# Galleria mellonella as a Model for Fungal Pathogenicity Testing

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# Chapter 33

# *Galleria mellonella* as a Model for Fungal Pathogenicity Testing

John Fallon, Judy Kelly, and Kevin Kavanagh

## Abstract

Insects are convenient models for assessing the virulence of microbial pathogens or for assessing the efficacy of antimicrobial drugs and give results comparable to those that can be obtained using mammals. *Galleria mellonella* larvae are easy to purchase and inoculate and provide results within 48 h. Various parameters may be used to monitor the effect of a pathogen on the insect and, as a consequence, measure its relative virulence. Larval death, changes in immune cells (haemocytes) numbers, or the extent of proliferation of the pathogen within the insect haemocoel are good indicators of virulence and of the insect's immune response. Analysing the humoral immune response also gives insight into the interaction of the pathogen with the insect. Changes in gene expression or the expression of key antimicrobial peptides provide data on this element of the insect's response and, through extrapolation, how the mammalian immune system might respond. *G. mellonella* larvae, therefore, provide a quick and convenient means of measuring microbial virulence and are a useful alternative to the use of mammals for this type of screening.

**Key words:** *Aspergillus*, *Candida*, Fungi, *Galleria*, Haemocytes, Microbial virulence, Mini-host

## 1. Introduction

The insect immune system shares a number of structural and functional similarities with the mammalian innate immune system and, as a consequence, insects may be used in place of mammals for screening microbial pathogens or for assessing the potency of antimicrobial drugs (1). Larvae of the greater wax moth *Galleria mellonella* are inexpensive to purchase, easy to inoculate, and can give results within 48 h. We have employed *G. mellonella* larvae to assess the virulence of yeast (2) and *Aspergillus fumigatus* (3), with strong correlations between the virulence in larvae and mice of *Candida albicans* (4) and *Cryptococcus* (5) demonstrated. The use of insects as a screening model is now well established for many pathogens

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(4–6), with *Galleria* and other insects, such as *Drosophila* and *Manduca*, often referred to as "mini-hosts" (7, 8).

Changes in *G. mellonella* larva viability can be used to measure the relative virulence of microbial pathogens and/or mutants. Monitoring the insect immune response to microbial pathogens can also be useful as this gives an insight into the virulence of "weak" pathogens and provides information on how the mammalian innate immune system may respond. Measuring changes in the haemocyte density and the fungal load gives information on the cellular immune response to the pathogen and the proliferation of the pathogen in the host, respectively (9). In addition, it is also possible to monitor the expression of genes for selected antimicrobial peptides as a means of assessing the humoral immune response of larvae (10). Analysing changes in the proteome of infected larvae can also be very useful for monitoring alterations in expression of low molecular weight antimicrobial peptides. We have used this latter technique to demonstrate that larvae mount a "proportionate" immune response when inoculated with pathogen-derived material (11).

Insects are also a useful in vivo screening system for assessing the potency of antimicrobial agents (12–15). In this application, larvae can be infected with the cells or conidia of a pathogen and then given a pre-determined dose of the antimicrobial agent. An alternative is possible where the larvae are given the agent in advance of the pathogen. It should be emphasised, however, that administration of an antimicrobial agent to larvae induces a protective immune response and that any protective effect could be due to the combined effect of the antimicrobial agent and the insect's enhanced immune response (15). Controls to account for the increased immune response of the insect must be included in order to see the actual effect of the drug in vivo.

The use of insects as in vivo models to assess fungal virulence has grown in popularity in recent years (1, 7, 8). Although there are many advantages to the use of insects, *G. mellonella* larvae in particular, it must be emphasised that the insect immune system is only analogous to the mammalian innate immune system, and therefore, insect models give no insight into the role of the adaptive immune system in combating infections in mammals. In addition, larvae lack many of the organs attacked specifically by fungal pathogens, e.g. brain, lungs, kidneys, and spleen, so they cannot be used to model organ-specific pathologies. However, *G. mellonella* does provide a convenient initial screening system for studying pathogens that cause systemic infection, although as with all mini-hosts, validation of results may require further confirmatory mammalian testing.

This chapter describes a range of methods for use with *G. mellonella* larvae for evaluating the virulence of fungal pathogens or

for assessing the efficacy of antimicrobial agents. It details techniques for measuring the effect of the pathogen or drug on the insect immune response and describes methods for quantifying the response of the larvae to the different agents.

## 2. Materials

### 2.1. Inoculation of Galleria mellonella Larvae with Candida albicans

1. Sixth instar *Galleria mellonella* larvae (Lepidoptera: pyralidae, greater wax moth) (Mealworm Company) (see Note 1).
2. Myjector syringe (Terumo Europe).
3. YEPD (2% (w/v) glucose (Sigma-Aldrich), 2% (w/v) bacto peptone (Difco), 1% (w/v) yeast extract (Oxoid)).
4. Stationary phase culture of *Candida albicans* ($1–2 \times 10^9$ cells/mL) grown in YEPD at 30°C and 200 rpm.
5. Phosphate buffered saline (PBS).
6. Petri dishes (9 cm).
7. Filter paper circles (9 cm).

### 2.2. Determination of Haemocyte Density of Galleria mellonella

1. Infected and control *G. mellonella* larvae.
2. Sterile needles (23 G, Terumo).
3. Sterile tubes (1.5 mL).
4. 1-Phenyl-3-(2-thiazolyl)-2-thiourea.
5. Ice-cold PBS containing 0.37% (v/v) 2-mercaptoethanol.
6. Haemocytometer.
7. Microscope.

### 2.3. Determination of Candida albicans Fungal Load in Infected Galleria mellonella Larvae

1. Sixth instar *G. mellonella* larvae.
2. Stationary phase culture of *C. albicans.*
3. Sterile (pre-autoclaved) pestle and mortar.
4. PBS.
5. YEPD-erythromycin agar plates: 2% (w/v) glucose, 2% (w/v) bacteriological peptone, 1% (w/v) yeast extract, and 2% (w/v) agar and 1 mg/mL erythromycin (Sigma-Aldrich).

### 2.4. Assessment of Caspofungin In Vivo Activity Against Candida albicans

1. *C. albicans*-infected *G. mellonella* larvae.
2. Caspofungin (Cancidas™, Merck & Co.).
3. PBS.
4. Petri dishes (9 cm).
5. Filter paper circles (9 cm).

### 2.5. Extraction of Galleria mellonella RNA and Antimicrobial Gene Expression Analysis

1. Sixth instar *G. mellonella* larvae.
2. Sterile (RNase-free) pestles and mortars.
3. Liquid nitrogen.
4. Sterile tubes (1.5 mL).
5. TRI® Reagent (Sigma-Aldrich).
6. Vortex mixer.
7. Chloroform.
8. Isopropanol.
9. 75% (v/v) ethanol.
10. DEPC-treated water.
11. Deoxyribonuclease I (AMP-D1) kit (Sigma-Aldrich).
12. RNase-free agarose.
13. 10× FA buffer (200 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 50 mM sodium acetate, 10 mM EDTA, pH 7).
14. 37% (v/v) formaldehyde.
15. 0.5% (w/v) SDS.
16. 10 mg/mL ethidium bromide.
17. Agarose gel electrophoresis equipment.
18. 5× RNA gel loading dye (for 10 mL: 80 µL 0.5 M EDTA, pH 8, 720 µL 37% (v/v) formaldehyde, 2 mL glycerol, 3.084 mL formamide, 4 mL 10× FA buffer, and 16 µL saturated aqueous bromophenol blue solution).
19. 1× TAE buffer (1/50 dilution of 50× stock: 24.2% (w/v) Tris-base, 5.71% (v/v) acetic acid, 0.05 M EDTA (pH 8)).
20. Nanodrop 1000 spectrophotometer.
21. Superscript III First-Strand Synthesis System (Invitrogen).
22. DMSO (molecular grade).
23. 10 mM dNTP mix (Promega).
24. Water (molecular grade).
25. Accutaq™ LA DNA polymerase.
26. 10 µM forward and reverse primers (Table 1, (10)).
27. Thermal cycler.
28. Blue/orange 6× loading dye (Promega).

### 2.6. Analysis of Proteomic Changes in Infected Larvae by 2D Gel Electrophoresis and LC/MS

1. Pre-chilled (–20°C) 1.5-mL tubes.
2. Sterile deionised water.
3. Bradford reagent.
4. IEF Buffer (8 M urea, 2 M thiourea, 4% (v/v) CHAPS, 1% Triton-X 100, 65 mM DTT, 10 mM tris base).

**Table 1**
**PCR primers and conditions for analysis of antimicrobial gene expression**

| Gene | Primer | Sequence 5′-3′ | Product size (bp) | PCR conditions |
|---|---|---|---|---|
| *Actin* | ACT1F | GGGACGATATGGAGAAGATCTG | 400 | |
| | ACT1R | CACGCTCTGTGAGGATCTTC | | 1 cycle: 98°C, 2 min |
| *Transferrin* | TRANSF | CCCGAAGATGAACGATCAC | 535 | |
| | TRANSR | CGAAAGGCCTAGAACGTTTG | | 35 cycles: 94°C, 1 min; 55°C, 1 min; 72°C, 1.5 min |
| *IMPI* | IMPIF | ATTTGTAACGGTGGACACGA | 409 | |
| | IMPIR | CGCAAATTGGTATGCATGG | | 1 cycle: 72°C, 10 min |
| *Galiomicin* | GALIOF | CCTCTGATTGCAATGCTGAGTG | 359 | |
| | GALIOR | GCTGCCAAGTTAGTCAACAGG | | Final hold: 10°C |
| *Gallerimycin* | GALLERF | GAAGATCGCTTTCATAGTCGC | 175 | |
| | GALLERR | TACTCCTGCAGTTAGCAATGC | | |

Primer sequences are taken from Bergin et al. (2007) (10)

5. IPG Buffer (ampholytes) pH 3–10 (GE Healthcare).
6. Bromophenol blue powder.
7. Iso-electric focusing (IEF) machine (Ettan IPGphor II, Amersham Biosciences).
8. IEF coffins (Amersham Biosciences).
9. IEF strips (GE Healthcare).
10. PlusOne strip cover fluid (GE Healthcare).
11. Test tubes (at least 13 cm long).
12. Dithiothreitol (DTT).
13. Iodoacetamide (IAA).
14. Equilibration buffer (30% glycerol, 2% SDS, 6 M urea, 50 mM tris base).
15. 1.5 M Tris–HCl, pH 8.8.
16. 10% SDS, filter-sterilised and stored at room temperature to prevent crystallisation.

17. 30% acrylamide.
18. 10% ammonium persulphate.
19. *N,N,N,N′*-Tetramethyl-ethylene diamine (TEMED).
20. SDS-PAGE standards (Bio-Rad).
21. 1× Running buffer (1 in 10 dilution of 10× stock: 30 g Trizma base, 144 g glycine, 10 g SDS in 1 L distilled water).
22. Agarose.
23. Coomassie stain.
24. Siliconised 1.5-mL centrifuge tubes washed with 100% acetonitrile.
25. Sterile scalpels or pipette tips washed in 100% methanol (scalpels should be used to excise larger spots or bands, with sterile pipette tips useful for excising smaller gel pieces).
26. Destaining buffer (100 mM ammonium bicarbonate ($NH_4HCO_3$): acetonitrile; 1:1).
27. 100% acetonitrile.
28. Trypsin digestion buffer (13 ng/μL sequencing grade trypsin (Promega) in 10 mM $NH_4HCO_3$, 10% (v/v) acetonitrile).
29. Extraction buffer (1:2 (v/v) 5% formic acid: 100% acetonitrile).
30. 0.1% formic acid made using LC/MS-grade water.
31. 0.22-μm cellulose filter tubes.
32. Liquid chromatography-mass spectrometry (LC-MS).

### 2.7. Extraction and Analysis of Peptides from Galleria mellonella Larvae

1. Peptide extraction buffer (HPLC grade methanol:glacial acetic acid: HPLC grade water, 9:0.1:0.9); chilled on ice prior to use.
2. Freeze drier for lyophilisation.
3. 0.1% (v/v) trifluoroacetic acid (TFA) made in HPLC-grade water.
4. *n*-Hexane.
5. Ethyl acetate.
6. Bradford reagent (Biorad).
7. 1.5-mL tubes.

## 3. Methods

### 3.1. Inoculation of Galleria mellonella Larvae with Candida albicans

1. Place ten healthy larvae on Whatman filter paper placed in sterile 9-cm Petri dishes.
2. Grow a *C. albicans* culture to stationary phase ($1–2 \times 10^9$/mL) in YEPD broth at 30°C, with shaking at 200 rpm. Harvest cells by centrifugation (2,056 × *g* for 5 min), wash in PBS and

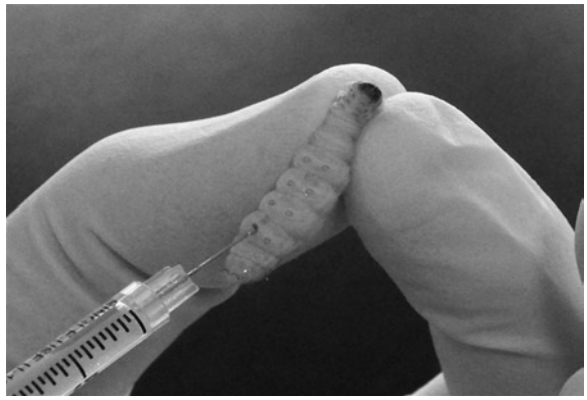


Fig. 1. Inoculation of *Galleria mellonella* larvae by injection through the proleg.

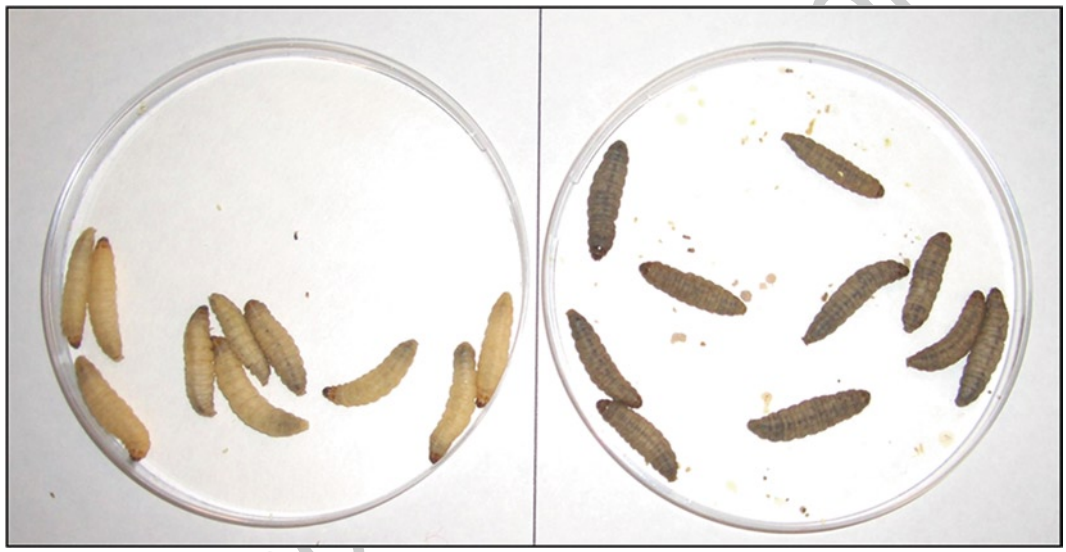


Fig. 2. Larval morphology. (**a**) Healthy *Galleria mellonella* larvae. (**b**) Larvae killed as a result of *Aspergillus fumigatus* infection 48 h previously. Note the dark colour of cadavers due to melanisation.

re-suspend in PBS at various cell densities, ranging from $5 \times 10^5$, $1 \times 10^6$, $2.5 \times 10^6$ to $5 \times 10^6$ per 20 μL (see Note 2).

3. Inoculate larvae by injecting 20 μL through the last left proleg into the haemocoel using a Myjector syringe (Fig. 1) and place at 30°C in the dark for up to 96 h (see Note 3). Untouched larvae and larvae injected with 20 μL of water or PBS should be included as controls.

4. Assess larvae at regular intervals (every 2 h) for viability and disease progression. For assessment of viability, larvae should be gently probed with a needle, and if no response is observed, the larvae may be considered to be dead. Changes in cuticle melanisation can also be used to monitor the severity of an infection (Fig. 2) (see Note 4).

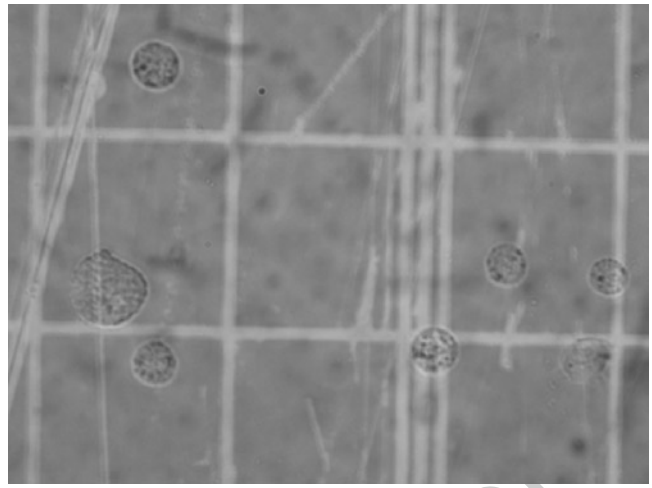


Fig. 3. *G. mellonella* haemocytes. *G. mellonella* haemocytes were extracted and viewed by light microscopy (40× objective). Note variations in size and granularity.

### 3.2. Determination of Haemocyte Density of Galleria mellonella

1. Pierce the backs of the anterior end ("head") of three randomly chosen larvae with a sterile needle and collect the yellow haemolymph ("blood") into a single pre-chilled tube containing a few grains of 1-phenyl-3-(2-thiazolyl)-2-thiourea (see Notes 5 and 6).
2. Dilute haemolymph 1 in 10 in cold PBS containing 0.37% (v/v) 2-mercaptoethanol to reduce clotting and melanisation. Mix gently by pipetting.
3. Count haemocytes on a haemocytometer (Fig. 3) and calculate the original density in the larvae (see Note 7).

### 3.3. Determination of Fungal Load of Galleria mellonella Inoculated with Candida albicans

1. Inoculate ten *G. mellonella* larvae with *C. albicans* ($5 \times 10^5$ cells/20 µL) and incubate for 24 h at 30°C.
2. Select three random larvae and place in a sterile pestle with 3 mL PBS and grind to a pulp with a mortar.
3. Dilute the resulting homogenate with PBS and plate 100-µL samples onto YEPD-erythromycin plates (see Notes 8 and 9).
4. Incubate plates at 30°C for 24 h and enumerate colony-forming units.
5. Calculate larval fungal load by multiplying the colony-forming units by the relevant dilution factor (see Note 10).

### 3.4. Assessment of In Vivo Activity of Caspofungin in C. albicans-Infected G. mellonella

1. Infect *G. mellonella* larvae with *C. albicans* as described above (see Note 11), including relevant control groups (see Note 12).
2. At 1 h post-infection, inoculate larvae with a 20-µL volume of caspofungin (e.g. 0.2, 0.1, or 0.05 µg/mL) or PBS (as control). Place at 30°C for up to 96 h.

3. Assess larval viability at 24-h intervals.
4. As an alternative to assess the effectiveness of caspofungin, inject larvae with caspofungin 1 or 4 h prior to infection with *C. albicans*.

### 3.5. Extraction of Galleria mellonella RNA and Antimicrobial Gene Expression Analysis

For RNA extractions, RNase-free materials should be used and precautions taken to minimise RNase contamination (see Note 13).

1. Place three *G. mellonella* larvae in a mortar (see Note 14); cover with liquid nitrogen (about 5 mL). Grind with a pestle until the larvae resemble a fine powder.
2. Add 3 mL TRI® reagent and mix. Leave to rise slightly in temperature until liquid becomes less viscous.
3. Transfer liquid to 1.5-mL tubes (1 mL in each) and centrifuge at 12,000 × *g* for 10 min at 4°C.
4. Transfer the supernatant to a new tube. Do not pool supernatants. To each tube, add 200 µL chloroform and vortex for 15 s. Leave to stand at room temperature for 10 min.
5. Centrifuge tubes as before and transfer the upper layer to a new tube and add 500 µL isopropanol. Invert the tubes several times and allow to stand at room temperature for 10 min.
6. Centrifuge tubes as before. Discard the supernatant and wash the pellet in 100 µL 75% ethanol by vortexing. Centrifuge tubes as before and remove ethanol completely. Allow tubes to air dry by placing in a Laminar flow hood on ice.
7. Re-suspend the pellet in 80 µL DEPC-treated water.
8. Remove contaminating DNA using the deoxyribonuclease I (AMP-D1) kit in accordance with the manufacturer's instructions to DNase treat samples.
9. Determine RNA concentration using the Nanodrop 1000 spectrophotometer.
10. Aliquot RNA and store at –80°C.
11. Prior to use, wash the gel rig and tank with 0.5% (w/v) SDS, rinse with DEPC-treated water followed by ethanol, and allow to air dry.
12. In order to visualise RNA, prepare 100 mL of 1% (w/v) agarose in 1× FA buffer. Heat the mixture until dissolved and allow to cool to hand-hot. Add 1.8 mL 37% (v/v) formaldehyde plus 1 µL of 10 mg/ml ethidium bromide prior to pouring the gel.
13. Prior to running, equilibrate the gel in 1× FA running buffer for at least 30 min.
14. Add 4 µL RNA to 4 µL 5× RNA gel loading dye; heat to 65°C for 5 min and chill on ice.

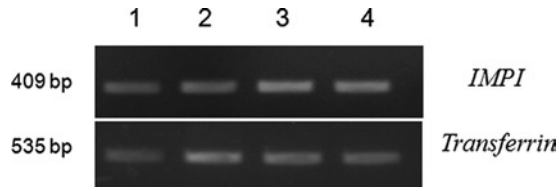



Fig. 4. Visualisation of antimicrobial gene expression. RNA extracted from infected larvae was used as the template for cDNA synthesis and subsequent PCR reactions to examine expression of antimicrobial peptide genes; (1) larvae injected with PBS, (2) larvae injected with 0.19 μg/mL caspofungin, (3) larvae injected with 0.095 μg/mL caspofungin, and (4) larvae injected with 0.048 μg/mL caspofungin.

15. Load the samples into the wells of the gel and run at 50 V in 1× FA buffer for approximately 40 min. Visualise bands using a UV transilluminator.
16. Synthesise cDNA using 1 μg RNA and the Superscript III First-Strand Synthesis System for RT-PCR kit according to manufacturer's instructions. cDNA should be stored at −20°C.
17. Analyse expression of *G. mellonella* antimicrobial genes by qRT-PCR using primers and PCR cycle conditions outlined in Table 1. The *Actin* housekeeping gene can be used as a control. Each PCR reaction (20 μL volume) contains 1 μg/μL cDNA, 2 μL 10× LA Buffer, 0.4 μL DMSO, 1 μL 10 mM dNTP mix, 14.6 μL water, 0.4 μL 10 μM forward primer, 0.4 μL 10 μM reverse primer, and 0.2 μL AccuTaq™ LA DNA polymerase. Solutions were mixed by pipetting and placed immediately into the thermal cycler.
18. Mix 4 μL of PCR product with 4 μL blue/orange 6× loading dye.
19. Visualise PCR products by running samples on a 1% (w/v) agarose gel (1 g agarose in 100 mL 1× TAE) with the addition of 2 μL 10 mg/mL ethidium bromide. Run gel at 50 V for 30 min and view using a UV transilluminator (Fig. 4).

### 3.6. Analysis of Proteomic Changes in Infected Larvae by 2D Gel Electrophoresis and LC/MS

The gel fragment tryptic digestion part of the protocol is a modification of the method described by Schevchenko et al. (17).

1. Bleed ten fungal-infected *G. mellonella* larvae into a pre-chilled 1.5-mL tube (as in subheading 3.2) and centrifuge at 800×*g* for 2 min at 4°C to pellet haemocytes. Transfer the cell-free supernatant to a fresh tube.
2. Make a 1 in 50 dilution of the crude cell-free haemolymph in deionised water and quantify protein content using Bradford method as per the manufacturer's instructions. Calculate the protein concentration of the neat cell-free haemolymph.

3. Add 350 µg protein to 100 µL IEF buffer and allow to solubilise for approximately 15 min at room temperature.
4. Add 2 µL ampholytes, pipette four to five times to mix, and allow to stand at room temperature for 15 min.
5. Add a further 150 µL IEF buffer and add a few grains of bromophenol blue to the solution and mix by pipetting.
6. Pipette 250 µL of the sample into an IEF coffin, tilt the IEF coffin to ensure an even distribution, and place a 13-cm IEF strip on top. Cover the strip with PlusOne DryStrip Cover Fluid.
7. Place the coffins into the IEF machine. Apply the following strip-focusing method per strip: 12 h at 50 V, 15 min at 250 V, increasing to 8,000 V with gradient increase over 5 h, and then hold at 8 h at 8,000 V. Once focusing is complete, strips may be stored at −70°C with the gel side of IEF strip facing upwards.
8. Aliquot 10 mL equilibration buffer into two separate test tubes. Dissolve 0.2 g DTT in the first equilibration tube and 0.4 g iodoacetamide in the second equilibration tube. Add a few grains of bromophenol blue to the second equilibration tube.
9. Transfer the IEF strip to the first equilibration solution, seal with paraffin film, and incubate horizontally on a rocking table for 15 min.
10. Transfer to the second equilibration solution and allow to equilibrate as in step 9.
11. Once equilibration is complete, rinse briefly with deionised water and place on top of the separating gel (see Note 15). Prepare a gel for 2D electrophoresis (see Note 16).
12. Place a piece of filter paper soaked in SDS-PAGE ladder at the corner of the gel and seal with a 1% agarose solution made with 1× SDS-PAGE running buffer.
13. Run gel at 80 V overnight and stain with Coomassie stain (Fig. 5).
14. Cut gel pieces from 2D gels (see Note 17) and transfer to individual siliconised tubes.
15. Wash stain from the gel piece using destaining buffer (200 µL) at room temperature and vortex occasionally. Repeat this step if required to remove all of the stain (see Note 18).
16. Remove all of the destaining buffer and resuspend the gel pieces in sufficient 100% acetonitrile to cover the gel piece (see Note 19).
17. Remove the acetonitrile. At this point, gel pieces can be stored at −20°C; alternatively proceed to step 20.
18. Add 60 µL trypsin digestion buffer to each tube containing gel pieces.

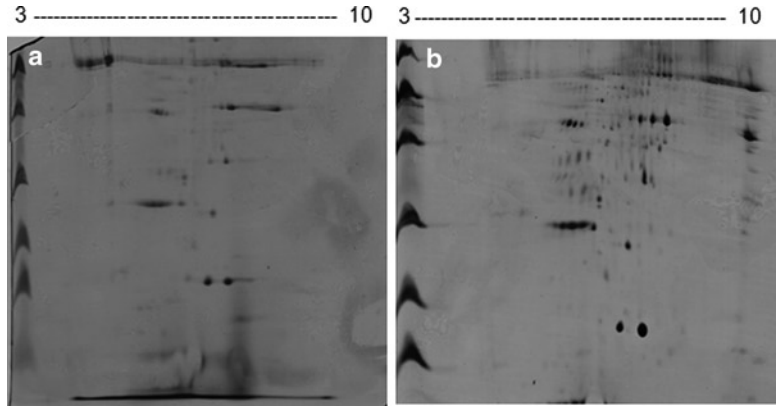



Fig. 5. *G. mellonella* larval haemolymph proteome. A control larvae and proteome of larvae challenged with beta-glucan. Note the increased expression of spots corresponding to specific proteins.

19. Place all tubes on ice for 30 min (see Note 20).
20. Add sufficient trypsin buffer to cover the gel pieces and keep on ice for approximately 60 min. Inspect all tubes to ensure that all the gel pieces remain covered.
21. Add 10 mM AmBic:10% vol/vol buffer to ensure that the gel plug is hydrated during enzymatic cleavage (see Note 21).
22. Incubate tubes at 37°C for 4–24 h to allow for tryptic digestion of protein (see Note 22).
23. Centrifuge the tryptic digests at 5,000 × *g* for 5 min and transfer the supernatant to a clean tube.
24. Add approximately 100 µL extraction buffer to the remaining gel piece pellet and incubate at 37°C for 15 min to extract peptides.
25. Centrifuge and transfer the supernatant to the tryptic digestion supernatant from step 23.
26. Vacuum dry the pooled supernatants to completion.
27. Store at –20°C until required for LC/MS analysis.
28. Prior to LC/MS analysis, resuspend the dried supernatants in 15 µL 0.1% formic acid and remove any debris by adding to a 0.22-µm cellulose acetate filter and centrifuging the tube at 20,000 × *g* for 3 min.
29. Transfer 10 µL of each sample to an LC/MS vial for analysis (see Note 23).

### 3.7. Extraction and Analysis of Peptides from G. mellonella Larvae

Peptide extraction is performed by the method of Cytrynska et al. (16) with slight modifications.

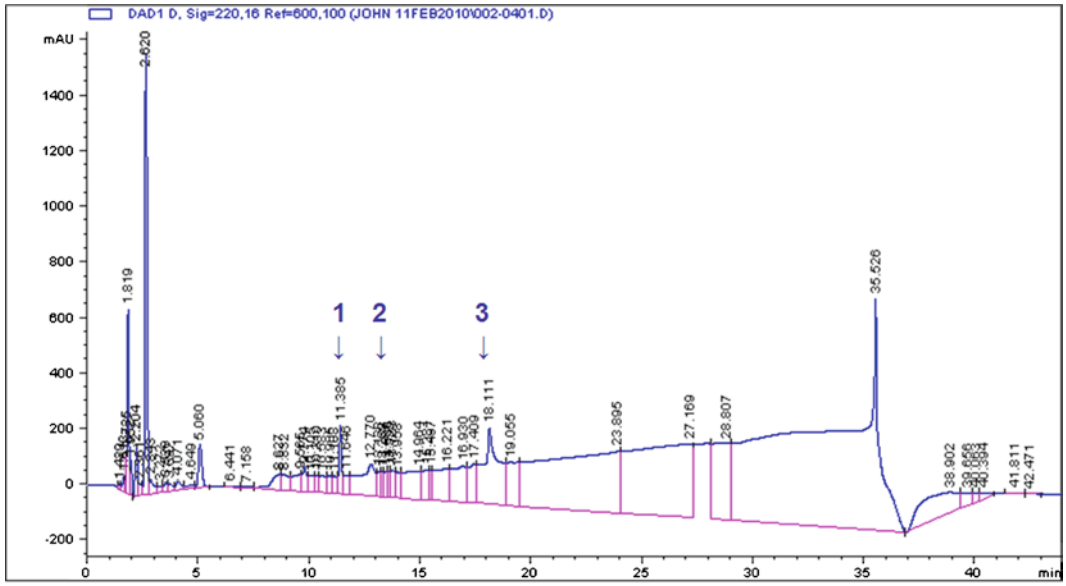
1. Bleed ten larvae into a pre-chilled 1.5-mL tube and centrifuge at 800 × *g* for 2 min at 4°C to pellet haemocytes. Transfer the cell-free supernatant to a fresh tube.

2. Make a 1/10 dilution of cell free haemolymph in peptide extraction buffer and leave to stand on ice for 30 min to precipitate high-molecular-weight proteins.
3. Centrifuge at 20,000 × *g* for 30 min at 4°C.
4. Transfer supernatant to a fresh tube and lyophilise in a freeze drier until dry.
5. Resuspend lyophilised extract in 200 µL 0.1% TFA.
6. Add an equal volume of *n*-hexane and mix thoroughly to remove lipid. Centrifuge at 20,000 × *g* for 10 min.
7. Remove the upper lipid-containing fraction and add an equal volume of ethyl acetate to the lower water fraction. Centrifuge at 20,000 × *g* for 10 min.
8. Remove the lower aqueous layer to a clean 1.5-mL tube.
9. Quantify the protein content by Bradford assay as per the manufacturer's instructions.
10. Equalise the protein concentration in a 100 µL volume to allow comparative analysis between different treatments.
11. Run samples on an HPLC using 220 nm as the reference wavelength for detection of peptide bonds. Where multiple wavelength analysis is possible, wavelengths of 254 and 280 nm should also be used for detection of disulphide bonds and aromatic residues, respectively. HPLC gradients should be as detailed in Table 2.
12. If possible, fractionate individual peaks according to the HPLC manufacturer's guidelines (Fig. 6).
13. Lyophilise individual peaks overnight and store at −20°C until tryptic digestion and characterisation by MALDI-ToF or LC/MS analysis (see Note 24).



**Table 2**
**HPLC conditions**

| Time (min) | Solvent | |
|---|---|---|
| 0–5 | 5% acetonitrile | Step and hold |
| 5–40 | 5–100% acetonitrile | Gradient |
| 40–42 | 100% acetonitrile | Step and hold |
| 42–44 | 100–5% acetonitrile | Gradient |




1. Larval haemolymph protein, 13.7kDa
2. Anionic antimicrobial peptide 2, 6.975kDa
3. Apolipophorin III, 20kDa

Fig. 6. Visualisation of *G. mellonella* antimicrobial peptides following HPLC fractionation.



## 4. Notes

1. *G. mellonella* larvae may be stored in wood shavings in the dark at 15°C prior to use. Larvae chosen for experiments should weigh between 0.2 and 0.4 g and used within 3–4 weeks of receipt. Larvae were discarded if they appeared dark in colour due to melanisation.
2. A range of doses is used to ascertain the lethal dose for different *C. albicans* strains.
3. The majority of larvae are dead after 96 h.
4. Larvae that melanise rapidly upon inoculation with a pathogen generally do not survive long, while those showing slow or little melanisation tend to survive.
5. Phenyl-3-(2-thiazolyl)-2-thiourea prevents melanisation of the haemolymph.
6. It should be possible to collect approximately 50–60 µL from each larva. Ensure all white flocular material is removed; this is the fat body and will impede counting.
7. Haemocyte density varies in response to different pathogens, and ascertaining the density can give an indication of the relative virulence of a pathogen (9).

8. Addition of erythromycin to the YEPD plates prevents bacterial growth.
9. For larvae infected 24 h previously, we generally dilute homogenate 1/2,000 in PBS prior to plating. After 24 h at 30°C, there are typically $10^6$ colonies per plate.
10. Virulent pathogens proliferate rapidly in the insect while weak or avirulent strains either grow slowly or are eliminated by the insect immune response (9).
11. .We routinely use group sizes of 30, with three plates each containing ten larvae.
12. Antimicrobial drugs can provoke an immune response in larvae so suitable control groups must be included in order to assess the actual antimicrobial activity of the test agent (15).
13. To remove contaminating RNases, glassware should be baked at 220°C for 12 h prior to use. Diethyl pyrocarbonate (DEPC) is a strong inhibitor of RNases. It can be used at a concentration of 0.1% (v/v) to treat water, which should be left stirring overnight, followed by incubation at 37°C for a minimum of 4 h prior to sterilisation by autoclaving. DEPC-treated water should be used to prepare all buffers required for RNA extraction. All bottle lids, O-rings, and magnetic stirrers should be soaked overnight in DEPC water and autoclaved prior use. All chemicals should be weighed without the use of a spatula. Gloves must be worn at all times and changed regularly. Pipette tips and tubes should be taken from freshly opened bags and autoclaved twice prior to use.
14. Three larvae are required to obtain sufficient RNA for gene expression analysis.
15. Separating gels should be prepared at least 5 h in advance to allow better polymerisation of the separating matrix.
16. To pour a gel, begin by thoroughly washing the glass plates with warm soapy water, rinse with 70% ethanol, and dry with lint-free tissue paper to remove any residual contamination on the glass. The glass plates that we routinely use are 200 mm wide and 200 mm long on the front and are 223 mm long at the back, and the gels (12.5% acrylamide) poured are approximately 190 mm × 160 mm and 1.5 mm thick. To prepare sufficient gel solution, mix 60 mL 1.5 M Tris–HCl, 76 mL deionised water, 100 mL 30% (w/v) acrylamide, 2.4 mL 10% (w/v) SDS, 1.5 mL 10% (w/v) APS, and 60 µL TEMED.
17. Ensure pieces are no more than 2-mm thick to allow better destaining and trypsin absorption.
18. This usually takes 1 h but may take longer depending on the size of the gel piece. This process can be made faster if larger pieces are sliced using a clean scalpel.

19. Upon addition of the acetonitrile, the gel pieces should turn white and shrink.
20. The 30-min incubation on ice allows the trypsin digestion buffer to penetrate the gel slices and also prevents trypsin autodigestion.
21. The volume added is judged by eye, but it is recommended to have at least 0.5 mm above the gel pieces.
22. . The incubation time is flexible, but the *best* results are usually achieved from an overnight (16 h) incubation.
23. LC/MS analysis will vary depending on the instrument used and the type of sensitivity required for analysis; therefore, it is not possible to write a specific protocol for this.
24. In our studies, we routinely use LC/MS for the characterisation of proteins.

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