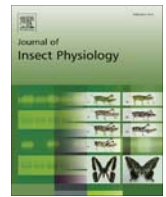


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## Thermal and physical stresses induce a short-term immune priming effect in *Galleria mellonella* larvae



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### ABSTRACT

Exposure of larvae of *Galleria mellonella* larvae to mild physical (i.e. shaking) or thermal stress for 24 h increased their ability to survive infection with *Aspergillus fumigatus* conidia however larvae stressed in a similar manner but incubated for 72 h prior to infection showed no elevation in their resistance to infection with *A. fumigatus*. Stressed larvae demonstrated an elevated haemocyte density 24 h after initiation of the stress event but this declined at 48 and 72 h. Larval proteins such as apolipoprotein, arylphorin and prophenoloxidase demonstrated elevated expression at 24 h but not at 72 h. Larvae maintained at 37 °C showed increased expression of a range of antimicrobial and immune-related proteins at 24 h but these decreased in expression thereafter. The results presented here indicate that *G. mellonella* larvae are capable of altering their immune response following exposure to mild thermal or physical stress to mount a response capable of counteracting microbial infection which reaches a peak 24 h after the initiation of the priming event and then declines by 72 h. A short-term immune priming effect may serve to prevent infection but maintaining an immune priming effect for longer periods may be metabolically costly and unnecessary while living within the colony of another insect.

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### 1. Introduction

Although lacking an adaptive immune response comparable to that of vertebrates, insects display a highly effective immune response that consists of interconnected cellular and humoral components. The cellular immune response of insects is mediated by haemocytes which can engulf, encapsulate or neutralize pathogens (Ratcliffe, 1993; Pech and Strand, 1996). Free floating haemocytes in the haemocoel are first to the site of infection and their numbers can be supplemented by the release of haemocytes bound to internal organs such as the fat body (Kavanagh and Reeves, 2004). The humoral immune response of insects is mediated by a variety of mechanisms including anti-microbial peptide production, (Ratcliffe, 1985) melanisation, haemolymph clotting (Kavanagh and Reeves, 2004) that impede the growth and development of the pathogen within the host.

Insects may encounter a number of stresses within their environment including infection, injury or abiotic factors such as temperature fluctuations and changes in nutrient availability. The gene responses in *Drosophila melanogaster* have been demonstrated to

be altered in response to invasion of a specific pathogen (De Gregorio et al., 2001; Irving et al., 2001). It has also been shown that insects may be primed against infection by certain pathogens based on prior exposure (Bergin et al., 2006; Little and Kraaijeveld, 2004). The availability of nutrients can also affect the insect immune response. Previous work demonstrated that dietary restriction can lead to an altered expression of a number of immune related genes and a delayed up-regulation of antimicrobial genes in *D. melanogaster* (Pletcher et al., 2005).

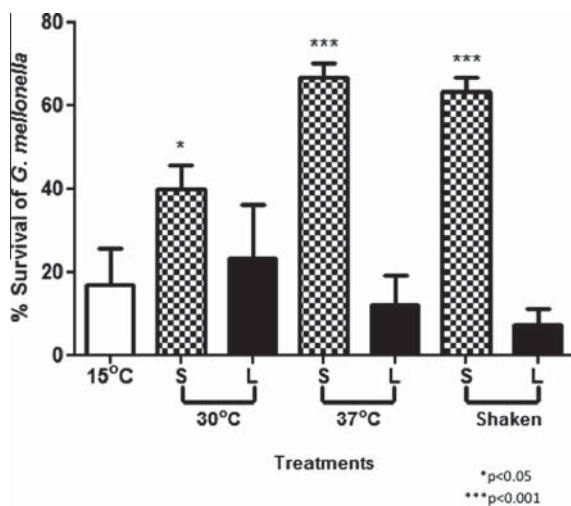
Immune priming has obvious survival advantages for the host and has been observed in a wide range of invertebrates including *D. melanogaster* (De Gregorio et al., 2001), *Anopheles* (Heard et al., 2005), *Caenorhabditis elegans* (Schulenburg et al., 2004) and Molluscs (Zhang and Loker, 2004). Inoculation of *D. melanogaster* with a sub-lethal dose of *Streptococcus pneumoniae* protected against a subsequent lethal inoculum and was mediated by a change in the density of circulating phagocytic cells (Pham et al., 2007). In *Bombus terrestris* the protection and specificity of immune priming can last up to 22 days, which is long after transcription and the elevated production of antimicrobial peptides has ended (Sadd and Schmid-Hempel, 2006).

Immune priming has been demonstrated in *Galleria mellonella* larvae following infection with sub-lethal doses of yeast (Bergin et al., 2006), fungal cell wall components (Mowlds et al., 2010),

Abbreviations: PO, phenoloxidase; PPO, prophenoloxidase.

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**Fig. 1.** Survival of *G. mellonella* larvae following incubation for 24 or 72 h prior to infection with *A. fumigatus* conidia. Larval survival was assessed 72 h post-infection. Control larvae were kept at 15 °C prior to inoculation. All larvae were incubated at 30 °C post-infection.

antifungal drugs (Rowan et al., 2009) and mild physical or thermal stress (Mowlds et al., 2008; Mowlds and Kavanagh 2008). Previous work demonstrated the differential expression of four genes in response to physical stress where *galiomicin* and *IMPI* demonstrated an increase in expression while *transferrin* and *gallerimycin* remained relatively unchanged (Mowlds et al., 2008). Thermally stressed larvae demonstrated elevated expression of a number of antimicrobial genes that enable increased resistance to infection (Mowlds et al., 2008; Wojda and Jakubowicz, 2007). Analysis of the effects of thermal and physical stress on *G. mellonella* larvae highlights the potential differential and proportional responses elicited by these stresses which appear to be similar to that observed in insects in response to microbial infections (Lemaitre et al., 1997; Bergin et al., 2006).

The immune system of insects shows strong structural and functional similarities to the innate immune system of mammals and results obtained using microbial pathogens in insects show a strong correlation to those generated using mammals (Jander et al., 2000; Mukherjee et al., 2013). *G. mellonella* larvae are widely used as model organisms for assessing the virulence of microbial pathogens or for determining the efficacy of antimicrobial agents (Kavanagh and Fallon, 2010). The aim of the work presented here was to establish whether an immune priming effect in *G. mellonella* larvae, induced as a result of exposure to mild physical or thermal stresses, was of short or long duration. Knowledge of how *G. mellonella* larvae respond to stress is important from a developmental point of view but would also inform how these insects could be used as *in vivo* models.

## 2. Materials and methods

### 2.1. Chemicals and reagents

All chemicals and reagents were of the highest purity and were purchased from Sigma–Aldrich Chemical Company Ltd (Dorset, UK) unless stated otherwise.

### 2.2. Fungal strain and culture conditions

*Aspergillus fumigatus* ATCC 26933 (obtained from the American Type Culture Collection) was grown on malt extract agar (MEA,

Oxoid) plates at 37 °C and conidia were harvested by washing with PBS Tween (0.01% v/v) solution.

### 2.3. Insect larvae

Sixth instar larvae of *G. mellonella* (Lepidoptera: Pyralidae, the Greater Wax Moth) (Mealworm Company, Sheffield, England) were stored in the dark at 15 °C to stop their development (Cotter et al., 2000, Hornsey and Wareham, 2011). Larvae of the same age and of equivalent weights ( $0.277 \pm 0.005$  g) were inoculated with  $1 \times 10^7$  *A. fumigatus* conidia per 20  $\mu$ l PBS through the last pro-leg into the haemolymph using a Myjector U100 insulin syringe (Terumo Europe, Leuven, Belgium).

### 2.4. Larval thermal and physical stress treatments

Prior to infection with *A. fumigatus* conidia larvae were maintained at 30 °C or 37 °C, or physically stressed by gentle shaking for 2 min and incubated at 30 °C, for 24 h (this is described as short term incubation (S)). Larvae were also stressed in a similar way to the short term incubated larvae but incubated at 30 °C for an extra 48 h prior to infection (this is described as long term incubation (L)). Control larvae were incubated at 15 °C for 24 h prior to inoculation. All larvae were placed at 30 °C once inoculated, and larval survival was assessed 72 h post infection.

Larvae were physically stressed by being gently shaken in cupped hands through a distance of 25 cm in a vertical motion at a frequency of 80 times per minute for a period of 2 min (Mowlds et al., 2008). This treatment had no effect on the survival of larvae. Ten larvae were used per treatment and all experiments were performed on three independent occasions.

### 2.5. Determination of haemocyte density

Haemocyte density was assessed by bleeding three larvae into a pre-chilled tube containing 0.37% (v/v) mercaptoethanol in sterile IPS (150 mM NaCl, 5 mM KCl, 0.1 M Tris–HCl, 10 mM EDTA and 30 mM Trisodium citrate in dH<sub>2</sub>O, pH 6.9) to prevent melanisation and the haemocyte density was assessed using a Neubauer haemocytometer. Experiments were performed on three independent occasions and the means  $\pm$  SE were determined.

### 2.6. 1D SDS–PAGE analysis of protein expression

Larvae were stressed as described and incubated at 15, 30 or 37 °C for 24, 48 or 72 h. Ten larvae per treatment were bled through the anterior region into pre-chilled micro-centrifuge tubes and the haemocytes were removed by centrifugation at 800g for 5 min at 4 °C. Cell free haemolymph (100  $\mu$ l) was diluted in ice-cold IPS and protein quantified by Bradford assay. Protein concentration was adjusted to 20 mg ( $\mu$ g) in 5 $\times$  solubilisation buffer (glycerol; 52%, 10% (v/v) SDS; 10.5%, 1.5 M Tris–HCl; 6.5%, bromophenol blue (0.5% w/v); 1.3% 2-mercaptoethanol; 2.63%) and incubated at 95 °C for 5 min. Samples were separated by 1-dimensional SDS–PAGE and visualized by Coomassie staining. Relative quantification of protein bands was carried out using Image J 1.46 software on each matching band.

### 2.7. 2D SDS–PAGE separation of haemolymph proteins and image analysis

Haemolymph (100  $\mu$ l) was collected from larvae that had been incubated at 37 °C for 24, 48 or 72 h by piercing the head of the insect and bleeding the haemolymph into a pre-chilled microcentrifuge tube. Protein concentration was determined by the Bradford method and 400  $\mu$ g of protein was acetone precipitated

per sample. Isoelectric focusing of protein samples on a pH 4–7 strip and 2D electrophoresis was performed as described previously (Bergin et al., 2006). Each 2D gel was scanned on a Hewlett Packard scanjet 5100c scanner and the images were analyzed using Progenesis SameSpot Software. The Progenesis software enabled the analysis of protein expression changes between gel replicates with significance determined using ANOVA. The program enables alignment of gels, with minor distortions between gels reduced by running all replicates at one time. The alignment software enables the user to link proteins manually between gels which in conjunction with the Auto alignment feature automatically match proteins between gels. A table of protein spots is built and every given protein has been linked to the matching proteins between the gels creating a list of proteins that can be cross referenced as a final check to ensure correct alignment.

### 2.8. LC/MS analysis of peptides

In-gel digestion was performed on 1-Dimensional gel bands or 2-Dimensional gel spots of interest from a reference gel with proteins migrated to the same point between gels. The gel pieces were trypsin digested as described by Shevchenko et al. (2006) and fragmented protein samples were eluted by LC/MS (Agilent 6340 Ion Trap) which determines the relative charge to mass ratio from detected ionized particles. These data were analysed using the mascot search engine to identify the protein, ([www.matrixscience.com](http://www.matrixscience.com)). MASCOT scores above 67 were deemed to have a significant match ( $p < 0.05$ ). The mass error tolerance was 1 Da allowing for a maximum of no more than two missed cleavages. Verification of protein sequences was confirmed by blasting the protein sequence on the Uniprot ([www.uniprot.org](http://www.uniprot.org)) and NCBI ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) websites.

### 2.9. Statistical analysis

All experiments were performed on three independent occasions and results are expressed as the mean  $\pm$  SE. Changes in larval survival were analysed with the log rank (Mantel-Cox) method using GraphPad Prism version 5.00. Analysis of changes in haemocyte density and protein expression were performed by Two-way ANOVA using GraphPad Prism version 5.00 for Windows 8, GraphPad Software, San Diego, California, USA, ([www.graphpad.com](http://www.graphpad.com)).

## 3. Results

### 3.1. Effect of physical and thermal stress on ability of *G. mellonella* larvae to survive fungal infection

Larvae of *G. mellonella* were physically or thermally stressed as described and incubated for a short (24 h) or long (72 h) period prior to infection with *A. fumigatus*. Larval survivals were assessed 72 h post infection. Larvae incubated at 30 °C for 24 h prior to infection showed  $40.0 \pm 5.8\%$  survival 72 h post-infection ( $p = 0.05$  vs control larvae) while those larvae incubated at 30 °C for 72 h prior to infection (L) showed  $23.1 \pm 12.8\%$  survival at the same time point. Larvae pre-incubated at 37 °C for 24 h prior to infection showed  $66.7 \pm 3.3\%$  ( $p = 0.001$ ) survival while those larvae that were incubated at 37 °C for 24 h and then at 30 °C for 48 h prior to infection showed  $12.0 \pm 7.23\%$  survival. Larvae physically stressed and incubated at 30 °C for 24 h demonstrated  $63.3 \pm 3.3\%$  survival ( $p = 0.001$ ) while those larvae stressed and incubated at 30 °C for 72 h showed  $7.3 \pm 3.7\%$  survival. These results indicate that larvae incubated at 30 or 37 °C, or physically stressed, for 24 h prior to infection showed increased survival

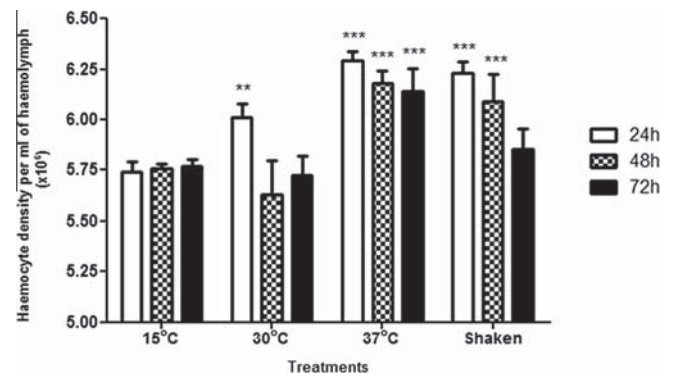


Fig. 2. Haemocyte density of larvae thermally (15 °C, 30 °C and 37 °C) or physically stressed (shaken) at 24, 48 or 72 h.

compared to larvae that received the same treatments but were infected 72 h after initiation of the stress event (Fig. 1).

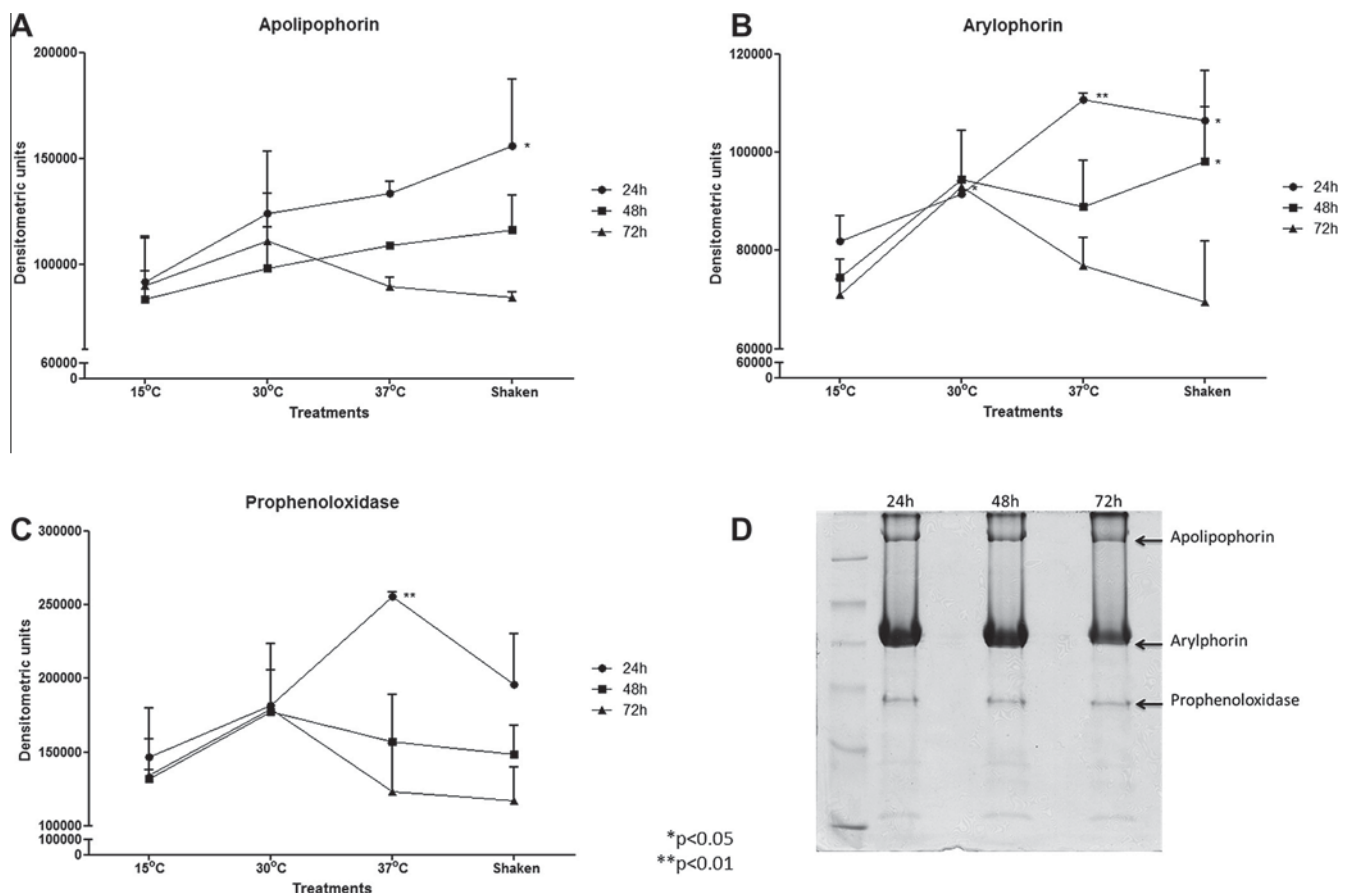
### 3.2. Effect of physical and thermal stress on haemocyte density

Exposing larvae to physical or thermal stresses lead to an increase in the density of circulating haemocytes in the haemolymph 24 h later (Fig. 2). In particular larvae incubated at 37 °C for 24 h showed  $6.23 \pm 0.04 \times 10^7$  haemocytes per ml haemolymph compared to  $5.97 \pm 0.04 \times 10^7$  per ml in larvae incubated at 15 °C ( $p = 0.001$ ). Those larvae that were physically stressed showed a similar increase in haemocyte density at 24 h i.e. ( $6.23 \pm 0.05 \times 10^7$ /ml). Interestingly the haemocyte density in larvae pre-incubated at 37 °C or physically stressed declined at 48 and 72 h possibly suggesting a return to pre-stress levels. Larvae incubated at 15 °C showed no significant alteration in haemocyte density over the 72 h.

### 3.3. Analysis of changes in protein expression in stressed larvae

Larvae were thermally or physically stressed as described and incubated for 24, 48 or 72 h at 15, 30 or 37 °C as appropriate. Protein was extracted from larvae and resolved by 1-D SDS-PAGE as described. Analysis of the alterations in the intensity of apolipoprotein (Accession No: AAT76806, 22% coverage, score 1241), prophenoloxidase (PPO) (Accession No: AAK64363, 5% coverage, score 91) and arylophorin (Accession No: AAA19801, 32% coverage, score 1113), revealed the increased intensity of all three proteins in sera from larvae incubated at 37 °C or physically stressed at 24 h (Fig. 3A–C). However the relative intensity of these three proteins was reduced at 48 and 72 h.

In order to examine whether there was a prolonged change in the expression of a range of proteins following a thermal stress haemolymph was extracted from larvae incubated at 37 °C for 24, 48 or 72 h and resolved by 2D SDS-PAGE (Fig. 4). A number of proteins with immune functions were shown to be increased in intensity at 24 h but declined thereafter (Table 1). The 26 kDa (spot 4) and 32 kDa ferritin (spot 5) proteins are important in maintaining the homeostasis of iron in the haemocoel and have important roles in the immune response (Levy et al., 2004). Both proteins show a 1.34–1.4-fold increase in intensity at 24 h but this declined at 48 and 72 h. Transferrin (spot 1) showed a 2.27-fold increase in expression at 24 h compared to the control, however the intensity declined to 1.62 and 1.25-fold at 48 and 72 h, respectively. Apolipoprotein III (spot 3) has several functions including lipid transport and a function in the innate immune response (Gupta et al., 2010) and showed a 1.82-fold increase in intensity at 24 h but this declined to 0.95 and 0.7-fold at 48 h and 72 h



**Fig. 3.** Relative expression of apolipophorin (A), arylophorin (B) and prophenoloxidase (C) in sera from *G. mellonella* larvae physically or thermally stressed at 24, 48 or 72 h. Representative gel of separated haemolymph proteins on 12.5% SDS-PAGE with proteins of interest indicated (D).

respectively. Arylophorin (spot 9) showed an increase in expression of 1.9-fold at 24 h but the intensity reduced to 0.53 and 0.59-fold at 48 and 72 h, respectively. Arylophorin is a storage protein for amino acids and aids in the immune defense of insects (Beresford et al., 1997). Serpin 1 (spot 6) demonstrated a 1.46-fold increase in expression at 24 h but its relative expression decreased thereafter. The expression of juvenile growth hormone precursor (spot 8) was maintained at a relatively constant level across the time points and may be considered as a loading control (Banville et al., 2012).

#### 4. Discussion

The results presented here indicate that mild physical and/or thermal stress can increase the resistance of *G. mellonella* larvae to infection by *A. fumigatus* if larvae are inoculated 24 h after the initiation of the stress event. Larvae thermally or physically stressed for 24 h prior to infection demonstrated an increase in haemocyte density and in the expression of a number of antimicrobial proteins. In contrast, those larvae inoculated 72 h after the initiation of the stress events demonstrated no enhanced resistance to infection by this pathogen.

The abundance of prophenoloxidase, arylophorin and apolipophorin was increased at 24 h in those larvae that had been thermally or physically stressed but the expression of these proteins declined in larvae incubated for 48 and 72 h. A short term increase in abundance of either prophenoloxidase or phenoloxidase (PO) may be due to two possible scenarios. PPO is the inactive storage form of the enzyme which may increase for an immune investment, whereas PO reflects the active enzyme used in active components of the immune response (Bocher et al., 2007). An increase in

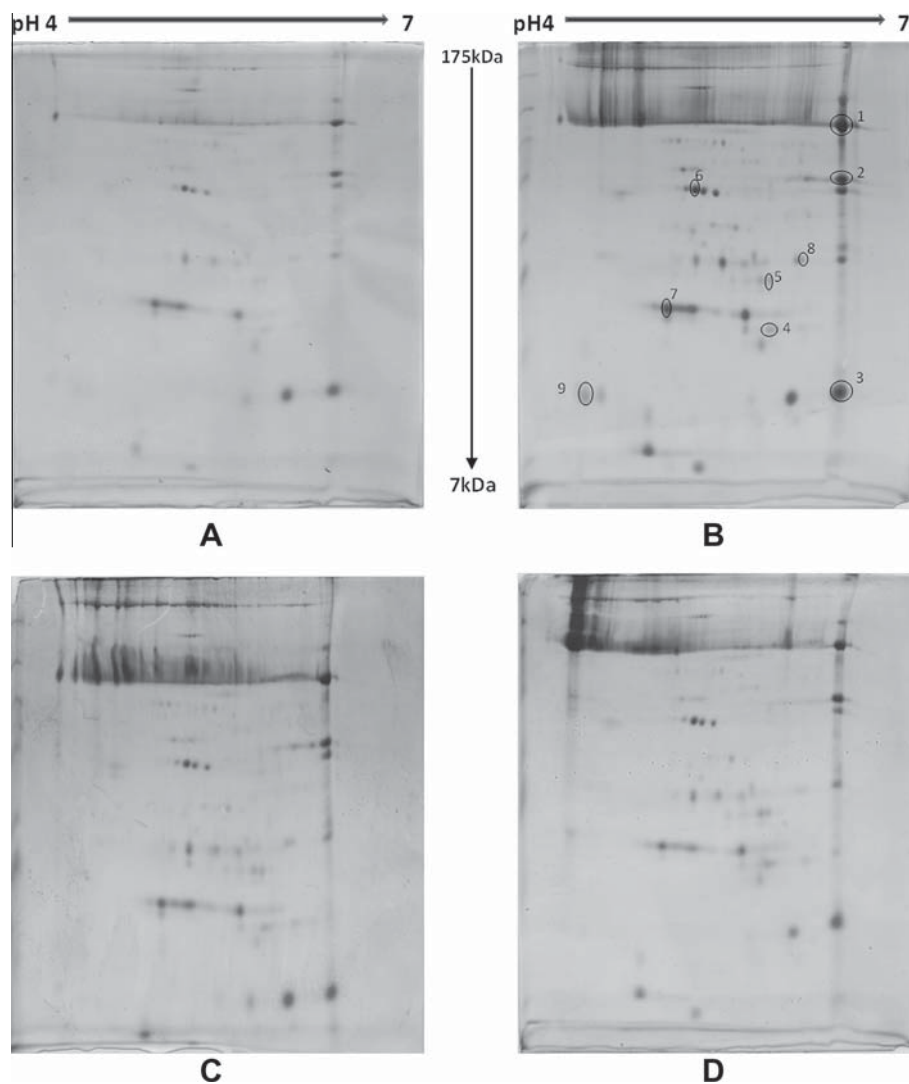
PPO could occur as part of priming for an immune response to a potential infection within the larvae whereas the activated PO would be required for dealing with an active infection within the larvae (Berisha et al., 2013).

The haemocyte density of larvae incubated at 37 °C or physically stressed reached the highest value at 24 h but declined at 48 and 72 h.

Immune priming is well characterized in insects and serves to protect the insect from a potentially lethal infection by raising immune defences following exposure to a sub-lethal infection. We previously established that non-lethal physical and thermal stress can lead to increased resistance of *G. mellonella* to infection with the yeast *Candida albicans* (Mowlds et al., 2008; Mowlds and Kavanagh 2008). In this work we have extended these observations and demonstrated that the priming effect in *G. mellonella* larvae is a short term event that declines after 24 h.

Immune priming in insects has the advantage of giving protection from a subsequent potentially lethal infection but is costly to maintain and can result in death if compensatory feeding is unavailable (Moret and Schmid-Hempel, 2000). Immune priming can also be affected by the social interaction and behaviour of challenged bees (Richard et al., 2008). Immune priming in *Formica selysi* workers following challenge with *Beauveria bassiana* is short term (Reber and Chapuisat, 2012) raising the possibility that colony living precludes the necessity of having a prolonged immune priming effect as other compensatory mechanisms may be operating in the colony. For example, in honey bee colonies an elevated nest temperature is generated as a colony-level response to prevent chalk brood (Starks et al., 2000). Social immunity in insects has been suggested in a number of colony based communities and it has been demonstrated that ants which are infected within





**Fig. 4.** 2D SDS-PAGE of separated haemolymph proteins from *G. mellonella* larvae incubated at 37 °C for 0 h (A), 24 h (B), 48 h (C) or 72 h (D). Proteins of interest (1–9) were excised and identified by LC–Mass spectrometry.

**Table 1**

Relative expression of proteins isolated from haemolymph of *G. mellonella* larvae incubated at 37 °C for 24, 48 or 72 h.

Protein identity	Organism	Score	Seq. coverage (%)	Accession number	0 h (control)	Relative expression		
						24 h	48 h	72 h
1 Transferrin precursor	<i>Galleria mellonella</i>	340	8	AAQ63970	1	1.93*	1.33	1.17
2 Imaginal disc growth factor-like protein	<i>Mamestra brassicae</i>	158	13	ABC79625	1	1.77	1.18	1.18
3 Apolipoprotein-3	<i>Galleria mellonella</i>	604	66	P80703	1	1.72	1.07	1.04
4 26 kDa ferritin subunit	<i>Galleria mellonella</i>	504	44	AAG41120	1	1.46	1.23	1.13
5 32 kDa ferritin subunit	<i>Galleria mellonella</i>	469	35	AAL47694	1	2.03***	1.13	0.97
6 Serpin 1	<i>Danaus plexippus</i>	86	6	EHJ75277	1	1.59	1.13	1.34
7 27 kDa glycoprotein precursor	<i>Bombyx mori</i>	93	7	Q8T113	1	1.99*	1.36	0.99
8 Juvenile hormone binding protein precursor	<i>Galleria mellonella</i>	262	24	AAS94224	1	1.21	1.11	0.99
9 Arylphorin	<i>Galleria mellonella</i>	168	5	AAA74229	1	1.91*	0.90	0.95

\*  $p < 0.05$ .

\*\*\*  $p < 0.001$ .

a colony can regurgitate droplets which were capable of transferring immune factors to other uninfected ants (Hamilton et al., 2011). Traniello et al. (2002) demonstrated that naïve termites reared alongside termites previously exposed to *Metarhizium anisopliae* can improve the subsequent resistance of the naïve termites to the fungal pathogen. A potential role for 'behavioural fever',

where insects alter their temperature through thermoregulatory behaviour, leading to an increased ability to withstand infection has been suggested (Watson et al., 1993; Blanford et al., 1998; Elliot et al., 2002).

The enhanced immune protection afforded by the priming events described here in *G. mellonella* larvae reached a peak 24 h

after the initiation of the stress event and then declined. The relatively short duration of the immune priming effect in *Galleria* may be due to the fact that in its normal habitat *G. mellonella* live in bee colonies where the high temperature may offer some degree of protection against pathogens thus not necessitating a long term, heightened immune response that may be metabolically costly to maintain. In contrast to other insects, ants do not show immune priming when challenged with *B. bassiana* and it has been suggested that behavioral or group level chemical defences may limit infection and thus remove the necessity for an immune priming response (Reber and Chapuisat, 2012). The observation of a short term (24 h) immune priming effect in *G. mellonella* larvae is of interest in that it may have evolved as a response to life within the colony of another insect. This raises the possibility of the immune response of one insect (i.e. *G. mellonella*) being modulated by living in the colony of another (i.e. *Apis mellifera*).

Insects, and larvae of *G. mellonella* in particular, are now widely used in industry and academia as *in vivo* models (Kavanagh and Fallon, 2010) and give results comparable to those that can be obtained using mammals (Jander et al., 2000). However no standardized incubation conditions have been accepted (Cook and McArthur, 2013). The results presented here demonstrate that larvae are sensitive to alteration in temperature and mild physical stress, factors that may affect the reliability of results generated using these larvae.

### Conflict of interest

The authors have no conflicts of interest to declare.

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