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Proteomic profiling of bacterial and fungal induced immune priming in *Galleria mellonella* larvae

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ABSTRACT

Some insects display immunological priming as a result of elevated humoral and cellular responses which give enhanced survival against subsequent infection. The humoral immune response of *Galleria mellonella* larvae following pre-exposure to heat killed *Staphylococcus aureus* or *Candida albicans* cells was determined by quantitative mass spectrometry in order to assess the relationship between the humoral immune response and resistance to subsequent bacterial or fungal infection. Larvae pre-exposed to heat killed *S. aureus* showed increased resistance to subsequent bacterial and fungal infection. Larvae displayed an increased hemocyte density ($14.08 \pm 2.14 \times 10^6$ larva^{-1} ($p < 0.05$) compared to the PBS injected control [$10.41 \pm 1.67 \times 10^6$ larva^{-1}]) and increased abundance of antimicrobial proteins (cecropin-D-like peptide (+22.23 fold), hdd11 (+12.61 fold) and prophenol oxidase activating enzyme 3 (+5.96 fold) in response to heat killed *S. aureus*. Larvae pre-exposed to heat killed *C. albicans* cells were resistant to subsequent fungal infection but not bacterial infection and showed a reduced hemocyte density ($6.01 \pm 1.63 \times 10^6$ larva^{-1} ($p < 0.01$) and increased abundance of hdd11 (+32.73 fold) and moricin-like peptide C1 (+16.76 fold). While immune priming is well recognised in *G. mellonella* larvae the results presented here indicate distinct differences in the response of larvae following exposure to heat killed bacterial and fungal cells.

1. Introduction

Insects do not possess an adaptive immune response, as seen in the jawed vertebrates, but some display immunological priming as a result of prior exposure which enhances survival to a subsequent insult mediated by increased humoral and cellular responses (Mowlds et al., 2010; Browne et al., 2013; Cooper & Eleftherianos, 2017). Immune priming can be associated with an elevation in the density of circulating hemocytes and the increased abundance of antimicrobial peptides (AMPs) which display potent antibacterial and/or antifungal activity (Yun & Lee, 2016; Yi et al., 2014; Fallon et al., 2011). The elevated hemocyte density arises due to the release of sessile hemocytes which are normally attached to the surface of internal organs and the inner surface of the cuticle rather than *de novo* synthesis (Matha and Áček, 2010; Bergin et al., 2006; Browne et al., 2014; Morton et al., 1987).

Pre-exposure of *Drosophila melanogaster* to a sub-lethal infection by *Streptococcus pneumoniae* (or *Beauveria bassiana*) induced protection against a subsequent otherwise-lethal infection by the same species (Pham et al., 2007). The protection was mediated by increased phagocyte activity and the Toll pathway, but not by activation of the imd pathway or elevated production of AMPs. Immune priming in *Bombyx mori* following exposure to *Photorhabdus luminescens* or *Bacillus thuringiensis* was accompanied by increased phagocytic activity of granular cells and enhanced anti-bacterial activity in the hemolymph (Wu et al., 2015a, 2015b). Over 75 genes were induced in *Tribolium castaneum* (red floor beetle) upon intra-hemocoel injection of LPS and included genes involved in signalling, immune defense, detoxification and stress response thus demonstrating the extent of the response as a result of immune priming to withstand a subsequent infection (Altincicek et al., 2008). Immune priming in *T. castaneum* was demonstrated to show a high degree of specificity following pre-exposure of beetles to a wide range of bacteria of different degrees of relatedness (Roth et al., 2009). However it was noted that immune priming was stronger following infection with certain strains, indicating that the response may be favoured against frequently encountered pathogens (e.g. *Bacillus thuringiensis*) rather than those rarely encountered (e.g. *Escherichia coli*).

Abbreviations: AMP, antimicrobial peptide; BP, biological processes; FDR, False Discovery Rates; GO, Gene ontology, SSDA, statistically significant differentially abundant; MP, molecular function; DEP, differentially expressed proteins.

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Elevated lysozyme activity was evident in hemolymph of *Galleria* larvae following intra hemocoel administration of LPS, *Escherichia coli*, zymosan or yeast cells and peaked 24 – 36 h after administration of dead or viable *Beauveria bassiana* spores (Vilcinskas & Matha, 1997). Interestingly, the size of the increase in lysozyme activity was different with the greatest increase being evident in those larvae administered viable spores.

Immune priming has obvious survival advantages in that it confers protection against a second potentially lethal infection but does have a significant metabolic cost to initiate and maintain (Moret & Schmid-Hempel, 2000). Many insects that live in colonies have colony level defences which limit the spread of a pathogen (Heinze & Walter, 2010) and consequently may not require an individual immune priming response. Immune priming in *Formica selysi* (ant) following challenge with *Beauveria bassiana* was short term (Reber et al., 2012) indicating that colony living removes some of the necessity of having a prolonged immune priming response. Honeybees (*Apis meliferra*) show less investment in a primed immune response since the elevated temperature in the hive can prevent infection by heat sensitive pathogens such as, *Ascosphaera apis* (Starks et al., 2000). Enhanced immune responses in early life can have a detrimental effect on insect physiology and accelerate ageing. *Tenebrio molitor* infected with *Staphylococcus aureus* showed increased phenoloxidase activity but reduced malpighian tubule activity and died faster than uninfected controls indicating that the initial immune response may protect against bacterial infection but that the tissue damage associated with it may ultimately reduce life expectancy (Khan et al., 2017)

The benefits of immune priming can be passed from mother or father to offspring thus ensuring their resistance to infection by a pathogen they have not encountered directly. Trans-generational immune priming was demonstrated in *Galleria* larvae that were fed non-pathogenic bacteria and the larvae demonstrated elevated abundance of gloverin and increased phenoloxidase and lysozyme activity in the hemolymph (Freitak et al., 2014). Eggs laid by females which had been fed these bacteria when in the larval form demonstrated increased expression of immune related genes and it was suggested that bacteria or bacterial fragments were deposited on the eggs in the oviduct prior to laying and this lead to this alteration (Freitak et al., 2014). 2D SDS PAGE was employed to visualise the proteins differentially abundant in the *G. mellonella* eggs from control mothers and those that had received bacteria prior to playing. Trans-generational immune priming in *Tribolium castaneum* has been characterised by monitoring host gene expression following exposure to *Bacillus thuringiensis* and revealed that larvae may be able to redirect their metabolism towards important physiological and immunological processes that achieve protection from infection but that these changes come at a cost of the host (Tate et al., 2017).

Changes in immune mediators as a result of priming have been linked with the first encounter with the microbial invader but is also observed in abiotically stressed insects (e.g. Wojda and Jakubowicz, 2007; Mowlds and Kavanagh, 2008). This has been observed in a number of insect species including *G. mellonella* (Bergin et al., 2006), *Drosophila melanogaster* (Irving et al., 2001) and *Anopheles gambiae*, and molluscs and sea urchins (Zhang and Gallo, 2016). Interestingly some antimicrobial drugs (e.g. caspofungin (Kelly and Kavanagh, 2011), silver based drugs (Rowan et al., 2009)) can provoke an immune response and induce increased resistance to pathogens against which these drugs have no inherent activity. This alteration in immune function can be mediated through changes in hemocyte density or population composition and alterations in humoral mediators.

In *G. mellonella* larvae resistance to *A. fumigatus* infection can be induced by mild physical and/or thermal stress and also following infection with a low dose of conidia and was mediated by an increase in hemocyte density and in the expression of a number of antimicrobial peptides (Fallon et al., 2011). Pre-exposure of *G. mellonella* larvae to a non-lethal dose of the yeast *Candida albicans* protected larvae from a subsequent infection with a dose of *C. albicans* which would normally prove fatal (Bergin et al., 2006).

Administration of components of microbial cell wall (e.g. laminarin (Bergin et al., 2006), β-glucan ((Mowlds et al., 2010), lipopolysaccharide (Wu et al., 2015a, 2015b)) can induce immune priming in *G. mellonella* larvae against subsequent infection by fungi or bacteria. *G. mellonella* larvae incubated at 37 °C displayed enhanced survival to a lethal *C. albicans* infection and this was attributed to an increase in hemocyte density and the expression of gallerimycin, transferrin, IMPI and galiomicin with the peak expression observed at 24 h post incubation (Mowlds and Kavanagh, 2008).

The aim of this work presented here was to characterise the cellular and humoral immune response of *G. mellonella* larvae primed by exposure to heat killed fungal and bacterial cells and to examine how the immune response may differ in order to establish if there was a degree of specificity in each priming response. The utilisation of 'Omic' technologies such as large scale MS-based proteomics has produced novel insights into insect immune responses and insect-pathogen interactions (Tuli and Ressom, 2009; Sheehan et al., 2019). In this work label free quantitative (LFQ) mass spectrometry was employed to compare the proteome of *G. mellonella* larvae following exposure to fungal or bacterial cells in order to characterise the changes that accompany the induction of immune priming.

2. Materials and methods

2.1. Larval culture and inoculation

Sixth instar larvae of the greater wax moth *G. mellonella* (Livefoods Direct Ltd, UK) were stored in the dark at 15 °C and maintained in wood chippings. Larvae weighing 0.22 ± 0.03 g were selected and used within two weeks of receipt. Ten healthy larvae per treatment and controls were placed in sterile 9 cm Petri dishes lined with Whatman filter paper and containing some wood chippings. Larvae were inoculated through the last left pro-leg into the hemocoel with a Myjector U-100 insulin syringe (Terumo Europe N.V., Belgium). Larvae were acclimatized to 30 °C for 1 h prior to all experiments and incubated at 30 °C where indicated. All experiments were performed independently on three separate occasions.

2.2. Strains and culture conditions

Candida albicans MEN was cultured in YEPD broth (2% (w/v) glucose, 2% (w/v) bactopeptone (Difco Laboratories), 1% (w/v) yeast extract (Oxoid Ltd., Basingstoke, England)). *S. aureus* (clinical isolate) was cultured in nutrient broth (Oxoid). Cultures were grown overnight at 37 °C and 200 rpm to the early stationary phase as described. Cells were harvested by centrifugation (2000*g*) and cell pellets were washed three times with phosphate-buffered saline (PBS) prior to injection into larvae. *C. albicans* and *S. aureus* cells were obtained by incubating at 95 °C for 15 min, and loss of viability was assessed by plating on YEPD- or nutrient agar plates, respectively. Stocks of *C. albicans* and *S. aureus* were maintained on YEPD agar plates (as above but supplemented with 2% (w/v) agar) and nutrient agar plates, respectively.

2.3. Immune priming of G. mellonella larvae

Larvae (n = 10) were injected with 20 μl of heat killed *C. albicans* (fungal priming; 1×10^6 20 μl^{-1}) or *S. aureus* (bacterial priming; 20 μl of a 0.1 OD_{600} heat killed *S. aureus* PBS solution (approx. 4×10^7 cells ml^{-1}). Control larvae were injected with 20 μl PBS and incubated at 30 °C for 24 h. For assessment of resistance of fungal or bacterial primed larvae to a subsequent fungal or bacterial infection, 24 h primed and control larvae were infected with live *C. albicans* (5×10^5 20 μl^{-1}) or *S. aureus* (20 μl of a 0.1 OD_{600} *S. aureus*) and survival of larvae was monitored over 96 h.

2.4. Determination of hemocyte density in primed G. mellonella larvae

Changes in hemocyte density were assessed by bleeding 40 µl of hemolymph from *G. mellonella* larvae (n = 5) into a micro-centrifuge tube on ice to prevent melanization. Hemolymph was diluted in 0.37% (v/v) mercaptoethanol supplemented PBS and cell density was determined using a hemocytometer. Hemocyte density was expressed in terms of hemocyte density larva^{-1}. Experiments were performed on three independent occasions and the means ± S.E. were determined.

2.5. Quantitative proteomics of immune primed larval hemolymph

Quantitative proteomics was conducted on hemocyte-free hemolymph from larvae primed by exposure to heat killed *C. albicans* (1×10^6 20 µl^{-1}) or heat killed *S. aureus* (20 µl of a 0.1 OD heat killed *S. aureus* PBS solution). Hemocyte-free hemolymph was used so as to optimise the characterisation of the protein component in hemolymph. Protein (75 µg) was prepared as described (Sheehan and Kavanagh, 2018). Protein identification from the MS/MS data was performed using the Andromeda search engine in MaxQuant (version 1.2.2.5; http://maxquant.org/) to correlate the data against a 6-frame translation of the EST contigs for *G. mellonella* (Cox et al., 2011; Vogel et al., 2011).

Results processing, statistical analyses and graphics generation were conducted using Perseus v. 1.5.5.3. LFQ intensities were $\log_2$-transformed and ANOVA of significance and t-tests between the hemolymph proteomes of control and primed larvae was performed using a p-value of 0.05 and significance was determined using FDR correction (Benjamini-Hochberg). Proteins that had non-existent values (indicative of absence or very low abundance in a sample) were also used in statistical analysis of the total differentially expressed group following imputation of the zero values using a number close to the lowest value of the range of proteins plus or minus the standard deviation. After data imputation these proteins were included in subsequent statistical analysis.

2.6. Data availability

The MS proteomics data and MaxQuant search output files have been deposited to the ProteomeXchange Consortium (Côté et al., 2012) via the PRIDE partner repository with the dataset identifier PXD014651.

2.7. Statistical analysis

All experiments were performed on three independent occasions and results are expressed as the mean ± Standard error. All statistical analysis listed performed using GraphPad Prism v 6.00 (Two-way ANOVA; Larval survival and alterations in circulating hemocyte density). Differences were considered significant at $p < 0.05$.

3. Results:

3.1. Immune priming of G. mellonella and resistance to infection

Larvae immune primed by exposure to heat killed fungal cells or heat killed bacteria (for 24 h) were infected with viable *C. albicans* (Fig. 1A) or *S. aureus* (Fig. 1B) and survival was assessed over 96 h.

Larvae that had been inoculated with heat killed *C. albicans* prior to infection with fungal viable cells displayed survival of 100 ± 3.33% at 24 h, 80 ± 3.33% $p < 0.01$ at 48 h, 70 ± 3.33%, $p < 0.001$ at 72 h and 50 ± 3.33, $p < 0.001$ at 96 h post infection relative to control larvae (24 h; 100 ± 3.33% , 48 h; 60 ± 6.66% 72 h; 20 ± 3.33%, 96 h; 10 ± 8.35%) incubated at 30 °C. Larvae primed with heat killed bacteria prior to infection with viable yeast cells displayed significantly enhanced survival (100 ± 6.66%, $p < 0.0001$ at 48 h, 100 ± 6.66%, $p < 0.0001$ at 72 h and 90 ± 4.28%, $p < 0.0001$ at 96 h) relative to control larvae (Fig. 1A).

Larvae infected with viable *S. aureus* cells following prior exposure to heat killed *S. aureus* showed enhanced survival (100 ± 5.29, $p < 0.01$ at 24 h, 80 ± 2.94, $p < 0.01$ at 48 h, 60 ± 7.82, $p < 0.01$ at 72 h and 50 ± 5.34%, $p < 0.001$) compared to control larvae (survival of 80 ± 5.63% at 24 h, 60 ± 3.33% at 48 h, 40 ± 3.33% at 72 h, 20 ± 3.33% at 96 h)

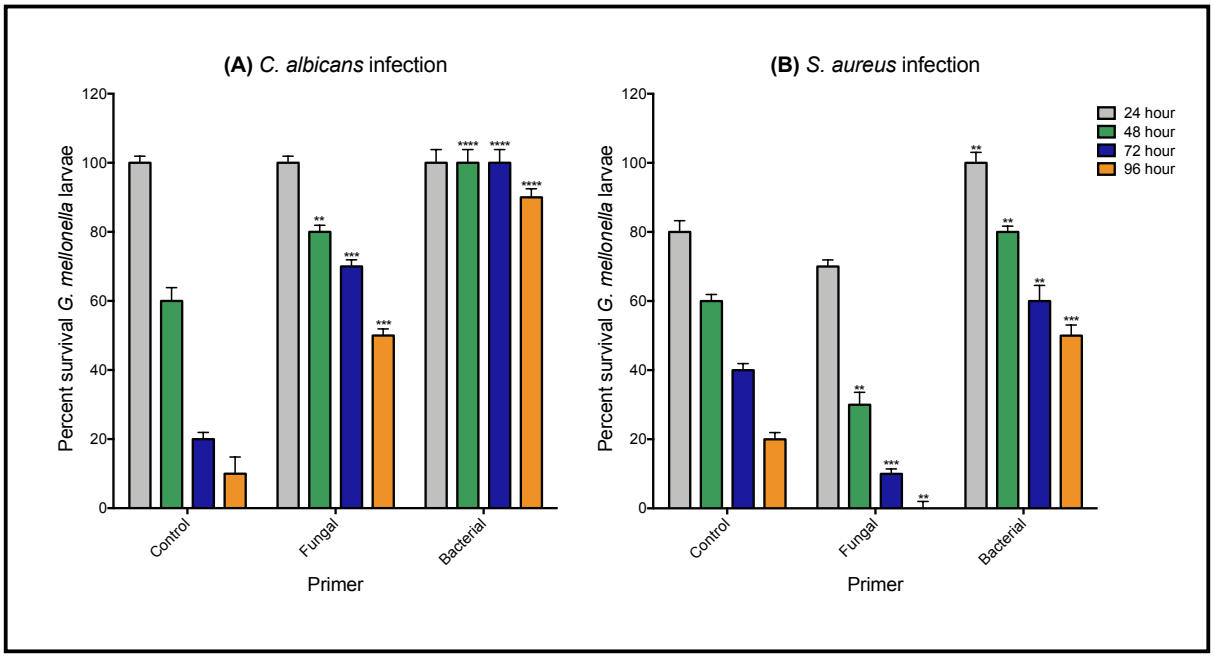

Fig. 1. Survival of *G. mellonella* larvae immune-primed (fungal and bacterial) and infected with *C. albicans* or *S. aureus*. Larvae were injected with 20 µl of heat killed *C. albicans* (Fungal; 1×10^6 20 µl^{-1}), *S. aureus* (Bacterial; 20 µl of a 0.1 OD heat killed *S. aureus* PBS solution) or PBS and incubated at 30 °C for 24 h. Larvae were then infected with 20 µl of live *C. albicans* (A; 5×10^5 20 µl^{-1}) or live *S. aureus* (B; 20 µl of a 0.1 OD_{600} *S. aureus* PBS solution). Survival of larvae was monitored over 96 h and statistical analysis was performed by comparing primed larval survival to control larvae at the relative time point (**; $p < 0.01$, ***; $p < 0.001$, ****; $p < 0.0001$). All values are the mean ± S.E. of three independent experiments.

(Fig. 1B). Larvae inoculated with heat killed *C. albicans* prior to *S. aureus* infection showed survival of 30 ± 6.24%, $p < 0.01$ at 48 h, 10 ± 2.45%, $p < 0.01$ at 72 h and 0 ± 3.33%, $p < 0.01$ at 96 h post infection (Fig. 1B).

3.2. Alterations in the density of circulation hemocytes following immune priming of G. mellonella larvae

The changes in circulating hemocyte density following immune priming with heat killed *C. albicans* or *S. aureus* were determined (Fig. 2). Administration of heat killed *C. albicans* resulted in a significant decrease in the number of circulating hemocytes at 24 h to 6.01 ± 1.63 × 10^6 larva^{-1} ($p < 0.01$) as compared to the hemocyte density in control larvae (10.41 ± 1.67 × 10^6 larva^{-1}). Injection of larvae with heat killed *S. aureus* resulted in a significant increase in the density of circulating hemocytes (14.08 ± 2.14 × 10^6 larva^{-1}, $p < 0.05$) as compared to control larvae.

3.3. Humoral immune proteome of immune primed G. mellonella larvae.

Shotgun proteomic analysis was performed on the cell free hemolymph proteome of *G. mellonella* larvae immune primed by fungal (Fig. 3A) or bacterial (Fig. 3B) exposure at 24 h. In total 1840 peptides were identified, representing 171 proteins with two or more peptides and 39 (fungal primed v control) and 24 (bacterial primed v control) proteins were determined to be differentially abundant (ANOVA, $p < 0.05$) with a fold change of > 1.5.

A principal component analysis (PCA) was performed on all filtered proteins and distinguished the proteome of control, fungal primed and bacterial primed samples (Fig S1). A total of 14, 73, 12 and proteins were deemed exclusive for control, fungal-primed, bacterial-primed larvae, respectively (Fig S2). These proteins were subsequently used to statistically analyse the total differentially expressed group after imputation of the zero values as described and were then included in statistical analysis after data imputation.

A range of proteins were increased in abundance in the hemolymph of *G. mellonella* previously inoculated with heat killed *C. albicans* cells relative to the proteome of control larvae and these consisted of gustatory receptor (+32.73 fold), hdd11 (+27.48 fold), moricin-like peptide C1 (+16.76 fold), peptidoglycan-recognition protein-LB (+15.85 fold) and prophenol oxidase activating enzyme 3 (+11.91 fold). There was also a range of proteins which was decreased in fungal-primed larvae relative to control larvae such as prophenoloxidase subunit 2 (−89.07 fold), Apolipophorin 2 (−3.08 fold), 3-dehydroecdysone 3beta-reductase (−1.75 fold) and methionine-rich storage protein 2 (−1.67 fold) (Fig. 3A, Fig S2, Table S1).

Proteins such as cecropin-D-like peptide (+22.23), hdd11 (+12.61), prophenol oxidase activating enzyme 3 (+5.96 fold), lysozyme-like protein 1 (+2.65 fold), hemolin (+2.32 fold) and peptidoglycan recognition protein (+2.13 fold) were increased in abundance in the hemolymph of *G. mellonella* larvae pre-exposed to heat killed *S. aureus* relative to control larvae. A range of proteins such as prophenoloxidase subunit 2 (−48.90), hexamerin storage protein PinSP1 (−2.73 fold), selenium-binding protein 1-like Protein (−2.08 fold), apolipophorin (2−1.88 fold) were decreased in abundance in the bacterial-primed *G. mellonella* larval hemolymph proteome relative to control larval proteome (Fig. 3B, Fig S2, Table S2).

Proteins that were exclusive to the proteomes of larvae previously exposed to heat killed yeast or bacterial inoculation (fungal and bacterial n = 9 i.e. not present in any replicate of control larvae) were serine protease inhibitor 12, N-acetylglucosamine-6-sulfatase isoform X1, cecropin-A1, conserved hypothetical protein, integument esterase 2 precursor, moricin-like peptide C3, moricin-like peptide C5, peptidoglycan-recognition protein-LB. Proteins exclusive (n = 12) to bacterial primed *G. mellonella* larval hemolymph were carboxylic ester hydrolase, growth-blocking peptide, chorion b-ZIP transcription factor, protease inhibitor-like protein, salivary cysteine-rich peptide, peptidoglycan-recognition protein-LB, antichymotrypsin-2, coatomer subunit gamma, pheromone binding protein, uncharacterized protein LOC113519625 [*G. mellonella*], lebocin-like anionic peptide 1, lebocin-5-like protein (Fig. 3B). Interestingly, there were 30 proteins SSDA (5 increased and 25 decreased) in *S. aureus* primed hemolymph as compared to *C. albicans* primed hemolymph (Fig. 3A) and 30 proteins SSDA (5 increased and 25 decreased) in *S. aureus* primed hemolymph as compared to *C. albicans* primed hemolymph.

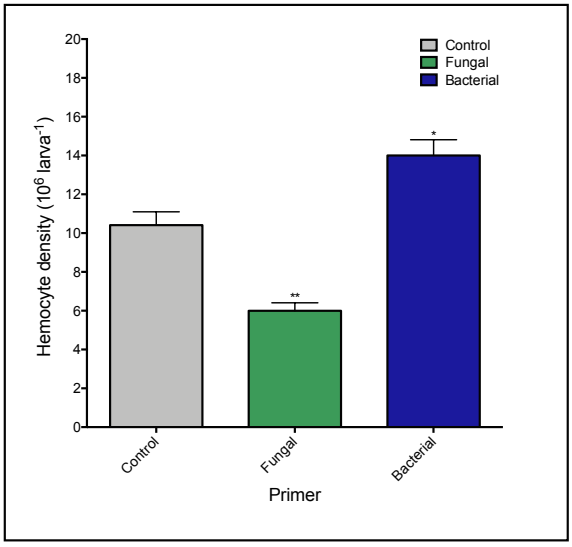

Fig. 2. Alterations in circulating hemocyte density from *G. mellonella* larvae immune-primed (fungal and bacterial) over 24 h. Larvae (n = 6) were injected with 20 µl of heat killed *C. albicans* (Fungal; 1 × 10^6 20 µl^{-1}), *S. aureus* (Bacterial; 20 µl of a 0.1 OD_{600} heat killed *S. aureus* PBS solution) or PBS and incubated at 30 °C for 24 h. Hemocytes were enumerated from primed and control larvae and statistical analysis was performed by comparing primed larvae to control larvae (*; $p < 0.05$, **; $p < 0.01$). All values are the mean ± S. E. of three independent experiments.

4. Discussion

Immune priming in insects offers the ability to protect against a subsequent infection but can be metabolically costly to induce and maintain (Sheehan *et al.*, 2020). Immune priming in *G. mellonella* has been well documented in recent years but the exact mechanism underlying this process and the level of specificity is poorly understood. Advances in quantitative shotgun proteomic technologies enable a greater level of sensitivity, the identification of a large number of proteins, accurate quantification and comparison of these proteins relative to a control (Tuli and Ressom, 2009). Alterations in hemocyte densities and proteomic profiles in immune primed larvae were assessed in order to characterise the cellular and humoral immune responses of *G. mellonella* larvae induced by exposure to heat killed bacterial or yeast cells. The utilisation of quantitative accurate mass spectrometry allowed the characterisation of the different responses induced by each stress and the determination of levels of specificity in each response. A single time point was used in this study (24 h) but it should be noted that the temporal response of insects to fungal and bacterial immune priming may differ (Tate and Graham, 2017) and future studies could use a wider array of timepoints to clarify this point.

Immune priming of larvae by exposure to heat killed *C. albicans* cells enhanced survival to subsequent *C. albicans* infection but not to *S. aureus* infection. In contrast priming of larvae with heat killed *S. aureus* enhanced larval survival to subsequent *S. aureus* and *C. albicans* infection. This may indicate that a bacterial infection may induce a robust immune response which displays broad spectrum anti-bacterial and anti-fungal activity however a fungal infection may only induce anti-

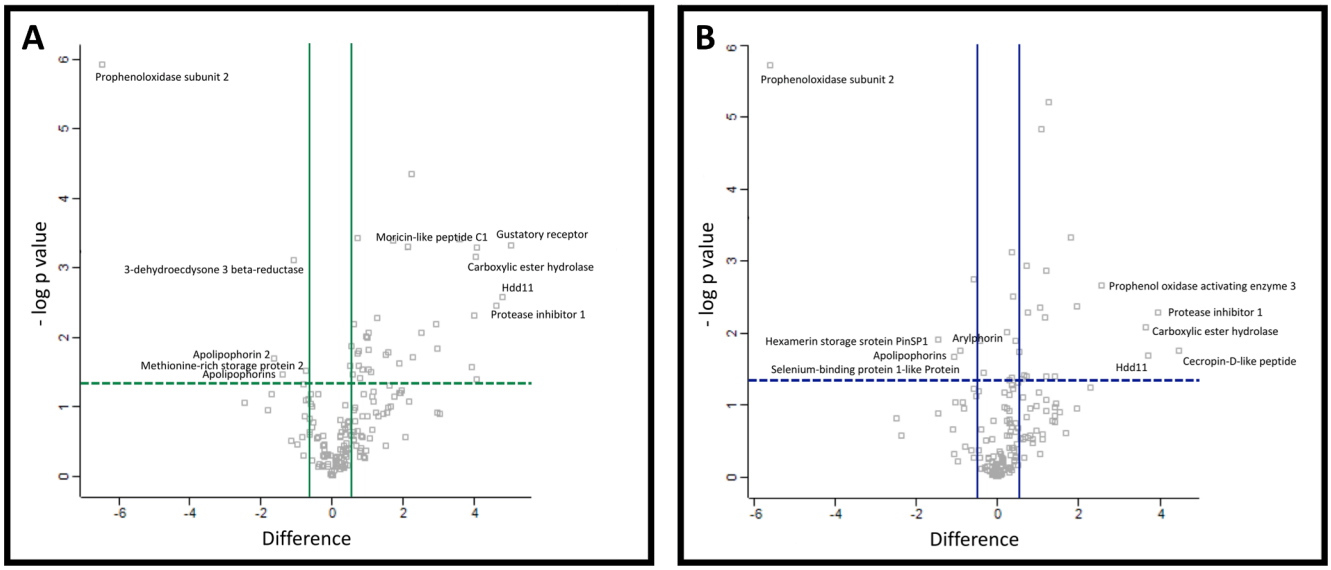

Fig. 3. Proteomic profiling of immune priming in *G. mellonella* larvae. Volcano plots (A; Fungal [heat killed *C. albicans*] and B; Bacterial [heat killed *S. aureus*] primed hemolymph proteomes as compared to the proteome of control [30 °C] larvae) represent protein intensity difference (- $\log_2$ mean intensity difference) and significance in differences (- log P-value) based on a two-sided *t*-test. Proteins above the line are considered statistically significant (p value < 0.05) and those to the right and left of the vertical lines indicate relative fold changes > 1.5. Annotations are given for the most differentially abundant proteins identified in hemolymph.

fungal immunity.

Hemocyte density was determined 24 h post-priming with heat killed *C. albicans or S. aureus,* to assess if the enhanced survival following subsequent infection may have been due to alterations in circulating hemocyte density. Interestingly, administration of heat killed fungal cells resulted in a significant decrease in hemocyte density, while administration of heat killed bacterial cells resulted in a significant increase. The reduction in hemocyte density in those larvae infected with heat killed *C. albicans* cells may be due to the binding of hemocytes to the yeast cells. Administration β-glucan to *G. mellonella* larvae resulted in an increase in survival which was correlated with increases in circulating hemocyte density but also alterations in the humoral immune response (Mowlds et al., 2010). Priming of larvae with LPS also resulted in increases in circulating hemocyte density, increased hemocyte phagocytic activity, increased encapsulation rate and increased bactericidal activity of cell free- hemolymph (Wu et al., 2015a, 2015b).

The humoral immune response of larvae immune primed by exposure to heat killed *C. albicans* or *S. aureus* for 24 h was assessed by tandem mass spectrometry. Larvae exposed to *C. albicans* demonstrated increased abundance of anti-fungal AMPs (moricin-like peptide C1, cobatoxin-like protein), proteins which may a role in binding of microbial components (e.g. peptidoglycan-recognition protein-LB, peptidoglycan recognition protein, peptidoglycan recognition-like protein B), proteins involved in phenoloxidase regulation (e.g. prophenoloxidase activating enzyme 3, serpin-4B, serpin-like protein, serpin-11, prophenoloxidase subunit 2) and proteins which play a role in the nodulation response (e.g. hdd11, hemolin, apolipoprotein D-like protein).

Moricins are secreted as pro-peptides and are activated via proteolysis and increase the permeability of bacterial and fungal membranes (Brown et al., 2008). The N-terminal residues (5–22) are amphipathic and responsible for bacterial membrane permeability, while the C-terminal residues (23–36), are hydrophobic and needed for full antimicrobial activity (Yi et al., 2014). *B. mori* moricin is active against *S. aureus*, targets the membrane and is induced by bacterial infection (Hara and Yamakawa, 1995). Cobatoxin was induced in *Helicoverpa armigera* in response to Gram-positive (*Bacillus thuringiensis*), Gram-negative (*Klebsiella pneumoniae)* and in response to yeast (*C. albicans*) (Wang et al., 2010). Cobatoxin from the *Centruroides noxius* scorpion is a toxin present in venom that blocks voltage-gated and Ca2 + -activated channels (Selisko et al., 1998).

Peptidoglycan recognition-like proteins bind to peptidoglycan via a conserved domain homologous to T4 bacteriophage lysozyme. Peptidoglycan recognition proteins from *H. diomphalia* (PGRP-SA) bind β-glucan and induced phenoloxidase activation (Seitz et al., 2003) and play an important role in bacterial cell identification and recognition by circulating immune cells (Dziarski, 2004). A range of components from the prophenoloxidase cascade was increased in response to *C. albicans* priming including a range of serpins. Serpins can limit the activity of phenoloxidase activating proteinases, thereby limiting the reaction speed and avoiding excessive melanization *in vivo* (Kanost, 1999; Kanost et al., 2004).

Previously, hdd11 was found to be up-regulated in *Hyphantria cunea* (mulberry moth) 2 h following inoculation with *Escherichia coli* (Gandhe et al., 2007). Hdd11 shares homology with noduler from *Antheraea mylitta* (Sarauer et al., 2003; Woon Shin et al., 1998) which plays an essential role in nodule formation, aggregation of yeast cells via binding β-glucan, bacterial cells via LPS, and RNAi knockdown of noduler results in increased fungal and bacterial burden during infection (Gandhe et al., 2007). Immunoglobulin superfamily member hemolin was induced by *Candida* challenge in *G. mellonella* larvae and has been shown to act as a pattern recognition receptor and opsonin in other insects (Shaik and Sehnal, 2009). Apolipoprotein D like protein was also found increased in response to heat killed *C. albicans*. Apolipoprotein functions as part of the lipophorin complex and is responsible for lipid transport (Niere et al., 2001, 1999) but it also augments the activity of lysozyme (Zdybicka-Barabas and Cytryńska, 2013), potentiates the activity of AMPs (Park et al., 2005), regulates phenoloxidase activity (Zdybicka-Barabas et al., 2014; Zdybicka-Barabas and Cytryńska, 2011) is a PRR and opsonin of lipopolysaccharide, lipoteichoic acids and fungal β-glucan (Wojda, 2017).

The response of the larval hemolymph to heat killed *S. aureus* was indicative of a bacterial immune response. A range of AMPs (e.g. lebocin-like anionic peptide 1, lebocin-5-like protein, cecropin-D-like peptide, lysozyme-like protein 1), proteins associated with microbial recognition (e.g. peptidoglycan recognition protein, peptidoglycan recognition-like protein B) and nodulation (e.g. hdd11, arylphorin, apolipophorin) were enriched within the dataset.

Lebocin is a proline-rich and O-glycosylated protein which is bioactivated by proteolytic cleavage of the precursor protein (Yi et al., 2014). Lebocin and Lebocin-like peptides display anti-*S. aureus* activity

but lacks anti-*Candida* activity (Cytryńska et al., 2007; Zhang et al., 2019). Cecropins are amphipathic α-helical AMPs of 11 amino acids in length that have the ability to target and kill bacteria (including *S. aureus*) and some fungi (Andrä et al., 2001; Bulet et al., 1999; Faruck et al., 2016; Lee et al., 2013). In insects, members of this family can be isolated from the hemolymph of moths and flies following bacterial infection (Qu et al., 1982; Mak et al., 2001; Kim et al., 2004; Mukherjee et al., 2011). In Gram-negative bacteria, the hydrophobic C-terminal of cecropin interacts with the phospholipid membrane of the bacteria leading to membrane disruption and bacterial cell death (Lee and Lee, 2015). Cecropin has antibacterial activity against multidrug resistant *Acinetobacter baumanii* and *Pseudomonas aeruginosa*, induces *C. albicans* apoptosis and recently has been shown to display immunomodulatory effects on human macrophages (Lee et al., 2015; Yun & Lee, 2016).

Lysozyme was also induced in larvae in response to infection with heat killed *S. aureus*. Lysozyme displays anti-fungal activity primarily at the cell surface (i.e. membrane or cell wall) which ultimately leads to osmotic imbalance and cell death in *C. albicans* (Woods et al., 2011; Wu et al., 1999). Lysozyme from *G. mellonella* has been shown to kill *C. albicans* by inducing apoptosis (Sowa-Jasilek et al., 2106). Lysozyme and cecropin fusion peptides display synergistic activity against *E. coli* (Lu et al., 2010). A range of proteins associated with microbial recognition and nodulation were also increased in abundance such as peptidoglycan recognition-like proteins and hdd11. Interestingly, there were no serpins increased in abundance in hemolymph in response to *S. aureus*, unlike the response to *C. albicans*.

Immune priming in larvae following administration of heat killed *S. aureus* produced inter-kingdom resistance to bacterial and fungal infection and this may be attributed to increases in circulating hemocyte density, the importance of this has been detailed in other studies (Mowlds et al., 2010; Wu et al., 2015a, 2015b). Alterations in the *S. aureus* primed- larval hemolymph proteome also provide evidence (i.e. cecropin, lysozyme, lebocin AMPs, microbial recognition proteins, decreased serpin abundance relative to *C. albicans* priming) as to why bacterial infection produced resistance to both bacterial and fungal infection. These proteins display both anti-bacterial and anti-fungal activity, and an increased abundance of microbial recognition protein possibly results in enhanced recognition and clearance of the infection (Seitz et al., 2003). The larval humoral immune response following administration of heat killed *C. albicans* is more specific and results in the increased abundance of primarily antifungal AMPs (moricin-like peptide C1), whereas the immune response to *S. aureus* is broad spectrum and results in the increased abundance of cecropin-D-like peptide which displays potent antibacterial and antifungal activity. Similar responses have been observed in *Tenebrio molitor* where challenge by Gram-positive or Gram-negative bacteria, but not fungi, induced transgenerational immune priming via increased antimicrobial peptide in eggs (Dubuffet et al., 2015). This indicates that the larval immune effector arsenal to bacterial infection may initiate a strong antimicrobial response which increases specific resistance to bacterial infection but also confers resistance to fungal infection.

Immune priming is an important survival strategy for certain groups of insects but it is metabolically costly to deploy and maintain. Priming offers the possibility of curtailing a potentially lethal infections shortly after exposure to a sublethal inoculum. The work presented here used a single time point for analysis but it should be noted that the response to bacterial and fungal stimulation may follow different timelines (Tate & Graham, 2017) so future work may look at multiple timepoints to see if each response peaks at an earlier or later time. The results presented here indicate that prior exposure to heat killed bacterial or yeast cells can induce resistance to subsequent potentially lethal infections but that bacterial-primed larvae show resistance to bacterial and fungal infection, while fungal primed larvae only showed resistance to subsequent fungal infection. This may indicate a level of specificity in the immune response to ensure maximum survival.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2021.104213.

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