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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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.
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 144h. The effects of these anti-mycobacterial drugs on phagocytosis and circulating hemocyte 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* 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 hemocytes and reduced the phagocytic rate of hemocytes 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 (TB) is now a global challenge with around 480,000 new MDR-TB cases 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* [12, 13], *M. marinum* [14, 15] and *M. aurum* [8, 11] 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 bacteria have no reporting requirements, but estimates of incidence are 4.65-5.99 cases per million persons for *M. fortuitum* [16] and 0.05-0.27 case per 100,000 adult patients for *M. marinum* [17]. These mycobacteria have similar host-pathogen interactions [7], cell wall profiles [8], membrane and efflux pump proteins [9], virulence genes [10] and drug resistance profiles [11] compared with *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 discovery of novel therapies for mycobacteria. Using wax moth larvae, *Galleria mellonella*, as an in vivo model to assess the virulence of 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 means they are accessible to most laboratories. Many experiments require minimal “specialised” 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 that 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 production of at least 18
antimicrobial peptides [20]. Vertebrate white blood cells and invertebrate hemocytes are not homologous, however they do have analogous roles including wound repair [21], cell clustering around foreign bodies [22], innate immunity [23], phagocytosis [24], and 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 advantage of using a model organism which negates the requirement of specialised 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 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 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.
**Materials and Methods**

**Reagents and larvae**

Reagents were purchased from Sigma-Aldrich Ltd (Dorset, UK) unless stated otherwise. Antibiotics were dissolved in sterile deionised 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). *M. marinum* R356933F was kindly provided by Michael Smith (Scottish Mycobacteria Reference Laboratory, Royal Infirmary of Edinburgh). Bacteria were cultured in a shaking incubator using Mueller-Hinton M7H9 broth enriched with albumin dextrose catalase (ADC) overnight at 37°C for *M. aurum* and *M. fortuitum* strains, and at 27°C for approximately 36h for *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.

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 minutes 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 manufacturer instructions. Cells were plated at 2.5 x 10⁶ c.f.u per 90mm plate and incubated at 27°C or 37°C as appropriate. The
MIC was performed in duplicate and determined as the point on the strip where bacteria no longer grew when in contact with the paper.

**Infection of G. mellonella larvae**

Experiments were performed in duplicate ($n = 15$) and 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 24h for 144h and recorded as dead when no longer responsive to touch. Survival was plotted as Kaplan-Meir survival curves and comparisons made using log-rank tests. Holm’s corrections for multiple comparisons [26] was applied.

Antibiotic toxicity was assessed by injecting larvae with 100 mg/kg of each and recording survival and levels of melanisation on the larval body for 144h. For antibiotic efficacy experiments, larvae were infected then given single treatments of antibiotic or PBS (mock-treated control) 2h post-infection. 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 $p \leq 0.05$ was considered significant and Holm’s correction was always applied to account for multiple comparisons.

**Measurement of the internal burden of mycobacteria inside infected larvae**

Forty larvae were infected with 10 µL of $4 \times 10^5$ c.f.u/mL *M. fortuitum* NCTC 10394 or $3 \times 10^7$ c.f.u/mL *M. marinum* Strain M. A single dose of antibiotic (10 mg/kg AMK or 50 mg/kg CIP for *M. fortuitum*, and 25 mg/kg AMK or 50 mg/kg INH for *M. marinum*) or PBS (mock-treated control) was administered 2h post-infection. Five larvae were randomly selected from each group and assessed for internal bacterial burden at 5, 24, 48, 96 and 144h post-infection.
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 microorganisms present in the larval gut but did not affect recovery of 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 then incubated at 27°C or 37°C as appropriate for 24 – 72h 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 FITC (FITC dissolved in 0.2M Na₂CO₃, 0.2M NaHCO₃, pH 9), and incubated in a 27°C shaking incubator for 30 minutes before washing three times with PBS.

Larvae received injections of PBS, AMK (50 and 5 mg/kg for *M. fortuitum*, 50 and 2.5 mg/kg for *M. marinum*) or EMB (50 mg/kg) as required, 10 minutes before injection with FITC-stained bacteria (4 x 10⁸ c.f.u/mL for *M. fortuitum*, 3 x 10⁷ c.f.u/mL for *M. marinum*). Larvae were incubated for 1h then injected with 30 µL Galleria saline [28] saturated with phenylthiol urea (PTU) and bled into individual sterile reaction tubes. The hemolymph was transferred to wells in a 24-well plate prefilled with 300 µL ice-cold Grace’s insect medium and 13mm round glass coverslips. The plate was incubated for 1h in the dark in a 27°C low speed shaking incubator. The supernatant was removed and 1 mL 3.7% formaldehyde added for 20 seconds, then 300 µL 0.4% trypan blue in PBS for 20 minutes. The coverslips were washed three times with PBS then mounted for examination at 40X magnification using a Delta Vision fluorescence microscope using differential interference contrast and filters for FITC (excitation 490 nm/emission 528 nm). Hemocytes were imaged through an appropriately sized z-stack to allow counting of internalised bacteria.
Ten larvae were examined for each condition, with 30 to 70 hemocytes counted per larvae. Phagocytic rate was calculated as the percentage of total hemocytes that had engulfed one or more bacteria. Multiple comparisons were assessed with Holm’s correction.

**Determination of circulating hemocyte numbers**

Adapted from Harding *et al.* [13]. 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 x 10^7 c.f.u/mL. This inoculum size was selected as it induced no lethality over the 48 h duration of the experiment because the collection of haemolymph from dead larvae was impractical. Following infection, larvae were inoculated 2h later with PBS, AMK (50 and 5 mg/kg for *M. fortuitum*, 50 and 2.5 mg/kg for *M. marinum*) or EMB (50 mg/kg for both species). Larvae were also mock-infected with 10 µL of PBS followed 2h later with AMK (50, 5 or 2.5 mg/kg) or EMB (50 mg/kg) to assess the effects of these antibiotics on circulating hemocyte 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 hours post-infection. Hemolymph was bled into individual sterile reaction tubes and 10 µL was loaded onto an improved Neubauer haemocytometer and the hemocytes 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, except *M. aurum* NCTC 10437, as the inoculum size increased larval survival was reduced (Fig 1a, b, d, e and 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 all 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 x 10^7 c.f.u/mL) 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 larvae were resistant to infection.

Administration of anti-mycobacterial antibiotics enhances 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. For all antibiotics tested, the MICs varied widely 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) 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% 144h post-injection, and no melanisation was observed on the larval body beyond the point of injection (data not shown).

The effects of a single dose 2h post-infection of the same antibiotics (Table 1) on survival of G. mellonella larvae 144h post-infection with mycobacteria are shown in Fig. 2. Complete Kaplan–Meier survival curves are available in Supplementary Data. To readily observe any therapeutic benefit of antibiotic treatment, larvae were infected with an inoculum of each Mycobacterium known to result in a high level of mortality (Fig 1). 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 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 for AMK of 2 and 1 mg/L respectively. 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 10mg/kg required for the more resistant M. marinum R356933F and a higher dose of 25 mg/kg 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 144h was almost invariably indistinguishable from the mock-treated control (PBS) (Fig. 2c). The 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 x 10^8 c.f.u/mL) 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 x 10^7 c.f.u/mL) led to only 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 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 144h post-infection 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* hemocytes 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 Methods). Nonetheless, FITC-labelling permitted the visualisation of internalised mycobacteria and a representative image of larval hemocytes that have phagocytosed bacteria is shown in Fig 4a. Groups of larvae were then administered a single 10 µL dose of either PBS, 2.5, 5 or 50 mg/kg AMK or 50 mg/kg EMB. These doses of AMK were selected because 2.5 and 5 mg/kg was shown to have no therapeutic benefit on infected larvae compared to 50 mg/kg that resulted in almost complete survival.
(Fig 2). Similarly, 50 mg/kg 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 hemocytes 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 x 10^8 c.f.u/mL) compared to *M. marinum* Strain M (3.0 x 10^7 c.f.u/mL). For both strains, exposure to a dose of AMK that was shown to be fully efficacious (50 mg/kg: 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 impact on phagocytosis compared to larvae exposed to PBS. In contrast, ineffective doses of 2.5 mg/kg AMK and 50 mg/kg EMB significantly reduced phagocytosis of *M. marinum* Strain M cells to a similar extent as the efficacious dose of 50 mg/kg AMK (Fig 4b).

In summary, the larval response in terms of phagocytosis was dependent on the infecting species of mycobacteria and phagocytosis was reduced by exposure to both efficacious and non-efficacious doses of antibiotics also 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 hemocytes.**

The reduction in phagocytosis induced by exposure to antibiotics could perhaps be explained by changes in the overall numbers of circulating hemocytes. Thus, the effect of exposure to the same doses of AMK and EMB administered in Fig 4 on circulating hemocytes was measured using microscopy (Fig 5 and 6).
Larvae were mock-infected with sterile PBS 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 hemocytes within the 3h after antibiotic administration that disappeared by 24 h. The transient increase in hemocyte 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 (Fig 4 and 5), 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 hemocytes but this was much less 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 hemocyte 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 hemocytes could mean that many may not encounter bacteria to phagocytose, thereby reducing the overall quantity of hemocytes containing bacteria.

**Antibiotic combination treatments provide greater therapeutic benefit to larvae infected with mycobacteria than antibiotic monotherapy.**

Treatment of *M. tuberculosis* infections usually involves 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 doses of each drug individually (Fig. 2). Thus, larval model reflects the enhanced efficacy of antibiotics administered in combinations as practiced in clinical settings.
Discussion

The data reported here shows 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 numbers of viable infecting bacteria correlates negatively with larval survival. The exception is *M. aurum* NCTC 10394, although *M. aurum* is exclusively reported as infectious in immuno-compromised humans [30, 31] (unlike *M. fortuitum* [32, 33] and *M. marinum* [17, 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 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* [17, 42, 43] *in vitro* and *in vivo*, although *in vivo* experiments predominantly use combination treatments. When used to treat human infections, amikacin can be used at 15 mg/kg for *M. fortuitum* infections [32] or 400 mg daily for *M. marinum* [44]. Ciprofloxacin has been used at approximately 8 mg/kg 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 and rifampicin at 600 mg is a well-established treatment for *M. marinum* [47] and isoniazid at 10 mg/kg has been shown to be an effective 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 therapy that included some of the ineffective antibiotics improved overall efficacy – some combinations gave almost 100% larval survival across the 144h 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 to the measured efficacy of the antibiotics is due to the direct inhibitory action of the drugs on the infecting bacteria, and what contribution is due to the apparent stimulation of the innate immune system (in the form of increased hemocyte numbers; Fig 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 if phagocytosis is enhanced, suppressed 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 hemocytes in the hours post-injection (Fig. 5). A similar response using antifungals has been shown with *G. mellonella* elsewhere [53, 54]. Considering 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 hemocytes has been shown to correlate with improved larval survival for fungal infections [55], yet here, the antibiotic EMB...
increased hemocyte 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 which 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.

Existing whole-organism models for mycobacterial, particularly *M. tuberculosis*, infections include mice, rats, guinea pigs, rabbits, cattle, and primates. However, all 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 [15].

*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 hemocytes engulf *M. marinum* which replicate inside the hemocytes and are eventually released, 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 hemocytes can only be collected from fly larvae, [60], meaning that the short term effects of infection on circulating hemocytes can only be examined in larval flies – 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 hemocytes can be collected from their hemolymph 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: None to declare


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Figure 1. Effect of infection with a range of mycobacteria species and strains on survival of *G. mellonella* larvae. The data is 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 used (heat killed, black open squares) are also shown. n = 30.

Figure 2. Effect of administration of a single dose of a range of antimycobacterial antibiotics on survival of *G. mellonella* larvae 144h post-infection with four mycobacteria species and strains. Larvae were infected with viable bacteria then treated 2 hours post-infection (M. *fortuitum* NCTC 10394, 4 x 10^8 c.f.u/mL; M. *fortuitum* NCTC 8573, 5 x 10^8 c.f.u/mL; M. *marinum* Strain M, 3 x 10^7 c.f.u/mL; M. *marinum* R356933F, 9 x 10^8 c.f.u/mL). Larval survival was monitored for 144h post-infection. The doses of antibiotic used are indicated below each bar in mg/kg. Each bar represents mean survival ± SEM after 144h. Mock-treated larvae were injected with PBS 2 hours post-infection. Any doses which conveyed a significant (*p* < 0.05, Log-Rank test on complete Kaplan-Meier curves) increase in larval survival across the full 144h 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.

Figure 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 x 10^8 c.f.u/mL) (a) and *M. marinum* Strain M (3 x 10^7 c.f.u/mL) (b). Antibiotics or PBS were administered 2h post-infection 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. Data shows mean ± SEM with each marker representing the average c.f.u/mL 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.

Figure 4. Effect of exposure to PBS or antibiotics on phagocytosis of FITC-labelled mycobacteria by *G. mellonella* hemocytes. Panel (a) represents a typical captured image of an optical slice through *G. mellonella* haemocytes showing internalised, fluorescent *M. marinum*. The proportion of larval hemocytes with phagocytosed *M. fortuitum* NCTC 10394 or *M. marinum* Strain M after 1h of incubation in the presence of efficacious or non-eficacious doses of AMK (50 mg/kg; 2.5 or 5 mg/kg, respectively) or a non-eficacious dose of EMB (50 mg/kg) and compared to a mock-treated control (PBS) is shown in (b). Data is from 10 larvae, 30 – 70 hemocytes examined per larvae, with mean ± SEM. n = 10. (*p* < 0.05, unpaired, two-tailed Student’s t-test with Holm’s correction applied for multiple comparisons). AMK, amikacin; EMB, ethambutol.
Figure 5. Effect of exposure to antibiotics on overall numbers of circulating *G. mellonella* hemocytes. Larvae were mock-infected with PBS followed 2 hours later by administration of PBS or doses of AMK (2.5, 5 or 50 mg/kg) or EMB (50 mg/kg). Numbers of hemocytes in the larval hemolymph were then counted at 5, 18.5, 24 and 48 h post the initial mock-infection with PBS. The number of hemocytes is presented relative to larvae mock-infected and treated with PBS. Data is from 9 larvae per time-point showing mean ± SEM. (*p* < 0.05, unpaired, two-tailed Student’s *t*-test with Holm’s correction applied for multiple comparisons) AMK, amikacin; EMB, ethambutol.

Figure 6. Effect of infection with mycobacteria and subsequent treatment with PBS or antibiotics on overall numbers of circulating *G. mellonella* hemocytes. Larvae were infected with *M. fortuitum* NCTC 10394 or *M. marinum* Strain M (1.0 x 10^7 c.f.u/mL) then 2 h post-infection were treated with efficacious or non-efficacious doses of AMK (50 mg/kg; 2.5 or 5 mg/kg, respectively) or a non-efficacious dose of EMB (50 mg/kg) or PBS as a mock-treated control. Numbers of hemocytes in the larval hemolymph were then counted at 5, 18.5, 24 and 48 h post-infection. The number of hemocytes is presented relative to larvae mock-infected and treated with PBS. (*p* < 0.05, unpaired, two-tailed Student’s *t*-test with Holm’s correction applied for multiple comparisons). Data is from 9 larvae per time-point showing mean ± SEM. AMK, amikacin; EMB, ethambutol.

Figure 7. Effect of antibiotic monotherapy or combination therapy on survival of *G. mellonella* larvae 144 h post-infection with mycobacteria. Larvae were infected with *M. fortuitum* NCTC 10394 (4 x 10^8 c.f.u/mL) (a) or *M. marinum* Strain M (3 x 10^7 c.f.u/mL) (b) followed by treatment 2 h post-infection with a single dose of either PBS, antibiotic monotherapy or antibiotic combinations. Antibiotic monotherapy doses for *M. fortuitum* were 5 mg/kg AMK, 2.5 mg/kg CIP, 100 mg/kg EMB, 5 mg/kg INH and 30 mg/kg RIF, and for *M. marinum* were 2.5 mg/kg AMK, 50 mg/kg CIP, 100 mg/kg EMB, 5 mg/kg INH and 10 mg/kg RIF. The combination treatments consisted of the same drug concentrations as above but pooled to make a single, combination dose. Data shows mean survival ± SEM after 144 h. * indicates a significant increase in survival of 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). Controls of larvae mock-infected with PBS are also shown. n = 30. AMK, amikacin; CIP, ciprofloxacin; EMB, ethambutol; INH, isoniazid; RIF, rifampicin.
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.

<table>
<thead>
<tr>
<th></th>
<th><em>M. fortuitum</em></th>
<th><em>M. marinum</em></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>NCTC 10394</td>
<td>NCTC 8573</td>
</tr>
<tr>
<td>AMK</td>
<td>0.12 – 0.25</td>
<td>0.12 – 0.25</td>
</tr>
<tr>
<td>CIP</td>
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<tr>
<td>EMB</td>
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</tr>
<tr>
<td>INH</td>
<td>1.0 – 2.0</td>
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</tr>
<tr>
<td>RIF</td>
<td>12.0</td>
<td>&lt;0.016</td>
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</tbody>
</table>

AMK, amikacin; CIP, ciprofloxacin; EMB, ethambutol; INH, isoniazid; RIF, rifampicin.
Figure 1

(a) *M. fortuitum* NCTC 10394  (b) *M. fortuitum* NCTC 8573  (c) *M. aurum* NCTC 10437

(d) *M. marinum* R356933F  (e) *M. marinum* Strain M  (f) *M. marinum* NCTC 2275
Figure 2

(a) AMK
(b) CIP
(c) EMB
(d) INH
(e) RIF

Survival (%) for different strains of Mycobacteria and antibiotics at various concentrations.
Figure 3

(a) *M. fortuitum* NCTC 10394

(b) *M. marinum* Strain M

<table>
<thead>
<tr>
<th>Time post-infection (h)</th>
<th>CFU/mL</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
</tr>
<tr>
<td>24</td>
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<tr>
<td>48</td>
<td></td>
</tr>
<tr>
<td>96</td>
<td></td>
</tr>
<tr>
<td>144</td>
<td></td>
</tr>
</tbody>
</table>

- PBS
- AMK 10 mg/kg
- CIP 50 mg/kg
- AMK 25 mg/kg
- INH 50 mg/kg

* denotes statistical significance.
**Figure 4**

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>5 mg/kg AMK</th>
<th>50 mg/kg AMK</th>
<th>50 mg/kg EMB</th>
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<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>*</td>
<td></td>
</tr>
<tr>
<td><strong>M. marinum Strain M</strong></td>
<td></td>
<td></td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

% hemocytes with phagocytosed bacteria

(a) 20 µm

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PBS: Phosphate-buffered saline
AMK: Amikacin
EMB: Erythromycin

* indicates significant difference.
Figure 5

Unmanipulated

PBS/PBS

PBS/AMK 50

PBS/AMK 5

PBS/AMK 2.5

PBS/EMB 50

Hemocytes/mL

Time post-infection (h)

-2.5E+05

2.5E+05

7.5E+05

1.3E+06

1.8E+06

2.3E+06

*
Figure 6

M. fortuitum NCTC 10394

M. marinum Strain M

Un-manipulated PBS/PBS

Time post-infection (h)
(a) *M. fortuitum* NCTC 10394

(b) *M. marinum* Strain M

Survival (%)
Supplementary data – Kaplan-Meier survival curves for antibiotic susceptibility experiments (pages 1-3) and antibiotic combinations (page 4)

The antibiotic used is at the top of each grouping, and the species and strain of *Mycobacteria* used at the top of each curve. AMK, amikacin; CIP, ciprofloxacin; EMB, ethambutol; INH, isoniazid; RMP, rifampicin.

The mock-treated larvae are indicated with a grey line without line markers. The highest antibiotic dose is an unbroken black line with black, filled square markers. Middle doses have a dashed grey line and filled grey triangle markers, and the lowest dose have a dotted black line with a filled black circle marker. A mock-infected control is shown with grey crossed markers and a dashed grey line.

The doses used in mg/kg are shown to the right of each curve. Any doses that increased larval survival significantly above the survival of the mock-treated control have an asterisk by the dose.
Kaplan-Meir survival curves for the antibiotic combination experiments. Each of the single antibiotic doses are shown in pale grey, with the initial of the antibiotic as the point marker. A, amikacin; C, ciprofloxacin; E, ethambutol; I, isoniazid; R, rifampicin. The combinations are shown in black, with the combinations used listed on the right hand side of the figure. The mg/kg are included (eg. A5 describes a 5 mg/kg dose of amikacin). An asterisk next to this label means that the survival is significantly higher than the mock-treated control (log rank test, Holm’s correction applied).