally very effective at improving larval survival (**Chapter Three**) – a high dose (50 mg/kg) was effective at recuing larval survival. Lower doses (5 mg/kg and 2.5 mg/kg) were less effective at improving survival, so were also examined here.

EMB was included as all Mycobacteria assessed in **Chapter Three** showed no improvements in survival after injections with EMB. A dose of 50 mg/kg was selected to be comparable to the highest AMK dose used here.

Three different doses of amikacin (AMK) were tested – only the highest dose (50 mg/kg) gave a statistically significant increase in the number of haemocytes in the 5 hours post-injection. The lower doses (5 and 2.5 mg/kg) also gave an increase in haemocyte numbers in this period, but not sufficiently to be significant. A single dose of ethambutol (EMB, 50 mg/kg)

was tested, which also gave a statistically significant increase in haemocyte numbers within 5 hours post-injection. For both antibiotics, the number of haemocytes was returned to the baseline, or close to baseline values, by the 24 hour time-point post-injection regardless of the dose given. Hence, the increase in haemocyte numbers triggered by injections with antibiotics was transient and seemingly scaled to the dose of antibiotic.

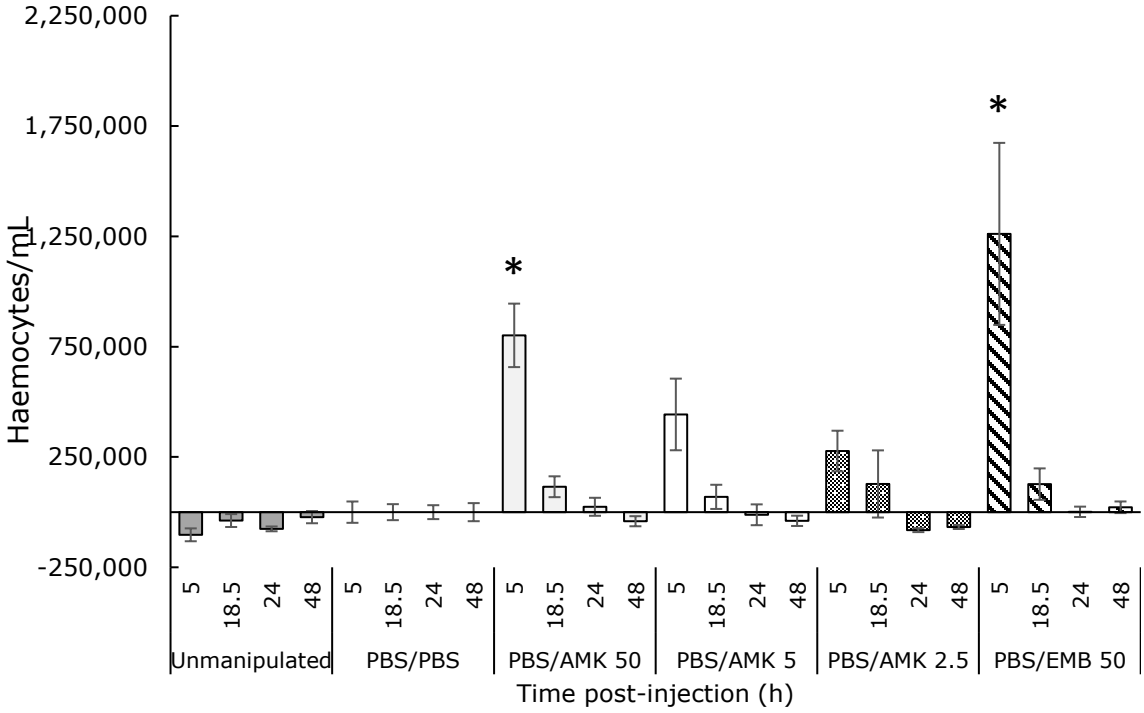

Figure 5.2 – Injecting larvae with antibiotics causes a transient increase in the number of circulating haemocytes in comparison to larvae injected with PBS. Larvae were injected with PBS as a mock-infection, then with PBS, AMK or EMB 2 hours later. The numbers of haemocytes in the larval haemolymph were then counted at 5, 18.5, 24 and 48 hours after the initial mock-infection with PBS. The number of haemocytes is presented relative to larvae mock-infected and mock-treated with PBS. The data are from nine larvae per timepoint, showing the mean ± SEM. $p<0.05$, unpaired, two-tailed Student's *t*-test with Holm's correction applied for multiple comparisons. AMK, amikacin; EMB, ethambutol; PBS, phosphate buffered saline.

5.5.1.2 Mycobacteria do not increase the number of circulating haemocytes in the hours post-injection but the number does increase when they are treated with antibiotics.

As with **Figure 5.2**, unmanipulated and PBS controls were both used in these experiments, and the data was normalised to the PBS control group (see **Figure 5.3**).

Infection with *M. fortuitum* NCTC 10394 or *M. marinum* Strain M does not increase the number of circulating haemocytes to a significant level. The number of haemocytes is consistently above the baseline for larvae infected with *M. marinum* Strain M, but this is not seen with *M. fortuitum* NCTC 10394.

Introducing antibiotics to treat the infected larvae shows a similar result as when the larvae were injected with antibiotics without infection (**Figure 5.2**) – there is a significant increase in the number of circulating haemocytes, particularly in the first few hours post-injection. However, in contrast to when the antibiotics were given alone, the increase in circulating haemocytes remains above the baseline for the remainder of the experiment. This increase is not significant, but is seen for both Mycobacteria used here, and for both AMK and EMB at all dose levels.

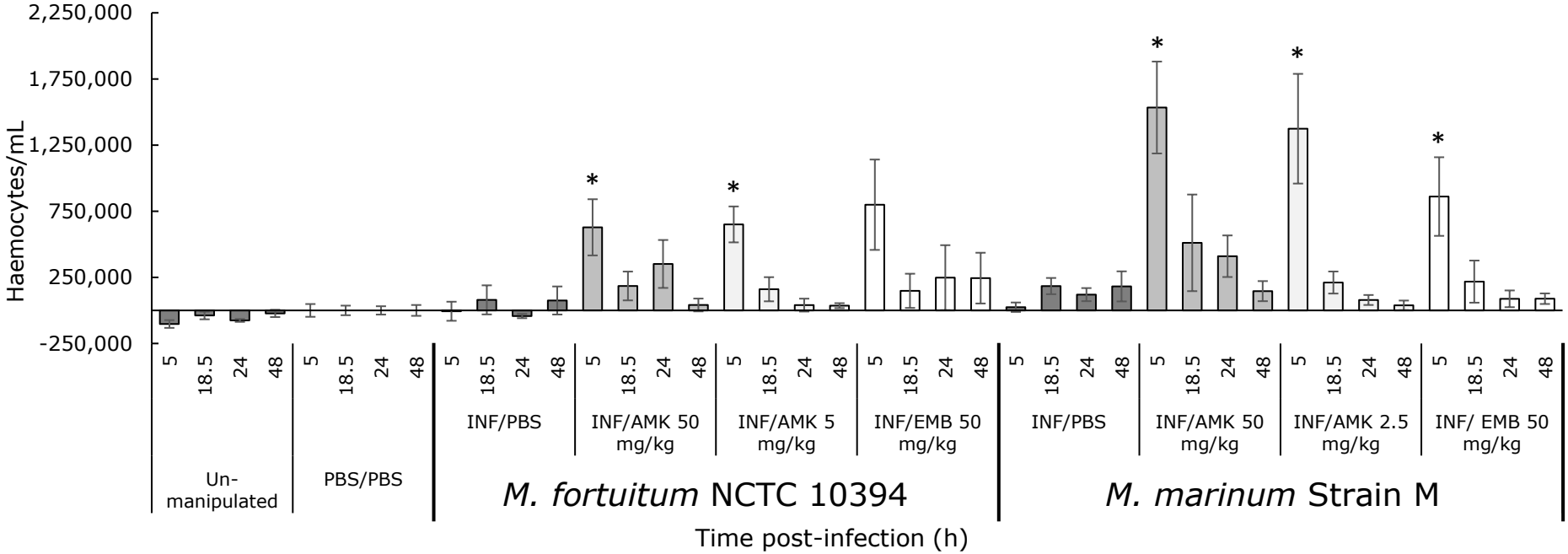

Figure 5.3 – Injecting larvae with bacteria does not increase the number of circulating haemocytes in comparison to larvae injected with PBS, but combining injections of bacteria with antibiotic treatments does increase the number of circulating haemocytes. Control larvae were injected with PBS as a mock-infection, then with PBS 2 hours later. The experimental larvae were infected with 1 x 10^7 c.f.u/mL of *M. fortuitum* NCTC 10394 or *M. marinum* Strain M, then injected 2 hours later with PBS or with an efficacious dose of AMK (50 mg/kg), a non-efficacious dose of AMK (5 or 2.5 mg/kg), or EMB (50 mg/kg). The numbers of haemocytes in the larval haemolymph were then counted at 5, 18.5, 24 and 48 hours after the initial mock-infection with PBS. The number of haemocytes is presented relative to larvae mock-infected and mock-treated with PBS. The data are from nine larvae per timepoint, showing the mean ± SEM. $p<0.05$, unpaired, two-tailed Student's *t*-test with Holm's correction applied for multiple comparisons. AMK, amikacin; EMB, ethambutol; PBS, phosphate buffered saline.

5.5.1.3 The number of circulating haemocytes increases when combinations of antibiotics are injected, and also when combinations are injected after infection with *M. fortuitum* and *M. marinum.*

Larvae were injected with combined treatments of antibiotics on their own (as in **Section 5.5.1.1**) and also after infection with bacteria (as in **Section 5.5.1.2**). The results are shown in **Figure 5.4**.

As with the single dose of antibiotics, injection with a combined dose of antibiotics causes a transient but significant increase in the number of circulating haemocytes. This increase is also seen when the antibiotics are injected after infections with *M. fortuitum* NCTC 10394 and *M. marinum* Strain M.

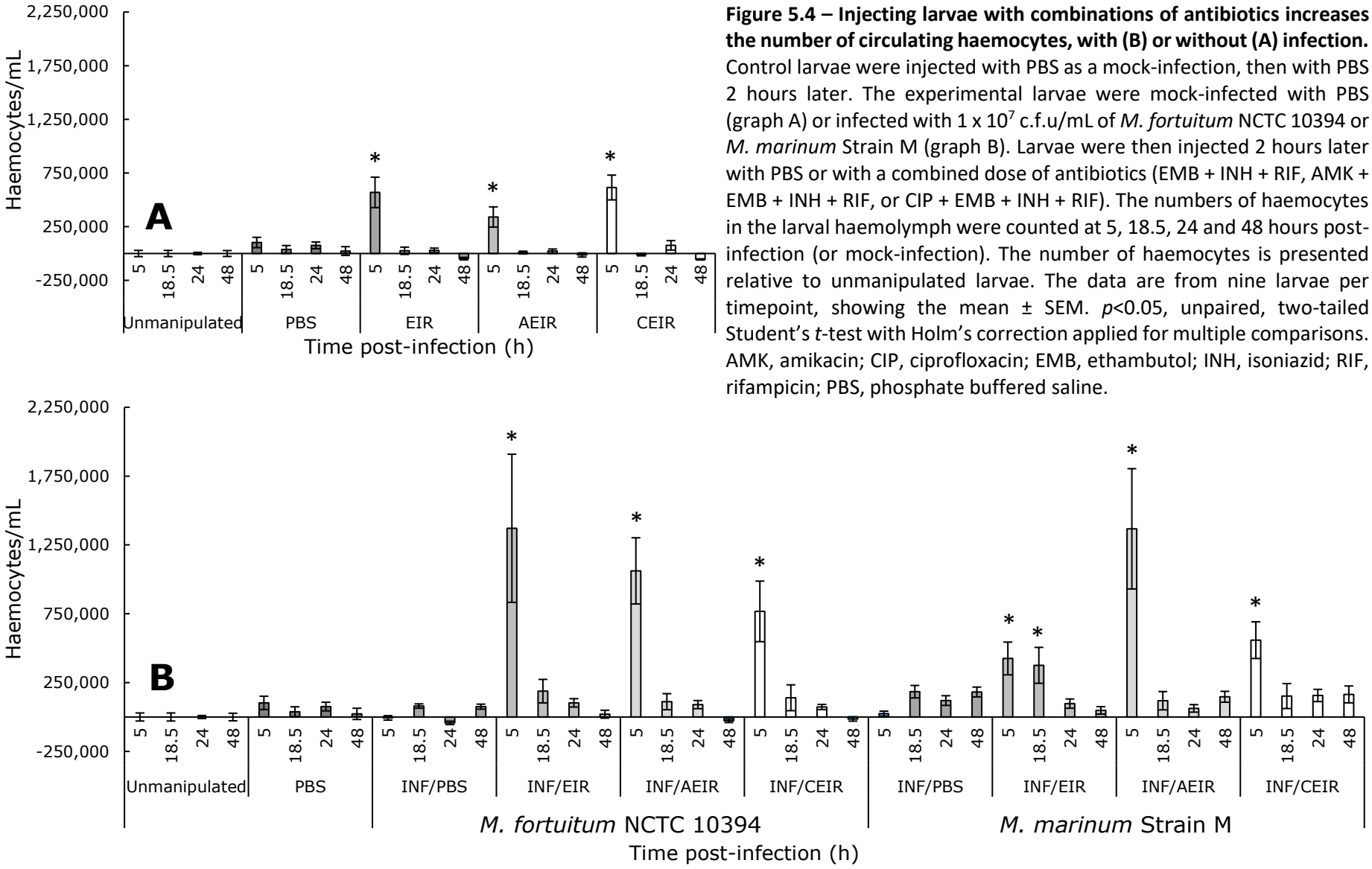

Figure 5.4 – Injecting larvae with combinations of antibiotics increases the number of circulating haemocytes, with (B) or without (A) infection. Control larvae were injected with PBS as a mock-infection, then with PBS 2 hours later. The experimental larvae were mock-infected with PBS (graph A) or infected with 1 x 10^7 c.f.u/mL of *M. fortuitum* NCTC 10394 or *M. marinum* Strain M (graph B). Larvae were then injected 2 hours later with PBS or with a combined dose of antibiotics (EMB + INH + RIF, AMK + EMB + INH + RIF, or CIP + EMB + INH + RIF). The numbers of haemocytes in the larval haemolymph were counted at 5, 18.5, 24 and 48 hours post-infection (or mock-infection). The number of haemocytes is presented relative to unmanipulated larvae. The data are from nine larvae per timepoint, showing the mean ± SEM. $p<0.05$, unpaired, two-tailed Student's *t*-test with Holm's correction applied for multiple comparisons. AMK, amikacin; CIP, ciprofloxacin; EMB, ethambutol; INH, isoniazid; RIF, rifampicin; PBS, phosphate buffered saline.

5.5.2 Both viable and heat-killed bacteria increase the numbers of nodules in the larval body

Unmanipulated and PBS controls were included for these experiments. All larvae were selected with the same criteria as in other experiments – cream, no evidence of melanisation, active and responsive to movement – yet both control groups contained nodules (see **Table 5.1**). The majority of these were the smallest type of single, "full stop" nodule, but there were a small number of small and large clumps of nodule. The values for the PBS group were set as background values and subtracted from the experimental groups. Larvae were assessed at 1, 2, 4 and 6 hours post-injection/infection. To see representative images of each type of nodule, please see **Figure 7.2**.

In terms of the larvae which were injected with bacteria, *M. fortuitum* NCTC 10394, *M. fortuitum* NCTC 8573, and *M. marinum* Strain M all had significant increases in nodulation (**Table 5.1**) and melanised "webs" of small, indiscriminate nodules (**Table 5.2**). There were also increases in these groups when the larvae were injected with heat-killed bacteria.

There was also an increase in the percentage of larvae with very light nodulation for the larvae infected with *M. marinum* R356933F (**Table 5.2**). However, this was the only group with a statically significant change from the PBS group, and the larvae injected with heat-killed *M. marinum* R356933F had no significant changes from the control group.

Table 5.1 – Viable and heat-killed bacteria are both capable of increasing the number of discrete, single "full stop" nodules and clumps of nodules in *G. mellonella* larvae. For each experimental condition, 35 larvae were prepared. Bacteria were injected at 1 x 10^7 c.f.u/mL. Five larvae were randomly selected for dissection at 1, 2, 4, and 6 hours post-injection. The experiments were repeated four times using larvae from different orders, meaning each timepoint has n = 20 larvae. Cells highlighted in yellow were statistically increased compared to the PBS uninfected controls (p<0.05, unpaired, two-tailed Student's t-test).

Conditions	Average # "full stop" nodules per larvae				Average # small clumps of nodules per larvae				Average # large clumps of nodules per larvae			
Unincubated, unmanipulated	11				2.555555556				1.125			
	Time post-injection											
	1	2	4	6	1	2	4	6	1	2	4	6
Uninjected, unmanipulated control	9.25	13.9	18.8	20.6	3.77	2.71	3.76	4.4	1.4	1	1.17	2.33
PBS uninfected control	12.8	15.4	20.3	13.6	3.57	3.63	4.27	4.42	1.75	1.56	1	1
***M. fortuitum* NCTC 8573**	25	15.3			7.88	6	11	10	2.83	2.6	2	
Heat-killed *M. fortuitum* NCTC 8573	21.5		50		13	3	14.8	7	2	1.5	1	1
***M. fortuitum* NCTC 10394**	26.2	31.1	20.2	22.6	8.94	6.89	8	6.6	1.43	2	1	1.6
Heat-killed *M. fortuitum* NCTC 10394	17.8	20.8	25.5	20.2	6.35	7.67	9.83	7.06	1.6	1	1.33	2
***M. marinum* Strain M**	33.6	17.1	21.6	26.3	7.47	5.44	6.88	11	1	2.33	1.13	1
Heat-killed *M. marinum* Strain M	15.8	15.1	16.6	14.4	4.3	9.06	4.8	5	1.38	2.25	1.78	2.33
***M. marinum* R356933F**	15.9	21	18.9	22.9	5.41	4.92	5.06	6.35	2	1.29	1	1
Heat-killed *M. marinum* R356933F	23.2	23.2	15.6	14.8	4.71	5.27	4.54	4	1.4	1	1.5	1.25

Table 5.2 – Viable and heat-killed bacteria are both capable of increasing the amount of melanised webbing and the percentages of larvae with nodulation. For each experimental condition, 35 larvae were prepared. Bacteria were injected at 1 x 10^7 c.f.u/mL. Five larvae were randomly selected for dissection at 1, 2, 4, and 6 hours post-injection. The experiments were repeated four times using larvae from different orders, meaning each timepoint has *n* = 20 larvae. The colour of the squares indicates how close the percentages in the cell are to 100% - cells with underlined percentages were were statistically increased compared to the PBS uninfected controls (*z*-score for two population proportions). AMK, amikacin.

Conditions	Percentage of larvae with light webbing				Percentage of larvae with heavy webbing				Percentage of larvae with very light nodulation				Percentage of larvae with light nodulation				Percentage of larvae with medium nodulation				Percentage of larvae with heavy nodulation			
Unincubated, unmanipulated	0%				0%				0%				0%				0%				0%			
	Time post-injection																							
	1	2	4	6	1	2	4	6	1	2	4	6	1	2	4	6	1	2	4	6	1	2	4	6
Uninjected, unmanipulated control	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	5%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
PBS uninfected control	0%	0%	0%	0%	0%	0%	0%	0%	5%	0%	0%	0%	0%	0%	0%	0%	0%	0%	5%	0%	0%	0%	0%	0%
M. fortuitum NCTC 8573	50%	25%	35%	30%	15%	25%	40%	50%	15%	20%	0%	0%	45%	35%	30%	10%	25%	30%	35%	40%	5%	15%	35%	40%
Heat-killed *M. fortuitum* NCTC 8573	55%	50%	35%	45%	30%	0%	15%	15%	5%	10%	0%	10%	40%	45%	50%	10%	40%	20%	45%	55%	10%	20%	5%	25%
M. fortuitum NCTC 10394	0%	0%	0%	0%	0%	0%	0%	0%	30%	40%	50%	45%	15%	20%	5%	20%	0%	0%	5%	0%	0%	0%	0%	0%
Heat-killed *M. fortuitum* NCTC 10394	0%	0%	0%	0%	0%	0%	0%	0%	45%	35%	30%	25%	5%	10%	10%	15%	0%	0%	5%	15%	0%	0%	0%	0%
M. marinum Strain M	0%	0%	5%	5%	0%	0%	0%	0%	35%	40%	40%	40%	10%	10%	25%	50%	0%	5%	10%	5%	0%	0%	0%	0%
Heat-killed *M. marinum* Strain M	0%	0%	5%	5%	0%	0%	0%	0%	35%	40%	35%	30%	10%	25%	10%	25%	0%	0%	0%	15%	0%	0%	0%	0%
M. marinum R356933F	0%	0%	0%	0%	0%	0%	0%	0%	10%	25%	20%	10%	5%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Heat-killed *M. marinum* R356933F	0%	0%	0%	0%	0%	0%	0%	0%	10%	0%	15%	10%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

Table 5.3 – Antibiotics decrease the number of discrete, single "full stop" nodules and clumps of nodules in *G. mellonella* larvae in comparison to infected but untreated controls. For each experimental condition, 35 larvae were prepared. Bacteria were injected at 1 x 10^7 c.f.u/mL. AMK at 50 mg/kg was injected either at the same time as the bacteria, or 30 minutes later. Five larvae were randomly selected for dissection at 1, 2, 4, and 6 hours post-injection. The experiments were repeated four times using larvae from different orders, meaning each timepoint has n = 20 larvae. Cells highlighted in yellow were statistically increased compared to the infected but untreated controls, cells in blue were statistically decreased (p<0.05, unpaired, two-tailed Student's t-test). AMK, amikacin.

Conditions	Average # "full stop" nodules per larvae				Average # small clumps of nodules per larvae				Average # large clumps of nodules per larvae			
	Time post-injection											
	1	2	4	6	1	2	4	6	1	2	4	6
AMK uninfected control	25.9	13.2	21.1	17.2	4.85	5.2	6.5	3.47	1.33	1	1	1
M. fortuitum NCTC 10394 w/AMK	12	17.4	19.2	16.9	4.88	5.5	6.75	7.79	1	2	1	1.6
M. fortuitum NCTC 10394 w/AMK 30m post-inf	28.4	32.4	21.6	21.7	6.83	3.46	6.11	6.92	1.8	1	1	1.6
M. fortuitum NCTC 8573 w/AMK	23.2	30.8	18.6	16.7	3.5	9.58	6.67	7.22	1.33	2	1.2	1.33
M. fortuitum NCTC 8573 w/AMK 30m post-inf	23.5	43.9	19.6	25.1	5.27	10.2	11.5	7.2	1.6	4	1	1.4
***M. marinum* Strain M w/AMK**	18	26.9	21.9	15.9	5.82	5.91	5.31	7.15	1	1	1.5	1.33
***M. marinum* Strain M w/AMK 30m post-inf**	17.8	15.1	15.3	12.8	4.2	5.55	5.31	5.43	1.5	2.25	1.67	1.5
***M. marinum* R356933Fw/AMK**	42.2	28.7	22.7	33.3	4.86	8.08	6.71	6.83	1.33	3.5	1	1
***M. marinum* R356933Fw/AMK 30m post-inf**	12.8	15.4	27.6	17.9	4.94	4.62	9.93	5.5	1.25	1	1.33	1.25

Table 5.4 – Antibiotics decrease the percentages of larvae with nodulation and melanised webbing in *G. mellonella* larvae in comparison to infected but untreated controls. For each experimental condition, 35 larvae were prepared. Bacteria were injected at 1 x 10^7 c.f.u/mL. AMK at 50 mg/kg was injected either at the same time as the bacteria, or 30 minutes later. Five larvae were randomly selected for dissection at 1, 2, 4, and 6 hours post-injection. The experiments were repeated four times using larvae from different orders, meaning each timepoint has *n* = 20 larvae. The colour of the squares indicates how close the percentages in the cell are to 100% - cells with underlined percentages were were statistically decreased compared to the infected but untreated controls (*z*-score for two population proportions). AMK, amikacin.

Conditions	Percentage of larvae with light webbing				Percentage of larvae with heavy webbing				Percentage of larvae with very light nodulation				Percentage of larvae with light nodulation				Percentage of larvae with medium nodulation				Percentage of larvae with heavy nodulation			
	Time post-injection																							
	1	2	4	6	1	2	4	6	1	2	4	6	1	2	4	6	1	2	4	6	1	2	4	6
AMK uninfected control	0%	0%	0%	0%	0%	0%	0%	0%	0%	10%	25%	15%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
M. fortuitum NCTC 10394 w/AMK	0%	0%	0%	0%	0%	0%	0%	0%	30%	30%	**20%**	**5%**	10%	5%	10%	20%	0%	0%	0%	5%	0%	0%	0%	0%
M. fortuitum NCTC 10394 w/AMK 30m post-inf	0%	0%	0%	0%	0%	0%	0%	0%	20%	30%	30%	**15%**	5%	5%	10%	20%	0%	0%	0%	5%	0%	0%	0%	0%
M. fortuitum NCTC 8573 w/AMK	25%	20%	**5%**	**5%**	0%	5%	**0%**	**5%**	10%	15%	**25%**	10%	20%	40%	30%	**50%**	10%	10%	25%	**10%**	0%	5%	**5%**	**5%**
M. fortuitum NCTC 8573 w/AMK 30m post-inf	**20%**	30%	15%	**5%**	0%	0%	0%	0%	15%	20%	**30%**	**25%**	25%	40%	25%	35%	10%	20%	15%	20%	10%	10%	15%	**0%**
***M. marinum* Strain M w/AMK**	0%	5%	0%	0%	0%	0%	0%	0%	**0%**	**10%**	20%	25%	0%	5%	**0%**	**0%**	0%	0%	0%	0%	0%	0%	0%	0%
***M. marinum* Strain M w/AMK 30m post-inf**	0%	0%	0%	0%	0%	0%	0%	0%	15%	20%	15%	15%	0%	10%	5%	**0%**	0%	5%	0%	0%	0%	0%	0%	0%
***M. marinum* R356933Fw/AMK**	0%	0%	0%	0%	0%	0%	0%	0%	0%	**0%**	10%	25%	0%	0%	0%	10%	0%	0%	0%	0%	0%	0%	0%	0%
***M. marinum* R356933Fw/AMK 30m post-inf**	0%	0%	0%	0%	0%	0%	0%	0%	0%	10%	40%	10%	0%	0%	0%	5%	0%	0%	0%	5%	0%	0%	0%	0%

5.5.2.1 Antibiotics can decrease the amount of nodulation in the larval body

When the larvae are injected with bacteria and treated with a dose of 50 mg/kg AMK there were significant decreases in nodulation and webbing (**Table 5.3** and **5.4**). This was seen when the antibiotic was injected at the same time as the bacteria, and when the antibiotic was administered 30 minutes post-infection. The bacteria which saw the greatest decrease in nodulation and webbing was *M. fortuitum* NCTC 85573 (**Table 5.4**) – this aligned with the reduction in melanisation in the antibiotic experiments described in **Chapter Three** (observed reduction in melanisation for larvae treated with effective antibiotic doses, data not shown).

5.5.3 Haemocytes can phagocytose Mycobacteria, although some staining protocols are more suitable for *G. mellonella* than others

Three different experimental techniques were employed to assess the interaction between the insect haemocytes and the Mycobacteria. Kinyoun staining is an adaptation of the acid-fast staining method developed by Robert Koch (see **Section 1.1.2**). FITC staining is a popular fluorescence-based stain but uses heat-killed bacteria. FDA/PI staining uses live bacteria.

5.5.3.1 Kinyoun staining is not well-suited to analysis of *G. mellonella* haemolymph

The results of a Kinyoun stain should show acid-fast organisms (Mycobacteria) as red, and non-acid-fast organisms will be blue. As shown in **Figure 5.5**, the Kinyoun staining procedure stained the bacteria and haemocytes as expected, but there were issues with preparing slides of a sufficient quality to observe the haemocytes and any internalised bacteria clearly.

Larval haemolymph melanises rapidly once it comes into contact with the air, and also begins to clot. Hence, air-drying the slides only creates a layer of black, sticky haemolymph which is poorly adhered to the glass and often cracks if over-dried – much like egg whites – because haemolymph has a high protein content.

Similar to egg whites, the haemolymph also cooks onto the slide during heat-fixing. This creates a thick, white, opaque layer which does not react to the staining and is not cooperative with light microscopy.

Hence, diluting the haemolymph with *Galleria* saline and phenylthiourea (PTU) was hoped to overcome the issues with melanisation, high-protein content, and opacity when heating. Experiments were also performed in centrifuging the haemocytes out of the haemolymph and resuspending them in saline with PTU. However, there were still issues with cracking (visible on the background of some of the images in **Figure 5.5**) and not being able to see the haemocytes and bacteria clearly enough to count bacteria inside clearly defined haemocyte cell boundaries. It is likely that this type of staining could be a useful, cheap, rapid tool for assessing the relationship between the haemocytes and Mycobacteria, as is shown with the other microscopy techniques used here, but requires further refinement.

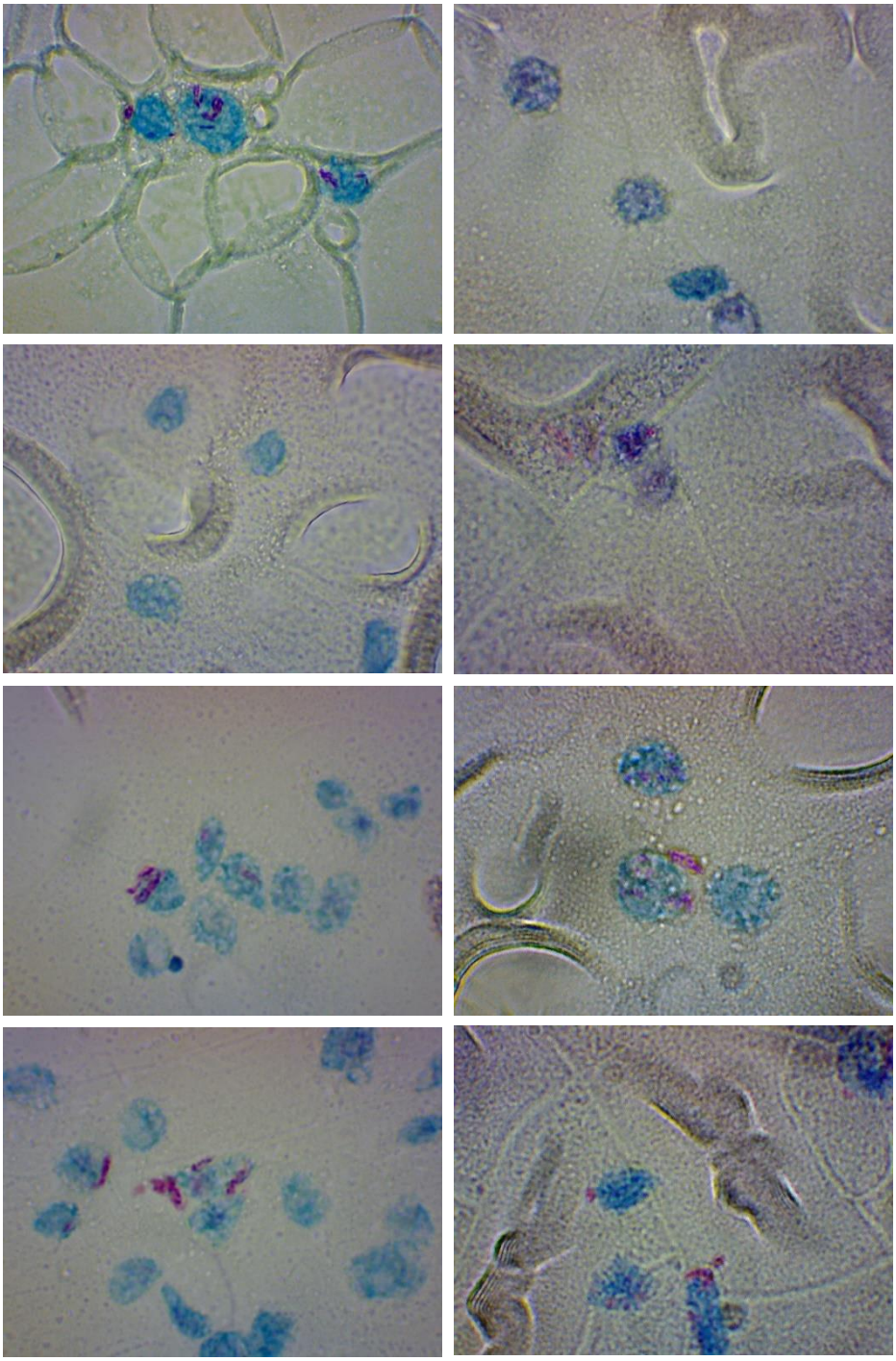

Figure 5.5 – Staining of haemocytes and Mycobacteria using the Kinyoun technique. Haemocytes stain blue and Mycobacteria stain red/pink. Slides are shown from a variety of experiments using both *M. fortuitum* and *M. marinum* bacteria, with dilutions of saline, haemocytes separated from haemolymph by centrifugation and resuspended in saline, and slides fixed by air-drying, heating, and ethanol. The difficulty in observing individual bacteria associated with haemocytes, whether the bacteria are internalised, and the "cloudiness" of the images through the haemolymph is represented in many of the images. The "cracking" of the dried haemolymph is also observed.

5.5.3.2 Successful FITC-staining of Mycobacteria allows analysis of rates of phagocytosis, and how they are affected by treatment with antibiotics

FITC-stained bacteria were readily visible inside haemocytes and moving through the z-plane (from the surface of the haemocyte closest to the lens to the surface closest to the slide, so moving through the haemocyte in three dimensions) allowed for confirmation that they were internalised within the haemocytes, rather than adhered to their surface. Examples of the images assessed to gather the data in **Figure 5.7** are shown in **Figure 5.6**.

Data was collected on the number of haemocytes which contained a phagocytosed bacterium, and the number of bacteria in these haemocytes. The data was then filtered into three analyses:

- Phagocytic rate, or the percentage of total haemocytes which have engulfed one or more bacterium
- Phagocytic index, or the average number of engulfed bacteria for all haemocytes, including ones which contain no bacteria
- Adjusted phagocytic index, or the average number of engulfed bacteria in the haemocytes which contain one or more bacterium

The results of these analyses are shown in **Figure 5.7**. The phagocytic rate shows that injecting a larger number of bacteria leads to a higher percentage of haemocytes containing one or more bacterium. *Mycobacterium fortuitum* NCTC 10394 showing a phagocytic rate of around 40% after 4 x 10^8 bacterial cells were injected, but *M. marinum* Strain M having a rate of around 20% after 3 x 10^7 bacterial cells were injected (**Figure 5.7 A**).

For both bacteria, injection with an effective dose of AMK (50 mg/kg) significantly reduced the percentage of haemocytes which contained one or more bacterium compared to the PBS controls (**Figure 5.7 B**). However, the response to the non-efficacious dose of AMK and the non-effective antibiotic (EMB) differed between the bacteria. The phagocytic

rate for these two experimental groups did not differ statistically from the control group when the larvae were injected with *M. fortuitum* NCTC 10394. The phagocytic rates for larvae injected with *M. marinum* Strain M were significantly lower than the rate for the control groups for both the low AMK dose and the EMB dose.

The phagocytic index values show a similar pattern for *M. marinum* – with both AMK doses and the EMB dose, a significant reduction compared to the control group was observed (**Figure 5.7 B**). This means that the there are fewer haemocytes which contain one or more bacterium, and the number of bacteria in each haemocyte is also reduced. However, the data for *M. fortuitum* is very different – the average number of bacteria in the haemocytes increases when AMK or EMB are injected, although this increase is only statistically significant for the higher AMK dose.

However, as the phagocytic index takes into account *all* of the haemocytes, including the ones which do not contain a bacterium, this data gives an inaccurate representation of the efficacy of the phagocytic haemocytes and will be affected by the number of haemocytes overall. **Section 5.5.1** shows that introducing antibiotics to the larval body will increase the number of circulating haemocytes, which will tangentially decrease the standard phagocytic index.

Only looking at the phagocytic haemocytes with an adjusted phagocytic index (**Figure 5.7 C**) gives a very different result to the phagocytic index. The haemocytes which phagocytosed *M. fortuitum* phagocytosed a larger number of bacteria when injected with AMK or EMB, although only the low AMK and EMB results were statistically significant. In contrast, the haemocytes which phagocytosed *M. marinum* Strain M had almost identical numbers of internalised bacteria between the high AMK, EMB and control group. However, the haemocytes from the larvae injected with a high dose of AMK phagocytosed significantly larger numbers of bacteria.

This means that antibiotics can reduce the number of haemocytes which contain one or more bacterium, but the haemocytes which do contain bacteria generally contain the same or more bacteria than the control groups. Hence, the rates of phagocytosis are reduced, but the efficacy of the phagocytosis is increased.

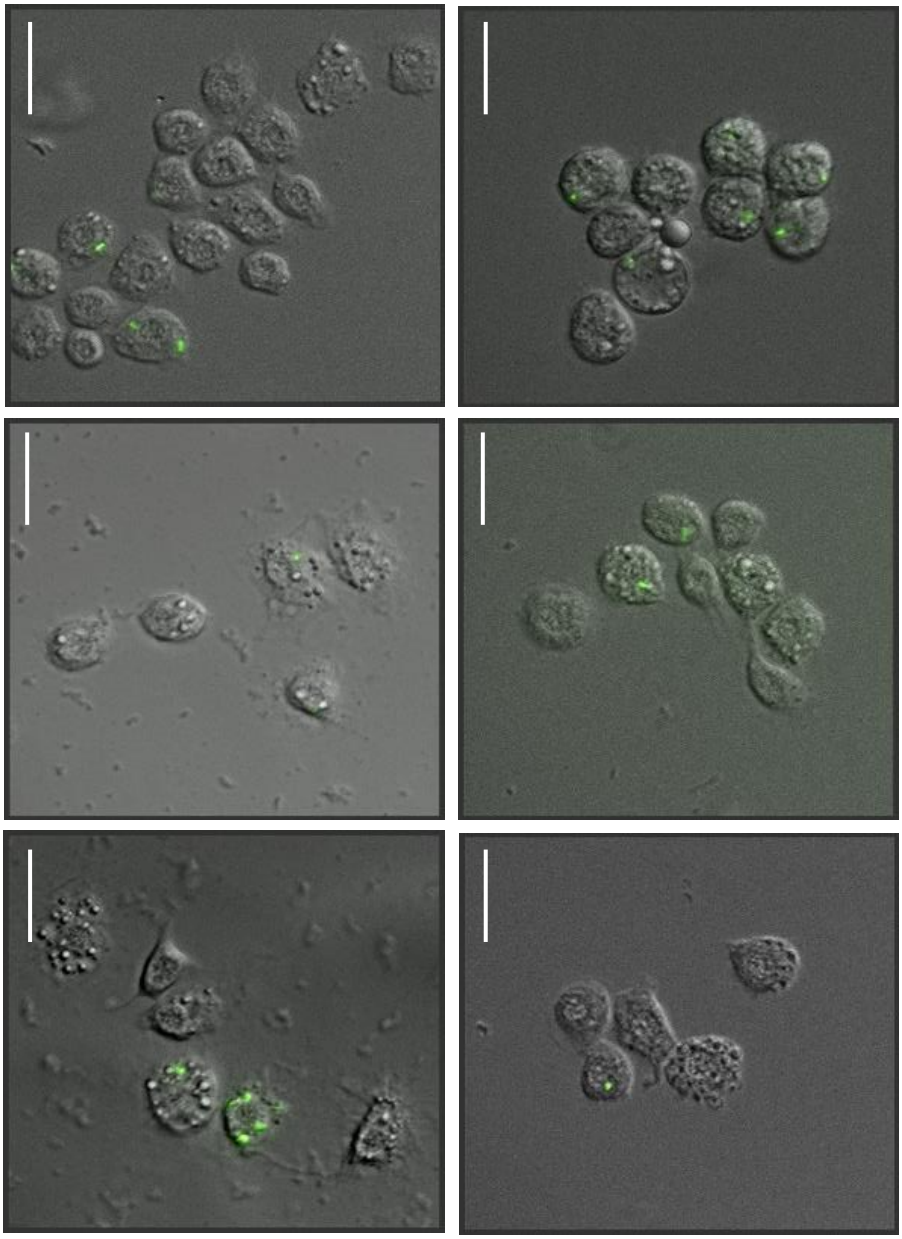

Figure 5.6 – Staining of haemocytes and Mycobacteria using FITC and fluorescence microscopy. Typical slides are shown from a variety of experiments using both *M. fortuitum* and *M. marinum* bacteria, with and without the injection of antibiotics before collection of the haemolymph. Cells were viewed in progressive slices through the z-plane to confirm that bacteria were internalised rather than adherent to the surface of the bacteria. Scale bars of 20 μm are shown on each image.

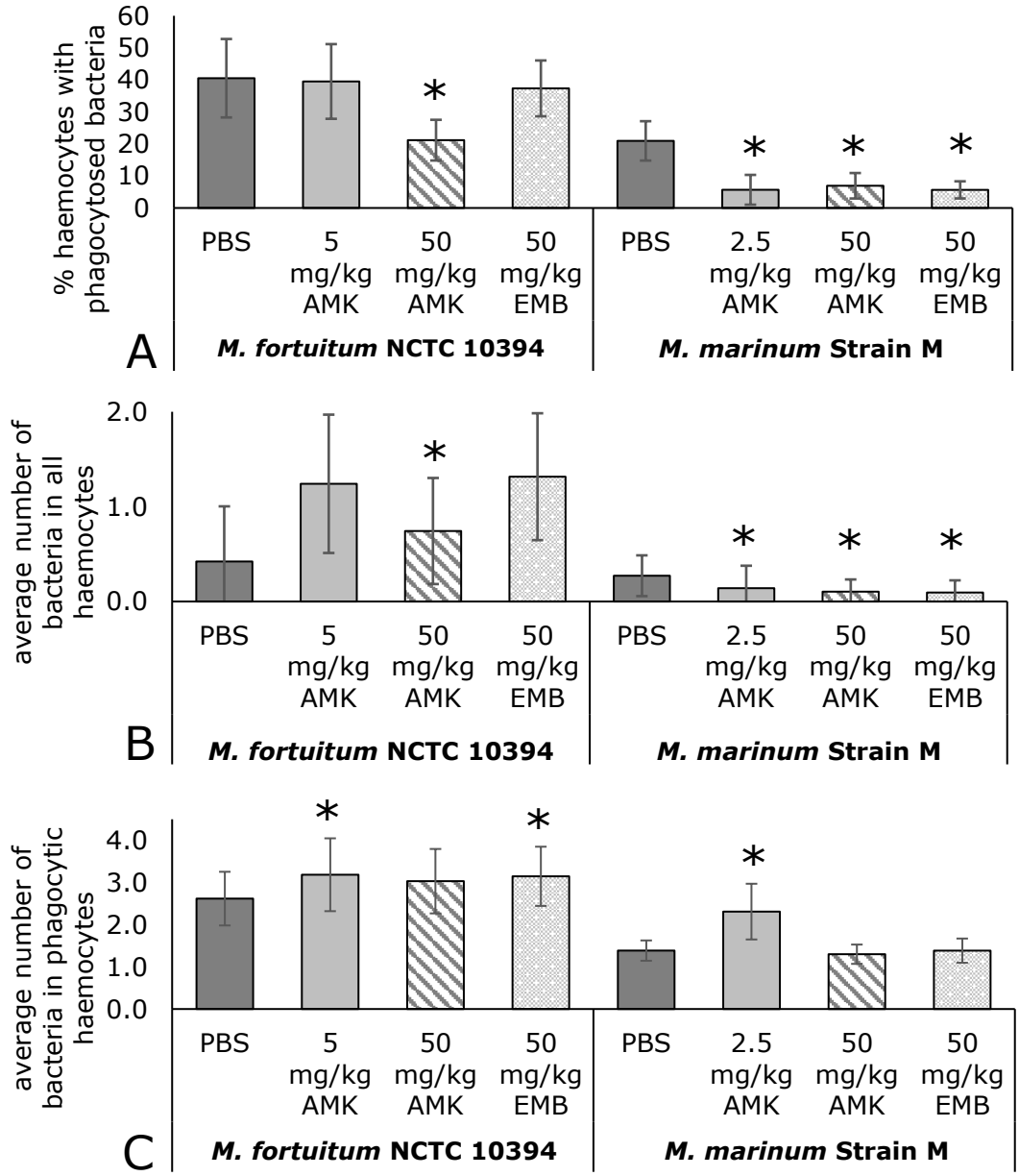

Figure 5.7 – The differences in phagocytic rate (A), phagocytic index (B), and adjusted phagocytic index (C) for larval haemocytes exposed to *M. fortuitum* NCTC 10394 and *M. marinum* Strain M. Larvae were injected with FITC labelled bacteria 10 minutes before injections with an efficacious dose of amikacin (50 mg/kg), a non-efficacious dose of AMK (5.0 or 2.5 mg/kg), a dose of a non-effective antibiotic (50 mg/kg ethambutol), or PBS. The larvae were incubated for 1 hour before collection of haemolymph. Results are from 10 larvae per condition, 30-70 haemocytes examined per larvae, showing the mean ± SEM. Asterix indicate $p<0.05$, unpaired, two-tailed Student's *t*-test with Holm's correction applied for multiple comparisons. AMK, amikacin; EMB, ethambutol; PBS, phosphate buffered saline.

5.5.3.3 Live/dead staining with FDA and PI

The percentages of dead and live Mycobacteria across an hour of exposure to *G. mellonella* haemocytes are shown in **Figure 5.8**. For all bacteria and strains used, the percentage of dead Mycobacteria liberated from lysed haemocytes remained at a low percentage across the experiment. The SEM values of each datapoint were also small. There is no obvious increase in the number of dead bacteria as time progresses for any of the bacteria or strains used here.

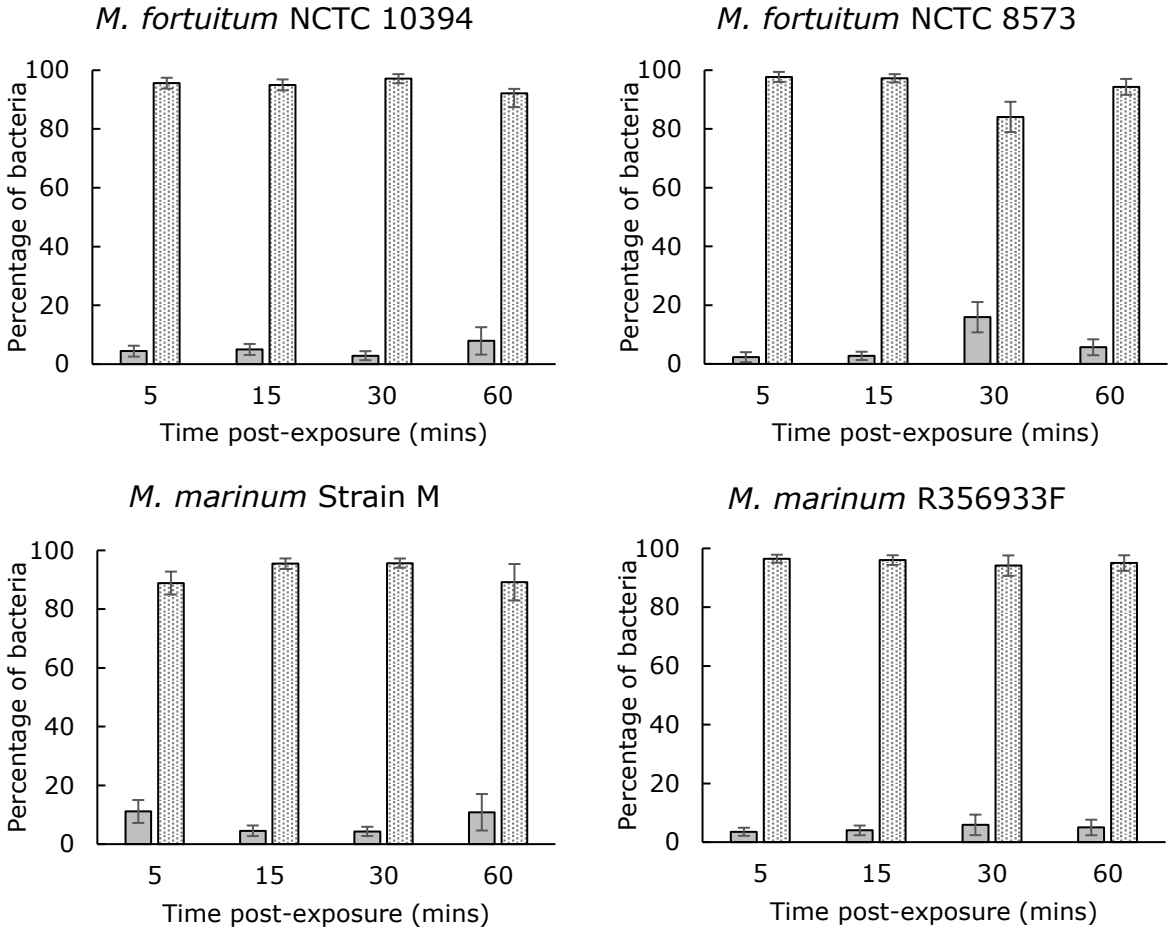

Figure 5.8 – The changes in bacterial survival after phagocytosis by *G. mellonella* haemocytes are minimal over a 6 hour period. Haemocytes were used from haemolymph diluted with *Galleria* saline and PTU. Haemolymph was combined in a 2:1 v/v ratio with bacterial suspensions. *M. fortuitum* NCTC 10394 was used at 4 x 10^8 c.f.u/mL, *M. fortuitum* NCTC 8573 at 6 x 10^8 c.f.u/mL, *M. marinum* Strain M at 3 x 10^7 c.f.u/mL, and *M. marinum* R356933F at 9 x 10^8 c.f.u/mL. Haemocytes were lysed using distilled water then stained with FDA and PI, washed, resuspended in GIM and visualised using fluorescence microscopy. Dead bacteria are solid grey bars, live bacteria are dotted grey and white bars. One slide was prepared per timepoint and for each bacterium, with 10 sections visualised per slide. FDA, fluorescein diacetate; GIM, Grace's insect medium; PI, propidium iodide; PTU, phenylthiourea.

5.5.4 Dexamethasone increases the susceptibility of the larvae to infections with Mycobacteria

The results comparing the survival curves for larvae infected with bacteria and those for larvae infected 10 minutes after an injection with 200 μg of dexamethasone are shown in **Figures 5.9** and **5.10**.

For *M. fortuitum* NCTC 8573, DEX decreased the survival of the larvae over the 144 hours compared to those given PBS (**Figure 5.9 C/D**), especially for 1 x 10^8 and 5 x 10^8 c.f.u/mL. Although the change was not statistically significant, 1 x 10^7 c.f.u/mL also caused more larval death after exposure to DEX.

For *M. fortuitum* NCTC 10394 (**Figure 5.9 A/B**) two of the groups had statistically significant difference between results – 2 x 10^8 and 1 x 10^9 c.f.u/mL. The larvae injected with 1 x 10^9 c.f.u/mL *M. fortuitum* NCTC 10394 after receiving DEX actually survived at a higher rate than those which received PBS, whereas those infected with 2 x 10^8 c.f.u/mL had a significantly lower rate of survival with DEX.

In contrast, DEX has no effect on decreasing larval survival for larvae infected with *M. aurum* (**Figure 5.9 E/F**). Survival was 100% when the larvae received PBS before infection, and practically 100% if they received DEX.

When infected with *M. marinum* strains, DEX has some efficacy in decreasing larval survival but the effects are reasonably subtle for *M. marinum* R356933F (**Figure 5.10 G/H**) and *M. marinum* Strain M (**Figure 5.10 I/J**). For *M. marinum* NCTC 2275, DEX clearly reduced larval survival, and larvae died much earlier in the experimental period (**Figure 5.9 K/L**).

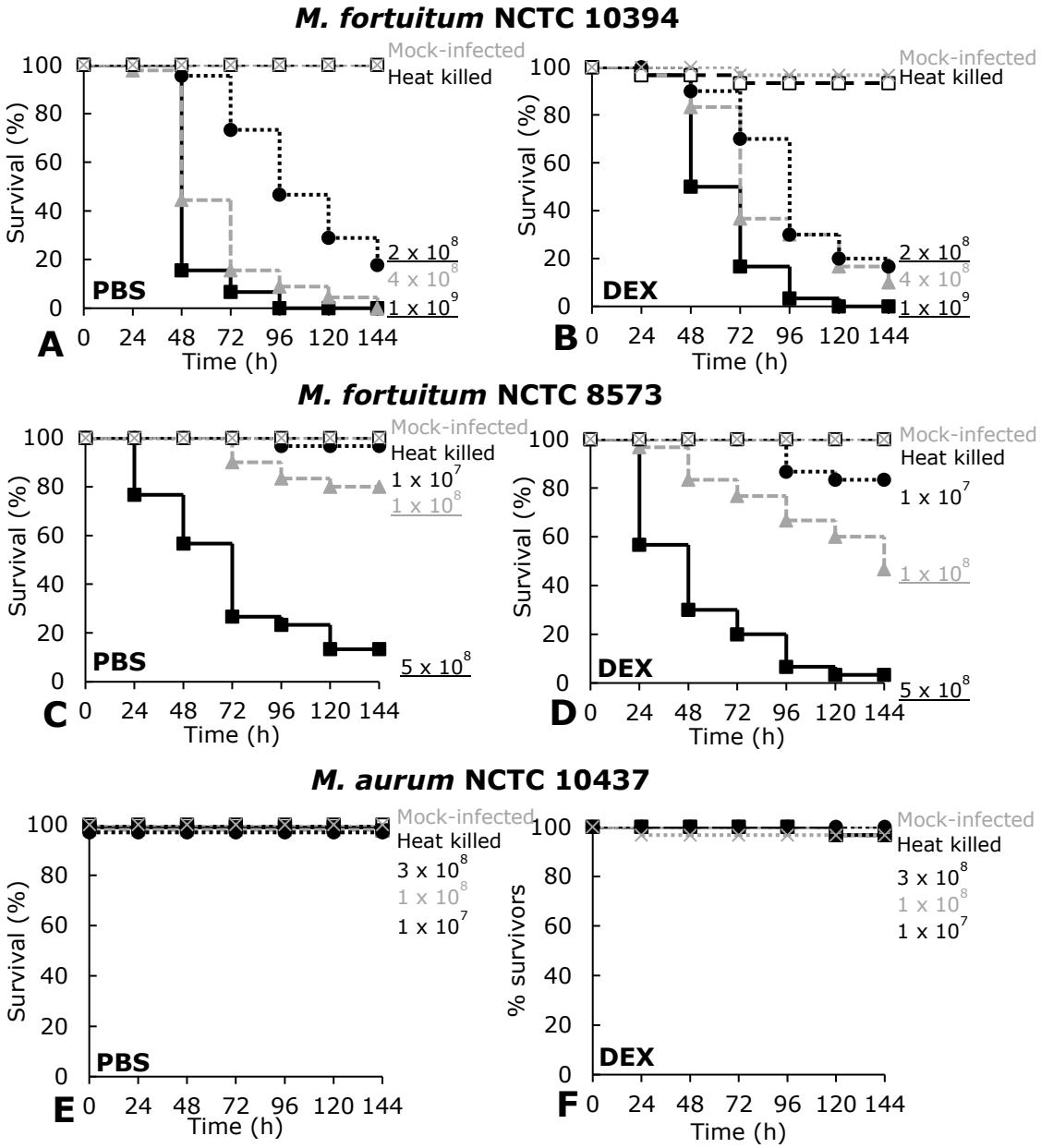

Figure 5.9 – Infections 10 minutes after injections with 200 µg of dexamethasone can cause a higher percentage of larval death than larvae not injected with DEX for *M. fortuitum* injections, but not for *M. aurum*. Larvae were injected with bacteria at the concentrations shown to the right of each survival curve (c.f.u/mL) – the species and strain of bacteria is shown above each figure. The curves on the left column (A, C and E) were injected with 4 µL of PBS before infection, those on the right (B, D and F) were injected with 200 µg DEX. Highest bacterial dose, solid black squares; middling bacterial dose, solid grey triangles; lowest bacterial dose, solid black circles. Controls of larvae mock-infected with PBS (grey crosses) and injected with heat-killed bacteria at an equivalent concentration to the highest c.f.u/mL used (heat-killed, black open squares) are also shown. When the difference in survival was statistically different, the relevant dose to the right of the curve is underlined ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons). $n = 30$. DEX, dexamethasone; PBS, phosphate buffered saline.

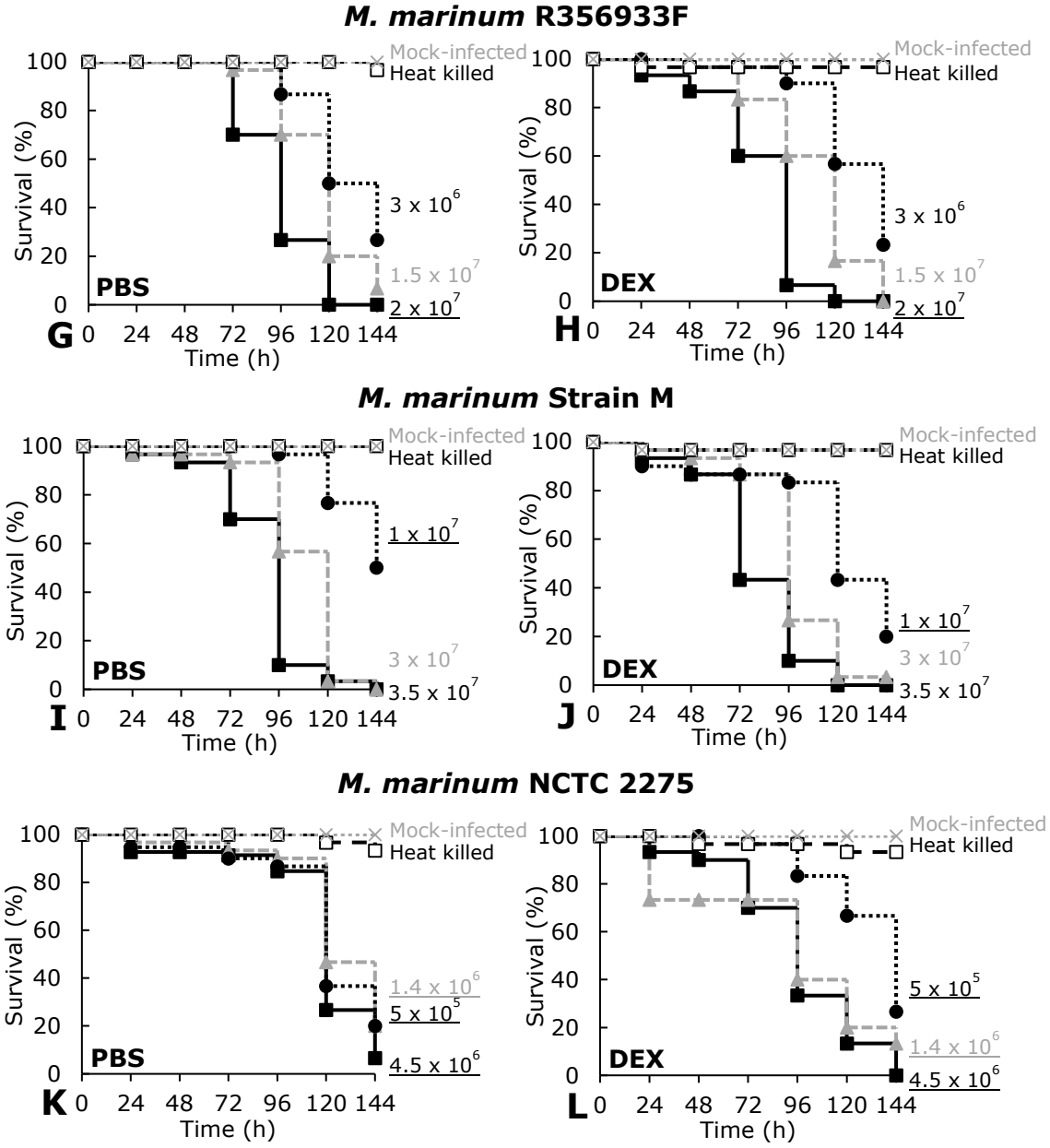

Figure 5.10 – Infections 10 minutes after injections with 200 µg of dexamethasone causes a higher percentage of larval death than larvae not injected with DEX for *M. marinum* injections. Larvae were injected with bacteria at the concentrations shown to the right of each survival curve (c.f.u/mL) – the strain of *M. marinum* is shown above each figure. The curves on the left column (G, I and K) were injected with 4 µL of PBS before infection, those on the right (H, J and L) were injected with 200 µg DEX. Highest bacterial dose, solid black squares; middling bacterial dose, solid grey triangles; lowest bacterial dose, solid black circles. Controls of larvae mock-infected with PBS (grey crosses) and injected with heat-killed bacteria at an equivalent concentration to the highest c.f.u/mL used (heat-killed, black open squares) are also shown. When the difference in survival was statistically different, the relevant dose to the right of the curve is underlined ($p < 0.05$, log-rank test with Holm's correction applied for multiple comparisons). $n = 30$. DEX, dexamethasone; PBS, phosphate buffered saline.

5.5.4.1 DEX reduces the phagocytic rate and adjusted phagocytic index of *G. mellonella* haemocytes

The same analyses were performed as those described in **Section 5.5.3.2**, although this section will focus on the phagocytic rate and adjusted phagocytic index.

- Phagocytic rate, or the percentage of total haemocytes which have engulfed one or more bacterium
- Adjusted phagocytic index, or the average number of engulfed bacteria in the haemocytes which contain one or more bacterium

Mycobacterium marinum Strain M and *M. fortuitum* NCTC 10394 were selected for experimentation as representative strains for both of these species, and *M. aurum* was examined due to its inability to cause larval death. The results of these experiments are shown in **Figure 5.11**.

DEX reduces the number of haemocytes which have phagocytosed one or more bacterium for all of the strains tested. These reductions are significant for *M. aurum* and *M. fortuitum* but are approaching significance for *M. marinum* as well ($p = 0.08$). Equally, DEX reduces the number of bacteria phagocytosed by the haemocytes which have engulfed one or more bacterium for *M. aurum* and *M. fortuitum*. The decrease is significant for *M. fortuitum.* There is a slight increase in the number of bacteria per phagocyte for *M. marinum,* but this increase is not statistically significant.

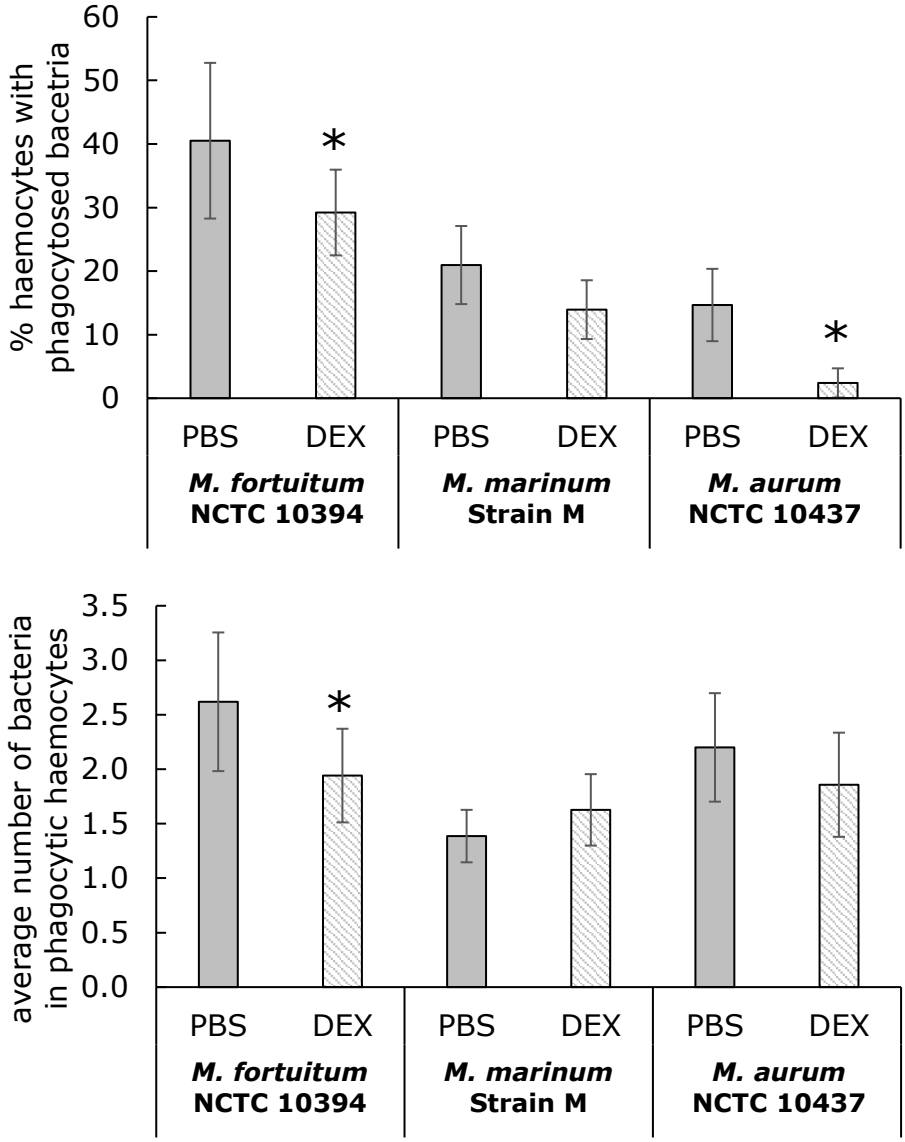

Figure 5.11 – The differences in phagocytic rate and adjusted phagocytic index for larval haemocytes exposed to *M. fortuitum* NCTC 10394, *M. marinum* Strain M and *M. aurum* NCTC 10437 when pre-injected with 200 µg DEX or PBS. Larvae were injected with 200 µg DEX or 4 µL PBS 10 minutes before FITC labelled bacteria. The larvae were incubated for 1 hour before collection of haemolymph. Results are from 10 larvae per condition, 30-70 haemocytes examined per larvae, showing the mean ± SEM. Asterix indicate $p<0.05$, unpaired, two-tailed Student's *t*-test with Holm's correction applied for multiple comparisons. DEX, dexamethasone; PBS, phosphate buffered saline.

5.5.4.3 The number of circulating haemocytes has a transient, significant increase after injection with 200 μg DEX, much like the result seen for single doses of antibiotic

The experiments were performed as described for the results given in **Section 5.5.1**, but larvae were injected with 200 μg of DEX as opposed to anti-Mycobacterial antibiotics. The results are shown in **Figure 5.12**.

Much in the same way as was seen with single and combined antibiotic doses with and without co-injection of bacteria, (**Figure 5.2, 5.3** and **5.4**), DEX causes a transient increase in the number of circulating haemocytes.

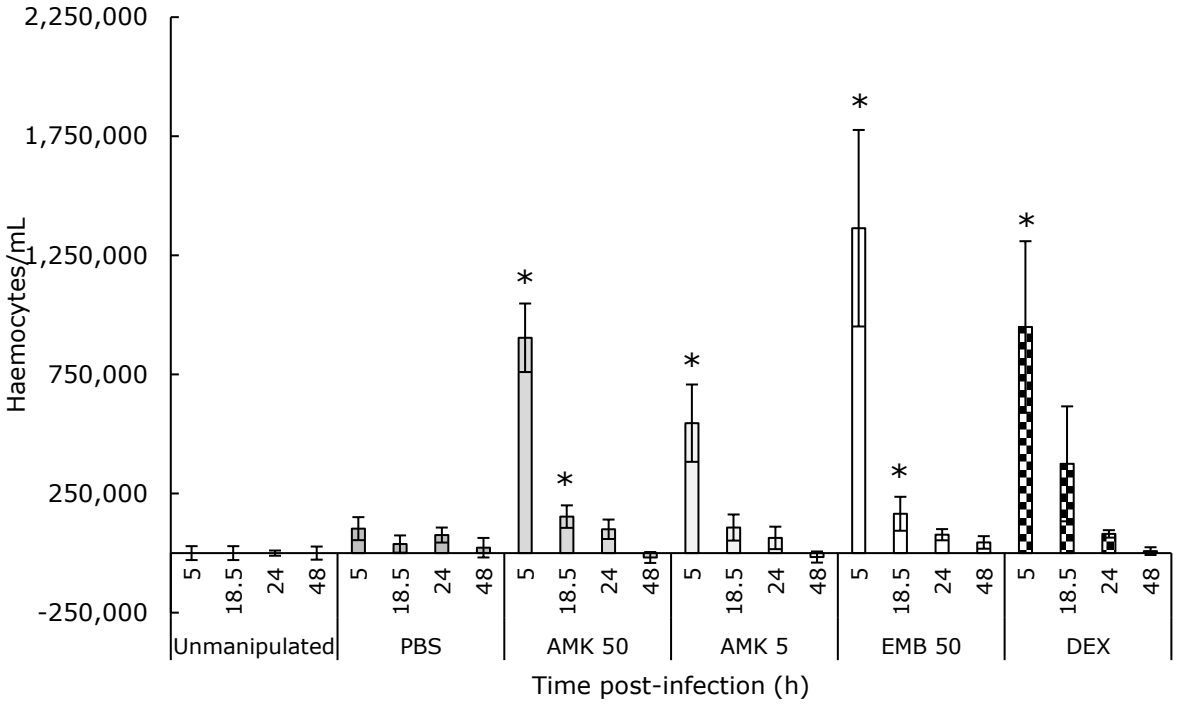

Figure 5.12 – Injecting larvae with DEX gives a similar haemocyte circulation profile to injections of antibiotics. Larvae were injected with PBS, single doses of antibiotic in the mg/kg shown on the x axis, or 200 µg of DEX. The numbers of haemocytes in the larval haemolymph were counted at 5, 18.5, 24 and 48 hours post-infection (or mock-infection). The number of haemocytes is presented relative to unmanipulated larvae. The data are from nine larvae per timepoint, showing the mean ± SEM. $p<0.05$, unpaired, two-tailed Student's *t*-test with Holm's correction applied for multiple comparisons. AMK, amikacin; EMB, ethambutol; DEX, dexamethasone; PBS, phosphate buffered saline.

5.6 Summary

The number of circulating haemocytes does not increase when larvae are injected with PBS or with viable bacteria (**Figure 5.3**). However, the number does increase when larvae are injected with antibiotics, either in single (**Figure 5.2**) or in combined doses (**Figure 5.4**). The number also increases when DEX is injected (**Figure 5.12**).

Infection with viable or heat-killed bacteria increases the number of discrete visible nodules, clumps of nodules, melanised webbing and microscopic nodules in the larvae (**Tables 5.1** and **5.2**). The amount of nodulation and webbing decreases when antibiotics are administered to the larvae (**Table 5.3** and **5.4**).

When assessing the interactions between haemocytes and Mycobacteria, Kinyoun staining was not particularly effective (**Figure 5.5**). Staining with FITC was much more effective (**Figure 5.6**) and fluorescence microscope images were analysed to determine phagocytic rate, phagocytic index, and an adjusted phagocytic index.

Antibiotics reduced the phagocytic rate of the haemocytes in some cases and may have increased the number of bacteria engulfed by each haemocyte (**Figure 5.7**). However, when a live/dead assay using FDA and PI was performed, the number of non-viable bacteria in the haemocytes remained at a very low percentage (**Figure 5.8**).

When the larval immune system was suppressed using injections of DEX, some concentrations of Mycobacteria caused higher rates of larval death compared to non-immunosuppressed larvae, but others had statistically comparable survival curves (**Figures 5.9** and **5.10**). Even when immunosuppressed, *M. aurum* was unable to have any meaningful effect on larval survival. DEX also reduced the phagocytic capabilities of larval haemocytes and affected the number of bacteria the haemocytes phagocytosed (**Figure 5.11**).

5.7 Discussion

The transient increase in circulating haemocyte numbers is an interesting response of the larvae to the injection of antibiotics. Non-pathogenic triggers such as ink particles and silica or latex beads[318,406,407] could be expected to cause an immune response due to their reasonably large physical size and the ability of the insect immune cells to recognise them as foreign. However, seeing a response to a compound suspended in sterile water might not have been anticipated.

Other groups have also noted that haemocyte density changes after larvae have been injected with a wide variety of compounds (described in **Section 5.1.3**), including antimicrobials[549,610,611]. However, it could also have been anticipated that the Mycobacteria would trigger an increase in circulating haemocytes, considering this has been observed with other pathogens[230,290,291,315,468,614–616]. The group in London who have been using *M. bovis* have shown that *M. bovis* also has no effect on circulating haemocyte numbers[379], but also report that a single 10 µL dose of isoniazid (5 mg/kg) or rifampicin (10 mg/kg) does not increase haemocyte density. This thesis looked at amikacin and ethambutol; INH and RIF were not examined. Yet the differences seen between these anti-Mycobacterials may be worth further investigation.

The number of circulating haemocytes for larvae infected with *M. fortuitum* or *M. marinum* is statistically comparable to larvae injected with PBS, which may be explained by the nodulation data in **Section 5.5.2**. Both heat-killed and viable bacteria stimulated a significant increase in the number of nodules in the 6 hour experimental period, which is well aligned with the first timepoint (5 hours) in the haemocyte density experiments. Hence, *M. fortuitum* and *M. marinum* may be increasing the number of circulating haemocytes, but these haemocytes are rapidly accumulated and then confined to nodules. The collection method used to collect haemolymph to test for changes in haemocyte density is unlikely to

allow for nodules to be represented in these counts, as nodules were observed in **Section 5.5.2** to be adherent to the larval body, as has been reported elsewhere[231,318].

In terms of observing the interactions between haemocytes and bacteria in more detail, it has been anticipated that Kinyoun staining would have been a useful tool to assess the phagocytosis of viable Mycobacteria as variations on this technique had been used elsewhere[262,378,380]. Although the staining clearly shows red bacteria and associated haemocytes (**Figure 5.5**), the slides were not of sufficient quality to determine which bacteria were phagocytosed. It is highly likely that the Kinyoun staining method can be applied to *G. mellonella* haemolymph with further modifications and refinement, and will be a rapid, cheap and efficient method for assessing the phagocytosis of acid-fast bacteria by haemocytes.

Using FITC-stained cells gave valuable insight into the phagocytosis of Mycobacteria, especially when correlated with the haemocyte density data. When larvae are injected with antibiotics, the number of circulating haemocytes increases (**Figure 5.2** and **5.3**), so the phagocytic rate goes down (**Figure 5.7 A**). There are more haemocytes but the same number of bacteria, so a larger number of haemocytes will not come into contact with a bacteria to phagocytose.

Interestingly, the antibiotics also seemed to increase the efficacy of phagocytosis by the haemocytes (**Figure 5.7 C**). Antibiotics increase the number of circulating haemocytes, increase the number of bacteria that haemocytes engulf, but reduces the number of nodules in the larval body compared to untreated infected controls (**Table 5.3** and **5.4**). Perhaps, as the haemocytes are more efficient at engulfing the bacteria, fewer nodules are required to isolate these bacteria.

The results from the FDA/PI live/dead assay also suggests that the Mycobacteria remain largely viable inside the haemocytes, at least for the hour of observation (**Figure 5.8**). These experiments would be a valuable starting-point to assess the survival of the bacteria in the haemocytes over a longer time period, and with antibiotics. Data from other groups suggests Mycobacteria should be able to survive inside the haemocytes[378,380], and may remain viable for the life-span of the moth[262]. It is not clear whether these larvae would be "contagious" and capable of infecting other larvae.

Using dexamethasone to supress the immune system of *G. mellonella* has been investigated by other groups[229,660–662] and the results here support that DEX can decrease the survival of larvae challenged by an infection (**Figure 5.9** and **5.10**). DEX also decreases the phagocytic rate of the larval haemocytes (**Figure 5.11**) but the effects on how efficient the haemocytes are at phagocytosis are not clear.

DEX triggers a transient increase in the number of circulating haemocytes (**Figure 5.12**), much as antibiotics will (**Figure 5.2**). This makes the results in **Figure 5.11** similar to those in **Figure 5.7** – antibiotics or DEX increases the number of haemocytes but the number of bacteria available to phagocytose is the same, meaning that many of the haemocytes will not encounter a bacterium to phagocytose. Hence, the decreased survival of the larvae in **Figures 5.9** and **5.10** must not be entirely due to an inability to phagocytose the bacteria.

Chapter Six

6.0 Conclusions and final discussion

This chapter summarises the overall findings of the work in this thesis, and conclusions that can be drawn from the results.

6.1 Overall findings of this thesis

This thesis represents one of the first investigations of *G. mellonella* and Mycobacteria in the modern age. At the time of commencement for this project, there were no modern modern publications describing *G. mellonella* as a model for Mycobacterial infections.

The primary aim was to demonstrate that a *Mycobacterium* can cause larval death, but that survival can be rescued with appropriate doses of antibiotics at clinically relevant concentrations. Secondary aims were to expand these experiments into protocols with multiple antibiotics doses, determine the effects of antibiotics on internal bacterial burdens, and assess non-standard treatment regimens. Tertiary aims looked at the immune system in more detail, specifically the changes in the numbers of haemocytes, nodulation, phagocytosis and the effect of an immunosuppressant (dexamethasone).

Ideally, this thesis would have been undertaken with the most clinically relevant *Mycobacterium – M. tuberculosis*. However, *M. tuberculosis* has to be handled in a biosafety level 3 laboratory and cultures take days, if not weeks, to be ready for use. The investment of time, equipment, and necessity to work in a secure lab renders many of the benefits of the *G. mellonella* model null, especially in a constrained timeline such as a doctoral degree.

Despite using non-tuberculous Mycobacteria as opposed to *M. tuberculosis*, the value of the experiments in this thesis is clear. *Mycobacterium fortuitum* and *M. marinum* are easy to grow and present no risk to immunocompetent workers. Experiments can be designed and

performed quickly, without harm to the researchers, and with minimal specialised equipment.

The larvae also have applications in toxicity testing and immune response assays. Finding new antibiotics requires multiple stages of *in vitro* and *in vivo* analysis, particularly of potential toxicity, and *G. mellonella* can be an informative bridge between *in vitro* and *in vivo* experiments. This has particular usefulness in the context of Mycobacteria – considering billions of people are infected with TB and there are millions of new cases each year, the search for improved or novel antibiotics against Mycobacterial infections is a key research concern.

Chapter Two addresses the initial aim of this thesis and showed that *M. fortuitum* and *M. marinum* are capable of causing larval death. Therefore, treatment protocols can be performed and assessed based purely on improvements in larval survival. However, *M. aurum* is not capable of causing larval death, and results in **Chapter Five** indicate that the lack of pathogenicity is maintained even when the larvae are immunosuppressed with dexamethasone.

The results in **Chapter Three** indicate an increase in the internal burden of viable bacteria in the larval body across the experimental period. This supports conclusions from other work indicating that Mycobacteria evade the immune system of their host and resist clearance.

Chapter Three also shows that established anti-Mycobacterial treatments are able to improve larval survival, although variations in efficacy were seen between Mycobacterial species and strains, and some antibiotics were ineffective for all Mycobacteria assessed in this thesis. This indicates the importance of testing multiple strains of a pathogen before drawing conclusions about an antibiotic's efficacy.

Considering that these anti-Mycobacterials are universally prescribed as combinations, it was imperative to show that combination treatments were effective at improving larval survival. Antibiotics combinations not only improved survival, but also improved the internal bacterial burden. Single doses of effective antibiotics also improved the internal bacterial burden.

Chapter Four expands the experiments into the secondary aims of this thesis. Efflux pump inhibitors were assessed with ineffective doses of anti-Mycobacterials, and β lactams were combined with β lactamase inhibitors. This chapter also indicates one of the key attributes for the model – thousands of larvae were used to assay these combinations, a number which would be impossible to replicate with a vertebrate model.

The results of β lactams combined with β lactamase inhibitors indicates that there is some value to these combinations, although many combinations were statistically ineffective, or gave survival rates which were comparable to the monotherapies. Similar results are seen for the efflux pump inhibitors – very few combinations were effective for restoring larval survival.

Finally, **Chapter Five** addressed the third aim – examining the interaction between the larval immune system and the Mycobacteria. The results from this chapter indicate that the larvae can initiate a strong immune response to infection – bacteria can be phagocytosed, isolated to melanised nodules, and the larvae increase the number of circulating haemocytes after infection. Yet **Chapter Two** shows that this does not prevent larval death, **Chapter Five** shows the bacteria can survive phagocytosis, and the dexamethasone experiments indicate that supressing the efficacy of the larval immune system can increase the rates of larval death.

In summary, this thesis sets the groundwork for experiments using non-tuberculous Mycobacteria in the *Galleria* model. There are plenty of avenues where a gate has been

opened, but the path itself has only been explored for a few steps. Yet preliminary results indicate that this pathogen/host combination will be a valuable research tool.

6.2 Suggested further work

The results in **Chapter Four** indicate a number of potentially useful combinations of novel compounds, both for the efflux pump inhibitor experiments and the β lactam/lactamase inhibitor experiments. It would be worthwhile to assess the effects of these combinations on internal bacterial burdens and expand these experiments to include a wider range of EPIs, β lactams, and β lactamase inhibitors. It would also be valuable to examine other avenues of interest, such as inhibiting ATPase, iron uptake and storage, cholesterol metabolism, or other "resistance, nodulation and cell division" proteins of the Mycobacteria.

Yet there were many more combinations of EPIs and β lactams/ β lactamase inhibitors which were unable to improve larval survival during the experimental period. It is not clear if the lack of efficacy for these combinations is due to one or both compounds being cleared from the larval system. Examining the pharmacokinetics of these combinations, and their successful counterparts, would help determine why these combinations did not improve survival while others were effective.

In **Chapter Five**, the survival of the Mycobacteria after phagocytosis was briefly assessed. This experiment could easily be expanded to determine the effects of antibiotics and dexamethasone on the data. It would also be useful to assess the haemocytes for a longer period, and look to see if viable bacteria remain in the larval haemocytes and nodules for the periods that were reported in much earlier papers[262].

In many parts of this thesis, examining the metabolism of the the various compounds used – whether they are "standard" anti-Mycobacterial antibiotics, efflux pump inhibitors, β lactams, β lactamase inhibitors, or dexamethasone – could give a valuable insight into how

the bioavailability of these drugs affects larval survival. Experiments using *G. mellonella* to assess drug metabolism and pharmacokinetics have been reported[393–395,471], so the suitability of applying these protocols to *G. mellonella* and Mycobacterial infections seems feasible.

While undertaking this work, a number of other groups have also used Mycobacteria in combination with the *G. mellonella* model, most notably a group at Imperial College in London[378,379] (see **Section 1.5**) who have utilised the model with *M. bovis* and will be expected to publish experiments using *M. tuberculosis* in the future. **Chapter Two** showed that *M. aurum* is not capable of causing larval death, and the Imperial group have shown similar results for *M. smegmatis.* However, there are a number of clinically relevant Mycobacteria which have only been assessed by one group, such as *M. abscessus*[380], or have never been assessed. These include *M. chimaera, M. chelonae,* and *M. ulcerans* (see **Section 1.2.1.1**). Although *M. tuberculosis* is certainly an enormous burden on the healthcare community and causes suffering and deaths for millions of patients each year, other Mycobacteria are also capable of causing harm and infection. *Galleria mellonella* can be just as useful for these bacteria as they are for a whole range of pathogens (see **Section 1.3.2**).

6.3 The changing role of *G. mellonella* in research

Briefly looking at the number of publications which use *Galleria mellonella* across time, it is clear that this model is becoming a well-established research tool, and the number of groups using moth larvae is increasing yearly. Great efforts are being made to standardise experiments and produce replicable data, but there are still many components of the *G. mellonella* model which are lacking. A genome for *Galleria* has only recently been published[332], there are only a small range of PCR primers and larvae-specific antibodies, and there are no regulations on how larvae should be raised, handled, stored, or transported.

However, the benefits of the model clearly outweigh the detriments, and moth larvae are well-aligned with modern attitudes to experimental design. The requirements for researchers to reduce the number of vertebrate organisms used in experimentation makes *G. mellonella* an attractive preliminary organism between *in vitro* and *in vivo* work. It is unlikely that insect larvae will become subject to increasingly tight regulations on *in vivo* experiments – for example, ethical approval must now be sought for experiments using cephalopods such as octopuses and squid.

Chapter Seven

7.0 Material and methods

This chapter will report the materials and methods associated with the experiments performed in this thesis.

7.1 Statistical analysis

Figures show results as mean values unless stated otherwise. All error bars represent standard error of the mean (SEM).

All survival curves are shown as Kaplan–Meir plots and significance determined using log-rank tests. Holm's corrections for multiple comparisons was applied when necessary[663]. For all experiments where statistical analysis was included, a p value of 0.05 or less was considered significant.

For burden assays, significance was determined using Mann-Whitney U tests[664] with a Holm's correction for comparison of multiple p values.

Haemocyte, nodule numbers, phagocytic rate and phagocytic index were compared using unpaired, two-tailed Student's t tests with Holm's correction. Percentages of nodules were determined for statistically significant variations using a z-score for two population proportions[665].

7.2 Reagents and larvae

7.2.1 Reagents

All reagents were purchased from Sigma Aldrich Ltd (which was purchased by Merck during the commission of this work and is now trading under this name) unless stated otherwise.

Antibiotics were dissolved in sterile deionised water and diluted to working concentrations with water. Stock solutions of antibiotic were stored in the conditions recommended by their datasheets, and new stocks made when solutions had been maintained for longer than recommended storage times.

7.2.2 Larvae

Larvae were purchased from UK Waxworm Ltd, stored in the sawdust they were delivered in at room temperature in the dark and were used within seven days of receipt. Larvae weighing between 0.25 and 0.3 g were used in all experiments. Larvae with any visible melanisation were not used, as were larvae which were cold to the touch or unresponsive to touch.

The larvae used in this study were raised without the use of antibiotics at any stage of the production process. They were provided with unlimited access to food until they reached the final instar, then stored in a temperature controlled room for 3-7 days before dispatch. The larval diet was cereal based, including bran, wheat, flour, honey, vegetable glycerine and beeswax (personal communication with supplier).

All injections were performed with a 25 µL Hamilton syringe with point style 2 – this needle type has a shorter bevel, meaning that the needle point does not need to penetrate as deeply under the larval cuticle to inject efficiently, which reduces the trauma to the larvae and reduces the risk of injecting into deeper structures. The syringe was cleaned between solutions by washing with 100% ethanol and rinsing with sterile PBS.

Injections were into the proleg of the larvae. In experiments which required multiple injections, injections were into a proleg which had not been injected previously.

After injections, larvae were maintained in plastic Petri dishes and not supplied with filter paper or sawdust to consume during incubation. As the diet and rate of eating effects

the immune system and haemolymph of the larvae[263], it allowed experiments to be more comparable to deny food to all larvae.

All larvae were incubated at 37 °C in the dark post-injection.

7.3 Bacteria and growth media

7.3.1 Growth media

All bacteria were cultured in a shaking incubator using sterile Mueller Hinton M7H9 broth enriched with albumin dextrose catalase (ADC) media supplement. Any agar plates used were M7H9 + ADC with 2% agar, except for antibiotics selective plates which also contained piperacillin (PIP) at 256 µg/mL.

Media was prepared by dissolving 2.35 g of media into 450 mL sterile, deionised water, and adding 500 µL Tween-80. If agar needed to be added, it was added to the bottle at this point. The media was then autoclaved at 121°C for 15 minutes and allowed to cool. As the supplement contains proteins which would be denatured at high temperatures, it was only added once the bottles were hand hot (approximately 50 °C) but when agar (if present) was still molten. Filter-sterilised PIP was also added at this timepoint if required.

7.3.2 Bacteria

Mycobacterium fortuitum NCTC 10394 and NCTC 8573, *M. marinum* Strain M and R356933F, and *M. aurum* NCTC 10437 were purchased from the National Collection of Type Cultures (Porton Down, Salisbury, UK). *Mycobacterium marinum* R356933F was kindly provided by Michael Smith (Scottish Mycobacteria Reference Laboratory, Royal Infirmary of Edinburgh, UK). Bacteria were cultured in a shaking incubator. *Mycobacterium aurum* and *M. fortuitum* strains were cultured overnight at 37 °C, and *M. marinum* strains at 27 °C for

36 – 48 hours. Aseptic technique was maintained by using a Bunsen burner for all experiments which used bacteria, and when preparing bacterial plates and cultures.

Stocks were maintained on M7H9 + ADC agar plates, and in glycerol stocks stored at minus 80 ^{o}C.

Prior to inoculation, all bacterial liquid cultures were washed twice and diluted to the required concentration using sterile PBS. Heat killing was performed in a 100 ^{o}C water bath for 10 minutes and loss of viability confirmed by plating the heat killed bacteria onto agar plates

7.3.3 Determining colony forming units per mL

Bacteria were grown in liquid cultures, then washed twice using sterile PBS. A 1:10 dilution was used to determine the concentration of bacteria using a UV-Vis spectrophotometer set to 600 nm. The optical density of the 1:10 dilution was extrapolated to the optical density of the original culture, and bacteria were then diluted to optical densities of 1.5, 1.0, and 0.5. These were then serially diluted (20 µL culture into 180 µL PBS) and 10 µL plated on agar plates. These were incubated at 27 ^{o}C or 37 ^{o}C as appropriate until colonies could be counted. The number of colonies across the serial dilution was then normalised and averaged, allowing the optical density of liquid cultures to be directly related to the c.f.u/mL.

7.4 Antibiotic susceptibility testing

7.4.1 Minimum inhibitory concentrations using gradient strips

Mycobacterium fortuitum NCTC 10394 and NCTC 8573, and *M. marinum* Strain M and R356933F were plated at 2.5 x 10^{6} c.f.u/mL spread onto a 90 mm agar plate and allowed to

dry before applying MIC strips according to manufacturer's instructions (AMK and CIP, Oxoid, UK; EMB, INH and RIF, Liofilchem, Italy).

Plates were incubated at 27 °C or 37 °C as appropriate until sufficient growth was seen to identify the appropriate MIC value. The distinctive "flame" shape of no-growth indicates the point on the strip where bacteria would no longer grow when in contact with the paper strip (see **Figure 7.1**).

MICs were performed in duplicate using three separate liquid cultures of bacteria.

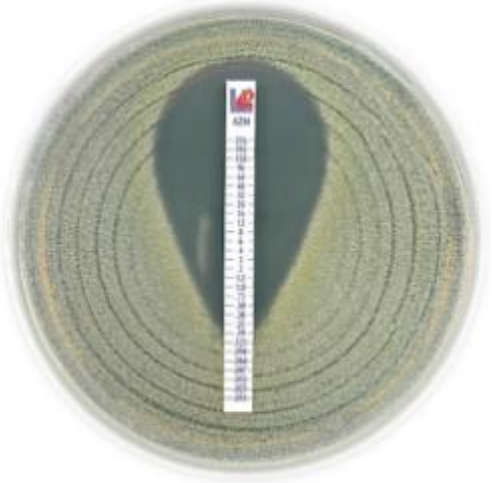

Figure 7.1 – "Flame" of no-growth on a bacterial lawn using an MIC test strip.
Image from Liofilchem product website.

7.4.2 Toxicity testing

Several reagents required testing to assess toxicity, including antibiotics, efflux pump inhibitors, β lactamase inhibitors, and dexamethasone. These reagents were prepared to 100 mg/kg and injected into duplicate groups of 15 larvae, with each group coming from separate orders, to give a total experimental group of 30 larvae (n = 30). The larvae were then incubated at 37 ^{o}C.

Every 24 hours for 144 hours post-injection, larval survival was assessed by physically moving the larvae with blunt, plastic tweezers and observing if they responded to touch. Those no longer responsive to touch were recorded as dead. Considering the production of melanin can also be an indication of toxicity, larvae were also evaluated for any signs of melanisation.

Survival was 100% for the duration of the experiment for all occasions of toxicity testing – it can be assumed for all experiments using these reagents that uninfected larvae injected with the reagent alone would have had 100% survival.

7.5 Injecting *G. mellonella* with bacteria and reagents

7.5.1 Infections based on bacterial optical density and colony forming units

Bacterial cultures were washed twice and diluted with PBS, then diluted to optical densities of 1.5, 1.0, and 0.5 using a UV-Vis spectrophotometer at 600 nm. Ten microlitres of these preparations were injected into larvae using a Hamilton syringe, Larvae injected with sterile PBS were included as mock-infected controls. Non-viable-bacteria control larvae received injections of heat-killed cells (**Section 7.3.2**) at an optical density of 1.5.

Larval survival was assessed every 24 hours for 144 hours and recorded as dead when they were no longer responsive to touch.

Once the larval survival with these bacterial concentrations had been collected and assessed, the experiment was repeated using bacteria diluted to the desired c.f.u/mL using the values determined in **Section 7.3.3**. Survival was shown as Kaplan–Meir survival curves.

7.5.2 Infections treated with single doses of anti-Mycobacterial antibiotics

The antibiotics used in this section are amikacin (AMK), ciprofloxacin (CIP), ethambutol (EMB), isoniazid (INH), and rifampicin (RIF).

A bacterial concentration which caused sufficiently high larval death was selected for each strain used, and prepared and injected as described in **Section 7.5.1.**

Antibiotics were diluted to the appropriate concentration using distilled water, and then injected in a 10 μL volume to give the desired internal antibiotic concentration in mg/kg. Injections were given 2 hours after infection. Groups of larvae were also mock-treated using PBS.

Survival data was collected, assessed and presented using the methods described in previous sections.

7.5.3 Infections treated with combined doses of anti-Mycobacterial antibiotics

The experimental design is very similar to that in **Section 7.5.2**, however treatments were prepared so that multiple antibiotics could be injected as a single 10 μL dose, meaning

that no additional trauma was affected to the larvae. The injections were also given 2 hours post-infection, with mock-infected larvae injected with PBS included as controls.

7.5.4 Assessing β lactam antibiotics combined with a β lactamase inhibitor

The β lactamase inhibitor used in these experiments is tazobactam (TZO; Santa Cruz Biotechnology, USA, and Fluorochem). The β lactams are amoxicillin (AMX; Fluorochem), cefalotin (CEF; Fluorochem), cefaclor (CEC; Fluorochem and Sigma Aldrich), and piperacillin (PIP). Both the β lactams and β lactamase inhibitor were used at 50 mg/kg in these experiments, and controls of each drug given as a single injection were included to demonstrate that any efficacy was due to the combination of both.

The experimental design is similar to **Section 7.5.2** but β lactams and tazobactam were injected simultaneously. Considering piperacillin and tazobactam are commonly used in a combined solution, the other β lactams were also prepared as combined solutions to allow administration as a single 10 µL injection 2 hours post-infection.

7.5.5 Assessing efflux pump inhibitors combined with anti-Mycobacterial antibiotics

The efflux pump inhibitors (EPIs) used in these experiments are verapamil (VPL; Santa Cruz Biotechnology, USA), flupenthixol (FLU; Santa Cruz Biotechnology, USA), chlorpromazine (CPZ; Santa Cruz Biotechnology, USA), thioridazine (THI; Santa Cruz Biotechnology, USA), and trifluoperazine (TFP; Santa Cruz Biotechnology, USA).

Initially, the combinations were tested in smaller groups of larvae (n = 10 or 15) than in other experiments, as the efflux pump inhibitors were tested at three different concentrations (0.1 mg/kg, 1 mg/kg, and 10 mg/kg) and would have required an enormous

number of larvae if tested in groups of 30 larvae. The anti-Mycobacterial doses were selected based on results from **Section 7.5.2** and chosen for conferring an insignificant or non-existent improvement to larval survival. The EPIs were injected 2 hours post-infection in combination with the appropriate antibiotic in a single 10 μL injection. Controls were included of each drug given as a single injection to show that any efficacy was due to the combined effect of the two drugs.

Any results which showed a significant or near-significant increase in larval survival over larvae which had only been treated with the antibiotic (no efflux pump inhibitor) were then tested in the standard *n* = 30 larval groups.

7.5.6 Using dexamethasone to inhibit immune cells

Dexamethasone was dissolved in sterile deionised water to 50 g/L. Injections of 4 μL were administered, meaning individual larvae received 200 μg of dexamethasone. These injections were given precisely 10 minutes before injections with bacteria. Survival was then assessed every 24 hours for the following 144 hours, as with previous experiments.

7.6 Assessment of the larval response to infection

7.6.1 Measuring the internal bacterial burden inside the larvae

Groups of 40 larvae were used for each bacterial and antibiotic combination. Forty larvae were injected with 10 μL of 4 x 10^8 c.f.u/mL *M. fortuitum* NCTC 10394, or 3 x 10^7 c.f.u/mL *M. marinum* Strain M. Antibiotics, or combinations of antibiotics, were injected 2 hour post-infection. One group was mock-treated with 10 μL of PBS.

Five larvae were randomly selected from each group and assessed for internal bacterial burden at 5, 24, 48, 96 and 144 hours post-infection. When there was a combination of live and dead larvae in the group, the five larvae selected were in a ratio

which represented the number of living and dead larvae in the group. For example, if there were 20 living larvae and 5 dead larvae, then 4 living larvae and 1 dead larvae were randomly selected at that time point.

Each larva was sterilised by washing in ethanol then placed in individual 1.5 mL Eppendorf tubes with 300 µL sterile PBS. They were then homogenised with plastic pestles which had been sterilised in ethanol and a further 300 µL of PBS added to the tubes. The homogenised larvae were then vortexed, and 20 µL of liquid removed from the tubes. Care was taken not to take up large pieces of material in the pipette tip. This 20 µL sample was then serially diluted in 180 µL PBS across six wells in sterile 96 well plates. From these diluted samples, 10ul was taken and spread onto PIP selective plates.

The plates were wrapped in Parafilm, and incubated upside down at 37 ^{o}C or 27 ^{o}C as appropriate for 24 to 72 hours until colonies were visible. Colonies were then counted, the dilutions taken into consideration, and then c.f.u/mL for each larvae used to generate figures.

7.6.2 Assessing the number of haemocytes circulating within the larval body post-injection

7.6.2.1 Haemocyte numbers for unmanipulated larvae and larvae injected with PBS or antibiotics, but no bacteria

Groups of 15 larvae were injected with 10 µL of sterile PBS, 10 µL of antibiotic, or left un-injected with minimal physical handling to act as an unmanipulated control, and were incubated at 37 ^{o}C in the dark in the same manner as larvae which had been infected in previous injection experiments. None of these larvae received any bacterial injections.

Three larvae were randomly selected and injected with 75 µL *Galleria* saline (186 mM NaCl, 13 mM KCl, 10 mM HEPES, 1 mM $NaHCO_3$, 2 mM $CaCl_2$; pH 6.8) saturated with phenylthiol urea (PTU) at 5, 18.5, 24 and 48 hours post-injection. For the unmanipulated larvae, the time points are relative to the time when the larvae were put into the incubator.

The larvae were cut with small surgical scissors which had been cleaned with ethanol, aiming for the distal dorsal side and shallow cuts to reduce the risk of interrupting the larval gut. Haemolymph was bled into individual sterile Eppendorf tubes. The PTU in the *Galleria* saline prevented immediate melanisation, but samples were immediately examined as melanisation would have occurred after a few hours and haemocytes may have lysed or degraded.

From each haemolymph sample, duplicates of 10 µL were loaded onto an improved Neubauer haemocytometer and the haemocytes counted to give duplicate haemocyte per mL values for each sample. The experiment was performed in triplicate, using different bacterial liquid cultures each time, to give n = 9 for each experimental condition. Holm's correction was applied for multiple comparisons.

7.6.2.2 Haemocyte numbers for larvae pre-injected with dexamethasone, then with sterile saline

As in **Section 7.6.2.1**, 15 larvae were used for each replicate of this experiment. Similar to **Section 7.5.6**, dexamethasone was given as a 4 µL injection to mean the larvae had received 200 µg of DEX and were then incubated and sampled as described in **Section 7.6.2.1**.

7.6.2.3 Haemocyte numbers for larvae injected with bacteria and then treated with single doses or combined doses of antibiotic

Fifteen larvae were used in each condition and replicate. Larvae were infected with 10 µL of *M. fortuitum* NCTC 10394 or *M. marinum* Strain M at an inoculum size of 1 x 10^7 c.f.u/mL. This number of bacteria was selected because haemolymph can only be harvested from living larvae, and the inoculum size inducted no lethality over the 48 hour experiment.

Two hours post-infection, larvae were then mock-treated with 10 µL of sterile PBS or given 10 µL of antibiotic. The larvae were then incubated and sampled as described in **Section 7.6.2.1**.

7.6.3 Evaluating the nodulation response of larvae

7.6.3.1 Nodulation and webbing for unmanipulated larvae and larvae injected with PBS or antibiotics, but no bacteria

Portions of this method are adapted from Lapointe *et al.*[231] For each experimental condition, 35 larvae were prepared. Five larvae were randomly selected for dissection at 1, 2, 4, and 6 hours post-injection. The experiments were repeated four times using larvae from different orders, meaning each timepoint has n = 20 larvae.

The larvae were anesthetised for 10 minutes in a Petri dish resting on ice to reduce movement. The haemocyte reactions in the larvae were arrested by injecting 50 µL of chilled 4% formaldehyde diluted in PBS and chilling the larvae for a further 10 minutes. The larvae were then dissected under a dissecting microscope at 40X magnification. During the dissection, the larvae were in a Petri dish resting on another dish filled with ice to help reduce the temperature. The lights on the dissecting microscope are quite warm, and during

the dissection the haemolymph and fat body of the larvae can become quite sticky and congealed. This isn't seen when the larvae are kept cool on ice.

An incision was made running between the prolegs from the anus, up between the true legs, and finishing between the mandibles. The larvae were then assessed using straight tipped forceps and a dissecting needle.

The larvae were examined for three different types of internal melanisation – the presence of light or heavy webbing, the sizes and number of nodules present, and the extent of nodulation. The nodules that were individually discrete and identifiable under the dissecting microscope were divided into three types – full stop nodules, small clumps, and large clumps. Examples of each of these types of nodule are shown in **Figure 7.2**.

Webbing was determined to be dense areas of melanisation where individual nodules were not visible under the dissection microscope – the webbing looked similar to spider webs. Nodulation was measured as very light, light, medium, and heavy, and is related but not limited to the types of nodule seen in **Figure 7.2** – the criteria for these nodule counts were for nodules which were not individually identifiable under the dissecting microscope. There were many larvae which exhibited nodulation, but not webbing.

For the types of nodules seen, the numbers could be collected quantitively and reported as an average across the five larvae and four replicates. The levels of nodulation and presence of webbing were collected as qualitative data ("yes" if present, "no" if not) and then represented as the percentage of larvae which showed that feature.

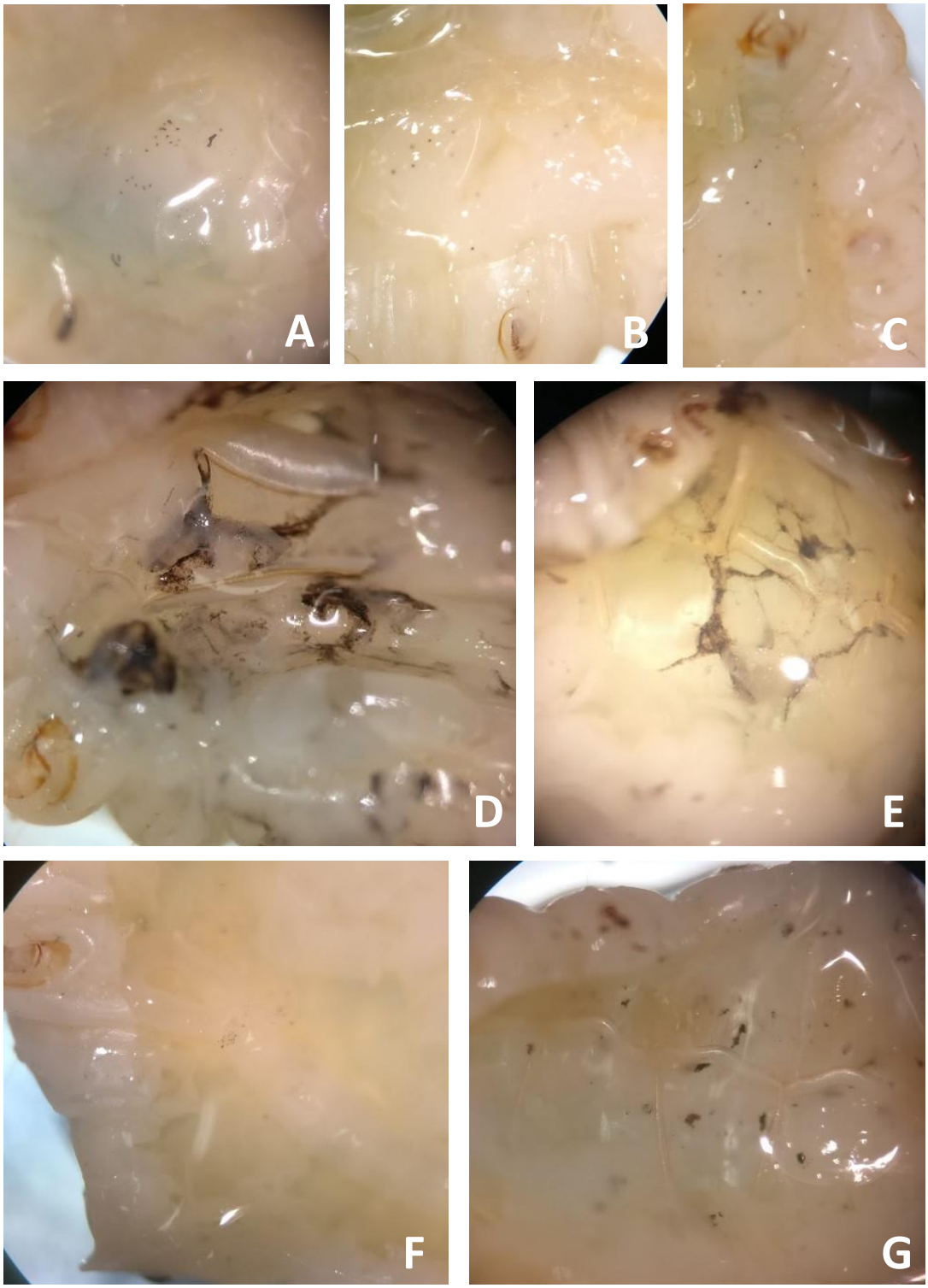

Figure 7.2 – Nodules and webbing in the larval body of infected *Galleria mellonella*. Images taken using a dissecting microscope. Images A, B and C all show discrete, individually identifiable "full stop" nodules. Images D and E show heavy webbing, where no individual nodules are visible but melanisation and nodulation are clearly present. Images F and G show nodulation where individual nodules were not readily identifiable but were clearly separated from one another (unlike in the webbing examples). These types of nodulation were divided into very light, light, medium and heavy nodulation.

7.6.3.2 Nodulation and webbing for larvae infected with bacteria and heat-killed bacteria, with and without antibiotics

The preparation process for these larvae was similar to that described in **Section 7.6.3.1**, although the larvae were injected with 1 x 10^7 c.f.u/mL of *M. fortuitum* NCTC 10394, *M. fortuitum* NCTC 8573, *M. marinum* Strain M or *M. marinum* R356933F before incubation. Larvae were also injected with heat-killed bacteria at the same c.f.u/mL.

Groups to be injected with antibiotics received injections either at the same time as the bacteria, or 30 minutes after the bacteria.

The results for these larvae were then compared to the mock-infected control group. A *t*-test was used for the nodulation type data, and a *z*-score for testing two population proportions was used for comparing the percentages of larvae showing webbing and the different levels of nodulation.

7.7 Assessment of the cellular response to infection by larval haemocytes

7.7.1 Kinyoun staining

Larvae which had been injected with *M. fortuitum* NCTC 10394 and *M. marinum* Strain M prepared to 4 x 10^8 c.f.u/mL and 3 x 10^7 c.f.u/mL respectively were incubated for an hour. Initially, haemolymph was bled directly onto ethanol-cleaned slides and allowed to air-dry, before staining was undertaken. In later iterations, haemolymph was collected, pooled, and diluted with *Galleria* saline saturated with PTU. The haemocytes were washed by centrifuging and resuspending in *Galleria* saline saturated with PTU. These were smeared onto slides and air-dried or heat-fixed over a Bunsen flame. Some slides were also exposed to methanol or formaldehyde to assist in fixing.

The Kinyoun staining was performed with a Kinyoun kit from Clin-Tech (Guildford, UK) using the supplied protocol. Briefly, the slide was flooded with carbol fuchsin for a minute, then washed gently with water. Excess dye was wiped from edges and back of the slide using tissue, and the slide was decolourised with a thin stream of acid alcohol for a period of no longer than 30 seconds. The slide was rinsed with water, then counterstained with methylene blue for around 30 seconds before a final wash. The slides were left to dry before examining on a light microscope with 1000X magnification. Images were captured using a Moticam X Wi-Fi-enabled microscope camera.

7.7.2 Phagocytosis of FITC-labelled bacteria

Liquid cultures of *M. fortuitum* NCTC 10394 and *M. marinum* Strain M were prepared to 4 x 10^8 c.f.u/mL and 3 x 10^7 c.f.u/mL respectively, then heat-killed and resuspended in 0.1 mg/mL FITC (fluorescein isothiocyanate dissolved in 0.2 M Na_2CO_3, 0.2 M $NaHCO_3$, pH 9) and incubated in a 27 ^oC shaking incubator for 30 minutes before being washed three times with PBS. The bacteria were kept in the dark to reduce the risk of the FITC being overexposed to light.

Ten larvae were examined for each condition, and received injections of PBS, AMK or EMB as required 10 minutes before receiving 10 µL injections of FITC-stained bacteria. The larvae were then incubated for 1 hour at 37 ^oC in the dark and injected with 30 µL *Galleria* saline saturated with PTU before bleeding their haemolymph into individual sterile Eppendorf tubes.

The haemolymph was then transferred into separate wells in a 24-well plate prefilled with 300 µL of ice-cold Grace's insect medium and 13 mm round glass coverslips which had been wiped clean with ethanol. The plate was incubated for 1 hour in the dark in a 27 ^oC shaking incubator set at a very low speed. The supernatant was then removed, and 1 mL of 3.7% formaldehyde diluted in PBS added for 20 seconds, before being removed and

replaced with 300 μL 0.4% trypan blue diluted in PBS, which was left on the cells for 20 minutes. The trypan blue reduces the fluorescence of any external bacteria which haven't been phagocytosed.

The coverslips were then washed three times with PBS and then mounted for examination at 400X magnification using a Delta Vision fluorescence microscope with differential interference contrast and filters for FITC (excitation 490 nm/emission 528 nm). The haemocytes were imaged through an appropriately sized z-stack to allow counting of internalized bacteria. A z stack essentially combines pictures ascending or descending through the z axis – each image has detail for the x and y axes, so stacking the images allows for analysis of the data across three planes, giving the location of the bacteria and the haemocytes in three dimention. Between 30 and 70 haemocytes were counted on each slide (i.e. per larvae). The phagocytic rate was calculated as the percentage of the total haemocytes that had engulfed one or more bacteria. Multiple comparisons were assessed with Holm's correction.

7.7.3 Survival of bacteria labelled with propidium iodide and fluorescein diacetate after exposure to haemocytes

Haemolymph was collected from around 30 larvae and combined with an equal volume of *Galleria* saline saturated with PTU. The detritus was allowed to settle, and clean, non-melanised haemolymph was collected from the homogenous layer above the detritus.

Liquid cultures of *M. fortuitum* NCTC 10394 (4×10^8 c.f.u/mL), *M. fortuitum* NCTC 8573 (6×10^8 c.f.u/mL), *M. marinum* Strain M (3×10^7 c.f.u/mL) and *M. marinum* R356933F (9×10^8 c.f.u/mL) were washed and prepared to the required concentration. A heat-killed vial of each bacteria was prepared. Heat killing was performed in a 100 ^{o}C water bath for 10 minutes.

In new, sterile Eppendorf tubes, 100 µL of clean haemolymph was combined with 50 µL of viable or heat-killed bacteria and incubated for the desired time period (5, 15, 30 or 60 minutes post-exposure). The tubes were centrifuged at approximately 7000 *g* for 3 minutes, resuspended in *Galleria* saline with PTU, centrifuged again, and resuspended in distilled water for 10 minutes to lyse the haemocytes. The tubes were then centrifuged again and resuspended in 100 µL of staining solution (prepared at 2.5 mL M7H9 + ADC media, 25 µL FDA and 25 µL PI).

FDA had been prepared by dissolving 5 mg of powdered FDA in 1 mL acetone, which was then stored at minus 20 ^{o}C. PI had been prepared by dissolving 2 mg powdered PI in 1 mL PBS and stored at 4 ^{o}C. These instructions were adapted from an ibidi application note[666].

The tubes were incubated in the dark for 10 minutes then washed three times in GIM, before a final resuspension in 50 µL GIM. This volume was loaded onto an ethanol cleaned slide and a coverslip adhered with nail polish. Slides were examined at 400X magnification using a Delta Vision fluorescence microscope with differential interference contrast and filters for FDA (excitation 490 nm/emission 528 nm) and PI (605 nm/660 nm).

One slide was prepared per timepoint for each bacterium, and the numbers of red and green bacteria were counted on 10 separate sections for each.

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Evaluation of greater wax moth larvae, *Galleria mellonella*, as a novel *in vivo* model for non-tuberculosis *Mycobacteria* infections and antibiotic treatments

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Abstract

Purpose. To evaluate the suitability of *Galleria mellonella* larvae as an *in vivo* model and drug-screening tool for mycobacteria infections.

Methodology. Larvae were infected using a range of inoculum sizes from a variety of rapid-growing mycobacteria, including strains of *M. fortuitum*, *M. marinum* and *M. aurum*. Larval survival, internal bacterial burden and the effects of amikacin, ciprofloxacin, ethambutol, isoniazid and rifampicin treatment on larval survival were measured over 144 h. The effects of these anti-mycobacterial drugs on phagocytosis and circulating haemocyte numbers were also examined using microscopy.

Results. Larval survival decreased after infection with *M. fortuitum* and *M. marinum* in a dose-dependent manner, but remained unaffected by *M. aurum*. Heat-killed bacteria did not cause larval death. Where antibiotic monotherapy was efficacious, larval survival post-infection increased in a dose-dependent fashion. However, efficacy varied between different antibiotics and species of infecting mycobacteria and, apart from rifampicin, efficacy *in vivo* correlated poorly with the *in vitro* minimum inhibitory concentrations (MICs). Combinations of antibiotics led to higher survival of infected larvae than antibiotic monotherapy. Selected antibiotic treatments that enhanced larval survival reduced the overall internal burden of infecting mycobacteria, but did not eradicate the pathogens. Administration of amikacin or ethambutol to uninfected larvae induced an initial transient increase in the numbers of circulating haemocytes and reduced the phagocytic rate of haemocytes in larvae infected with *M. marinum*.

Conclusions. This report demonstrates the potential of employing a wax moth larvae model for studying fast-growing mycobacteria infections, and as a cheap, effective system for initial screening of novel treatments.

INTRODUCTION

Mycobacteria species, primarily *Mycobacterium tuberculosis*, are major human pathogens and a significant cause of morbidity and mortality. Multidrug-resistant tuberculosis (MDR-TB) is now a global challenge, with around 480 000 new MDR-TB cases occurring in 2015 [1]. Extensively drug-resistant cases were reported in 96 countries in 2012 [2] and 'totally drug-resistant' cases have been described in several countries [3–6]. Using *M. fortuitum* [7, 8], *M. marinum* [9, 10] and *M. aurum* [11, 12] as surrogates for *M. tuberculosis* is well established, both *in vitro* and *in vivo*, but *M. fortuitum* and *M. marinum* are important infectious bacteria in their own right. Infections with either bacterium have no reporting requirements, but estimates of incidence are 4.65–5.99 cases per million persons for *M. fortuitum* [13] and 0.05–0.27 case per 100 000 adult patients for *M. marinum* [14]. These mycobacteria have similar host–pathogen interactions [15], cell-wall profiles [11], membrane and efflux pump proteins [16], virulence genes [17] and drug-resistance profiles [12] to *M. tuberculosis*, but are much less capable of infecting an immunocompetent human and generally have a much faster growth rate.

A rapid screening model is needed to assess effective treatment combinations and expedite the discovery of novel therapies for mycobacteria. Using wax moth larvae, *Galleria mellonella*, as an *in vivo* model to assess the virulence of

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Keywords: *Galleria mellonella*; *Mycobacterium fortuitum*; *Mycobacterium marinum*; *Mycobacterium tuberculosis*; invertebrate infection model; antibiotic susceptibility.
Abbreviations: AMK, amikacin; ADC, albumin dextrose catalase; CIP, ciprofloxacin; EMB, ethambutol; INH, isoniazid; MDR-TB, multi-drug resistant tuberculosis; MIC, minimum inhibitory concentration; PIP, piperacillin; RIF, rifampicin; TB, tuberculosis.
Supplementary material is available with the online version of this article.

pathogens and the efficacy of antimicrobials is well established (reviewed in [18, 19]). Their small size, low purchase and maintenance costs, and reduced ethical controls mean that they are accessible to most laboratories. Many experiments require minimal specialized equipment, and larvae can be incubated at 37 °C and easily inoculated with precise volumes – a significant advantage over other invertebrate models, such as fruit flies and nematodes, which are not viable at 37 °C for extended incubations and are awkward to inoculate. The wax moth larvae model can be a valuable screening tool to highlight treatment combinations, novel compounds and alternative therapies that can then be assessed in mammalian trials.

Compared to mammals, *G. mellonella* lacks an adaptive immune system, yet the invertebrate innate immune system is very robust, with complex cellular defences and the production of at least 18 antimicrobial peptides [20]. Vertebrate white blood cells and invertebrate haemocytes are not homologous, although they do have analogous roles, including wound repair [21], cell clustering around foreign bodies [22], innate immunity [23], phagocytosis [24] and the production of reactive oxygen species as a defence mechanism [23].

Using a cheap and rapid *in vivo* model to reliably model mycobacterial treatments is clearly attractive. Considering the advantages of using a model organism that negates the requirement for specialized laboratories and ethical approval, it is equally advantageous (for the reasons stated above) to use non-tuberculosis mycobacteria species such as *M. fortuitum, M. marinum* and *M. aurum*, since *M. tuberculosis* is a highly pathogenic bacterium that is capable of infecting immunocompetent individuals [25] and requires weeks of incubation before cultures are ready to use. This report aims to demonstrate the suitability of *G. mellonella* as a model for mycobacteria infections, allowing the application of the model as a cheap, efficient and rapid *in vivo* screen for antibiotic combinations and novel treatments that may be effective against *M. tuberculosis* infections.

METHODS

Reagents and larvae

Reagents were purchased from Sigma-Aldrich Ltd (Dorset, UK) unless stated otherwise. Antibiotics were dissolved in sterile deionized water. Larvae were purchased from UK Waxworms Ltd (Sheffield, UK), stored in the dark at 20 °C and used within 7 days of receipt. Injections were performed with a 25 µl Hamilton syringe. Larvae were incubated at 37 °C in Petri dishes in all experiments.

Bacteria and growth media

M. fortuitum NCTC 10394 and NCTC 8573, *M. marinum* strain M and R356933F, and *M. aurum* NCTC 10437 were purchased from the National Collection of Type Cultures (Porton Down, Salisbury, UK). *M. marinum* R356933F was kindly provided by Michael Smith (Scottish Mycobacteria Reference Laboratory, Royal Infirmary of Edinburgh, UK). The bacteria were cultured in a shaking incubator using Mueller–Hinton M7H9 broth enriched with albumin dextrose catalase (ADC) overnight at 37 °C for the *M. aurum* and *M. fortuitum* strains, and at 27 °C for approximately 36 h for the *M. marinum* strains. Any agar plates used were Mueller–Hinton M7H9 broth enriched with ADC supplement and 10 % agar, except for the selective plates used in the burden experiment, which included piperacillin (PIP) at 256 mg l^{-1}.

Prior to all inoculations, all bacteria were washed twice and diluted to the required concentration using PBS. Heat-killing was performed in a 100 °C water bath for 10 min and loss of viability confirmed by plating out as above.

Antibiotic susceptibility testing

The minimum inhibitory concentration (MIC) of each antibiotic against *M. fortuitum* NCTC 10394 and NCTC 8573, and *M. marinum* strain M and R356933F was determined using MIC strips (AMK and CIP, Oxoid, UK; EMB, INH and RIF, Liofilchem, Italy) according to the manufacturer's instructions. Cells were plated at 2.5×10^6 c.f.u. per 90 mm plate and incubated at 27 °C or 37 °C as appropriate. The MIC was performed in triplicate and determined as the point on the strip where bacteria no longer grew when in contact with the paper.

Infection of *G. mellonella* larvae

The experiments were performed in duplicate (n=15) and the results pooled (n=30). Larvae were infected with varying inoculum sizes for each mycobacteria strain. Larvae injected with sterile PBS were included as mock-infected controls. Non-viable-bacteria control larvae received injections of heat-killed cells at a concentration equal to the largest viable cell inoculum tested. Larval survival was assessed every 24 h for 144 h and larvae were recorded as dead when they were no longer responsive to touch. Survival was plotted as Kaplan–Meir survival curves and comparisons were made using log-rank tests. Holm's correction for multiple comparisons [26] was applied.

Antibiotic toxicity was assessed by injecting larvae with 100 mg kg^{-1} of each and recording the survival and levels of melanization on the larval body for 144 h. For antibiotic efficacy experiments, larvae were infected and then given single treatments of antibiotic or PBS (mock-treated control) 2 h post-infection (p.i.). For combination experiments, antibiotics were prepared as a single 10 µl dose. Comparisons of survival were made using log-rank tests on Kaplan–Meir survival curves. In all tests $P\leq0.05$ was considered significant and Holm's correction was applied to account for multiple comparisons.

Measurement of the internal burden of mycobacteria inside infected larvae

Forty larvae were infected with 10 µl of 4×10^8 c.f.u. ml^{-1} *M. fortuitum* NCTC 10394 or 3×10^7 c.f.u. ml^{-1} *M. marinum* strain M. A single dose of antibiotic (10 mg kg^{-1} AMK or 50 mg kg^{-1} CIP for *M. fortuitum*, and 25 mg kg^{-1} AMK or

50 mg kg^{-1} INH for *M. marinum*) or PBS (mock-treated control) was administered 2 h p.i. Five larvae were randomly selected from each group and assessed for internal bacterial burden at 5, 24, 48, 96 and 144 h p.i.

Preparation of larval suspension for plating was performed as described previously [27]. Piperacillin (PIP) was selected from a range of antimicrobials to use in selective agar plates as it inhibited the *in vitro* growth of micro-organisms present in the larval gut but did not affect the recovery of the colony-forming units of any of the mycobacteria used in this study (data not shown). Larval suspensions were serially diluted and plated on M7H9+ADC+PIP selective agar plates and then incubated at 27 °C or 37 °C as appropriate for 24–72 h until colonies were visible.

Measuring phagocytosis of FITC-labelled mycobacteria

M. fortuitum NCTC 10394 and *M. marinum* strain M cultures were heat-killed, resuspended in 0.1 mg ml^{-1} FITC (FITC dissolved in 0.2 M Na_2CO_3, 0.2 M $NaHCO_3$, pH 9) and incubated in a 27 °C shaking incubator for 30 min before being washed three times with PBS.

The larvae received injections of PBS, AMK (50 and 5 mg kg^{-1} for *M. fortuitum*, 50 and 2.5 mg kg^{-1} for *M. marinum*) or EMB (50 mg kg^{-1}) as required, 10 min before injection with FITC-stained bacteria (4×10^8 c.f.u. ml^{-1} for *M. fortuitum* and 3×10^7 c.f.u. ml^{-1} for *M. marinum*). The larvae were incubated for 1 h and then injected with 30 µl *Galleria* saline [28] saturated with phenylthiol urea (PTU) and bled into individual sterile reaction tubes. The haemolymph was transferred to wells in a 24-well plate prefilled with 300 µl ice-cold Grace's insect medium and 13 mm round glass coverslips. The plate was incubated for 1 h in the dark in a 27 °C low-speed shaking incubator. The supernatant was removed and 1 ml 3.7 % formaldehyde was added for 20 s before 300 µl 0.4 % trypan blue in PBS was added for 20 min. The coverslips were washed three times with PBS and then mounted for examination at 40× magnification using a Delta Vision fluorescence microscope with differential interference contrast and filters for FITC (excitation 490 nm/emission 528 nm). The haemocytes were imaged through an appropriately sized z-stack to allow counting of internalized bacteria.

Ten larvae were examined for each condition, with 30 to 70 haemocytes being counted per larvae. The phagocytic rate was calculated as the percentage of the total haemocytes that had engulfed one or more bacteria. Multiple comparisons were assessed with Holm's correction.

Determination of circulating haemocyte numbers

Fifteen larvae were injected per tested condition. Larvae were infected with 10 µl of *M. fortuitum* NCTC 10394 or *M. marinum* strain M at an inoculum size of 1.0×10^7 c.f.u. ml^{-1}. This inoculum size was selected as it induced no lethality over the 48 h duration of the experiment and the collection of haemolymph from dead larvae was impractical. Larvae were inoculated 2 h after infection with PBS, AMK (50 and 5 mg kg^{-1} for *M. fortuitum*, 50 and 2.5 mg kg^{-1} for *M. marinum*) or EMB (50 mg kg^{-1} for both species). Larvae were also mock-infected with 10 µl of PBS and then 2 h later with AMK (50, 5 or 2.5 mg kg^{-1}) or EMB (50 mg kg^{-1}) to assess the effects of these antibiotics on circulating haemocyte numbers in the absence of infection. Unmanipulated controls were included. Three larvae were randomly selected and injected with 75 µl *Galleria* saline [28] at 5, 18.5, 24 and 48 h p.i. Haemolymph was bled into individual sterile reaction tubes and 10 µl was loaded onto an improved Neubauer haemocytometer and the haemocytes counted with duplicates for each sample. This experiment was performed in triplicate to give n=9 for each experimental condition. Holm's correction was applied to multiple comparisons.

RESULTS

Infection of *G. mellonella* larvae with viable mycobacteria results in inoculum size-dependent lethality

Larvae were infected with a range of inoculum sizes of different mycobacteria. In all cases, with the exception of *M. aurum* NCTC 10437, the larval survival decreased as the inoculum size increased (Fig. 1a, b and d–f). *M. aurum* NCTC 10437 had no detrimental effect on larval survival at any of the inoculum sizes tested (Fig. 1c). Notably, heat-killed bacteria had no detrimental effect on larval survival in any of the experiments, indicating that larval death was caused by infection with viable *M. fortuitum* or *M. marinum*. There were variations between *M. fortuitum* and *M. marinum* virulence – *M. marinum* required fewer bacteria (~3×10^7 c.f.u. ml^{-1}) to decrease larval survival substantially, compared to *M. fortuitum*, which required up to 30 times more bacteria to have the same effect.

M. marinum NCTC 2275 was not selected for continued experimentation as of the three *M. marinum* strains used it had the weakest virulence. *M. aurum* NCTC 10437 was also not investigated further as the larvae were resistant to infection.

Administration of anti-mycobacterial antibiotics enhances the survival of *G. mellonella* larvae infected with mycobacteria

The MICs of a range of anti-mycobacterial antibiotics (AMK, CIP, EMB, INH and RIF) were measured for each of the mycobacteria to allow comparison of *in vitro* antibiotic susceptibility with efficacy of the same drugs versus infected larvae *in vivo*. The *in vitro* MICs are described in Table 1. The MICs varied widely for all antibiotics tested, even between strains of the same species.

Prior to studying the efficacy of the same antibiotics *in vivo*, larvae were administered a high dose (100 mg kg^{-1}) of each antibiotic alone to determine if any were toxic. Compared to larvae administered PBS alone, there was no evidence of toxicity to the larvae from any of the antibiotics – survival was 100 % 144 h p.i., and no melanization was observed on

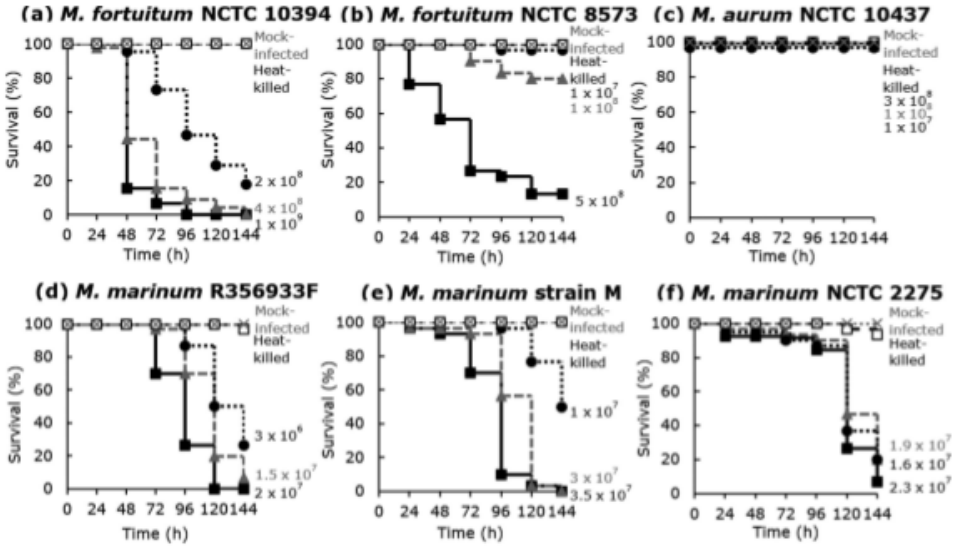

Fig. 1. Effect of infection with a range of mycobacteria species and strains on survival of *G. mellonella* larvae. The data are presented as Kaplan–Meir survival curves. The number of cells injected (10 µl) into each larva is shown beside each panel as colony-forming units (c.f.u.) per ml (highest cell concentrations, black filled squares; middle concentration, grey filled triangles; lowest concentration, black filled circles). Controls of larvae mock-infected with PBS (uninfected, grey crosses) and injected with heat-killed bacteria at an equivalent concentration to the highest c.f.u. ml^{-1} used (heat-killed, black open squares) are also shown. $n = 30$.

the larval body beyond the point of injection (data not shown).

The effects of a single dose 2 h p.i. of the same antibiotics (Table 1) on survival of *G. mellonella* larvae 144 h p.i. with mycobacteria are shown in Fig. 2. Complete Kaplan–Meier survival curves are available in the Supplementary Data (available in the online version of this article). To readily observe any therapeutic benefit from antibiotic treatment, the larvae were infected with an inoculum of each *Mycobacterium* known to result in a high level of mortality (Fig. 1).

Table 1. MICs of five antibiotics for *M. fortuitum* NCTC 10394, *M. fortuitum* NCTC 8573, *M. marinum* strain M and *M. marinum* R356933F using three independent biological replicates

	mg l^{-1}			
	M. fortuitum		*M. marinum*	
	NCTC 10394	NCTC 8573	Strain M	R356933F
AMK	0.12–0.25	0.12–0.25	1.0	2.0
CIP	0.008	0.05	0.5	0.25–0.5
EMB	2.0	>256	0.125–0.19	0.19–0.25
INH	1.0–2.0	>256	4.0–6.0	2.0–3.0
RIF	12.0	<0.016	6.0–8.0	0.38–0.5

AMK, amikacin; CIP, ciprofloxacin; EMB, ethambutol; INH, isoniazid; RIF, rifampicin.

The antibiotic doses administered were selected on the basis of pilot experiments that screened a wide range of doses for therapeutic benefit (data not shown). An upper threshold dose of 100 mg kg^{-1} was selected for all the antibiotics tested.

AMK was the most successful antibiotic tested, conferring significant therapeutic benefit against infections by all four mycobacteria tested (Fig. 2a). Comparison of the effectiveness of AMK *in vivo* with the *in vitro* MICs of AMK for each bacterial strain (Table 1) revealed poor correlation. For example, *M. marinum* R356933F and *M. marinum* strain M had MICs of 2 and 1 mg l^{-1}, respectively, for AMK. In contrast, the doses of AMK required to successfully treat the *in vivo* infections with the same strains were reversed, with a single-dose of only 10 mg kg^{-1} being required for the more resistant *M. marinum* R356933F and a higher dose of 25 mg kg^{-1} being required for the more sensitive *M. marinum* strain M.

CIP treatment (Fig. 2b) was effective for larvae infected with the *M. fortuitum* strains, but displayed little efficacy versus larvae infected with either strain of *M. marinum*. Unlike with AMK, this observation did correlate with the MIC values for each strain (Table 1), as both *M. fortuitum* strains were far more sensitive to CIP than the *M. marinum* strains.

EMB was the least effective antibiotic in these experiments – survival at 144 h was almost invariably indistinguishable from that for the mock-treated control (PBS) (Fig. 2c). The

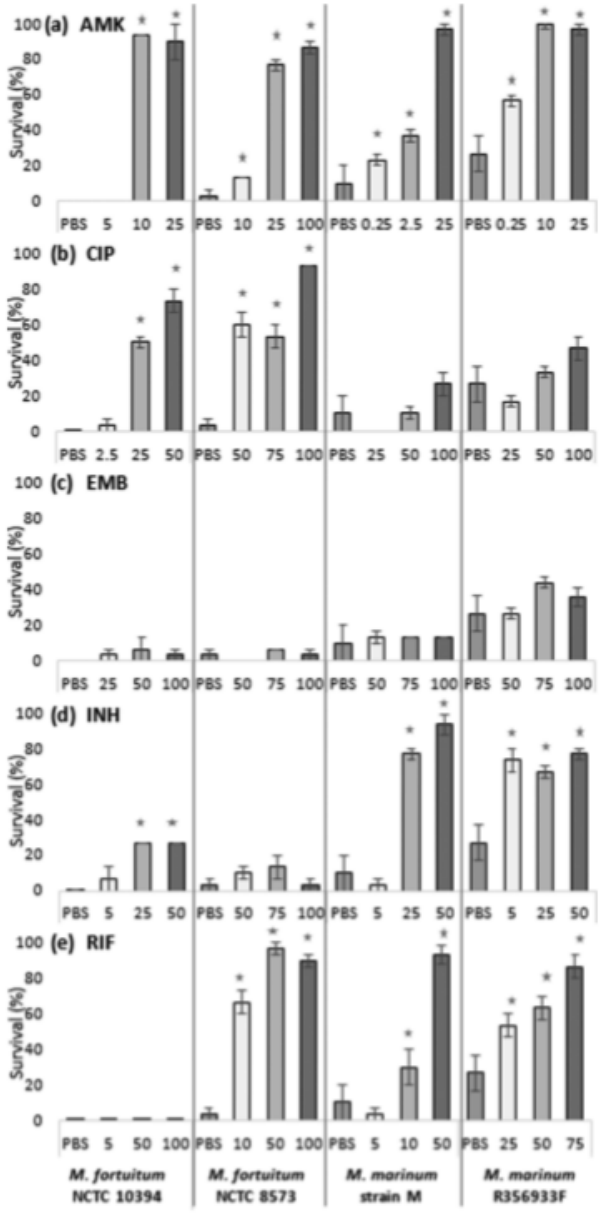

Fig. 2. Effect of the administration of a single dose of a range of antimycobacterial antibiotics on survival of *G. mellonella* larvae 144 h p.i. with four mycobacteria species and strains. Larvae were infected with viable bacteria and then treated 2 h p.i. (*M. fortuitum* NCTC 10394, 4×10^8 c.f.u. ml^{-1}; *M. fortuitum* NCTC 8573, 5×10^8 c.f.u. ml^{-1}; *M. marinum* strain M, 3×10^7 c.f.u. ml^{-1}; *M. marinum* R356933F, 9×10^8 c.f.u. ml^{-1}). Larval survival was monitored for 144 h p.i. The doses of antibiotic used are indicated below each bar in mg kg^{-1}. Each bar represents mean survival±SEM after 144 h. Mock-treated larvae were injected with PBS 2 h p.i. Any doses that conveyed a significant ($P<0.05$, log-rank test on complete Kaplan–Meier curves) increase in larval survival across the full 144 h observation are indicated with a star (*). Multiple comparisons were corrected with Holm's correction. $n=30$. AMK, amikacin; CIP, ciprofloxacin; EMB, ethambutol; INH, isoniazid; RIF, rifampicin.

correlation between the *in vitro* and *in vivo* efficacies of EMB was poor.

INH is largely ineffective for larvae infected with *M. fortuitum* NCTC 8573 (Fig. 2d), which is supported by the *in vitro* data (Table 1). Survival of larvae infected with *M. fortuitum* NCTC 10394 is statistically significant, and the MIC results suggest sensitivity to INH, but overall survival was low even at the highest doses tested. This contrasts with larvae infected with *M. marinum* – both strains responded positively to INH treatment, and high doses conveyed a long-lasting survival advantage, supported by the sensitivity to INH *in vitro*.

RIF significantly improved larval survival after infection with *M. marinum* (Fig. 2e) and this correlated with the *in vitro* MICs (Table 1). However, the *M. fortuitum* strains had differing responses to RIF. *Mycobacterium fortuitum* NCTC 10394 is unresponsive even at high doses, whereas larval survival significantly improved when *M. fortuitum* NCTC 8573 was treated with similar doses. Notably, this did correlate with the MICs because *M. fortuitum* NCTC 8573 was highly sensitive to RIF *in vitro*, whilst *M. fortuitum* NCTC 10394 had the highest MIC of any of the mycobacteria strains tested.

To conclude, in cases where the administered antibiotic enhanced larval survival *in vivo*, this occurred in a dose-dependent manner, with some doses resulting in near 100 % survival compared to mock-treated controls. However, not all antibiotics were efficacious *in vivo* and the degree of correlation between the *in vitro* sensitivity of the mycobacteria to the drugs and their ability to enhance larval survival *in vivo* was variable.

In subsequent experiments, *M. fortuitum* NCTC 10394 and *M. marinum* strain M were used, as they are the type strains for their species.

Administration of AMK, CIP or INH to larvae infected with mycobacteria reduces the overall bacterial burden within the larvae

The effect that efficacious doses of antibiotics had on the bacterial burden within the larvae was measured by enumerating viable bacteria in homogenates of larvae that had been exposed to bacteria and PBS or antibiotic. Doses of AMK, CIP and INH were selected that were previously shown to confer almost full survival (Fig. 2) on larvae infected with inoculum sizes of *M. fortuitum* NCTC 10394 or *M. marinum* strain M that were shown to be lethal to untreated larvae over a period of 144 h (Fig. 1).

Infection with *M. fortuitum* NCTC10394 (4.0×10^8 c.f.u. ml^{-1}) resulted in a large drop in viable bacteria over the first 5 h p.i. (Fig. 3a). In contrast, infection with *M. marinum* strain M (3.0×10^7 c.f.u. ml^{-1}) only led to a minor decrease in bacterial numbers over the same time period (Fig. 3b). This obvious difference in the ability of the innate immune system to eliminate the two mycobacteria strains could explain why larvae need a higher inoculum of *M. fortuitum* cells to cause significant larval death compared to the *M. marinum* species (Fig. 1). Nonetheless, viable mycobacteria were recovered from both mock (PBS) and

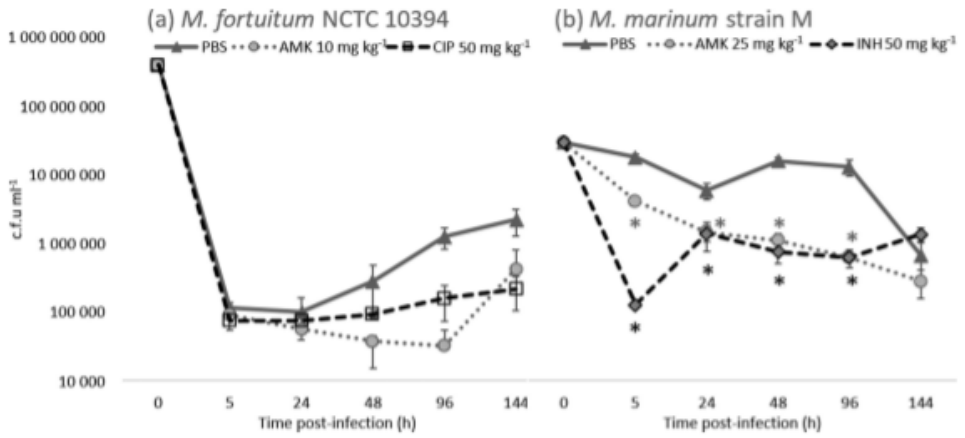

Fig. 3. The effect of infection with mycobacteria and subsequent mock-treatment with PBS or antibiotics on the bacterial burden within *G. mellonella* larvae. Larvae were infected with *M. fortuitum* NCTC 10394 (4×10^8 c.f.u. ml^{-1}) (a) and *M. marinum* strain M (3×10^7 c.f.u. ml^{-1}) (b). Antibiotics or PBS were administered 2 h p.i. at the doses indicated. Mock-treated larvae are represented by grey filled triangles. Larvae treated with amikacin, ciprofloxacin and isoniazid are represented with grey circles, open black squares and black hatched diamonds, respectively. The data show the mean±SEM, with each marker representing the average c.f.u. ml^{-1} for five larvae in each condition at each time point. Asterisks indicate significant differences between groups treated with an antibiotic and those that received PBS ($P<0.05$, Mann–Whitney U-test). Multiple comparisons were corrected with Holm's correction. AMK, amikacin; CIP, ciprofloxacin; INH, isoniazid.

antibiotic-treated larvae throughout the entire duration of the experiment (Fig. 3), despite the fact that the doses of antibiotics administered permitted almost full survival of infected larvae (Fig. 2). Bacterial burden is mitigated by treatment with the selected antibiotics, but viable bacteria were still detected 144 h p.i. with all antibiotics tested. At some time points, the difference in internal mycobacterial burden between antibiotic-treated and mock-treated larvae was significantly reduced, potentially accounting for the observed efficacy of these antibiotics (Fig. 2).

Infecting mycobacteria are phagocytosed by *G. mellonella* haemocytes, but phagocytosis is reduced by exposure to antibiotics

To determine the fate of mycobacteria in *G. mellonella* larvae, and how this was influenced by antibiotic therapy, bacterial phagocytosis was measured by microscopy (Fig. 4). The procedure required to label the mycobacteria with FITC meant that the infecting bacteria were dead (see the Methods section). Nonetheless, FITC-labelling permitted the visualization of internalized mycobacteria and a representative image of larval haemocytes that have phagocytosed bacteria is shown in Fig. 4(a). Groups of larvae were administered a single 10 µl dose of either PBS, 2.5, 5 or 50 mg kg^{-1} AMK, or 50 mg kg^{-1} EMB. These doses of AMK were selected because 2.5 and 5 mg kg^{-1} were shown to have no therapeutic benefit for infected larvae compared to 50 mg kg^{-1}, which resulted in almost complete survival (Fig. 2). Similarly, 50 mg kg^{-1} EMB was selected because this dose had little therapeutic benefit. In this way the effect of therapeutic and non-therapeutic doses of antibiotics on phagocytosis could be measured. After 10 min the larvae administered with either 10 µl PBS or antibiotics were then inoculated with FITC-labelled mycobacteria and phagocytosis was measured after 1 h at 37 °C (Fig. 4b).

In larvae exposed to PBS only, over 40 % of haemocytes compared to 21 % had engulfed one or more *M. fortuitum* NCTC 10394 or *M. marinum* strain M cells, respectively (Fig. 4b). This reflected the higher infecting inoculum of *M. fortuitum* NCTC 10394 (4.0×10^8 c.f.u. ml^{-1}) compared to *M. marinum* strain M (3.0×10^7 c.f.u. ml^{-1}). For both strains, exposure to a dose of AMK that was shown to be fully efficacious (50 mg kg^{-1}: Fig. 2) resulted in a significant reduction in phagocytosis compared to the PBS-treated controls. Phagocytosis dropped to 21 and 6.9 % for *M. fortuitum* NCTC 10394 or *M. marinum* strain M cells, respectively. The effect of non-efficacious doses of AMK and EMB on phagocytosis differed between *M. fortuitum* NCTC 10394 and *M. marinum* strain M cells. For *M. fortuitum* NCTC 10394, these doses had no

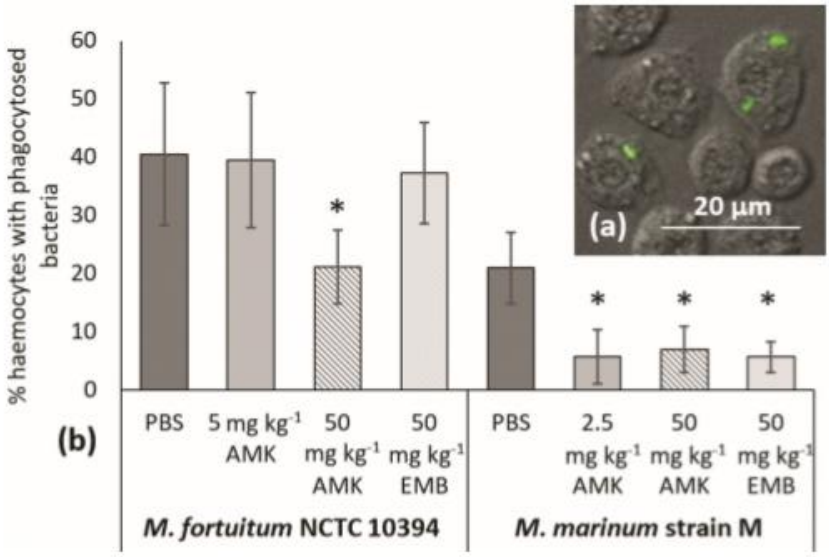

Fig. 4. Effect of exposure to PBS or antibiotics on phagocytosis of FITC-labelled mycobacteria by *G. mellonella* haemocytes. (a) A typical image of an optical slice through *G. mellonella* haemocytes showing internalized fluorescent *M. marinum*. (b) The proportion of larval haemocytes with phagocytosed *M. fortuitum* NCTC 10394 or *M. marinum* strain M after 1 h of incubation in the presence of efficacious or non-efficacious doses of AMK (50 mg kg^{-1}, or 2.5 or 5 mg kg^{-1}, respectively) or a non-efficacious dose of EMB (50 mg kg^{-1}) and compared to a mock-treated control (PBS). The data are from 10 larvae, with 30–70 haemocytes examined per larvae and mean±SEM, n=10. (*) indicates significant difference between groups treated with an antibiotic compared with those that received PBS. P<0.05, unpaired, two-tailed Student's t-test with Holm's correction applied for multiple comparisons. AMK, amikacin; EMB, ethambutol.

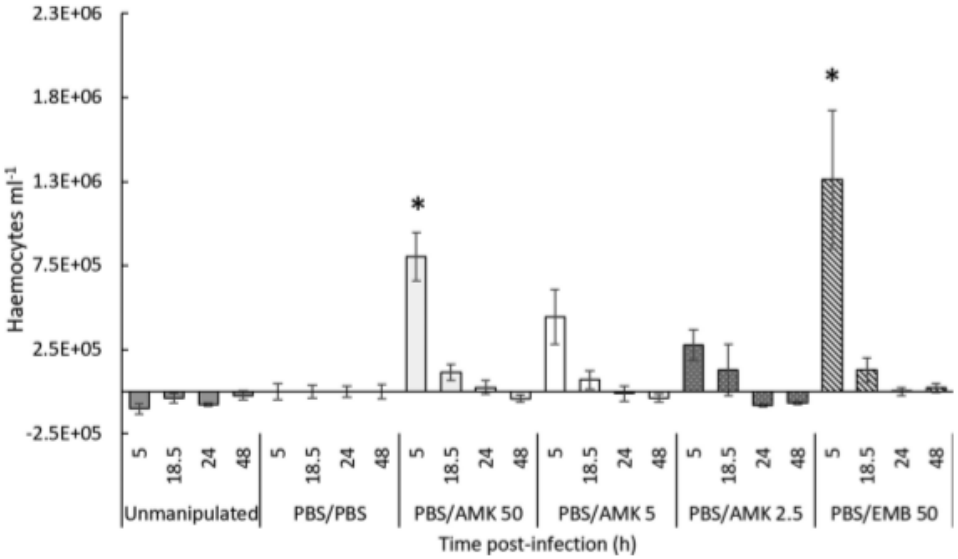

Fig. 5. Effect of exposure to antibiotics on overall numbers of circulating *G. mellonella* haemocytes. Larvae were mock-infected with PBS and this was followed 2 h later by administration of PBS or doses of AMK (2.5, 5 or 50 mg kg^{-1}) or EMB (50 mg kg^{-1}). The numbers of haemocytes in the larval haemolymph were then counted at 5, 18.5, 24 and 48 h post the initial mock-infection with PBS. The number of haemocytes is presented relative to larvae mock-infected and treated with PBS. The data are from nine larvae per time-point, showing the mean±SEM. (*) indicates significant difference between groups administered antibiotics compared with those administered PBS. $P<0.05$, unpaired, two-tailed Student's *t*-test with Holm's correction applied for multiple comparisons. AMK, amikacin; EMB, ethambutol.

impact on phagocytosis compared to larvae exposed to PBS. In contrast, ineffective doses of 2.5 mg kg^{-1} AMK and 50 mg kg^{-1} EMB reduced phagocytosis of *M. marinum* strain M cells to a similar extent as the efficacious dose of 50 mg kg^{-1} AMK (Fig. 4b).

In summary, the larval response in terms of phagocytosis was dependent on the infecting species of mycobacteria, and phagocytosis was also reduced by exposure to both efficacious and non-efficacious doses of antibiotics in a species-dependent fashion.

Administration of antibiotics to uninfected larvae and larvae infected with mycobacteria induces a significant increase in the number of circulating haemocytes

The reduction in phagocytosis induced by exposure to antibiotics could perhaps be explained by changes in the overall numbers of circulating haemocytes. Thus, the effect of exposure to the same doses of AMK and EMB administered in Fig. 4 on circulating haemocytes was measured using microscopy (Figs 5 and 6).

Larvae were mock-infected with sterile PBS and then injected 2 h later with PBS, AMK or EMB. Relative to PBS treatment, exposure to all doses of each of the antibiotics resulted in a significant, transient increase in the number of circulating haemocytes within the 3 h after antibiotic administration that disappeared by 24 h (Fig. 5). The transient increase in haemocyte number induced by AMK after 5 h was dose-dependent.

Similarly, when larvae were infected with a viable inoculum of *M. fortuitum* NCTC 10394 and *M. marinum* strain M cells, followed by administration of PBS or the same antibiotic doses of AMK and EMB used previously (Figs 4 and 5), the circulating haemocyte numbers were again significantly higher after 5 h than in larvae mock-infected with PBS (Fig. 6). Mock-treatment with PBS also resulted in an increase in haemocytes, but this was much smaller than that induced by antibiotic treatment. As before, the antibiotic-induced increase in haemocyte numbers peaked at 5 h and declined noticeably thereafter (Fig. 6).

In summary, the previous data revealing that antibiotics reduced the phagocytic rate of FITC-labelled dead mycobacteria (Fig. 4) could perhaps be explained by the increase in circulating haemocyte numbers that was induced by exposure to the antibiotics alone (Fig. 5) or by antibiotics administered to larvae infected with viable mycobacteria (Fig. 6). For example, increased numbers of haemocytes could mean that many may not encounter bacteria to phagocytose, thereby reducing the overall quantity of haemocytes containing bacteria.

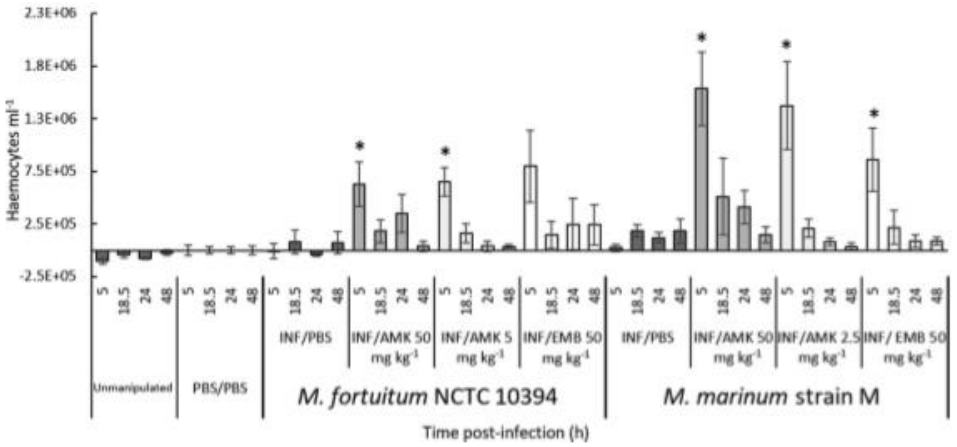

Fig. 6. Effect of infection with mycobacteria and subsequent treatment with PBS or antibiotics on overall numbers of circulating *G. mellonella* haemocytes. Larvae were infected with *M. fortuitum* NCTC 10394 or *M. marinum* strain M (1.0×10^7 c.f.u. ml^{-1}) and then 2 h p.i. were treated with efficacious or non-efficacious doses of AMK (50 mg kg^{-1}, or 2.5 or 5 mg kg^{-1}, respectively) or a non-efficacious dose of EMB (50 mg kg^{-1}) or PBS as a mock-treated control. The numbers of haemocytes in the larval haemolymph were then counted at 5, 18.5, 24 and 48 h post-infection. The number of haemocytes is presented relative to larvae mock-infected and treated with PBS. (*) indicates significant difference between groups treated with antibiotics compared to those administered PBS. $P<0.05$, unpaired, two-tailed Student's *t*-test with Holm's correction applied for multiple comparisons. The data are from nine larvae per time-point and show the mean±SEM. AMK, amikacin; EMB, ethambutol.

Antibiotic combination treatments provide greater therapeutic benefit to larvae infected with mycobacteria than antibiotic monotherapy

Treatment of *M. tuberculosis* infections usually involves the administration of combinations of antibiotics for optimal therapy. To mimic combination therapy with infected *G. mellonella* larvae, and to observe whether typical combination treatments were also more efficacious in this model system, three antibiotic combinations were selected using WHO treatment guidelines [29]. Thus, the therapeutic benefit conferred by three multiple-drug combinations was studied: one triple combination (EMB+INH+ RIF) and two quadruple combinations (AMK+EMB+INH+RIF and CIP+EMB+INH+RIF) (Fig. 7). The doses of the individual antibiotics administered within the combination regimens were selected on the basis that they provided minimal therapeutic benefit when given as monotherapy to larvae infected with mycobacteria (Fig. 2). When these non-efficacious doses were combined, larval survival improved significantly compared to mock-treated groups (PBS) (Fig. 7) and to larvae administered with a dose of each individual drug (Fig. 2). Thus, the larval model reflects the enhanced efficacy of antibiotics administered in combinations as practised in clinical settings.

DISCUSSION

The data reported here show that *G. mellonella* larvae could be employed as a valuable *in vivo* infection model for several mycobacteria species and allow the therapeutic effects of antibiotic treatments to be quantified.

The majority of the mycobacteria examined can kill *G. mellonella* larvae, and the number of viable infecting bacteria correlates negatively with larval survival. The exception is *M. aurum* NCTC 10394, although *M. aurum* is exclusively reported as being infectious in immunocompromised humans [30, 31] (unlike *M. fortuitum* [32, 33] and *M. marinum* [14, 34]), so non-pathogenicity in immunocompetent larvae is perhaps unsurprising.

Ethambutol, isoniazid and rifampicin were selected for their historic inclusion in standard TB treatment regimens [35], and amikacin and ciprofloxacin were selected for their use as second-line treatments for MDR-TB [36]. All five have been used with *M. fortuitum* [32, 37–41] and *M. marinum* [14, 42, 43] *in vitro* and *in vivo*, although the *in vivo* experiments have predominantly used combination treatments. When used to treat human infections, amikacin can be used at 15 mg kg^{-1} for *M. fortuitum* infections [32] or 400 mg daily for *M. marinum* [44]. Ciprofloxacin has been used at approximately 8 mg kg^{-1} for *M. fortuitum* infections [45] and 500 mg twice daily for *M. marinum* [46]. Ethambutol, isoniazid and rifampicin are not prescribed for *M. fortuitum* infections, although daily doses of ethambutol at 25 mg kg^{-1} and rifampicin at 600 mg are a well-established treatment for *M. marinum* [47], and isoniazid at 10 mg kg^{-1} has been shown to be an effective

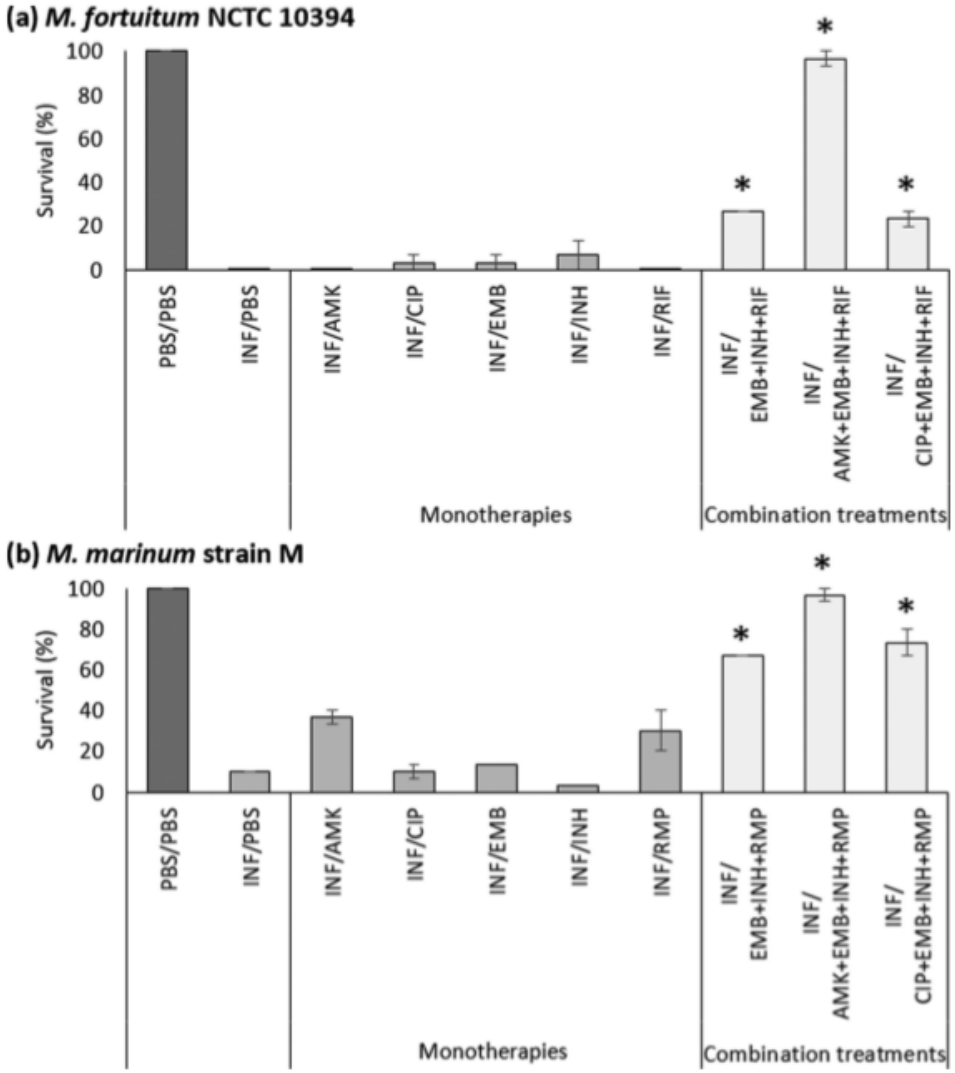

Fig. 7. Effect of antibiotic monotherapy or combination therapy on survival of *G. mellonella* larvae 144 h p.i. with mycobacteria. Larvae were infected with *M. fortuitum* NCTC 10394 (4×10^8 c.f.u. ml^{-1}) (a) or *M. marinum* strain M (3×10^7 c.f.u. ml^{-1}) (b), followed by treatment 2 h p.i. with a single dose of either PBS, antibiotic monotherapy or antibiotic combinations. The antibiotic monotherapy doses for *M. fortuitum* were 5 mg kg^{-1} AMK, 2.5 mg kg^{-1} CIP, 100 mg kg^{-1} EMB, 5 mg kg^{-1} INH and 30 mg kg^{-1} RIF, and for *M. marinum* they were 2.5 mg kg^{-1} AMK, 50 mg kg^{-1} CIP, 100 mg kg^{-1} EMB, 5 mg kg^{-1} INH and 10 mg kg^{-1} RIF. The combination treatments consisted of the same drug concentrations as above, but pooled to a make a single combination dose. The data show the mean survival ±SEM after 144 h. * indicates a significant increase in survival for combination therapy compared to each individual monotherapy. $P<0.05$, log-rank test on complete Kaplan–Meier curves with Holm's correction applied for multiple comparisons.

treatment for *M. marinum* in zebrafish [48]. Whilst the efficacious doses of these drugs in *G. mellonella* larvae were different, they were at least comparable and of a similar order of magnitude.

The effective antibiotics generally improved larval survival in a dose-dependent manner compared with mock-treated larvae (Fig. 2). However, varied sensitivity to each antibiotic was observed between strains and several antibiotics had no

therapeutic effect at the highest dose tested. Combination therapies that included some of the ineffective antibiotics improved overall efficacy – some combinations gave almost 100 % larval survival across the 144 h experiment. Considering the necessity of employing antibiotic combinations when treating patients infected with mycobacteria, this result is key to indicating the potential role of *G. mellonella* in testing further novel combination treatments. In addition, measurement of the bacterial burden (Fig. 3) indicated that efficacious doses of antibiotics that permitted almost full survival of the infected larvae only reduced the overall numbers of bacteria within the larvae without eliminating them. This implies that a certain threshold number of mycobacteria are required to cause larval death and that the larvae are readily able to survive despite still being infected with bacterial numbers that are below this apparent lethal threshold. It is not clear from this work what contribution the direct inhibitory action of the drugs on the infecting bacteria makes to the measured efficacy of the antibiotics, and what contribution is made by the apparent stimulation of the innate immune system (in the form of increased haemocyte numbers; Figs 5 and 6) that is triggered upon exposure to the antibiotics. It would seem likely that both actions contribute to the measured efficacy of the drugs.

Human phagocytic cells have been assessed against a multitude of antimicrobials and pathogens to determine whether phagocytosis is enhanced, supressed or unaffected (reviewed in [49]), and is it well established that a number of medications can reduce [50] or increase [51] the number of circulating immune cells in humans. A reduction in the number of circulating white blood cells is a very rare side-effect when using anti-tuberculosis medications [52] – there is no suggestion that any of the drugs in this study cause a reliable change in the number of circulating immune cells in humans. Interactions between antibiotics and the larval immune system may explain why antibiotics cause such a significant increase in the number of circulating haemocytes in the hours post-injection (Fig. 5). A similar response has been shown when using antifungals with *G. mellonella* elsewhere [53, 54]. Given that the increase is not seen when larvae are mock-treated with PBS, this suggests that antimycobacterials trigger a non-specific immune response. Increased numbers of circulating haemocytes has been shown to correlate with improved larval survival for fungal infections [55], yet here the antibiotic EMB increased haemocyte density but did little to improve larval survival, so this correlation may not be universally applicable.

The lack of correlation between the *in vitro* efficacy of the antibiotics (Table 1) and the *in vivo* data (Fig. 2) is not necessarily detrimental to the conclusions of this study. A useful application of this model is to test drugs that have only been examined *in vitro* to determine possible variances in the *in vivo* results, and it is vital not to overly extrapolate *in vitro* data to predict *in vivo* results, as there are often discrepancies between the two.

The existing whole-organism models for mycobacterial, particularly *M. tuberculosis*, infections include mice, rats, guinea pigs, rabbits, cattle and primates. However, all of these present problems with cost, housing requirements, operating regulations and ethical concerns. Non-mammalian models can also be a useful tool. For example, zebrafish are not natural hosts of *M. tuberculosis*, but *M. marinum* infections eventually produce lipid-rich and necrotic granulomas [56] in adult and larval fish, despite the immature fish larvae having no adaptive immune system at that developmental stage [10].

Drosophila melanogaster (fruit fly) is becoming a well-established model for *M. marinum* [57]. Infected flies show evidence of 'wasting' [58], a key symptom in humans. Fly haemocytes engulf *M. marinum* that replicate inside the haemocytes and are eventually released and then found extracellularly to the host cells [59] similar to *M. tuberculosis* with human immune cells. Unfortunately, fruit flies are reared at 18–29 °C and are difficult to inoculate with specific volumes. Circulating haemocytes can only be collected from fly larvae, [60], meaning that the short-term effects of infection on circulating haemocytes can only be examined in larvae – long-term experiments must use adult flies [59].

For these reasons, *Galleria mellonella* larvae are an attractive model organism for screening novel compounds against mycobacterial infections. They thrive at 37 °C, can be easily and consistently inoculated with specific quantities of bacteria and drugs, and haemocytes can be collected from their haemolymph long after infection. Similarly to *D. melanogaster*, experiments are limited to the life cycle of the larvae, and larvae are unlikely to be of use in persistence models of latent TB infections. However, considering the pressure to locate new and novel compounds to treat mycobacterial infections, especially drug-resistant strains, having access to a convenient *in vivo* model early in the drug discovery and development timeline could be invaluable. We suggest that *G. mellonella* would be a valuable tool for testing compounds with efficacy against mycobacteria and may provide useful evidence to support further work with clinically relevant cell lines or small-mammal trials.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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