

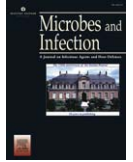


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Original article

Physical stress primes the immune response of *Galleria mellonella* larvae to infection by *Candida albicans*

Peter Mowlds, Aoife Barron, Kevin Kavanagh*

Department of Biology, Medical Mycology Unit, NICB, National University of Ireland Maynooth, Co. Kildare, Ireland

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Abstract

Larvae of the greater wax moth (*Galleria mellonella*) that had been subjected to physical stress by shaking in cupped hands for 2 min showed reduced susceptibility to infection by *Candida albicans* when infected 24 h after the stress event. Physically stressed larvae demonstrated an increase in haemocyte density and elevated mRNA levels of *galiomicin* and an *inducible metalloproteinase inhibitor (IMPI)* but not *transferrin* or *gallerimycin*. In contrast, previous work has demonstrated that microbial priming of larvae resulted in the induction of all four genes. Examination of the expression of proteins in the insect haemolymph using 2D electrophoresis and MALDI TOF analysis revealed an increase in the intensity of a number of peptides showing some similarities with proteins associated with the insect immune response to infection. This study demonstrates that non-lethal physical stress primes the immune response of *G. mellonella* and this is mediated by elevated haemocyte numbers, increased mRNA levels of genes coding for two antimicrobial peptides and the appearance of novel peptides in the haemolymph. This work demonstrates that physical priming increases the insect immune response but the mechanism of this priming is different to that induced by low level exposure to microbial pathogens.

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Keywords: *Galleria mellonella*; Antimicrobial proteins; *Candida*; Host defence; 2D gel electrophoresis; MALDI TOF analysis; Gene expression

1. Introduction

The cellular immune response of insects is mediated by haemocytes while the humoral response is mediated by the production of a range of antimicrobial peptides (AMPs) and melanisation [1]. The insect immune response bears a number of similarities to the innate immune response of mammals and as a result various insects have been utilised for assessing the pathogenicity of bacterial [2] and fungal isolates [3] and for assessing the efficacy of a variety of antimicrobial drugs. Positive correlations between the virulence of microbial pathogens in insects and mammals [4,5] have been established and the use of insects for screening microbial mutants or antimicrobial drugs is now recognized as a valuable approach to reduce the need to use mammals for this type of testing [6].

Although insects lack an immune response equivalent to the adaptive immune response of vertebrates they are still capable of dealing with the entry of microbial pathogens and parasites in a rapid and effective manner [1]. The cellular immune response consists of haemocytes which function to phagocytose and encapsulate invading cells, or form nodules around larger structures (e.g. eggs of parasitic wasps). The humoral immune response is mediated by the production of a range of antimicrobial peptides, the formation of melanin or the coagulation of haemolymph to seal ruptures in the cuticle or retard the passage of pathogens. AMPs are released from a range of organs and cells [7,8] into the haemolymph of the insect where they diffuse to the site of infection and attack components of the bacterial or fungal cell wall [9].

Although lacking an adaptive immune response it is well established that exposure of insects to low levels of a pathogen [10] or pathogen associated material (e.g. glucan, LPS) leads to priming of the immune response and enhanced protection against a subsequent infection with a potentially lethal dose of

* Corresponding author. Tel.: +353 1 708 3859; fax: +353 1 708 3845.
E-mail address: kevin.kavanagh@nuim.ie (K. Kavanagh).

pathogen [11,12]. We have previously established that exposure of larvae of *Galleria mellonella* to low doses of yeast or fungal cell wall material induces genes coding for antimicrobial peptides and the appearance of novel immune related peptides in the haemolymph [12]. While immunological priming has clear benefits for an insect and may allow it to withstand a subsequent lethal infection it does carry a biological cost and, in the absence of compensatory feeding, can be fatal [13].

In this work the immune response of physically stressed *G. mellonella* larvae to infection with *Candida albicans* was examined to determine whether physical stress could prime the immune response and to ascertain whether the nature of the priming was different to that previously observed in microbially challenged larvae [12].

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. Yeast strains and culture conditions

C. albicans MEN (kindly donated by Dr. D. Kerridge, Cambridge, UK) was employed in this work and yeast cultures were grown as described previously [12].

2.3. Insect larvae

Sixth instar larvae of *G. mellonella* (Lepidoptera: Pyralidae, the greater wax moth) (Mealworm Company, Sheffield, England) were stored in wood shavings in the dark at 15 °C [3]. Larvae (0.2 and 0.4 g) were randomly chosen and all experiments were performed on three independent occasions. Larvae were inoculated with 5×10^5 yeast cells per 20 μ l through the last pro-leg into the haemolymph as described previously [12].

2.4. Physical stress treatments of insect larvae

Larvae were shaken in cupped hands through a distance of 25 cm in a vertical motion back and forth at a frequency of 80 times per minute for a period of 2 min before being transferred to 30 °C for 24 h prior to inoculation with *C. albicans*. Post-inoculation, larvae infected with *C. albicans* were incubated at 30 °C. Ten larvae were used per treatment and all the 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 microcentrifuge tube containing a few granules of phenylthiourea to prevent melanisation. Dilutions were made by mixing the haemolymph with 0.37% (v/v) mercaptoethanol in sterile PBS and the haemocyte density was assessed with the aid of a Neubauer haemocytometer.

Experiments were performed on three independent occasions and the means \pm SE were determined.

2.6. Induction of immune relevant proteins of *G. mellonella* by RT-PCR

Larval RNA was extracted using TRI-reagent at 0, 4, 18, 24 and 48 h post the stress event. RNA (2 mg) was treated with DNase I prior to cDNA synthesis using the SuperScript Kit (Invitrogen) with oligo(dT) primers. RNA was extracted from each treatment on three independent occasions and quantification was performed using gel analysis and Gene tools software (Syngene).

PCR amplification of target genes was performed with primers and following the same conditions as listed in Ref. [12] and cycled 35 times. All samples were normalised to the corresponding β actin value. The highest level of expression in a series was set to 100% and other values of that series are given as the percentage relative expression [10].

2.7. Two-dimensional gel electrophoretic separation of haemolymph proteins

Haemolymph was collected from control and physically stressed larvae into a pre-chilled microcentrifuge tube. A cocktail of protease inhibitors (10 μ g/ml leupeptin, pepstatin A, aprotinin and tosyl-lysylchloromethane (TLCK)) was added to the haemolymph, which was concentrated at $800 \times g$ for 5 min to remove haemocytes and cell debris. Haemolymph was solubilised in IEF buffer for 30–60 min at room temperature with occasional vortexing.

Iso-electric focusing (IEF) was performed with 0.3 mg of haemolymph protein loaded on 13 cm IPG strips (Amersham Biosciences UK Ltd) as described previously [12]. The IPG strips were applied to a 10% SDS-PAGE slab gel in a Biorad protean gel rig. Running buffer (5X) was placed in the internal chamber of the gel rig and gels were electrophoresed overnight at 50 V at room temperature. Separated proteins were visualised by Coomassie staining.

2.8. Image acquisition and analysis

Each 2D gel was scanned on a Hewlett Packard Scanjet 5100c Scanner and the images analysed with ImageMaster 2D Platinum v7 software (Amersham Biosciences UK Ltd). The protein spots of interest on each gel were detected, normalised, edited and manually matched to a reference gel. Proteins separated by 2D electrophoresis were quantified in terms of their relative volume (% vol). Proteins that were up- or down-regulated and proteins that appeared or disappeared under one condition or another were selected for analysis with MS. The mean increase/decrease in spot intensity was determined based on three independent gels per treatment.

2.9. Protein identification by MALDI TOF analysis

Mass spectrometry of trypsin digested proteins was performed using an Ettan™ MALDI-TOF spectrometer

(Amersham Biosciences, GmbH, Freiburg, Germany) as described previously [12]. The resulting mass list from the tryptic-digested protein was analysed using ProFound peptide mapping version 4.10.5 developed by Rockefeller University (<http://www.unb.br/cbsp/paginiciais/profound.htm>). The taxonomy used to identify tryptic fingerprint was *Drosophila* and other Metazoa with a tolerance mass error of 1.0 Da. Verification of sequences was performed using a BLAST search on the NCBI website (<http://www.ncbi.nlm.nih.gov>) to identify conserved domains of protein families. A Z score greater than 1.65 represents a 95% confidence level.

2.10. Statistical analysis

All assays were performed on three independent occasions. Results are expressed as the mean \pm SE. Survival curves were analysed with the log rank (Mantel–Cox) method utilising GraphPad Prism version 5 software. The variations in mRNA levels were compared by *t* test (two tailed, variance of 3 method) using Sigma Stat Statistical analysis Package Version 1.00 (SPSS Inc, Chicago, IL, USA). Differences were considered significant at $p \leq 0.05$.

3. Results

3.1. Effect of physical stress on the susceptibility of *Galleria* larvae to fungal infection

Previous work has established that exposure of larvae of *G. mellonella* to fungal cells or fungal cell wall material induces an immune response that reduces the susceptibility of larvae to subsequent infection by *C. albicans* [12]. In the work presented here larvae were subjected to mild physical stress as described and incubated for 24 h at 30 °C before being inoculated with *C. albicans* at a density of 5×10^5 /larva. Shaking of larvae had no adverse effect on survival over a 72 h period. The results (Fig. 1) indicate that the survival of control larvae (i.e. unstressed and infected) was $23.3 \pm 7.7\%$, $20 \pm 7.3\%$ and $10 \pm 5.4\%$ at 24, 48 and 72 h, respectively. In contrast, physically stressed larvae showed survival rates of $80 \pm 7.3\%$, $53.3 \pm 9.1\%$ and $50 \pm 9.1\%$ at the same time points. Analysis of the survival curves indicated a statistically greater survival in stressed larvae, compared to unstressed larvae, at $p < 0.0001$ using the log rank (Mantel Cox) test. These results indicate that physically stressing larvae 24 h prior to inoculation with *C. albicans* reduces the sensitivity of larvae to microbial infection.

3.2. Analysis of the effect of physical stress on haemocyte density in *G. mellonella* larvae

The insect immune response consists of interacting cellular and humoral components [1]. Haemocytes represent the main element of the cellular response and the numbers of circulating haemocytes can vary depending upon the stage of the insect's lifecycle and in response to infection. The number of haemocytes in larvae that had been physically stressed was assessed

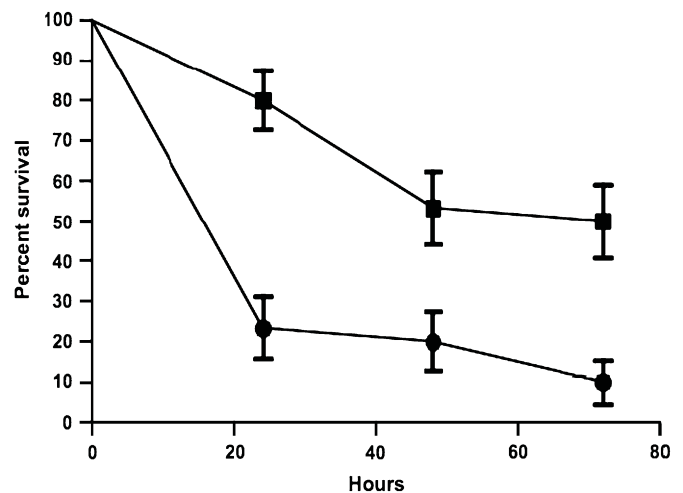


Fig. 1. Survival (%) of physically stressed *G. mellonella* larvae inoculated with *C. albicans*. Larvae were physically stressed by shaking for 2 min 24 h prior to inoculation with 5×10^5 cells of *C. albicans*. All values represent the mean \pm SE from three independent experiments. (Control (unstressed and infected) larvae: circles; stressed and infected larvae: squares.)

24 and 48 h after the stress event. The number of haemocytes in physically stressed larvae was twice as high at 24 h ($6.6 \pm 0.37 \times 10^5$ haemocytes/larva) compared to the control ($3.2 \pm 0.25 \times 10^5$ haemocytes/larva) ($p = 0.002$). At 48 h the haemocyte density in physically stressed larvae was $6.76 \pm 0.39 \times 10^5$ haemocytes/larva while that of the control was $2.9 \pm 0.45 \times 10^5$ haemocytes/larva ($p = 0.003$). These results indicate that physical stress induces an increase in the number of circulating haemocytes which may play a role in the increased survivability demonstrated following yeast or bacterial infection.

3.3. Gene expression of proteins involved in an immune response

During an immune reaction to microbes, the up-regulation of proteins of the insect immune system has been observed [10]. We have previously demonstrated that pre-exposure of *G. mellonella* larvae to a sub-lethal dose of *C. albicans* induces a number of genes coding for antimicrobial peptides [12].

The mRNA levels of genes coding for proteins and peptides involved in this protective immune response within larvae following mild physical stress were assessed. Larvae were physically stressed as described, RNA was extracted from whole larvae over 48 h and cDNA was generated. The genes that were examined coded for *galiomicin*, a defensin identified in *G. mellonella* [14], a cysteine rich antifungal peptide *gallerimycin* [15], *transferrin*, an iron binding protein and an *inducible metalloproteinase inhibitor (IMPI)* from *G. mellonella* [16] (Fig. 2).

In the case of *galiomicin*, physical stress induced a relative expression of $76.2 \pm 3.2\%$ after 1 h rising to a level of $92.1 \pm 7.3\%$ at 24 h (Fig. 3). In contrast expression of *galiomicin* in control larvae remained below $26.1 \pm 0.3\%$ throughout the duration of the experiment. The mRNA levels of *galiomicin* at 48 h were statistically greater in physically stressed

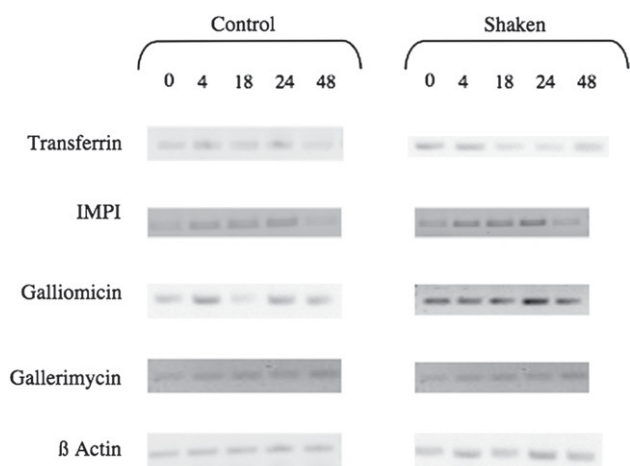


Fig. 2. RT-PCR analysis of *G. mellonella* cDNA from control and stressed larvae on a 1% agarose gel. Three independent RNA extractions were performed and PCR was carried out using primers for *galliomycin*, *IMPI*, *transferrin*, *gallerimycin*, and β actin (housekeeping gene) on cDNA.

larvae than in controls at $p = 0.004$. The expression of *IMPI* (Fig. 3) in physically stressed larvae is greater at all time points compared to that in the control larvae. Peak mRNA levels are reached at 24 h in both control and physically stressed larvae but the value is $58.2 \pm 7.1\%$ in the former group of larvae and $92 \pm 7.2\%$ in the latter group. mRNA

levels of *IMPI* at 48 h were statistically greater in physically stressed larvae than in controls at $p = 0.002$.

The expression of *transferrin* (Fig. 3) in control and physically treated larvae was variable with the peak expression in control larvae being recorded at 24 h while the peak expression in physically challenged larvae was found at 1 h. However, overall there was no statistically significant difference between the mRNA levels in both treated and untreated larvae and at 48 h ($p = 0.365$). The mRNA levels of *gallerimycin* (Fig. 3) over 48 h were marginally higher in physically stressed larvae compared to controls but this was not deemed significantly different ($p = 0.07$).

Although the effect of physical stress on absolute levels of larval mRNA was not determined here these results indicate that the mRNA levels of *galliomycin* and *IMPI* are elevated in larvae that have been physically challenged peak at 24 h and this corresponds to the time at which the larvae are normally inoculated with microbial cells. mRNA levels of *transferrin* and *gallerimycin* in treated larvae are not significantly different to those in control larvae.

3.4. Proteomic analysis of immune response of physically stressed larvae

Two-dimensional electrophoresis has been successfully employed in *Drosophila melanogaster* in constructing a 2D

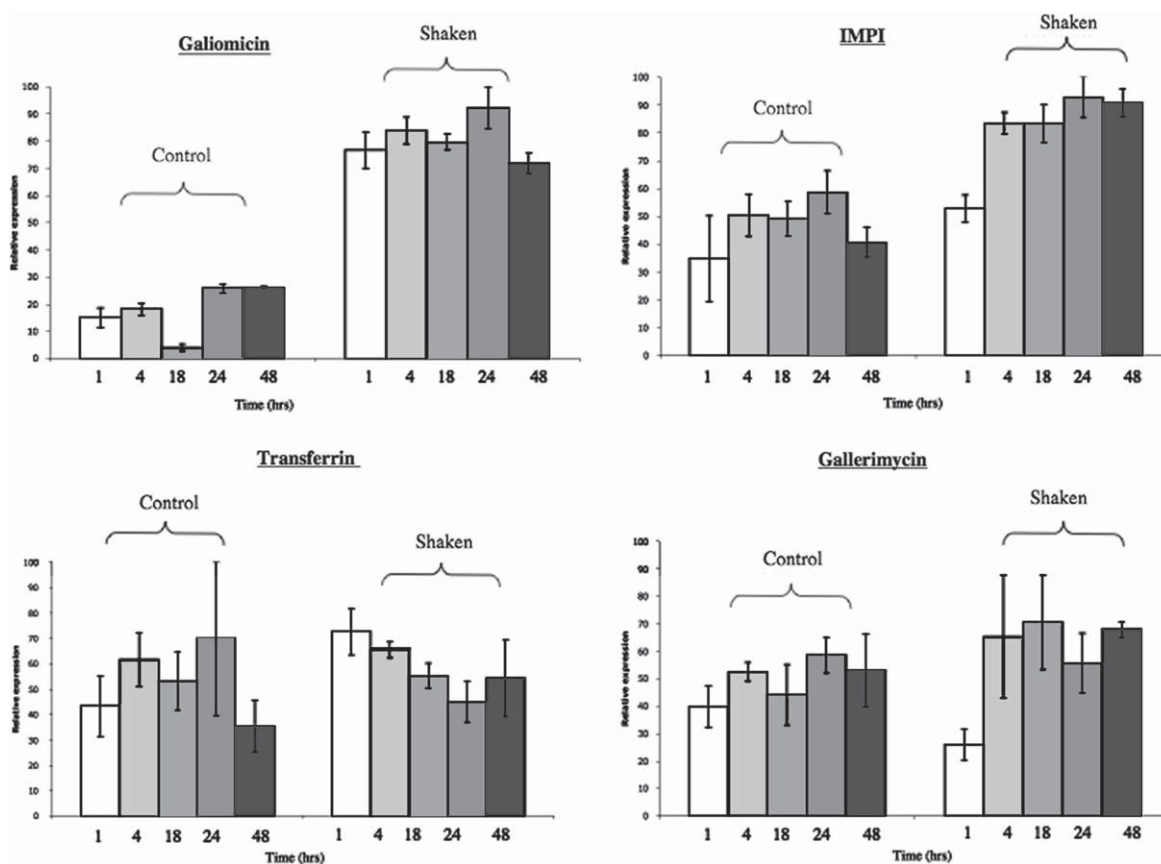


Fig. 3. Quantification of RT-PCR from control and physically challenged larvae. Densitometric quantification of PCR products from unsaturated images of RT-PCR was performed using Gene tools software (Syngene). Values were then normalised with the corresponding value of β actin. The treatment that gave the highest level of expression was normalised to 100 and the remaining results are expressed as relative expression (%).

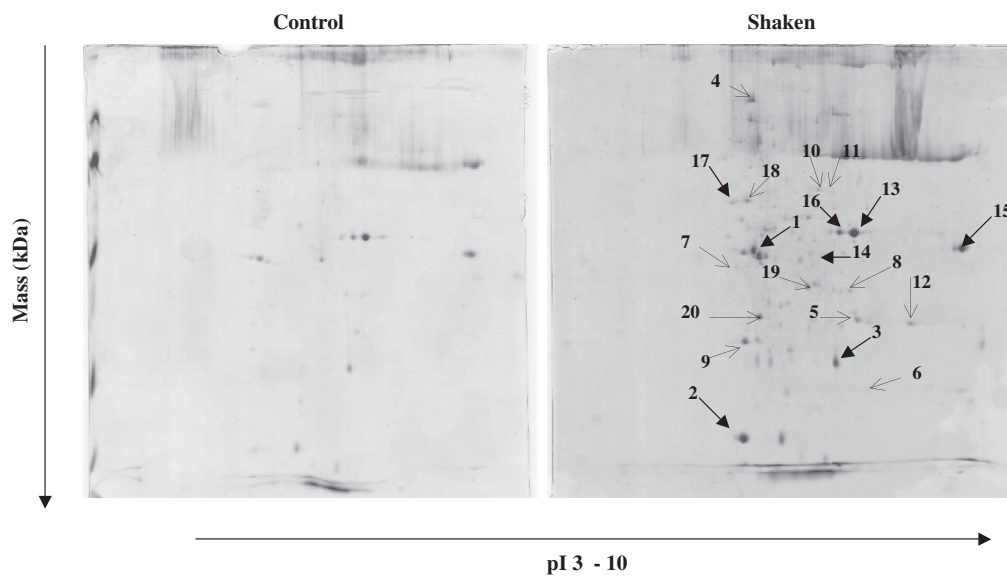


Fig. 4. Two-dimensional analysis of haemolymph samples from control and physically stressed larvae. Protein spots found in both gels with altered intensity are indicated by closed arrow. Newly induced protein spots in stressed larval haemolymph are indicated by open arrows (→).

database of haemolymph proteins [17] and this method has been utilised to analyse the immune response of *Drosophila* [18–20] and *Bombyx mori* [21]. To investigate the changes in haemolymph protein composition of larval haemolymph due to physical challenge, 2D gel electrophoresis was employed and the relevant proteins were excised and digested with trypsin for MALDI TOF analysis. Control haemolymph was obtained from larvae that had received a PBS injection. From the ImageMaster 2D analysis of the gels 19 spots were analysed for their altered spot intensities (Fig. 4). The intensity of eight peptide spots was shown to be altered in treated larvae while 11 new peptides were observed in treated larvae. Proteins were identified through ProFound peptide mapping and several proteins were identified within the haemolymph of *G. mellonella* with a wide range of functions (Table 1).

Of the spots that demonstrated alterations in intensity in treated larvae, seven showed increased intensity while one peptide (spot 14, Z score 0.43) showed a 2.19 fold decrease in intensity. Many of the peptides identified showed potential functions

in the insect's immune response. Spot 2 (Z score 1.2) showed the greatest increase in intensity (7.08 fold relative to that in control larvae) and showed homology to cyclophilin 1 which displays protein folding abilities. The intensity of spot 3 (Z score 0.55) was increased by 1.89 fold in physically challenged larvae and showed some similarities to a cysteine protease. A protein (spot 13, Z score 0.86) showing homology to notch inhibitor was identified in challenged larvae and its intensity was increased by 3.11 fold. In *Drosophila* this protein is essential in the normal development and regulation of cell growth and differentiation and may play a role in haemocyte regulation [22]. The intensity of spot 14 (Z score 0.43) was decreased by 2.19 fold and this peptide shows similarities to myosin 1B which has been associated with the control of protein traffic in multi-vesicular endosomes most probably through its ability to modulate with actin [23]. The intensity of spot 16 (Z score 1.32) was increased by 2.27 fold in treated larvae and this protein shows similarities to flotillin which controls the intracellular immunity of *Dictyostelium* to *Mycobacterium marinum* infection [24].

Table 1
Summary of proteins identified on Coomassie stained 2D gels of haemolymph from physically stressed larvae

Function	Identified proteins	Spot number	NCBI accession number	% Protein coverage	Z score	Fold expression level Shake
Immune receptor	Beta tubulin fragment	1	AAF98251	18	0.88	+2.11
Protein folding assisting enzyme	Cyclophilin 1 CG9916-PA	2	NP523366	26	1.2	+7.08
Electrons transfer reactions	Cysteine protease	3	XP767345	15	0.55	+1.89
Notch inhibitor and tumor suppressor gene	Lethal (2) giant larvae gene	13	AA28671	22	0.86	+3.11
Believed to be responsible for contraction of cell vacuole	Myosin IB	14	CAA15454	14	0.43	-2.19
Translocation of substrates across the membrane	EAL31705 ABC transporter family	15	GA15454	10	0.4	+3.56
Uptake of pathogenic bacteria (caveolae) scaffolding protein	Flotillin-1	16	CG8200	23	1.32	+2.27
Synthesis of RNA	DNA directed-RNA polymerase I largest subunit	17	P9185	9	0.69	+1.5

Table 2
Summary of induced proteins identified on Coomassie stained 2D gels of haemolymph from physically stressed larvae

Function	Identified proteins	Spot number	NCBI accession number	% Protein coverage	Z score
Lysine biosynthesis; important in enzyme production	Dihydrodipicolinate synthase	4	NP861157	23	0.91
Regulation of nucleotide cyclisation	GGDEF domain protein	5	YP271328	12	0.39
Clotting protein	ENSANGP0000002783	6	XP_552073	14	0.56
Regulation of translation of RNA into proteins	TRNA methyltransferase	7	NP501316	8	0.31
Receptor for microbial pathogens	T-cell receptor joining region	8	CAA70369	11	0.89
Breaks down connective tissue	Membrane bound metallopeptidase	9	NP_848322	31	1.12
Outer membrane protein	P44 paralogue	10	AA032044	24	1.01
Control of transcription	Zinc finger CCCH-type transcription factor	11	NP_001010888	34	1.56
Unknown function	Hypothetical protein	12	XP675265	18	0.65
Signal transduction (GTPase family)	GA19685-PA	18	EAL27299	24	0.85
Important in the elongation step of protein synthesis. 60s ribosomal protein.	CG17521	19	AAG22453	9	0.53

Eleven novel peptides were observed on gels prepared from haemolymph from physically stressed larvae that were not visible on gels from control larvae (Table 2). Many of these proteins have similarities with the proteins associated with cellular homeostasis (spot 4, 5, 7, 19) and immune response (spot 6, 8, 9, 18). Spot 8 (Z score 0.89) was identified as a peptide showing homology with T-cell receptor joining regions and spot 9 (Z score 1.12) was identified as showing similarities to a membrane bound metallopeptidase. Metallopeptidase expression has been found to be increased in mammals in response to injury [25]. Spot 6 (Z score 0.56) demonstrated similarities with ENSANGP00000019647 which contains fibrinogen-related domains involved in blood clotting which increases in *Bio-mphalaria glabrata* upon infection and is thought to be a lectin involved in the innate immune response [26].

4. Discussion

The insect immune system consists of cellular and humoral components and, despite the absence of a system analogous to the adaptive immune system of mammals, utilises a wide range of mechanisms to deal rapidly and effectively with pathogens and parasites [1]. Previous work has established that priming the insect immune response by exposure to a sub-lethal dose of a pathogen or microbial material (e.g. LPS, glucan) protects against subsequent infection from a potentially lethal dose of pathogen [12,27]. While immunological priming has clear advantages in terms of combating a subsequent larger infection it can have an adverse affect on survival especially when resources are limited [13]. While insects lack an adaptive immune response the ability of insects to react to a low level microbial insult gives them the opportunity to increase antimicrobial defences in order to ensure survival following a larger infection within a short period of time (e.g. 24–48 h).

In the work presented here we sought to establish whether mild physical stress altered the insect immune response to infection and if it offered any protective benefit to the insect. Larvae were gently shaken for 2 min in cupped hands and then incubated for 24 h prior to inoculation. This treatment had no adverse effect on insect survival. Our results indicate that this

treatment increases the ability of larvae to survive subsequent infection with *C. albicans*. Analysis of the haemocyte density showed higher numbers in treated larvae and this has previously been correlated with a greater ability to combat infection [28]. It is possible that the elevated levels of haemocytes were due to the release of haemocytes attached to internal organs within the insect rather than the *de novo* synthesis new cells.

The elevated mRNA levels of genes coding for four antimicrobial peptides have been recorded previously in larvae challenged with yeast cells [12]. Elevated levels of *galiomicin* (a defensin) and *IMPI* mRNA were recorded here; however the levels of *transferrin* and *gallerimycin* mRNA were not significantly altered when compared to those in the control larvae. Differential expression of antimicrobial peptide transcripts has been recorded previously when *Drosophila* was subjected to infection with various microbes [10]. In terms of the cellular immune response, recent work has highlighted the appearance of a super-spreading haemocyte after microbial challenge of *Manduca sexta* but not following wounding [29]. These findings indicate that insects can modulate their immune responses depending upon the nature of the microbial challenge or physical stress (shaking, wounding). When exposed to a microbial pathogen the expression of a wide range of AMPs may be required whereas the response to a physical challenge (e.g. shaking, wounding) may only require the induction of a subset of antimicrobial peptides. Presumably a microbial infection is a greater threat in that the microbe may grow within the insect while a mild physical stress (e.g. damage to the cuticle, shaking) may be self-limiting, unless there is the entry of a pathogen, and may not pose an immediate threat to the insect's survival.

Analysis of the peptides in the haemolymph of physically challenged larvae revealed that the intensity of seven peptide spots was elevated, one was decreased and that 11 novel peptides were recorded in the haemolymph from treated larvae. Many of the peptides (spot 1, 2, 3 and 16) which demonstrated an increase in intensity showed similarities to proteins which play a role in the insect immune response or pathogen recognition. A number of induced peptides found in the haemolymph of physically challenged larvae showed similarities to proteins with immune response functions (spot 6, 8 and 9)

while others were similar to the proteins involved in signal transduction (spot 18) and protein synthesis (spot 19).

The results presented here indicate that physically challenging larvae of *G. mellonella* by shaking primes the immune response and results in a reduced susceptibility to infection by yeast (*C. albicans*) cells. Shaking of larvae resulted in an increase in the number of circulating haemocytes which may aid the insect in combating subsequent infection. The levels of mRNA of genes coding for two antimicrobial peptides (*galiomycin* and *IMPI*) were increased but those of two other genes (*transferrin* and *gallerimycin*) were not significantly altered possibly indicating that physical stress activates a different response pathway compared to microbial infection [10]. Analysis of protein expression revealed the increased intensity of a number of peptides showing similarity with known proteins involved in cellular homeostasis or immune response. A number of induced peptides (spot 6, 8 and 9) demonstrated varying levels of homology with proteins with immune functions.

While physical stress primes the immune response of *G. mellonella* to infection, the nature of this response appears to be distinct to that induced by exposure to sub-lethal doses of microbial pathogens. Further work to elucidate the pathways controlling the differential response of insects to microbial and physical challenge is warranted as it may give insights into the complexity of the regulation governing the insect immune response. It is evident that this differential response is mediated through variations in the cellular [29] and humoral [10] components of the insect immune response.

Acknowledgements

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