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REVIEW

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# *Galleria mellonella* infection models for the study of bacterial diseases and for antimicrobial drug testing

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**ABSTRACT**

*Galleria mellonella* (greater wax moth or honeycomb moth) has been introduced as an alternative model to study microbial infections. *G. mellonella* larvae can be easily and inexpensively obtained in large numbers and are simple to use as they don't require special lab equipment. There are no ethical constraints and their short life cycle makes them ideal for large-scale studies. Although insects lack an adaptive immune response, their innate immune response shows remarkable similarities with the immune response in vertebrates.

This review gives a current update of what is known about the immune system of *G. mellonella* and provides an extensive overview of how *G. mellonella* is used to study the virulence of Gram-positive and Gram-negative bacteria. In addition, the use of *G. mellonella* to evaluate the efficacy of antimicrobial agents and experimental phage therapy are also discussed. The review concludes with a critical assessment of the current limitatons of *G. mellonella* infection models.

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## Introduction

One of the most commonly used models for studying microbial infections is the murine model. However, there are ethical, budgetary and logistical hurdles associated with the use of rodents as infection models. Firstly, maintaining a sufficient number of animals required to obtain statistically relevant data is expensive and often regarded as ethically objectionable. Secondly, mammals have lengthy reproduction times, which slow the progress of experimentation. More recently, *Galleria mellonella* (greater wax moth or honeycomb moth) has been introduced as an alternative model to study microbial infections. More than 1000 articles have been published on PubMed about the *G. mellonella* infection model, of which >200 were published in 2014–2015 alone demonstrating the increasing popularity of this infection model.

*G. mellonella* is an insect from the order *Lepidoptera* and the family *Pyralidae* (snout moths).[1] It is in fact the caterpillar larvae, or wax worm, and not the adult moth that is used as an animal model. When compared with traditional mammalian model hosts, *G. mellonella* larvae are cheaper to establish and easier to maintain, as they don't require special lab equipment.[2] Additionally, the use of *G. mellonella* does not require ethical approval and their short life span makes them ideal for high-throughput studies.

With the completion of many microbial genome projects over the last decade, there is now a large number of so-called "hypothetical proteins" in the databases. These annotations are based on identified open reading frames, sometimes with predicted functions after bioinformatics analyses, but often without an experimentally confirmed function. Many of these novel proteins are believed to be virulence factors and deciphering their functions will increase our understanding in disease mechanisms and ultimately provide a base for the development of novel therapeutic agents. Unlike other invertebrate models such as *Caenorhabditis elegans* and *Drosophila melanogaster*, *G. mellonella* larvae can survive at 37°C and therefore allow the investigation of temperature-dependent microbial virulence factors.[3,4]

Insects have diverged from vertebrates approximately 500 million years ago. Although vertebrates have developed an adaptive immune response, their innate immune response still retains remarkable similarities with the

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immune response in insects.[5] Over recent years, *G. mellonella* has been widely used as an infection model to study bacterial and fungal infections and for assessing the efficacy of novel antimicrobial drugs.

## The *Galleria mellonella* immune system

The innate immune response of insects consists of 2 major parts, the cellular and the humoral immune response. The cellular response is mediated by phagocytic cells, termed hemocytes. These are found within the hemolymph, which functions analogously to mammalian blood. These cells are not only involved in phagocytosis, but also in encapsulation and clotting. The humoral response is orchestrated by soluble effector molecules that immobilize or kill the pathogen and includes complement-like proteins, melanin, and antimicrobial peptides (summarized in Table 1).

### *Cellular immune response*

At least 8 types of hemocytes are found in insects, of which 6 different types have been identified in *G. mellonella*.[6] These include prohemocytes, plasmatocytes, granular cells, coagulocytes, spherulocytes and oenocytoids. In *G. mellonella*, plasmatocytes and granular cells play a key role in cellular defense and are involved in phagocytosis, nodule formation and encapsulation.[7] Plasmatocytes are the most common hemocyte and are characterized by a leaf-like shape and lysosomal enzymes within their cytoplasm. Granular cells are smaller and contain many granules in the cytoplasm. Encapsulation begins with the attachment of granular cells to the foreign target triggering the release of material (e.g. plasmatocyte spreading peptides, PSP) that promotes the attachment of multiple layers of plasmatocytes around the foreign target resulting in a smooth capsule.[8,9] Encapsulation is mainly associated with immune responses against larger microbes, such as protozoa and nematodes (including eggs and larvae).[6]

Phagocytosis in insects and mammals is believed to be very similar and involves both plasmatocytes and granular cells.[7] *G. mellonella* expresses on hemocytes a protein with high homology to human calreticulin found on neutrophils, which is believed to be involved in non-self recognition in cellular defense reactions.[10] Once phagocytosed, pathogens are killed by several mechanisms including reactive oxygen species (e.g., superoxide) generated by the oxidative burst, which is initiated by the NADPH oxidase complex. In neutrophils, the functional NADPH complex is formed after translocation of proteins $p47^{phox}$ and $p67^{phox}$ from the cytosol to the plasma membrane. Homologous proteins p47 and p67 were identified in *G. mellonella* hemocytes and it was shown that superoxide production in both hemocytes and human neutrophils could be triggered by 12-myristate 13 acetate (PMA) and inhibited by diphenyleneiodonium chloride.[11,12]

**Table 1.** Components of the *G. mellonella* innate immune response.

| a) cellular response | | |
|---|---|---|
| hemocytes | prohemocytes | 5 |
| | plasmatocytes | 5,7 |
| | granular cells | 5,7 |
| | coagulocytes | 5 |
| | spherulocytes | 5 |
| | oenocytoids | 5,43 |
| b) humoral response | | |
| opsonins | apolipophorin-III (apoL-III) | 13-15,18-20,121 |
| | peptidoglycan recognition proteins (PGRPs) | 22 |
| | cationic protein 8 (GmCP8) | 23 |
| | hemolin | 25,26 |
| antimicrobial peptides (AMPs) | lysozyme | 27,29,41,70 |
| | cecropin | 27,30,61,89 |
| | moricin-like peptides | 27,31 |
| | gloverin | 22,27,34,89 |
| | galiomycin | 27,70 |
| | gallerimycin | 27,35,70 |
| | *Galleria* defensin | 27,28,33,41 |
| | Gm proline-rich peptides 1 and 2 | 27,33,41 |
| | Gm anionic peptide 1 and 2 | 27,29,33,41 |
| | inducible serine protease inhibitor 2 | 27 |
| | heliocin-like peptide | 27 |
| | x-tox | 27,36 |
| | Gm apolipophoricin | 33 |
| melanization | phenoloxidase pathway | 37-39,122 |

### *Humoral immune response*

#### *Opsonins*

*G. mellonella* produces several plasma proteins that serve as opsonins that recognize and bind to conserved microbial components similar to pattern recognition receptors in mammals. Apolipophorin-III (apoLp-III), a major exchangeable lipid transport molecule plays a crucial role in the innate immune response as a pattern recognition molecule. ApoLp-III shows high affinity for hydrophobic ligands such as bacterial lipopolysaccharide (LPS) and lipoteichoic acid (LTA).[13,14] Furthermore, binding to $\beta$-1, 3 glucan and to fungal conidia resulting in increased cellular encapsulation was reported.[15] ApoLp-III shows high homology with mammalian apolipoprotein E (apoE), which is involved in LPS detoxification, the stimulation of phagocytosis and nitric oxide (NO) release from platelets.[16,17] A multifunctional role has also been demonstrated for apoLp-III. For example, apoLp-III stimulates increases in hemolymph antibacterial activity and superoxide production by hemocytes[18] and enhances the activity of the antimicrobial peptide cecropin.[19] More recently, it was shown that apoLp-III acts

synergistically with *G. mellonella* lysozyme increasing the permeabilizing activity of lysozyme against Gram-negative bacteria.[20]

Peptidoglycan recognition proteins (PGRPs) bind to peptidoglycan via a conserved domain homologous to T4 bacteriophage lysozyme.[21] *G. mellonella* PGRPs were identified during the analysis of LPS induced genes in hemocytes using subtractive hybridization.[22] PGRPs in some other insect species are also able to hydrolyze peptidoglycan, but this has not been demonstrated for PGRPs from *G. mellonella*.

A novel opsonin with homology to the cationic protein 8 (CP8) of *Manduca sexta* (tobacco hornworm) has been identified from the hemolymph of the *G. mellonella* larvae and termed GmCP8 (*G. mellonella* CP8). GmCP8, which is produced in the fat body (a biosynthetic organ analogous to the mammalian liver), midgut, and integument and is secreted into the hemolymph showed marked binding activity to LPS, LTA, and $\beta$-1,3-glucan.[23]

Hemolin, a member of the immunoglobulin protein superfamily binds to LPS and LTA and associates with hemocytes.[24] Hemolin is found in several lepidopteran species, including *G. mellonella*, where it is expressed in several organs, including the silk gland of the larvae and is up-regulated during bacterial infection[25] or after exposure to low doses of $\beta$-glucan (3.75$\mu$g/larva).[26]

### Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs), or host defense peptides, are found among all classes of life and play a major part in innate immunity showing broad-spectrum microbicidal activity. An analysis of the AMP repertoire of *G. mellonella* hemolymph identified 18 known or putative AMPs: lysozyme, 5 moricin-like peptides, 2 cecropins, gloverin, Gm proline-rich peptides 1 and 2, Gm anionic peptide 1 and 2, galiomycin, gallerimycin, inducible serine protease inhibitor 2, x-tox and heliocin-like peptide.[27] Another AMP, an insect defensin, named *Galleria* defensin, was purified from the larval hemolymph of *G. mellonella* immunized against *E. coli*.[28] Insect AMPs are mainly produced in the fat body, hemocytes, the digestive tract, salivary glands and the reproductive tract. In mammals, AMPs are secreted from epithelial surfaces and from phagocytic cells, where they are stored within intracellular granules.

Lysozyme degrades cell wall peptidoglycan by hydrolyzing the $\beta$-1, 4 linkage between N-acetylglucosamine and N-acetylmuramic acid. In addition, *G. mellonella* lysozyme also shows non-enzymatic activity against fungi resembling the mode of action of cationic defense peptides. The exact mechanism is unclear, but a synergistic effect with anionic peptide 2 (AP2) has been suggested.[29]

Cecropins and moricins both belong to the family of amphipathic $\alpha$-helical AMPs, which penetrate bacterial cell walls and form cytoplasmic membrane pores resulting in ion leakage. They are active against both Gram-negative and Gram-positive bacteria. *G. mellonella* cecropin is expressed as a prepropeptide, with a putative 22-residue signal peptide, a 4-residue propeptide and a 39-residue mature peptide.[30] Moricin-like peptides in *G. mellonella* were first identified by Brown et al, who showed that these AMPs have a particular strong activity against filamentous fungi.[31] Defensins are cysteine-rich cationic peptides that act by forming voltage-dependent ion channels in the cytoplasmic membrane resulting in ion leakage and cell lysis. Insect defensins act against Gram-positive and certain Gram-negative bacteria.[32]

Proline-rich peptides are small peptides between 2–4 KDa and appear to increase membrane permeability of bacteria. Gm proline-rich peptide 1 was also shown to inhibit the growth of yeast.[33] Gloverin belongs to the family of glycin-rich AMPs that binds to LPS on Gram-negative bacteria and inhibits the synthesis of vital outer membrane proteins resulting in a permeable outer membrane.[34] Gallerimycin is a defensin-like antifungal peptide, which was first cloned in *G. mellonella* from hemocytes isolated from LPS-pretreated larvae. It has no measurable effects on Gram-positive and Gram-negative bacteria or yeast, but shows activity against filamentous fungi.[35] X-tox is an atypical inducible defensin-like peptide that lacks detectable antimicrobial activity suggesting a yet unknown immune function.[36]

A comparative study with 8 *G. mellonella* AMPs (Gm proline-rich peptides 1 and 2, *Galleria* defensin, Gm defensin-like peptide, Gm anionic peptides 1 and 2, Gm cecropin and Gm apolipophoricin) revealed varying activity against Gram-positive bacteria, fungi and yeast. The most effective was Gm defensin-like peptide, which inhibited yeast, fungi and sensitive bacteria at concentrations of $<3$ $\mu$M. In contrast, Gm apolipophoricin and Gm proline-rich peptide 2 showed the lowest antimicrobial activity.[33]

### Phenoloxidase pathway and melanization

The melanization response can be described as the synthesis and deposition of melanin to encapsulate pathogens at the wound site followed by hemolymph coagulation and opsonization and is analogous to abscess formation in mammalian infections.[37] Melanin formation is catalyzed by phenoloxidase (PO), which is produced as the inactive zymogen pro-phenoloxidase (ProPO) in hemocytes.[38] Insect ProPO is an important innate immunity protein due to its involvement in cellular and humoral defense (reviewed in[39]).

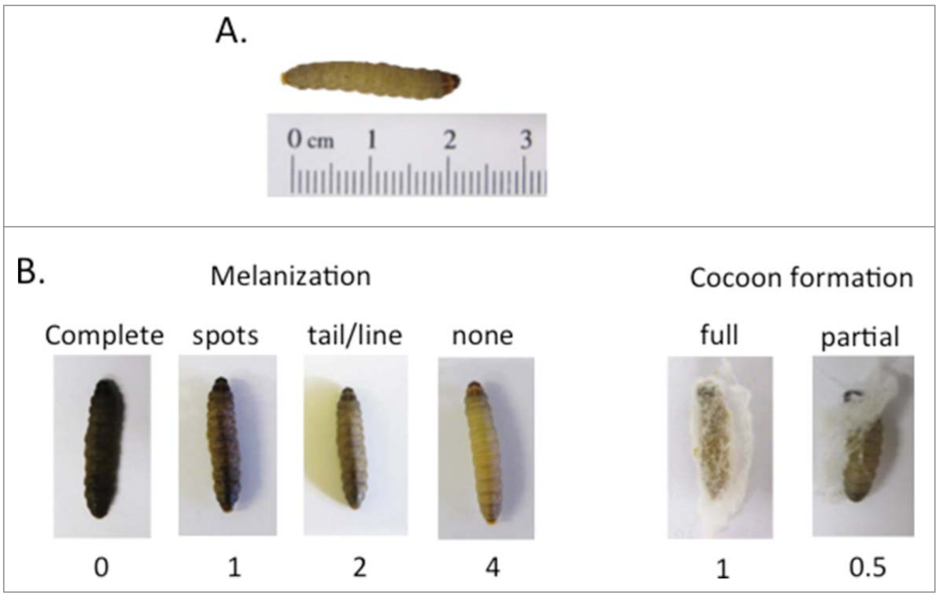


**Figure 1.** Photographic images of *G. mellonella* larvae. A: Image of a healthy *G. mellonella* last instar larvae with a typical creamy color and a size of 2 to 2.5 cm. B: Images of infected larvae showing different stages of disease. Melanization, which comprises the synthesis and deposition of melanin to encapsulate pathogens at the wound site followed by hemolymph coagulation and opsonization typically starts with distinctive black spots on the cream colored larvae (third image from left). Complete melanization (black larvae, left image) correlates with death of the larvae soon after. A decrease in cocoon formation can also be used as a marker for disease in *G. mellonella* larvae (right image) The numbers on the bottom of Fig. 1B are the health index scores (see also Table 2).

Melanization is initiated after the engagement of soluble PRRs with target surfaces, such as LTA[13] or thermolysin,[40] triggering the serine protease cascade that results in the cleavage of ProPO to PO. The activated PO converts monophenols and phenols to quinines, which polymerize non-enzymatically to form melanin around invading pathogens and wounds. PO can also produce cell damaging reactive oxygen species and therefore PO activation is highly controlled by protease inhibitors.[38] It has recently been reported that lysozyme, *Galleria* defensin, proline-rich peptide 1, and anionic peptide 2 decreased the hemolymph PO activity considerably suggesting a role of these AMPs in immune modulation.[41]

### *Extracellular nucleic acid traps*

Upon stimulation with LPS, PMA or interleukin-8 (IL-8), neutrophils release chromosomal DNA spiked with bactericidal proteins to form an extracellular fibril matrix known as neutrophil extracellular traps (NETs) due to their ability to trap and kill bacteria.[42] Microscopic ex-vivo analyses of *G. mellonella* hemolymph clotting reactions has shown that oenocytoids represent a source of endogenously derived extracellular nucleic acids. It has been suggested that actively released nucleic acid from neutrophils and hemocytes have comparable roles in entrapping pathogens and enhancing innate immune responses.[43]

## Experimental aspects of *G. mellonella* infection models

The last instar larvae, which develop from the egg in about 5 weeks are used for experimental studies. The larvae are 2 to 2.5 cm long and have a creamy color (Fig. 1). The larvae can be stored at 15°C before use and it is recommended to starve the larvae for 24h before infection.[2] The most common infection route is by intrahemocoelic injection through the last left pro-leg[44] or through the skin.[2] Oral infection has also been described,[45] but has the draw back that exact infection doses are difficult to obtain. This problem can be overcome by using a technically more challenging force-feeding method.[2] Microbial inoculums should be washed prior to infection to minimize the introduction of virulence factors secreted during in-vitro growth of the microorganism. It is also recommended to apply a placebo inoculum as a control for potential physical trauma due to the injection.[46] At least 10–20 larvae for each experimental condition should be used. After infection, the larvae may be maintained at temperatures up to 37°C.[3,4]

Microbial virulence in the *G. mellonella* infection model is typically assessed within 5 d and the most commonly used end point is the survival rate at different time points. When larvae are inoculated with a variety of doses, a half-maximum lethal dose ($LD_{50}$) can be calculated. Other end points include the expression of anti-microbial proteins in response to the infection[47]

**Table 2.** The *G. mellonella* Health Index Scoring System.[49]

| Category | Description | Score |
|---|---|---|
| activity | no movement | 0 |
| | minimal movement on stimulation | 1 |
| | move when stimulated | 2 |
| | move without stimulation | 3 |
| cocoon formation | no cocoon | 0 |
| | partial cocoon | 0.5 |
| | full cocoon | 1 |
| melanization | black larvae | 0 |
| | black spots on brown larvae | 1 |
| | $\geq$3 spots on beige larvae | 2 |
| | $<$3 spots on beige larvae | 3 |
| | no melanization | 4 |
| survival | dead | 0 |
| | alive | 2 |

and production of lactate dehydrogenase as a marker of cell damage.[48] More recently, a health index scoring system (Table 2) was introduced which assesses the larvae health status by assigning scores according to 4 major observations: larvae mobility, cocoon formation, melanization and survival.[49] Melanization typically starts with distinctive black spots on the cream colored larvae. Complete melanization (black larvae) correlates with death of the larvae soon after (Fig. 1).

Microbial virulence can also be assessed by measuring the proliferation of the microorganism inside the larvae during infection. This is typically done by plating larval extracts on agar plates for enumeration[48,49] or, more recently, by using bioluminescent microorganisms to detect the pathogen load by biophotonic imaging.[50,51]

## *G. mellonella* larvae as a model to study virulence of gram-positive bacteria

The *G. mellonella* infection model has been used to study a variety of Gram-positive bacteria, including *Streptococcus pyogenes* (group A streptococcus),[49,52] *Streptococcus pneumoniae* (pneumococcus),[53] *Enterococus faecalis*,[50,51,54-62] *Enterococcus faecium*,[63-65] *Staphylococcus aureus*[46,66,67] and *Listeria monocytogenes*[68-72] (summarized in Table S1).

Infection of larvae with the *S. pyogenes* reference strain SF370 (a serotype M1 strain) killed the larvae in a dose-dependent manner with a $LD_{50}$ of 6 × $10^6$ CFU.[49] Infection with different M-type strains (serotypes M1, M2, M3, M4, M6, M18, M28, M49) gave a wide range of responses in the *G. mellonella* model with serotype M18 being the most virulent and serotype M2 the least virulent strains.[49] There were also significant differences between a M3 carrier strain (MGAS12501) and a M3 invasive strain (MGAS315), which showed higher killing at doses of $10^6$ CFU and a more rapidly progressing melanin accumulation and hemolymph coagulation. Approximately 45% of larvae survived after 24h and ~25% after 96h when infected with the invasive strain, compared to survival rates of ~75% after 24h and ~55% after 96h for larvae infected with the carrier strain. Interestingly, survival in *G. mellonella* strongly correlated with survival in mice. Furthermore, a new GAS strain lineage (subclone 8 strains), which is associated with necrotizing fasciitis had significantly higher mortality compared with subclone 5 strains, which are epidemiologically associated with decreased necrotizing fasciitis cases in humans and have a significantly decreased capacity to cause necrotizing fasciitis in mice.[52] The *G. mellonella/S. pyogenes* model was further improved by the introduction of a health index that enabled measurement of more subtle differences, including cocoon formation and melanization[49] (see also under Experimental aspects of *G. mellonella* infection models, Fig. 1 and Table 2).

Differences in virulence between *S. pneumoniae* serotypes (2, 4, 14, 19A, 19F) could also be observed in the *G. mellonella* model and correlated with the presence or absence of known virulence factors. $LD_{50}$ values ranged from $1.56\times10^6$ CFU (England$^{14}$-9 strain) to $1.36\times10^5$ CFU (strain Portugal$^{19F}$-21), when determined 48h after inoculation with ~$10^6$ CFU, and were generally higher than those that have been observed in murine models of infection.[53]

The first article describing a *G. mellonella* infection model with *E. faecalis* was published in 2007. In this study, larvae were infected via injection of $5\times10^5$ CFU *E. faecalis* into the hemocoel resulting in intense melanization within 5 min and death 30 min later. Extracellular gelatinase (GelE) was identified as a major virulence factor in *G. mellonella* infections. Purified GelE injected into the hemolymph degraded the inducible antimicrobial peptide Gm cecropin analogous to its ability to degrade human complement proteins.[61]

Recently, the *E. faecalis/G. mellonella* infection model was improved by the introduction of a bioluminescent marker allowing for visualization of the infecting bacteria by biophotonic imaging. The luxABCDE operon was introduced into *E. facalis* by a plasmid that carries a toxin-antitoxin cassette providing segregational stability in the absence of antibiotic selection.[51] No difference in killing was detected between larvae infected with wild-type strain and the bioluminescent strain. A decrease of bioluminescence was observed in the first 2 hours, before the signal increased and reached a peak after 4 hours consistent with disease progression. The signal then remained stable until the death of the larvae after 24h. By expressing the lux-cassette under the control of either the cytolysin or the gelatinase promotor, this model was

later used to demonstrate temporal regulation of gelatinase and cytolysin in response to the host environment.[50]

In contrast to *E. faecalis, Enterococcus faecium* causes only weak lethality as demonstrated by monitoring the bacterial load after infection of *G. mellonella*. However, virulence was significantly increased when the antibiotic and stress response regulator (AsrR) was deleted.[65] The parenteral load markedly decreased from $1\times10^6$ to $3\times10^4$ CFU after 72h, whereas the *asrR*$^-$ mutant load only slightly decreases to $2.8\times10^5$ CFU.

Desbois and Coote have shown that *G. mellonella* larvae infected with *S. aureus* were killed in a dose-dependent manner. Infection with $1\times10^7$ CFU resulted in complete killing after 24h, whereas infection with $1\times10^5$ CFU resulted in ~20% killing after 120h. Virulence was temperature-dependent with more efficient killing at increasing temperatures when tested at 25°C, 30°C, and 37°C.[46] Over recent years, the *G. mellonella* model was mainly used for screening potential antistaphylococcal drugs (see section 6), but analysis of selected virulence factors has also been reported. Deletion of the Sec pathway (SecDF$^-$) significantly reduced virulence in the *G. mellonella* model and this was consistent with reduced cytotoxicity and invasion of human umbilical vein endothelial cells.[67]

Mukherjee et al. investigated the suitability of the *G. mellonella* model to study *L. monocytogenes* infections and found that at infection doses of $10^6$ CFU, the model could distinguish between pathogenic *L. monocytogenes* and nonpathogenic Listeria species, such as *L. innocua*. The model also proved to be a valuable tool to study attenuated *L. monocytogenes* strains. Deletion mutants with deficiencies in phospholipase B (PLB), listeriolysin (LLO, Hly), metalloproteinase Mpl (an important spreading factor) and Act (a protein that directs polymerization of actin in the host cell), as well as isogenic mutants lacking the virulence gene cluster (*vgc*) and the virulence gene regulator PrfA all showed increased survival rates of *G. mellonella* larvae. Virulence of the mutant strains correlated well with previous results obtained from mouse models.[70] Infection of larvae with *L. monocytogenes* results in increased production of lysozyme, galiomycin, gallerimycin and insect metalloproteinase inhibitor (IMPI),[70] activation of the phenoloxidase system and hemocyte destruction.[68] Increased expression of cecropin D has also been reported and these AMPs had a strong inhibitory effect on *L. monocytogenes*.[69] *L. monocytogenes* also elicits a cellular immune response with formation of nodules (melanized cellular aggregates) that contained entrapped bacteria on the surface of the brains. These nodules are similar to structures on the brain of humans infected with *L. monocytogenes*.

## *G. mellonella* larvae as a model to study virulence of gram-negative bacteria

Gram-negative bacteria investigated by using the *G. mellonella* infection model include *Pseudomonas aerogi-nosa*,[73-86] *Escherichia coli*,[87-91] *Klebsiella pneumonia*,[48,92-94] *Legionella pneumophila*,[95-97] *Francisella tularensis*,[98,99] *Acinetobacter baumanii*[100,101] and various species of *Burkholderia*[102-106] (summarized in Table S1).

One of the first studies of *P. aeruginosa* in a *G. mellonella* infection model was published in 1975 and demonstrated that rough mutants (mutants with deficiencies in LPS expression) of 2 *P. aeruginosa* strains were 8-62-fold less pathogenic than the smooth wt strains.[79] Since then, this model was used in several studies to analyze *P. aeruginosa* virulence and *G. mellonella* immune defense mechanisms. *P. aeruginosa* is highly virulent in *G. mellonella* and it was shown that injection of as little as 25 CFU of strain NCTC13437 resulted in 100% killing of the larvae after 24h.[77] Elastase B injected into the larvae at sublethal doses resulted in an increase in antibacterial activity and up-regulation of lysozyme and AMPs, in particular apoLp-III.[74] Similarly, apoLp-III levels are increased after infection of *G. mellonella* with *P. aeruginosa* followed by proteolytic degradation. In vitro and in-vivo studies suggest that both elastase B and serine protease IV play a role in apoLp-III degradation.[73,75] A more recent study using an entomopathogenic and 2 clinical strains found differences in the humoral immune responses, in particular the levels of lysozyme, phenoloxidase and AMPs. Notably, high levels of elastase A activity was detected in the entomopathogenic strain, but not in the 2 clinical strains.[76] Infection of *G. mellonella* with an entomopathogenic *P. aeruginosa* strain also showed significant changes in morphology and spreading ability and eventually apoptotic death of granylocytes and plasmatocytes.[82]

The first study reporting the use of the *G. mellonella* infection model to study pathogenic *E. coli* was published in 2012.[89] Leuko and Raivio demonstrated that *G. mellonella* larvae could be killed by enteropathogenic *E. coli* (EPEC) in a dose-dependent manner with a $LD_{50}$ value of $2.57\times10^3$ CFU at 48h post-infection. The bacteria were injected into the hemocoel, but disappeared shortly thereafter and became localized to melanized capsules. Infections resulted in an increase in the AMPs gloverin and cecropin.

A recent study showed a remarkable correlation between virulence gene repertoire and virulence potential of extraintestinal pathogenic *E. coli* (ExPEC) in the

*G. mellonella* model. ExPEC isolates with higher number of virulence genes resulted in significantly faster killing of the larvae.[91] In a similar study, 40 well-characterized ExPEC strains were analyzed in a *G. mellonella* infection model. The study which measured larvae survival, melanization, and cell damage found increased virulence in isolates from community-associated infections, complicated urinary tract infections (UTIs) and urinary-sourced bacteremia in particular in isolates belonging to the ST131 lineage.[88]

The suitability of *G. mellonella* as an infection model for *K. pneumoniae* has only recently been demonstrated. Survival of infected larvae was dose and strain dependent. For example, infection with $10^6$ CFU of the O1:K2 serotype strain 52145 caused 75% death after 24h and 100% death after 72h, whereas infection with the same dose of the O1:K2 serotype strain 43816 caused 95% death after 24h. Infections resulted in host responses similar to innate immune responses in mouse pneumonia models including cell death associated with bacterial replication and inhibition of phagocytosis and AMP production.[93] Similar results were reported from another study that compared a selection of 50 clinical isolates and reference strains at challenge doses of $10^5$ CFU. Survival rates after 24h ranged from 0% (3 isolates) to almost 100%, and 68% of the strains caused greater than 50% mortality. In addition, lactate dehydrogenase as a marker of cell damage, melanisation, and bacterial proliferation was analyzed and broadly correlated with survival rates.[48]

Comparison of 15 clinical *K. pneumoniae* producing carbapenemase (KPC(+) strains with 60 KPC(−) strains revealed decreased virulence of KPC(+) strains in the *G. mellonella* model, which was opposite from disease severity found in patients. This suggests some limitation of the *G. mellonella* model for *K. pneumoniae* infections.[94] However, a more recent study has shown a strong variability in virulence among carbapenem-resistant *K. pneumoniae* (CR-Kp). The differences were associated with the type of KPC gene and the capsular polysaccharide (CPS) type, and differences in serum resistance correlated with virulence in *G. mellonella*.[92]

The *G. mellonella* infection model has only recently been used to study virulence and pathogenesis of *L. pneumophila*. Harding et al. have demonstrated that 3 commonly used serogroup 1 strains caused death of at least 70% of the larvae that was strain, infectious dose and growth phase dependent. After infection, *L. pneumophila* was found within hemocytes inside a vacuole that showed resemblance with the *Legionella*-containing vacuole (LCD) observed in macrophages. Severe organ damage accompanied by melanization, nodule formation and increased AMP production was also observed.[96]

A *G. mellonella* infection model for *F. tularensis* was established by Aperis *et al.* using a live vaccine strain (LVS).[99] Infection of larvae with $3\times10^5$ CFU resulted in 100% mortality after 3 days, whereas infection with $3\times10^4$ CFU led to ~70% mortality after 10 d. The authors also observed that >90% of hemocytes were associated with bacteria 48 hours post-infection, which correlates well with the infection of macrophages in mammals. Virulence was found to be higher when larvae were incubated at 37°C compared to 30°C. Until today, this model has mainly been used for screening antimicrobial agents effective against *F. tularensis* (see section 6).

The utility of the *G. mellonella* infection model to study *A. baumanii* virulence was investigated by Peleg et al. Using the reference strain ATCC 17978, the authors showed that 75% of larvae infected with $3.5\times10^5$ CFU died within 48h.[101] A study comparing 5 different *A. baumanii* isolates found significant differences in virulence. Six days post-infection with $1\times10^5$ CFU, larvae survival rates were between 16% (isolate AB5075) and 85% (AB5711). Notably, AB5075 was also the most virulent strain in a mouse pulmonary infection model.[100]

*B. mallei* and *B. pseudomallei* were found to be highly virulent in the *G. mellonella* model. Only 10 CFU of *B. mallei* killed >90% of larvae after 4 days, whereas 10 CFU of *B. pseudomallei* killed >80% larvae after 2 d Both species caused severe paralysis starting 12 hours before death, similar to what can be observed in infected hamsters. At the time of death, ~$10^6$ CFU/ml of hemolymph were recovered indicating a very fast growth rate of the bacteria. In contrast, infection with $10^5$ CFU of a cystic fibrosis epidemic *B. cenocepacia* strain only resulted in 15% mortality after 6 days.[103] Strong virulence in the *G. mellonella* model was also found in other *Burkholderia* species. Two strains of *B. cepacia* showed $LD_{50}$ values of 1 CFU and 30 CFU, respectively, 48h postinfection, whereas 2 *B. cenocepacia* strains had $LD_{50}$ values of 900 CFU and 4000 CFU, respectively.[104] Varying virulence within the same species was also reported for *B. pseudomallei* and *B. thailandensis*. Two *B. pseudomallei* strains (576 and K96243) with low median dose values in mice had 100% mortality rates 24h after the challenge of *G. mellonella* larvae with 100 CFU, whereas a third strain (708a), which is attenuated in mice, was avirulent (100% survival). Similar, 2 *B. thailandensis* strains (CDC272 and CDC301) were highly virulent (100% mortality), whereas strains Phuket and E264 were significantly less virulent (80% and 50% mortality, respectively) when infected with 100 CFU and the differences reflect the observed virulence in mouse infection models.[106] *B. thailandensis* is rapidly phagocytosed by

hemocytes; bacteria were shown to be associated with hemocytes at 0.5 hours post-infection, and intracellular bacteria could be seen 1 hour after infection.[105]

## Use of *G. mellonella* to evaluate efficacy of antimicrobial agents

The rate of antibiotic resistance among important human pathogens, such as *Pseudomonas, Klebsiella* and *Acinetobacter* species, has accelerated dramatically in recent years. The discovery and development of novel antimicrobial agents is therefore of the utmost importance. Novel compounds are generally screened in vitro first to assess their effectiveness and potential toxicity. Eventually, successful candidates will have to be evaluated in an animal model, usually in murine and other rodent models, before their potential application in humans. In vivo tests are important to identify potential loss of activity due to host factors, e.g. degradation by host enzymes and the effect of physiological conditions such as pH.[107] However, experiments using mammalian hosts are time-consuming, expensive and often regarded as ethically objectionable. In contrast, the *G. mellonella* model is a simple and inexpensive alternative for the rapid evaluation of antimicrobial drug effectiveness in vivo and reduces the likelihood of an antimicrobial agent that performed well in in vitro studies from progressing to an unsuccessful performance in a mammalian model. The *G. mellonella* model can therefore serve as an additional pre-screening experiment to lower the number of antimicrobial drugs proceeding to tests in mammalian models (summarized in Table S2).

*G. mellonella* larvae can be accurately injected with defined doses of bacteria resulting in consistent survival/mortality rates. It is therefore easy to determine a dose that will not kill the larvae immediately, but leads to increased mortality over an appropriate time course, e.g., one to 3 d. Antimicrobial agents can be administered in different treatment regimes, including the total dose given, the number of doses and the treatment schedule. Most studies have used single treatment doses, usually given between 30min and 2 hours after infection of the larvae with the test pathogen. In some cases, the agent was given immediately after infection[108,109] or even before the infection.[105] The antimicrobial agent can be delivered systemically by injecting it directly into the hemocoel, which closely mimics the conventional administration route used in mammalian models.

Several studies have used combinations of antibiotics and shown synergism when administered to infected *G. mellonella* larvae. For example, gentamycin and daptomycin injected 1h after infection with vancomycin-sensitive *E. faecalis* or vancomycin-resistant *E. faecium* were significantly more effective than either antibiotic given alone at the same doses.[110] Krezdorn et al. have tested a variety of single, dual and triple antibiotic combinations against the multidrug-resistant *P. aeruginosa* strain NCTC13437 and showed synergistic effects for several combinations, like cefotaxime and piperacillin, amikacin and meropenem, or the triple combination of piperacillin, amikacin and meropenem, which was particularly effective.[111] Interestingly, there was little correlation between antibiotic combinations that showed synergy in in-vitro screens and those that showed enhanced effects in the *G. mellonella* model. This further emphasizes the role of the *G. mellonella* model as a useful tool for large-scale screen of antibiotic efficacy in an in-vivo model. The authors have also shown that antibiotic efficacy can be increased with administration of multiple doses. For example, larvae infected with $2.5\times10^3$ CFU and treated with a single dose of amikacin plus meropenem 2 hours post-infection showed increased survival after 24h (100%) and 48h (20%) compared to no survival after 24h in untreated larvae. However, treatment of larvae infected with $2.5\times10^6$ CFU and treated with a triple dose of amikacin plus meropenem 2 hours, 5 hours and 8 hours post-infection had survival rates of >50 % after 72h.[111] The importance of the correct timing of drug administration was shown for *G. mellonella* larvae infected with *F. tularensis*. Although, azithromycin, ciprofloxacin, levofloxacin and streptomycin all increased survival of larvae when injected as a single dose 2 hours after infection, only ciprofloxacin and streptomycin were effective when administered as 2 doses 24h and 48h after infection.[99]

Some more unconventional compounds tested in the *G. mellonella* model include the antibiofilm compound hamamelitannin, the estrogen receptor antagonist tamoxifen, the antihistamine terfenadine, the antimicrobial peptide LL-37 (and the enantiomer D-LL-37), the quorum sensing inhibitor and antibiofilm compound baicalin hydrate, cinnamaldehyde (isolated from cinnamon oil), a mutant form of *P. aeruginosa* acyl-homoserine lactone acylase PvdQ, a carbene silver(I) acetate derivative (SBC3), and metal ions (silver and zinc). See Table S2.

PvdQ is an effective quorum-quenching enzyme from *P. aeruginosa* and it has recently been shown that a variant with 2 point mutations, PvdQ (L$\alpha$146W, F$\beta$24Y), showed strong activity toward C8-HSL, the major communication molecule expressed by *Burkholderia* species. Injection of PvdQ (L$\alpha$146W, F$\beta$24Y) into *G. mellonella* larvae 1h before infection with *B. cenocepacia* significantly increased the survival of the larvae compared to untreated control animals.[102]

Of particular interest are the results obtained with hamamelitannin and baicalin hydrate. These are drugs that reduce the activity of bacterial virulence factors, in this case the ability to form biofilms, without possessing direct antimicrobial activity. The survival rates of *G. mellonella* larvae were further increased when these antivirulence agents were combined with antibiotics (hamamelitannin with vancomycin in larvae infected with *S. aureus* and baicalin hydrate with tobramycin in larvae infected with *B. cenocepacia*).[112] In many cases the expression of virulence factors by the pathogens is controlled by temperature.[3,4] *G. mellonella* is therefore perfectly suited for the screening of antivirulence drug, as the larvae can be incubated at a variety of temperatures that are necessary for expression of virulence factors.

The *G. mellonella* model has also been used to evaluate the combination of antibiotic therapy and antimicrobial photodynamic therapy (aPDT). PDT is based on photoactive dye molecules (photosensitizers) that produce reactive oxygen species when irradiated with visible light. Injection of methylene blue into larvae infected with *E. faecium* followed by whole body illumination increased the survival rates of the larvae compared to untreated controls. In addition, treatment of larvae infected with vancomycin-resistant *E. faecium* with aPDT followed by administration of vancomycin significantly reduced mortality rates when compared to aPDT or antibiotic treatment alone.[63] The photosensitizer can be injected into the haemocoel of the larvae and their relatively translucent body facilitates light delivery activating the photosensitizer, which makes *G. mellonella* an excellent model to evaluate these forms of therapy.

## Use of *G. mellonella* to evaluate experimental phage therapy

Phage therapy describes the therapeutic application of bacteriophages to treat pathogenic bacterial infections. Multiple studies have demonstrated the effectiveness of phage therapy in animal models for the treatment of various bacterial pathogens. However, apart from Russia and Georgia, this treatment has not been approved to treat infections in humans. The *G. mellonella* model was used to assess the efficacy of phage therapy in *Burkholderia cenocepacia* and in *Pseudomonas aeruginosa*. Injection of phages KS4-M and KS12 immediately after infection with a lethal dose of *B. cenocepacia* strain K56-2 resulted in significantly improved survival of the larvae. A similar result was obtained with phage KS14 after infection with strain C6433.[113] More recently, it was observed that the efficacy of experimental phage therapy was improved in the presence of sublethal concentrations of certain antibiotics and this effect has been termed 'phage-antibiotic synergy' (PAS).[114] Supporting evidence was provided by Kamal & Dennis, who demonstrated that the use of low-dose meropenem increased the survival rates of *G. mellonella* larvae infected with *B. cenocepacia* and treated with phage K12 over controls treated with antibiotic or phage alone.[115] Phage therapy was also analyzed with 29 phages and 121 *P. aeruginosa* isolates from cystic fibrosis patients and the protective efficacy of 2 selected phages (KT28 and PA5oct) against *P. aeruginosa* was confirmed using the *G. mellonella* model.[116]

## Limitations in the use of *G. mellonella* as an infectious disease model

As discussed in the previous sections, *G. mellonella* is an excellent model for assessing the virulence for a range of microrganisms. Although, it will not replace mammalian models, *G. mellonella* provides a rapid and cost-effective alternative to collect initial data. However, one has to consider that the *G. mellonella* infection model is still in its infancy and not as well established as some other invertebrate models, such as the nematode (*Caenorhabditis elegans*) or the fruit fly (*Drosophila melanogaster*).[117] The *G. mellonella* genome has not been fully sequenced and there is no established method for generating mutant strains and no access to microarrays or RNA interference libraries. Most importantly, there are no stock centers for *G. mellonella*, like the Drosophila stock centers where researchers can purchase specific genotypes that were raised under standard conditions. *G. mellonella* larvae are usually purchased from a wide range of independent breeders who sell the larvae as pet food. Differences in genotypes, breeding conditions or maintenance of the animals might well influence their susceptibility to infections. Even after the larvae are purchased, treatment conditions might vary between research labs, e.g. housing temperature, light sources and diet. It was shown that pre-exposure of larvae to heat induces their immune response,[118] whereas starvation results in reduction of immune responses and increased susceptibility to infection.[119] Furthermore, the size of the inoculum was shown to have an effect on cellular and humoral immune responses.[120]

Variations in supplier, breeding conditions, maintenance and handling of *G. mellonella* larvae might easily result in differences in mortality rates after infection with pathogens. This might explain conflicting results with some reference strains that induced variable mortality in larvae in different research labs. For example, in a study conducted in the US larvae infected with $10^6$ CFU of the *S. pyogenes* serotype M3 strain MGAS315 resulted in ~45% survival after 24h and 25% survival after 96h.[52]

In contrast, we have used the same strain and observed lower virulence (90% survival after 24h and 70% survival after 96h) when infected with a higher dose of $8\times10^6$ CFU.[49] The larvae for these studies were purchased from different suppliers (Best Bet Inc., Blackduck, MN, USA and Biosuppliers, Auckland, New Zealand) and there were also differences in maintenance conditions. The larvae from Best Bet Inc. were stored at 10-12°C without food for up to 10 d and, after infection, were incubated in the presence of 0.5% $CO_2$. The larvae from Biosuppliers were stored at room temperature with food and infected larvae were incubated under normal atmospheric conditions. These problems might be solved with the establishment of stock centers that supply reference populations of well-defined *G. mellonella* genotypes.

## Conclusion

Over recent years, *G. mellonella* has become increasingly popular as a surrogate host to study infectious diseases, as well as a screening platform for antibiotics. However, this model is still in its infancy. Major hurdles are the lack of stock centers that supply reference strains raised under standard conditions to enable comparable experiments carried out by different research groups and the limited availability of genomic information on *G. mellonella*. Furthermore, experimental conditions often differ between individual research labs and need to be standardized to minimize ambiguity. It is probably only a question of time until these issues are addressed, which will help to advance *G. mellonella* to a powerful and reliable infection model.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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