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Effect of stress on heat shock protein levels, immune response and survival to fungal infection of *Mamestra brassicae* larvae.

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Abstract

Although the utilisation of fungal biological control agents to kill insect pests is desirable, it is known that the outcome of infection may be influenced by a number of criteria, including whether or not the target insect is stressed. In the current work, topical treatment of larvae of the lepidopteran pest, *Mamestra brassicae*, with conidia of *Beauveria bassiana*, followed by a heat stress (HS; 37^{o}C for 1 h) 48 h later, resulted in a similar level of larval survival to that occurring for no heat stress (No-HS), fungus-treated larvae. By contrast, when the HS was applied 24 h after fungal treatment, larval survival was significantly increased, indicating that the HS is protecting the larvae from *B. bassiana*. Similarly, exposure of larvae to a HS provided protection against *Metarhizium brunneum* (V275) at 48 h (but not 24 h) after fungal treatment.

To elucidate the mechanism(s) that might contribute to HS-induced increases in larval survival against fungal infection, the effects of a HS on key cellular and humoral immune responses and on the level of selected heat shock proteins (HSP) were assessed. When larvae were kept under control (No HS) conditions, there was no significant difference in the haemocyte number per ml of haemolymph over a 24 h period. However, exposure of larvae to a HS, significantly increased the haemocyte density immediately after (t= 0 h) and 4 h after HS compared to the No HS controls, whilst it returned to control levels at t=24 h. In addition, *in vitro* assays indicated that haemocytes harvested from larvae immediately after (0 h) and 4 h (but not 24 h) after a HS exhibited higher rates of phagocytosis of FITC-labelled *B. bassiana* conidia compared to haemocytes harvested from non-HS larvae. Interestingly, the HS did not appear to increase anti-fungal activity in larval plasma. Western blot analysis using antibodies which cross react with *Drosophila melanogaster* HSP, resulted in a relatively strong signal for HSP 70 and HSP 90 from extracts of 50,000 and 100,000 haemocytes, respectively, harvested from No-HS larvae. By contrast, for HSP 60, a lysate

derived from 200,000 haemocytes resulted in a relatively weak signal. When larvae were exposed to a HS, the level of all three HSP increased compared to the No HS control 4 h and 16 h after the HS. However, 24 h after treatment, any heat stress-mediated increase in HSP levels was minimal and not consistently detected. Similar results were obtained when HSP 90, 70, and 60 levels were assessed in fat body harvested from heat stressed and non-heat stressed larvae. With regard to HSP 27, no signal was obtained even when a lysate from 200,000 haemocytes or three times the amount of fat body were processed, suggesting that the anti-HSP 27 antibody utilised does not cross-react with the *M. brassicae* HSP. The results suggest that a HS-mediated increase in haemocyte density and phagocytic activity, together with an upregulation of HSP 90 and 70, may contribute to increasing the survival of *M. brassicae* larvae treated with *B. bassiana* and *M. brunneum* (V275).

Key Words. *Mamestra brassicae*, *Beauveria bassiana*, *Metarhizium*, heat shock proteins, haemocytes, immune responses.

Abbreviations. BCA : biological control agent; HS : Heat stress; No HS : No heat stress; HSP : Heat shock protein; Bb : *Beauveria bassiana*; CE : constant environment room; PDA : Potato dextrose agar; PD : Potato dextrose; DPBS : Dulbeccos's phosphate buffered saline; FITC : Fluorescein isothiocyanate; GLMM : Generalized linear mixed model; EPF: entomopathogenic fungus.

1. Introduction

There is an urgent need to find replacements for many chemical pesticides currently used to control insect pests of relevance to the agricultural, horticultural and forestry sectors. One approach focuses on the use of insect-specific biological control agents (BCA), including viruses, bacteria, and entomopathogenic fungi (EPF) (see Lacey et al., 2015 and references therein). Different strategies may employ these BCA on their own, or as part of an integrated pest management regime (Butt et al., 2001; Sandhu et al., 2012; Lacey et al., 2015). With regard to EPF, strains of *Beauveria spp*. and *Metarhizium spp*. are often utilised. Both of these fungi have a broad host range and have been used to control a variety of crop pests, including European corn borer, termites, whitefly, green leafhoppers, locusts, grasshoppers, etc. (Butt et al., 2001; Sandhu et al., 2012; Lacey et al., 2015). They also show potential for controlling insect pests of trees, including the Asian longhorn beetle (Dubois et al., 2004; Shanley et al., 2009) and the pine processionary moth (Er et al., 2007; Sevim et al., 2010).

Despite the desire to use EPF to control insect pests, it is generally acknowledged that the interaction of a fungus with any given insect host/target is complex and that the outcome of infection (death of the insect pest or the EPF) may be influenced by a variety of factors. For instance, apart from evolving physical barriers to prevent cuticular penetration by the fungus (Ortiz-Urquiza and Keyhani, 2013), a major response of an insect to fungal infection is the mobilisation of haemocyte-mediated immune responses (including phagocytosis and encapsulation), and humoral immune responses (including the upregulation of anti-fungal molecules, phenoloxidase activity, reactive oxygen species, etc.) in order to protect itself from the fungus (Lemaitre and Hoffmann, 2007; Stokes et al., 2015; Butt et al., 2016). Similarly, fungi have evolved a number of strategies that enable them to infect and disable insects, including the production of molecules designed to suppress insect immune responses (Bulet and Stocklin, 2005; Ortiz-Urquiza and Keyhani, 2013; Butt et al., 2016).

In view of this, an active area of research is focussed on elucidating the molecular mechanisms that influence the efficacy of fungal BCA for any given pest insect. For instance, the efficacy of EPF may be improved by increasing their virulence through genetic modification (Wang and St. Leger, 2007; St. Leger and Wang, 2010), by suppressing relevant immune responses in the target pest insect (Dean et al., 2002; Richards et al., 2011, 2013), and/or by utilising other BCA that act synergistically with the fungal BCA (Ansari et al., 2008). By contrast, other stresses may decrease the efficacy of EPF. For example, in the wax moth, *Galleria mellonella* (an insect that lives communally in bee hives, where temperatures may reach 40^{o}C), it was demonstrated that exposure of the larvae to a heat shock of 43^{o}C for 15 min after natural infection with *B. bassiana*, positively affected their survival by extending the life time compared to larvae left at a culturing temperature of 28^{o}C (Wojda et al., 2009). Moreover, it was shown that the increase in survival was not due to a deleterious effect of heat shock on the fungus as similar results were obtained when larvae were given a heat shock first and then injected with *B. bassiana*. Interestingly, the heat stress (in conjunction with fungal infection), also increased the level of certain anti-microbial peptides in the haemolymph, and it was concluded that this likely accounted for the increased survival rate of the heat shocked larvae (Wojda et al., 2009). These results are corroborated and extended by studies that demonstrate that exposure of *G. mellonella* to a mild physical stress (shaking) and/or thermal stress resulted in short-term immune priming, which correlates with protection against infection by *Candida albicans* and *Aspergillus fumigatus* (Mowlds and Kavanagh 2008; Mowlds et al., 2008; Browne et al., 2014).

The molecular mechanisms or pathways activated by heat shock or physical stress and how these culminate in increases in insect immune responses and survival, are not clear at present. Although, in *G. mellonella*, it has been hypothesised that stress-induced heat shock protein (HSP) 90 and/or HSP 90 derivatives may play a role (Wojda and Jakubowicz, 2007;

Dubovskiy et al., 2013). HSP 90 is one of several HSP families that are grouped according to molecular weight (*e.g.* HSP 90, 70, 60, and the small HSP) (Parsell and Lindquist, 1993; Sun and MacRae, 2005; Richter et al., 2010). HSP are present in all cells in all forms of life. Under normal (unstressed) conditions, they function primarily as molecular chaperones and ensure the proper folding of nascent polypeptides. Following cellular stress, the appearance of denatured proteins and polypeptides stimulates an upregulation in gene expression of HSP, such that their level within the cell increases markedly. In addition to being induced by heat shock, HSP may also be up-regulated in response to a variety of stresses. In insects, such stresses may include diapause, anoxia, desiccation, different developmental stages, ageing, and exposure of insects to UV radiation, drought, oxidation, parasitoid envenomation, and a wide range of chemicals and contaminants (including heavy metals and ethanol) (*e.g.* Sonoda et al., 2007; Shim et al., 2008; Lopez-Martinez et al., 2009; Nguyen et al., 2009; Zhang and Denlinger, 2010; Michaud et al., 2011; Tower, 2011; Zhao and Jones, 2012; Kim et al., 2015). The hypothesis that *G. mellonella* HSP 90 and/or its derivatives stimulate immune responses and contribute to survival of the larvae against pathogens (Wojda et al., 2007; 2009; Dubovskiy et al., 2013) is supported by studies in other insects. For instance, heat shock has also been shown to restrict virus infection in *Drosophila melanogaster* (Merkling et al., 2015), whereas in *Spodoptera frugiperda* Sf9 cells, induced and cognate HSP 70s were found at high levels in cells infected with *Autographa californica multiple nucleopolyhedrovirus* (Lyupina et al., 2011). In the red flour beetle, *Tribolium castaneum*, injection with crude lipopolysaccharides (LPS) induced strong expression of HSP mRNA transcripts (Altincicek et al., 2008). Also, eicosanoids have been shown to mediate small HSP gene response to biotic stress (including virus particles and *B. bassiana*) (Zhang et al., 2015a, b). These studies and others, suggest that in insects, the stress and immune responses are interlinked possibly sharing certain signal transduction pathways (Altincicek et al., 2008;

Adamo, 2008; Wojda and Taszlow, 2013; Eggert et al., 2015; Zhang et al., 2015a, b). Moreover, physical stress can induce HSP and/or immune responses in other invertebrates (*e.g.* Singh and Aballay, 2006; Malagoli et al., 2007), whilst over the last two decades or so, a significant role for HSP in the immune system of mammals has emerged (Binder, 2014). This work raises the possibility that (under certain circumstances), RNAi-mediated knockdown of key HSP genes in pest insects could lead to a state of immunosuppression, which would increase their susceptibility to BCAs, including insect-specific EPF.

In the current work, the major aim was to gain an insight into how larvae of the lepidopteran pest, *M. brassicae*, respond to stress at the molecular level and whether exposure of larvae to stress can alter their susceptibility to fungal BCAs. More specifically, in view of the work performed previously using *G. mellonella*, and because *M. brassicae* larvae do not usually live at such relatively high temperatures (up to 40^{o}C), the study sought to determine the effect of a non-lethal heat stress on *M. brassicae* haemocyte number, and on humoral and haemocyte-mediated immune responses. Utilising a proteomic approach, the effect of heat stress on the levels of (selected) HSP in two immunocompetent tissues, fat body and haemocytes, was also examined. In addition, the virulence of two *M. brunneum* strains (4556 and V275 [AKA Met 52]) and one *B. bassiana* strain were compared for efficacy against *M. brassicae* larvae, and then the two most virulent strains were utilised in bioassays to determine if heat treatment of the larvae affects their susceptibility to the EPF. It is envisaged that results gained using *M. brassicae* larvae will be applicable to other insect pest species, including pests of trees and forestry.

2. Materials and Methods

2.1. Chemicals.

All chemicals were obtained from Sigma-Aldrich unless indicated otherwise.

2.2. Insects

Mamestra brassicae larvae were reared in a controlled environment (CE) room under standard conditions of 20°C, 70 % relative humidity, and a light:dark cycle of LD 16 h:8 h, and fed on artificial diet (Bio-Serv, New Jersey).

2.3. Preparation of fungi and dose-response assays

To identify a fungal strain that is highly virulent towards *M. brassicae* larvae following topical application (and thus suitable for use in subsequent proteomic and molecular work), dose-response assays were undertaken using three different fungal strains. With regard to *B. bassiana*, the effect of this strain on the mortality of *M. brassicae* larvae has been investigated previously (Richards et al., 2011). However, as the virulence of a fungus can be influenced by batch, storage, method of application, etc., the ability of *B. bassiana* to kill *M. brassicae* larvae was re-tested for the current work. Moreover, this was done using exactly the same procedure as that being used for the *M. brunneum* strains in order to ensure that the results obtained for the different fungi would be directly comparable.

Freeze-dried *B. bassiana* conidia (isolate IMI 386367; from CABI Bioscience, Egham, Surrey, UK), were prepared, and dose-response assays for topically applied fungus performed, as described in Richards et al., (2011), with codacide (a vegetable oil adjuvant) included in the final solution for dipping the larvae (1/100 v/v, final concentration;

Tween80/codacide). Note that an assessment period of 14 days was selected for these assays. This is because 'healthy' larvae start to pupate by day 14 onwards. Thus, if the assessment period is too short, some mortality will be missed, whilst there is little to be gained by prolonging an assay beyond day 14. Assays were repeated on three separate occasions; for each assay, 15 larvae were used for each control and treatment category. The number of viable conidia in the fungal suspension was determined by plating out 10-fold dilutions of the stock suspension (in triplicate) on potato dextrose agar (PDA) and then counting the number of colonies formed 2 to 3 days later. Note that because viability is determined 2 to 3 days after the larvae are dipped into the fungal suspension, this procedure only allows us to accept an assay if the viability is suitable (*i.e.* at least 90 %) or abandon it (if viability is less than 90 %). However, for all the assays conducted, the viability of the fungi in the 'dipping solution' was determined to be at least 90 %, thus, none of the assays were abandoned. For *M. brunneum* strains, dose-response assays for topically applied fungus were performed using two strains, V275 (= BIPESCO 5, F52, Met52) and ARSEF4556 (= 4556). These fungi were grown on PDA plates (at 26^{o}C) and passaged intermittently through *M. brassicae* larvae to maintain virulence. Conidia, harvested from 12 to 14 day old fungal colonies, were processed and dose-response assays performed as for *B. bassiana*. The viability of the all fungal conidia utilised in the assays was greater than 90 %.

Dose-response assays for injected fungus were performed using *B. bassiana* only. Basically, a conidial suspension was prepared in 0.05 % Tween 80 (as described above but without codacide) and then diluted as required (the exact dilution varied because the concentration of the stock varied, but was always at least 1/2000) with sterile Dulbecco's Phosphate buffered saline (DPBS). *Mamestra brassicae* larvae were anaesthetised by immersing them in water and then injected with 2 µl of DPBS either with or without a known number of *B. bassiana*

208 conidia. After treatment, the larvae were incubated and assessed as described above. The
209 viability of the conidia utilised was also determined as described above.
210
211 *2.4. Combined stress bioassays; topically applied fungus and heat stress*
212
213 These assays were undertaken using *B. bassiana* and V275 only. Suspensions of conidia were
214 prepared as described above and adjusted to $4x10^6$ conidia ml^{-1}. Twenty four h and 48 h after
215 topical application of the fungus to *M. brassicae* larvae (as described above), the larvae were
216 either left in the CE room at 20°C (= no heat stress control; No HS) or subjected to a heat
217 stress of 37°C for 1 h and then returned to the CE room (note that as the larvae were left in
218 their individual pots during the heat stress, time is required for the temperature to equilibrate
219 to the heat stress temperature). Larval mortality was then assessed as described above. For
220 each control and treatment category, 15 larvae were used and assays were repeated on three
221 separate occasions. The effect of the heat stress on the viability of fungal conidia was
222 determined by exposing an aliquot of the conidial suspension prepared for topical application
223 to the same heat stress or non-heat stress conditions as used for the larvae, followed by
224 plating out of the conidia on nutrient agar plates (see above).
225
226 *2.5. Combined stress bioassays; heat stress and injected fungus*
227
228 These assays were undertaken using *B. bassiana* only. A conidial suspension was prepared
229 and diluted, and fungal viability determined, as described above. *Mamestra brassicae* larvae
230 were either subjected to a heat stress of 37°C for 1 h, or left at CE room conditions. Four
231 hours after treatment, larvae were injected with 2 µl of DPBS either with or without
232 approximately 1.0×10^2 *B. bassiana* conidia. After treatment, the larvae were incubated and

assessed as described above. As described previously (Richards and Dani, 2010; Richards et al., 2011), the data were analysed using a Cox proportional hazard model (Cox, 1972) to compare the overall (*i.e.* the whole pattern) mortality of larvae in different treatments.

2.6. Preparation of immunocompetent tissues (haemocytes, plasma and fat body) from non-heat stressed and heat stressed larvae

Larvae were exposed to a heat stress of 37^{o}C for 1 h, or left at no-heat stress (CE room) conditions. The larvae were then returned to the CE room. To prepare haemocytes at different time points after treatment, larvae were cooled on ice for 15 min. The cuticle was then swabbed with 70 % alcohol, pierced with a sterile 19 gauge needle and the haemolymph collected in a sterile Eppendorf tube lightly dusted with phenylthiocarbamide (to prevent activation of the phenoloxidase cascade). Routinely, for each control and treatment category, haemolymph from three to five larvae was pooled (usually 50 to 100 µl was collected) and an aliquot of this was diluted 1 in 5 with TC-100 (a lepidopteran tissue culture medium). Diluted haemolymph was then added to each of two Neubauer haemocytometers and the haemocyte number per ml determined for each sample. Assays were repeated on four to six separate occasions. To analyse the data, three linear mixed models were built, wherein 'assays' were used as random effects while the fixed effects that were examined were the treatments. While the haemocyte number per ml was being determined, the remainder of each haemolymph sample was centrifuged (254 g, 4^{o}C for 8 min). The plasma was then transferred to a clean tube, centrifuged (13,000 g, 5 min, RT), and either used immediately in *in vitro* anti-fungal assays (see below) or stored at -80^{o}C. With regard to the haemocyte pellet, after removal of plasma, an appropriate volume of protein loading buffer (National Diagnostics; containing lithium dodecyl sulphate and 1,4-dithiothreitol at a final concentration of 1.6 % and 100 mM,

respectively) was added and the sample (*i.e.* haemocyte pellet in sample buffer) was either used immediately in western blotting assays (see below) or stored at -80°C.

To prepare fat body from heat stressed (HS) and non-heat stressed (No-HS) larvae at different times after treatment, decapitated larvae were dissected under DPBS and dissected fat body placed into a pre-weighed sterile Eppendorf tube on ice. Routinely for each treatment, fat body from three to five larvae was pooled. After dissection, the fat body was centrifuged (254 g, 4°C for 8 min), any liquid removed, and the tube weighed again so that the weight of fat body could be determined. An appropriate volume of protein loading buffer was then added so that each sample contained approximately 27 µl of SDS sample buffer per mg of fat body. Samples were stored at -80°C until required for western blotting (see below).

2.7. Phagocytosis assays

Beauveria bassiana conidia were labelled with Fluorescein isothiocyanate (FITC) as described previously (Richards et al., 2013). *Mamestra brassicae* larvae were exposed to a heat stress (37°C for 1 h) or left under CE room conditions. Haemolymph from non-heat stressed and heat stressed larvae collected immediately after heat stress (t=0 h), 4 h and 24 h after heat stress, was used to prepare monolayers of haemocytes for phagocytosis assays, performed as described previously (Richards et al., 2013). Note that due to the relatively small size of the larvae, for each control and treatment category, haemolymph collected from five to 10 larvae was pooled and duplicate monolayers prepared from this. Routinely, for each monolayer, 50 µl of Tris buffered saline (with 10 mM $CaCl_2$) containing $1x10^6$ FITC-labelled *B. bassiana* conidia was added, and the percentage of haemocytes that had phagocytosed one or more conidia was determined. Assays were repeated on four occasions,

and (as indicated above) each replicate was formed from a pooled sample from a separate set of individual insects. For each monolayer, groups of 50 haemocytes in 8 to 10 areas (*i.e.* 400 to 500 haemocytes per monolayer) were examined. The data were analysed as described in Richards et al., 2013, using a Generalized Linear Mixed Model (GLMM).

2.8. Determination of anti-fungal activity in plasma from heat stressed and non-heat stressed larvae

Beauveria bassiana conidia were prepared as described above and the stock solution diluted to 4 x 10^4 conidia ml^{-1} using potato dextrose (PD) broth containing 100 µg $µl^{-1}$ ampicillin (routinely, this represented a dilution of about 1/2000). One hundred µl of this suspension was added per well of a 96-well tissue culture plate (Corning Inc., Corning, New York, USA), followed by 100 µl of plasma prepared from larvae that had been exposed to a heat stress or kept under control (no heat stress) conditions (see above). Plasma was also added to wells containing the diluent for the fungus without conidia, whilst addition of fungal suspension to 100 µl of PD broth served as a blank (*i.e.* control for fungal growth). Plates were then incubated in the CE room for 24 h. At the end of this period, the contents of each well was mixed and aliquots of equal volume removed from each well and diluted to 100 µl using PD broth. This was then spread on PDA plates which were subsequently incubated in the CE room. For each treatment, plates were prepared in triplicate and the number of fungal colonies on each plate was determined after 2.5 days. In this way, the effect of a heat stress on the humoral (anti-fungal) response in plasma could be determined. Assays were repeated on three separate occasions. Results were analysed by fitting a GLMM (Poisson distribution, log link) to the counts observed at each time with treatment as a fixed effect and assay as a random effect.

308 *2.9. Western blotting*

309

310 To detect the presence of different stress proteins (HSP 90, 70, 60 and 27) in haemocyte and
311 fat body lysates from heat stressed and no-heat stressed larvae, western blotting was utilised.
312 Discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)
313 was performed as described by Laemmli (1970) using a 4 % stacking gel and a 12.5 %
314 separating gel under reducing conditions (the protein loading buffer contained a final
315 concentration of 100 mM DTT). Chemilumenscent western blot analysis was performed, as
316 described previously (Dani et al. 2003). Blots were probed using primary antibodies (Abcam,
317 Cambridge, UK) which cross react with *Drosophila melanogaster* HSPs. These included a rat
318 monoclonal to HSP 90 (ab13494; 1/500), a mouse monoclonal to HSP 70 (ab5439; 1/1,000),
319 a mouse monoclonal to HSP 60 (ab59457; 1/1,000) and mouse monoclonal to Turkey HSP 27
320 (ab49919; 1/500) (Abcam, Cambridge, UK). The secondary antibodies consisted of
321 peroxidase-labelled anti-rat or anti-mouse, as appropriate (Sigma-Aldrich) (all 1/5,000).
322 Bound antibodies were visualised using ECL™ Western Blotting Detection Reagent (GE
323 Healthcare, Little Chalfont, UK). Tissue samples were prepared as described above.
324 Routinely, samples of tissues harvested from larvae subjected to different treatments on three
325 separate occasions were prepared, and western blotting performed twice for each sample.

326

327 **3. Results**

328 *3.1. Effect of topically applied fungi on mortality of* M. brassicae *larvae*

329

330 As shown in Figure 1 (a to c), *B. bassiana* is the most virulent fungus towards *M. brassicae*
331 larvae, followed by V275, then 4556. For instance, dipping larvae in a suspension of 3 x 10^7
332 *B. bassiana* conidia per ml results in about 90 % mortality on day 14 of the assay, whereas

333 the same dose kills only 38.3 % and 50.9 % of larvae treated with 4556 and V275,
334 respectively (Fig. 1 a, b and c). Moreover, larvae treated with *B. bassiana* were killed more
335 quickly than those treated with either of the *M. brunneum* strains. For example, a dose of 3 x
336 10^7 *B. bassiana* conidia per ml, kills 55.0 % of the larvae by day 7 of the assay, whilst the
337 same dose of V275 and 4556 kills 21.4 % and 0 % of the larvae, respectively, at the same
338 time point (Fig. 1a to c). Due to the relatively low level of mortality caused by 4556, this
339 fungus was excluded from further study.
340
341 *3.2. Effect of heat shock on the ability of* M. brassicae *larvae to survive (natural) fungal*
342 *infection*
343
344 These assays were undertaken using *B. bassiana* and V275 only. Figure 2a and b show the
345 probability of survival of larvae in the different treatment groups as a function of time; the
346 expected percentage survival of larvae for each treatment at the end of the assay (day 14) is
347 also given. Note that when *M. brassicae* larvae were not treated with *B. bassiana* or V275
348 and not exposed to a heat stress, there was no mortality (data not shown). Similarly, when
349 larvae treated with a control solution (*i.e.* without *B. bassiana* or V275 conidia) were exposed
350 to a heat stress 24 h or 48 h later, there was no mortality (data not shown). In view of this,
351 these treatment groups were removed from the statistical analysis.
352
353 Immersion of *M. brassicae* larvae in a suspension of $4x10^6$ *B. bassiana* per ml (Bb / No-HS)
354 resulted in 34.3 % survival on day 14 of the assay. When larvae are dipped in *B. bassiana* and
355 then exposed to a heat stress (37^{o}C for 1 h) 48 h later (Bb / HS 48 h), expected survival is
356 34.7 % and, utilising the Cox proportional hazard model, it was determined that there is no
357 significant difference between these treatments. By contrast, if the heat stress is applied 24 h

after topical application of the fungus (Bb / HS 24h), the expected larval survival on day 14 of the assay is 57.1 %, and this treatment was determined to be significantly different to the no heat stress control (Bb / No HS). More specifically, the hazard ratio of Bb / HS 24 h compared to Bb / No-HS is 0.5238 ($P<0.00712$; with confidence interval limits of 0.8388 [upper] and 0.3271 [lower]), whereas the hazard ratio of Bb / HS 48 h compared to Bb / No-HS is 1.016 ($P<0.941$; with confidence interval limits of 1.511 [upper] and 0.6656 [lower]). These results indicate that subjecting larvae to a heat stress 24 h after treatment with *B. bassiana* is protecting them in some way from this fungus. Exposure of *M. brassicae* larvae to a heat stress also provides some protection against V275 (Fig. 2b). However, as shown in Figure 2b, a reduction in the efficacy of V275 occurs only when the HS is applied 48 h (but not 24 h) after treatment with the fungus. Similarly, when larvae are dipped in V275 and then exposed to a heat stress (37^{o}C for 1 h) 24 h later (V275 / HS 24 h), expected survival is 44.4 % and, utilising the Cox proportional hazard model, it was determined that there is no significant difference between these treatments. By contrast, if the heat stress is applied 48 h after topical application of the fungus (V275 / HS 48h), the expected larval survival on day 14 of the assay is 65.7 %, and this treatment was determined to be (borderline) significantly different to the no heat stress control (V275 / No HS). More specifically, the hazard ratio of V275 / HS 48 h compared to V275 / No HS is 0.5549 ($P<0.0495$; with confidence interval limits of 0.9987 [upper] and 0.3083 [lower]), whereas the hazard ratio of V275 / HS 24 h compared to V275 / no-HS is 1.129 ($P<0.941$; with confidence interval limits of 1.909 [upper] and 0.667 [lower]). These results indicate that subjecting larvae to a heat stress 48 h after treatment with V275 is somehow protecting them from this fungus.

Fungal viability (colony forming) assays performed on PDA plates indicated that the viability of non-heat stressed *B. bassiana* and V275 conidia was 94.4 ± 2.5 % and 92.1 ± 4.2 % (mean

+/- SE , n=4), respectively. Subjecting *B. bassiana* and V275 conidia to a HS of 37°C for 1 h prior to plating out conidia on nutrient agar plates, resulted in a viability of 94.6 ± 4.5 % and 89.7 ± 6.6 % (mean +/- SE , n=4), respectively. The very low level of reduction in conidial viability as a result of heat stress is highly unlikely to affect results obtained in the combined stress bioassays (see above).

3.3. Effect of injected B. bassiana *on mortality of* M. brassicae *larvae, and combined stress bioassays for heat stress and injected fungus*

As indicated in Figure 3, increasing the number of conidia injected into *M. brassicae* larvae results in an increase in larval mortality. However, the increase in mortality observed between injection of 100 conidia and 1,000 conidia, was minimal. The results indicate that injection of 100 *B. bassiana* conidia kills about 42 ± 7 % of the larvae and this dose was selected for further combined stress (heat stress and injected fungus) assays.

To further confirm that the heat stress-induced protection against topically applied fungal infection (see above) is not due to heat stress-mediated damage to fungal conidia, larvae were subjected to a heat stress (37°C, 1 h), then fungal conidia injected into the larvae 4 h later. These bioassays (and subsequent proteomic work, see below), were performed using *B. bassiana* only. As shown in Figure 4, the heat stress 'protects' the larvae from the fungus. For instance, following injection of no heat stressed *M. brassicae* larvae with 100 *B. bassiana* conidia (Bb / No-HS), about 49 % of the larvae survive to day 14 of the assay. By contrast, when injection of conidia is followed by a heat stress (F / HS), survival is increased to 72.4 % (Fig. 4). Moreover, the hazard ratio of Bb / No HS compared to DPBS / No HS is 16.2 ($P<0.000179$; with confidence interval limits of 69.45 [upper] and 3.775 [lower]), whereas the hazard ratio of Bb / HS compared to DPBS / No HS is 7.466 ($P<0.00851$; with confidence

interval limits of 33.38 [upper] and 1.67 [lower]). These results indicate that the heat stress is somehow protecting the larvae from *B. bassiana*.

3.4. Effect of heat stress on haemocyte number in larval haemolymph

As indicated in Table 1, the number of haemocytes per ml of haemolymph from larvae not exposed to a heat stress (No HS) at t=0 h, t=4 h and t=24 h is very similar. By contrast, exposure of larvae to a heat stress (HS) results in a significant increase in the number of haemocytes per ml compared to the no heat stress controls straight after (t= 0 h) and 4 h after heat stress. However, there is no significant difference in the number of haemocytes per ml for heat stressed and no heat stressed larvae at the 24 h time point (Table 1). Note that the mean weight of larvae exposed to a heat stress was the same as those left under non-HS conditions (data not shown). Thus, changes in haemocyte number are not attributed to changes in haemolymph volume.

3.5. Effect of heat stress on phagocytosis

As indicated in Figure 5 and Table 2, when monolayers of haemocytes harvested from control (no heat stressed; No-HS) larvae are presented with FITC-labelled *B. bassiana* conidia, approximately 18 to 21 % of the haemocytes phagocytose one or more conidia. By contrast, when monolayers are prepared from haemocytes harvested from larvae 0 h and 4 h after a heat stress, phagocytosis is highly significantly increased compared to the corresponding non-heat stressed controls (Figure 5, Table 2). Twenty four hours after the heat stress, however, phagocytosis is slightly reduced in response to the heat stress and there is no

433 significant difference in the level of phagocytosis occurring for haemocytes from heat
434 stressed and non-heat stressed larvae (Fig. 5, Table 2).
435
436 *3.6. Effect of heat stress on anti-fungal activity in larval plasma*
437
438 When plasma from non-heat stressed (No HS) *M. brassicae* larvae is incubated with the
439 diluent used to prepare *B. bassiana* conidia for 24 h and then then plated out on PD agar, no
440 colonies grow, indicating that the plasma is not contaminated with fungus (data not shown).
441 As indicated in Figure 6 and Table 3, when *B. bassiana* is incubated in PD broth for 24 h and
442 then plated out on PD agar (B), more colonies grow on the agar compared to when *B.*
443 *bassiana* is incubated in plasma from no heat stressed (No-HS) or heat stressed larvae. Thus,
444 the conidia grow better in PD broth compared to plasma (*i.e.* the former offers more optimal
445 growth conditions). Moreover, heat stress was not found to have a significant effect on the
446 number of fungal colonies counted compared to the no heat stress controls. The worst case
447 (95% confidence) effect of heat shock was estimated to be a reduction in the expected count
448 by no more than 12%.
449
450 *3.7. Effect of heat stress on the levels of selected heat shock proteins in haemocytes and fat*
451 *body of* M. brassicae *larvae*
452
453 A representative western blot indicating the levels of HSP 90, 70 and 60 in haemocytes and
454 fat body from non-heat stressed and heat stressed *M. brassicae* larvae 4 h after treatment is
455 shown in Fig. 7. These western blotting assays were performed primarily to determine if
456 commercially available antibodies against *Drosophila* HSP 90, 70, 60 and 27 can cross react
457 with *M. brassicae* HSPs, and whether an increase in HSP levels can be detected following

458 exposure of the larvae to heat stress. With regard to *M. brassicae* haemocytes harvested from
459 non-heat stressed larvae, processing of a haemocyte lysate derived from 50,000 haemocytes
460 resulted in a relatively strong signal for HSP 70 (Fig. 7). For HSP 90, processing of 100,000
461 resulted in a clear signal but one that was not as strong as that obtained for HSP 70. For HSP
462 60, processing of a lysate derived from 200,000 haemocytes resulted in a relatively weak
463 signal. When *M. brassicae* larvae were exposed to a heat stress (37^{o}C for 1 h), the level of all
464 these HSP (HSP 90, 70 and 60) increased compared to the non-heat stressed control. With
465 regard to HSP 27, no signal could be obtained for any of the samples, even when a lysate
466 from 200,000 haemocytes was processed. With regard to fat body, as indicated in Figure 7,
467 when equal amounts of fat body derived from non-heat stressed larvae was processed
468 (equivalent of 730 µg per sample), the signal obtained for HSP 90 and 70 was similar,
469 whereas a much weaker signal was obtained for HSP 60. As observed for HSP in
470 haemocytes, however, when an equivalent amount of fat body derived from heat stressed
471 larvae was processed, there was an upregulation for all the HSP (Fig. 7). Also, no signal for
472 HSP 27 was ever obtained even when four times as much sample was processed.

473 When western blotting was performed using tissues prepared from heat stressed and non-heat
474 stressed larvae 16 h after treatment, the results were similar to those obtained from tissues
475 harvested 4 h after treatment (data not shown). However, 24 h after treatment, any heat stress-
476 mediated increase in HSP levels was minimal and not consistently detected (data not shown).
477 Moreover, when larvae were subjected to a heat stress of 30^{o}C for 1 h, HSP 90, 70 and 60
478 were detected in haemocytes and fat body harvested from non-heat stressed and heat stressed
479 larvae, but there was no discernible upregulation of these HSP in response to the heat stress.

480 **4. Discussion**

481 It is well documented that the ability of different fungal strains to infect and kill different
482 insect species varies considerably and is influenced by a number of factors associated with
483 both the fungus and insect (Lemaitre and Hoffmann, 2007; Ortiz-Urquiza and Keyhani, 2013;
484 Stokes et al., 2015; Butt et al., 2016). In the current work, the virulence of three different
485 fungal strains (two *M. brunneum* strains [V275 and 4556]; and one *B. bassiana* strain) against
486 larvae of the lepidopteran pest, *M. brassicae*, was tested. Dose-response assays indicated that
487 a relatively low level of mortality was obtained using 4556; as a result it was deemed
488 unsuitable for use in further bioassays and subsequent proteomic and molecular work. By
489 contrast, the higher level of larval mortality obtained with both V275 and *B. bassiana*,
490 indicated that they were suitable for use in bioassays, whilst the greater virulence of *B.*
491 *bassiana* indicated this to be the most suitable fungus to utilise in future proteomic and
492 molecular work. This is primarily because the relatively small size of the *M. brassicae* larvae
493 used in assays means that pooled samples of tissue (derived from three to eight larvae,
494 depending on the assay) are required for assessment. Thus, for fungus-treated insects, it is
495 necessary to ensure that as many larvae as possible are infected in order to avoid dilution of
496 the pooled sample with material from uninfected larvae, which could result in a (false)
497 negative result. Interestingly, with regard to the virulence of *B. bassiana*, the batch of conidia
498 used in the current work possesses a slightly higher virulence for *M. brassicae* larvae
499 compared to that used in previous studies (Richards et al., 2011). Whether this is due to
500 subtle changes to the fungus, to the insects, or both is not clear.
501
502 The finding that treatment of *M. brassicae* larvae with a heat stress following natural
503 infection by *B. bassiana* and V275 increases survival of the larvae (compared to non-heat
504 stressed controls), suggests that this stress is somehow protecting the larvae from the fungi.
505 One possibility is that the increase in temperature is having an adverse effect on the fungal

conidia. However, colony forming assays on nutrient agar plates indicate that the heat stress does not reduce the ability of the conidia to germinate and produce colonies *in vitro* compared to non-heat treated conidia. Thus, the heat stress does not appear to be high enough and/or long enough to affect fungal growth adversely. In view of this, it may be concluded that the increase in survival observed in heat stressed larvae is due to stress-induced alterations in the physiology of the larvae that somehow confer protection from fungal-mediated mortality. This conclusion is supported by the fact that a heat stress also protects *M. brassicae* larvae from *B. bassiana*-induced mortality even when the heat stress is applied four hours before injection of fungal conidia (*i.e.* under experimental conditions where the conidia are not at any time subjected to a heat stress). Similar results have been reported for heat-stressed *G. mellonella* larvae challenged with pathogens (Wojda and Jakubowicz, 2007; Wojda et al., 2009; Mowlds and Kavanagh, 2008; Wojda and Taszlow, 2013; Browne et al., 2014). Moreover, the current work, and that performed using *G. mellonella*, indicate that the degree of protection afforded is influenced by the magnitude and duration of the heat stress. Interestingly, the current work also indicates that the timing of application of the two stresses (*i.e.* fungal infection and heat stress) is important since the 'protective effect' exerted by the heat stress was manifest when it was applied 24 h and 48 h after topical application of *B. bassiana* and V275, respectively. An explanation for this likely relates to the time taken for the different fungal strains to penetrate the cuticle of *M. brassicae* larvae and to enter the haemocoel. Thus, under the experimental conditions utilised here, *B. bassiana* can be detected in the haemocoel 18 to 24 h after topical application, whereas V275 is first detected 30 to 48 h later (data not shown). In support of this, the dose-response assays using *B. bassiana* and V275 (Figures 1a and 1c) along with the combined stress bioassays for these fungi (Figures 2a and 2b), indicate that *B. bassiana* kills *M. brassica* larvae more quickly than V275. These results suggest that the time at which physiological changes induced by the

heat stress occur relative to fungal infection, is critical for protection. As far as we are aware, this is the first report where two different fungal strains have been directly compared in this way. In addition to heat stress, other types of stress have been shown to protect *G. mellonella* larvae from infection by pathogens. For example, it has been demonstrated that shaking of *G. mellonella* larvae (in a cupped hand), reduces their susceptibility to infection by *Candida albicans* and *Aspergillus fumigatus*, compared to non-stressed larvae (Mowlds et al., 2008; Browne et al., 2014). The effect of physical stress on survival of *M. brassicae* larvae treated with *B. bassiana* or V275, has not yet been investigated. Moreover, in the current work, although it is clear that immersing larvae in water to anaesthetise them (as required in the fungal injection bioassays) does not kill them, it is not known if this procedure may contribute to the heat stress-mediated reduction in fungal mortality observed.

The precise way in which a heat stress can alter the physiology of *M. brassicae* larvae in order to make them better withstand infection by *B. bassiana* and V275 is unclear. However, it is well documented that subjecting an organism to a heat stress usually upregulates expression of protective HSP (see below). As a first step in investigating if HSP play a role in protecting *M. brassicae* larvae from fungal infection, a proteomic approach was utilised to assess the levels of key HSP in larvae under non-heat stress and heat stress conditions. The *M. brassicae* larvae used in the current work were routinely cultured at 20^{o}C. Using antibodies raised against *Drosophila* HSP and a western blotting approach, HSP 90 and 70 were readily detected in haemocytes and fat body under such non-heat stress conditions. HSP 60 was also detected but the signal was much weaker (and more sample and a much longer exposure time of film to blot was required to visualise it). Clearly, as we do not know how well each of the commercially available anti-HSP utilised cross reacts with *M. brassicae* HSP, it is not possible to make conclusions about the relative abundance of the different

556 proteins present in *M. brassicae* tissues. It is clear, however, that subjecting the larvae to a
557 heat stress of 37^{o}C for 1 h (an increase of 17^{o}C), represents a significant, but non-lethal stress
558 and that the level of HSP 90, 70 and 60 is upregulated in response to this. Note that in the
559 current work, the heat stress was administered to larvae in deli pots and thus the increase in
560 temperature occurred gradually. If the increase in temperature had occurred more suddenly, it
561 is likely that the fold increase in induced HSP levels would have been greater. By contrast, a
562 heat stress of 30^{o}C for 30 min is not sufficient to increase HSP levels. The response of cells
563 to heat stress is extremely well characterized, and it is known that induced (and constitutive)
564 HSP protect the cell from the damaging effects of a heat stress by effectively removing
565 damaged/denatured proteins and polypeptides from the cell (*e.g.* by refolding them and/or by
566 directing them to a 'dismantling' pathway) (Parsell and Lindquist, 1993; Sun and MacRae,
567 2005; Richter et al., 2010). The finding that HSP 90, 70 and 60 are upregulated in *M.*
568 *brassicae* haemocytes and fat body in response to heat stress, suggests that these HSP play a
569 key role in protecting these cells from the damaging effects of heat. The possibility that they
570 may also protect the larvae from fungal infection, is discussed below. With regard to HSP 27,
571 it is known that small HSP are widely distributed in insects under non-stress conditions and
572 that they are upregulated in response to a variety of stresses (*e.g.* Sonoda et al., 2007; Shim et
573 al., 2008; Lopez-Martinez et al., 2009; Michaud et al., 2011; Zhang et al., 2015a, b; King and
574 MacRae, 2015). The lack of signal obtained for *M. brassicae* HSP 27 utilising a *Drosophila*
575 anti-HSP 27 antibody, suggests that this antibody does not cross react with the *M. brassicae*
576 protein.
577
578 It is known that the virulence of a fungus may be influenced by the ability of the insect to
579 mount effective immune responses against it (Lemaitre and Hoffmann, 2007; Ortiz-Urquiza
580 and Keyhani, 2013; Stokes et al., 2015; Butt et al., 2016). Moreover, in mammals, a role for

(mostly extracellular) HSP in signalling tissue damage or cellular stress (including heat stress), and activating the innate and/or adaptive immune systems, has been demonstrated (see below and Wallin et al., 2002; Giuliano et al., 2011). In view of this and the fact that a heat stress increases the level of HSP 90, 70 and 60 in *M. brassicae* larvae, the current study sought to determine the effect of a heat stress on *M. brassicae* haemocyte number, on a haemocyte-mediated immune response (*i.e.* phagocytosis), and on humoral (anti-fungal) immune responses.

With regard to humoral immunity, under the experimental conditions utilised, the results indicate that anti-fungal activity against *B. bassiana* was not detected in plasma from unstressed or heat stressed *M. brassicae* larvae. Thus, although the heat stress clearly increases HSP levels in these insects (see above), it does not appear to be able to induce anti-fungal activity. Similarly, in *G. mellonella* larvae, a heat stress alone did not increase anti-fungal and lysozyme activity in plasma, although heat stress and injection of *B. bassiana* did (Wojda et al., 2009). In the current study, the aim was to focus on how heat stress alone affects larval physiology, thus, the effect of two stresses (*i.e.* heat stress and fungal infection) on anti-fungal activity in *M. brassicae* remains to be investigated.

With regard to the effect of a heat stress on haemocyte density (*i.e.* haemocyte number per ml of haemolymph), it is clear that this is increased significantly compared to the controls immediately after (t=0 h) and 4 h after application of the heat stress, but not 24 h later. Similarly, with regard to haemocyte-mediated immunity, phagocytosis assays indicate that the heat stress significantly increases the ability of *M. brassicae* haemocytes to phagocytose FITC-labelled fungal conidia when they are harvested from larvae immediately after (t=0 h) and 4 h after the heat stress, but not 24 h after the stress. These results, together with those

606 obtained from bioassays, allow us to hypothesise that subjecting *M. brassicae* larvae to a heat
607 stress at the time when a fungus is first entering the larval haemocoel, protects the larva from
608 the fungus by increasing both the number of haemocytes present and their phagocytic
609 activity. This hypothesis is further supported by the fact that *M. brassicae* haemocytes can
610 phagocytose *B. bassiana* conidia (Richards et al., 2013), and other studies that indicate that
611 insect haemocytes play an important role in protecting insects from fungal infection through
612 their participation in wound healing, and coagulation and immune responses, including
613 phagocytosis (Lemaitre and Hoffmann, 2007; Ortiz-Urquiza and Keyhani, 2013; Stokes et al.,
614 2015; Butt et al., 2016). To our knowledge, this is the first time that a heat stress has been
615 demonstrated to increase phagocytic activity of insect haemocytes.
616
617 Exactly which molecular mechanisms underlie the heat stress-mediated increase in survival
618 of *M. brassicae* larvae to fungal infection, is not clear at present, although research using
619 mammalian models indicates that HSP and phagocytes interact to protect cells and
620 organisms. For instance, in murine macrophages, heat stress and (extracellular) HSP 70 have
621 been shown to elevate phagocytosis following binding to the lipid raft microdomain on the
622 plasma membrane of the cells, but not by acting as an opsonin (Vega and De Maio, 2005;
623 Kovalchin et al., 2006; Wang et al., 2006a; b). In the current work, the time which elapsed
624 from the beginning of the heat stress to the time taken to actually assess haemocyte number
625 and phagocytosis is as much as 1 h 30 min to 1h 45 min, respectively. Thus, it is possible that
626 constitutively expressed and induced HSP 70 (and other HSP) in *M. brassicae* haemocytes
627 may utilise a similar (extracellular) mechanism to contribute to the increase in phagocytosis
628 observed. This hypothesis could be investigated by using HSP-specific antibodies (as used
629 for western blotting) to determine if HSP are present in *M. brassicae* plasma or on the outer
630 surface of haemocytes, and whether the antibodies can block phagocytosis.

631

632 Another possibility (not investigated in the current work), is that heat shock proteins induced
633 in *M. brassicae* larvae by a heat stress (including HSP 90, 70 and/or 60), may increase
634 survival of the larvae by effectively reducing the ability of the fungal conidia to penetrate the
635 cuticle. In support of this, recent work indicates that in mammals, HSP (especially HSP 90
636 and 70) secreted from cells in response to tissue injury, infection and cell damage, may play a
637 role in promoting cell motility (a crucial event for wound closure/healing), and activation of
638 immune responses (see above and Wallin et al., 2002; Basi et al., 2003; Atalay et al., 2009;
639 De Maio, 2011; Li et al., 2012; Bellaye er al., 2014). It remains to be determined if HSP
640 secreted by *M. brassicae* haemocytes (especially those localised at areas where the
641 cuticle/epidermis are damaged by fungal penetration), can contribute to wound healing at
642 these sites.

643

644 To conclude, although the current work has shed light on the effects of a heat stress on the
645 susceptibility of insects to pathogens and the possible role of HSP in these events, it is clear
646 that many more questions have been raised. Many of these will be addressed in future work
647 with the aim of elucidating the molecular mechanisms involved in the response of an insect to
648 stress, including infection with BCA. In particular, since HSP may be induced in response to
649 a variety of stresses (not just a heat stress), the results of the current work have implications
650 for pest management strategies utilising BCA in conjunction with any other agent (*e.g.*
651 another BCA, a chemical pesticide, a botanical, etc.). RNAi-mediated knock-down of
652 selected HSP will undoubtedly help to clarify the role of these proteins in larval survival
653 following exposure to stress.

654

655

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Table 1. Effect of heat stress (HS) on haemocyte number per ml of haemolymph. The number of haemocytes per ml with corresponding 95 % confidence intervals and P-value comparison with no heat stress (No HS) controls is presented.

[a]Time (hours)	Number of haemocyte × 10^7 (with 95% confidence intervals [lower; upper])		P-value comparison with No HS
	Without heat stress	With heat stress	
0	1.215 (0.942; 1.488)	2.355 (2.082; 2.628)	0.000003
4	1.300 (0.788; 1.812)	2.358 (1.845; 2.870)	0.000754
24	1.193 (0.998; 1.388)	1.378 (1.183; 1.573)	0.0711

889

a: Number of hours after exposure of larvae to heat stress or no heat stress conditions.

Table 2. Probability of phagocytosis for treatments with and without heat stress, with corresponding P-values

[a]Time (hours)	Probability of phagocytosis with lower limit and upper limit with 95% confidence interval		P-value of treatment
	Without heat stress	With heat stress	
0	21.2% (19.6%,22.7%)	29.0% (27.2%,31.0%)	$<1.3x10^{-14}$
4	18.1% (15.8%,20.7%)	27.2% (24.2%,30.4%)	$<2.2x10^{-16}$
24	18.8% (17.3%,20.4%)	19.9% (18.4%,21.5%)	0.279

.

a: Number of hours after exposure of larvae to heat stress or no heat stress conditions.

Table 3. Effect of heat stress on anti-fungal activity in *M. brassicae* plasma.

[a]Time (h)	Mean count (95 % CI)			'p' comparison with no heat shock		
	[b]Broth	[c]No HS	[c]With HS	Broth	No HS	With HS
0	180 (166; 194)	167 (155; 181)	165 (152; 178)	0.044	NA	0.670
4	175 (165; 185)	131 (123; 140)	128 (120; 137)	<0.0001	NA	0.521
24	205 (183; 230)	178 (158; 200)	168 (150; 189)	<0.0001	NA	0.127

a: Number of hours after exposure of larvae to heat stress (HS) or no heat stress (No HS) conditions.

b: *Beauveria bassiana* conidia were incubated with PD broth for 24 h, then plated out on PD agar plates and the number of colonies per plate enumerated.

c: *Beauveria bassiana* conidia were incubated with plasma from heat stressed (HS) or non-heat stressed (No HS) larvae for 24 h, then plated out on PD agar plates and the number of colonies per plate enumerated.

Figure Legends

Figure 1. Dose-response assays for mortality in *Mamestra brassicae* resulting from three different strains of topically applied fungi. Larvae were dipped into a Tween 80 / codacide solution containing a known number of *Beauveria bassiana* (a), 4556 (b), or V275 (c) conidia. For the control (No F), larvae were dipped into a Tween 80/codacide solution without fungus. For all graphs, each point represents the mean percentage of dead larvae ± SE.

Figure 2. Effect of heat stress on survival of *Mamestra brassicae* larvae infected with *B. bassiana* (a), or *Metarhizium brunneum* V275 (b). Larvae were dipped in a Tween 80 / codacide solution either without or with (*B. bassiana* [Bb] or V275) fungal conidia. Larvae

905 were then subjected to a heat stress (HS) either 24 h or 48 h later, or left at CE room
906 conditions (No-HS). The letters in superscript indicate significant differences between
907 treatments and the figure after each treatment represents the expected percentage survival of
908 larvae at the end of the assay.
909
910 **Figure 3.** Effect of injection of *Beauveria bassiana* on mortality of *Mamestra brassicae*
911 larvae: dose-response assays. Larvae were injected with 2 µl of Dulbecco's phosphate-
912 buffered saline (DPBS) either without (DPBS) or with 100, 1000, or 3000 *B. bassiana*
913 conidia. Each point represents the mean percentage of dead larvae ± SE.
914
915 **Figure 4**. Effect of heat stress on survival of *Mamestra brassicae* larvae injected with
916 *Beauveria bassiana*. Larvae were either subjected to a heat stress (HS) or left at CE room
917 conditions (No-HS). Four hours later, larvae were injected with 2 µl of DPBS either with
918 $1x10^2$ *B. bassiana* conidia (Bb) or without conidia (DPBS). The letters in superscript indicate
919 significant differences between treatments and the figure after each treatment represents the
920 expected percentage survival of larvae at the end of the assay.
921
922 **Figure 5.** Effect of heat stress on the ability of *Mamestra brassicae* haemocytes to
923 phagocytose FITC-labelled *Beauveria bassiana* conidia. Monolayers of haemocytes were
924 prepared from no-heat stressed (No-HS) larvae, or from larvae immediately after the HS (t=0
925 h), or 4 h (t=4 h) or 24 h after treatment (t=24 h). The monolayers of haemocytes were
926 overlaid with $1x10^6$ FITC-labelled *B. bassiana* conidia for 90 min, and then the number of
927 haemocytes phagocytosing one or more conidia determined. The values presented represent
928 means and 95 % confidence intervals of haemocytes ingesting one or more conidia.

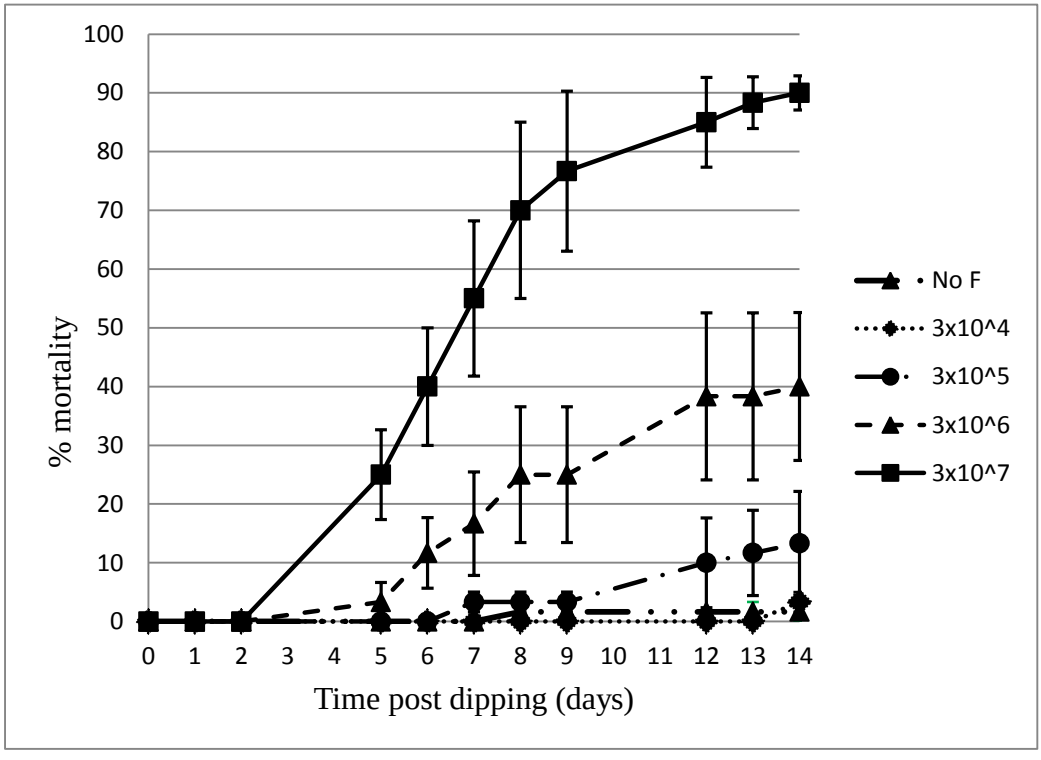
929

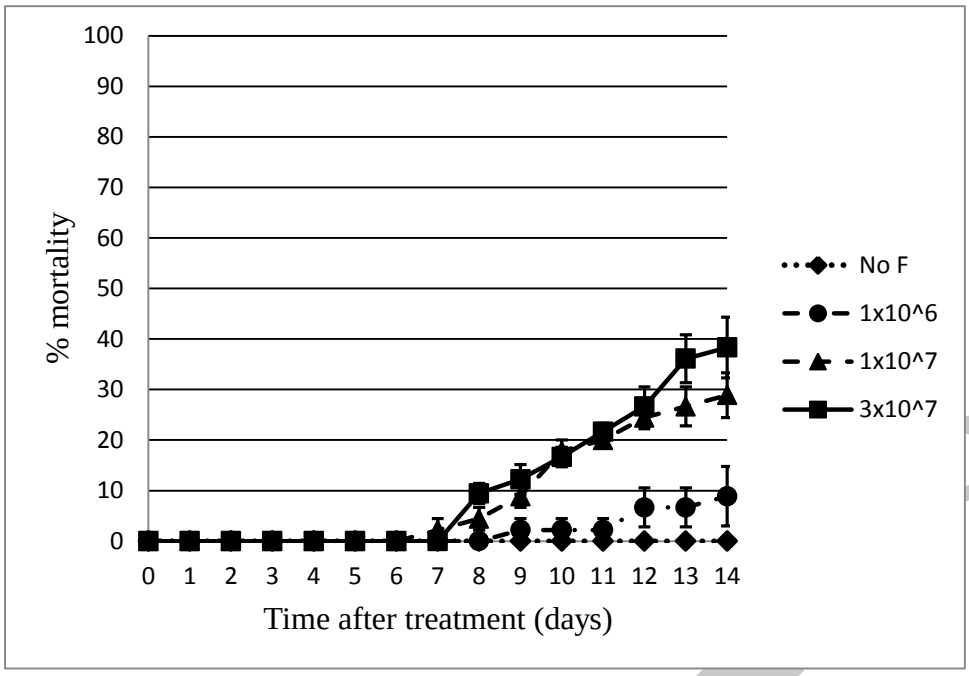
930 **Figure 6.** Effect of heat stress on anti-fungal activity in larval plasma. Plasma prepared from
931 heat stressed (HS) and non-heat stressed (No-HS) larvae was added to *Beauveria bassiana*
932 conidia in the wells of a 96-well plate. Conidia in broth served as a blank (B) control for
933 fungal growth. After 24 h, equal aliquots were taken from each well, plated out on nutrient
934 agar plates and the number of colonies formed determined. The values presented represent
935 means and 95 % confidence intervals of number of fungal colonies per plate.

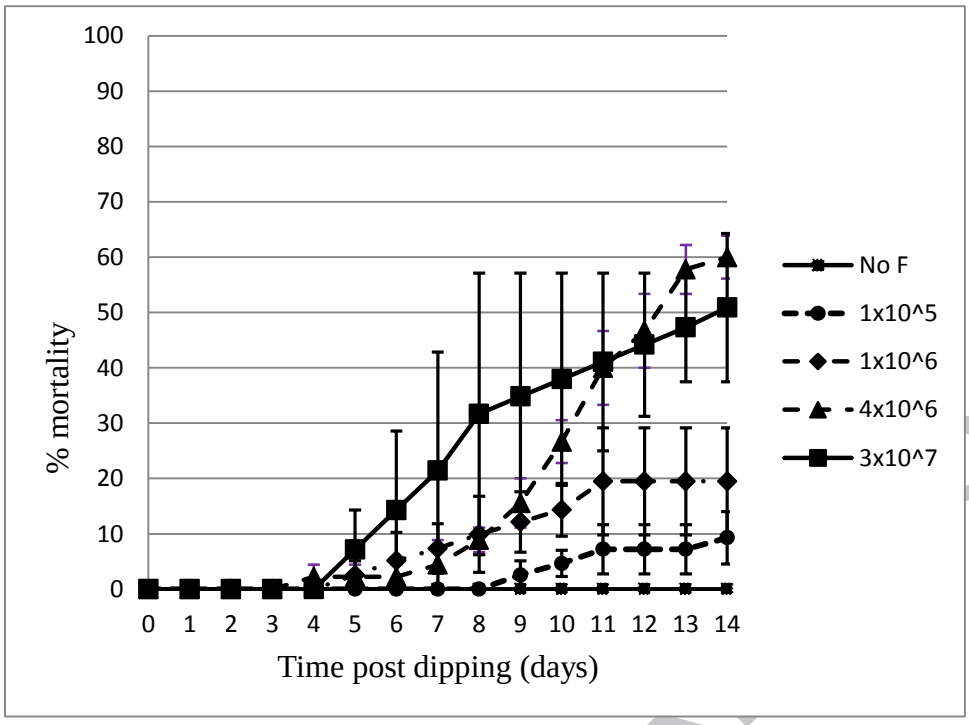
936

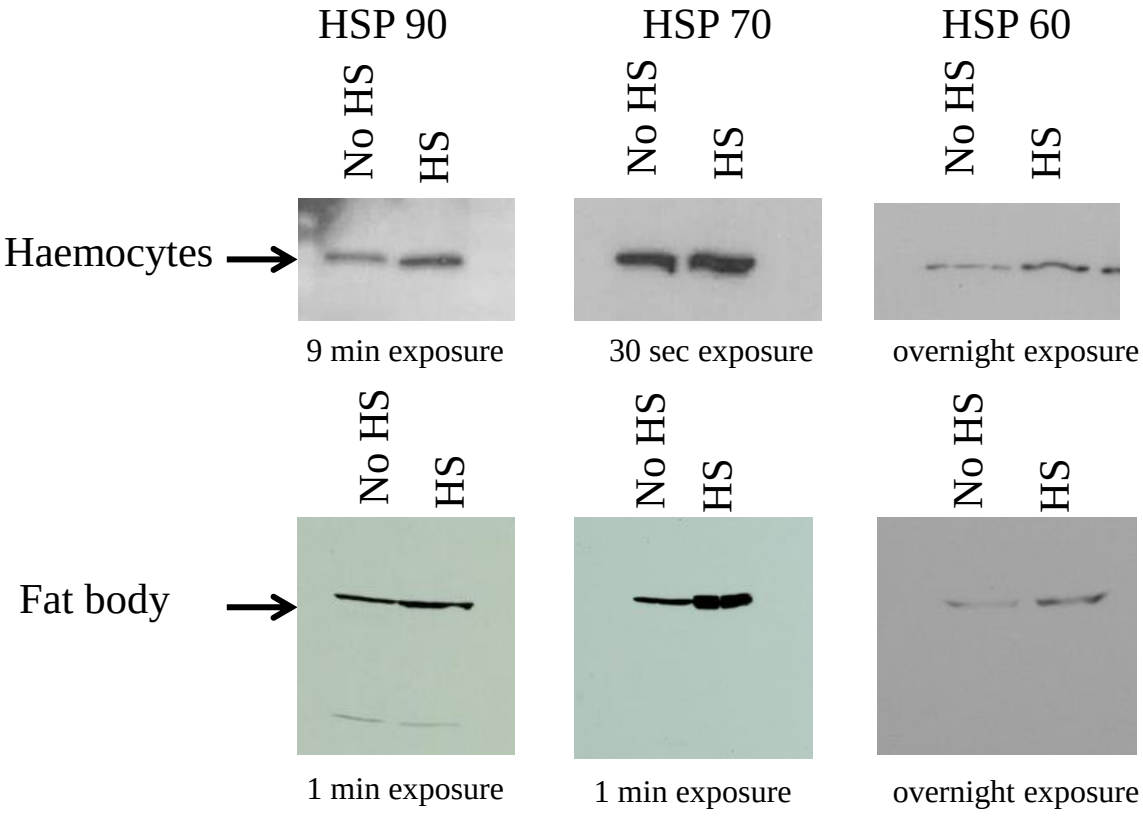
937 **Figure 7.** Western blot showing levels of HSP 90, HSP 70 and HSP 60 in *Mamestra*
938 *brassicae* haemocytes and fat body under no heat stress (No-HS) and heat stress (HS)
939 conditions. For haemocytes, to detect HSP 90, HSP 70 and HSP 60, the equivalent of
940 100,000, 50,000 and 200,000 haemocytes were loaded, respectively, (for both HS and NHS
941 samples). For fat body, to detect HSP 90, HSP 70 and HSP 60, the equivalent of 0.73 mg of
942 fat body were loaded. For all blots, The time that the x-ray film was exposed to the blots is
943 indicated below the images.

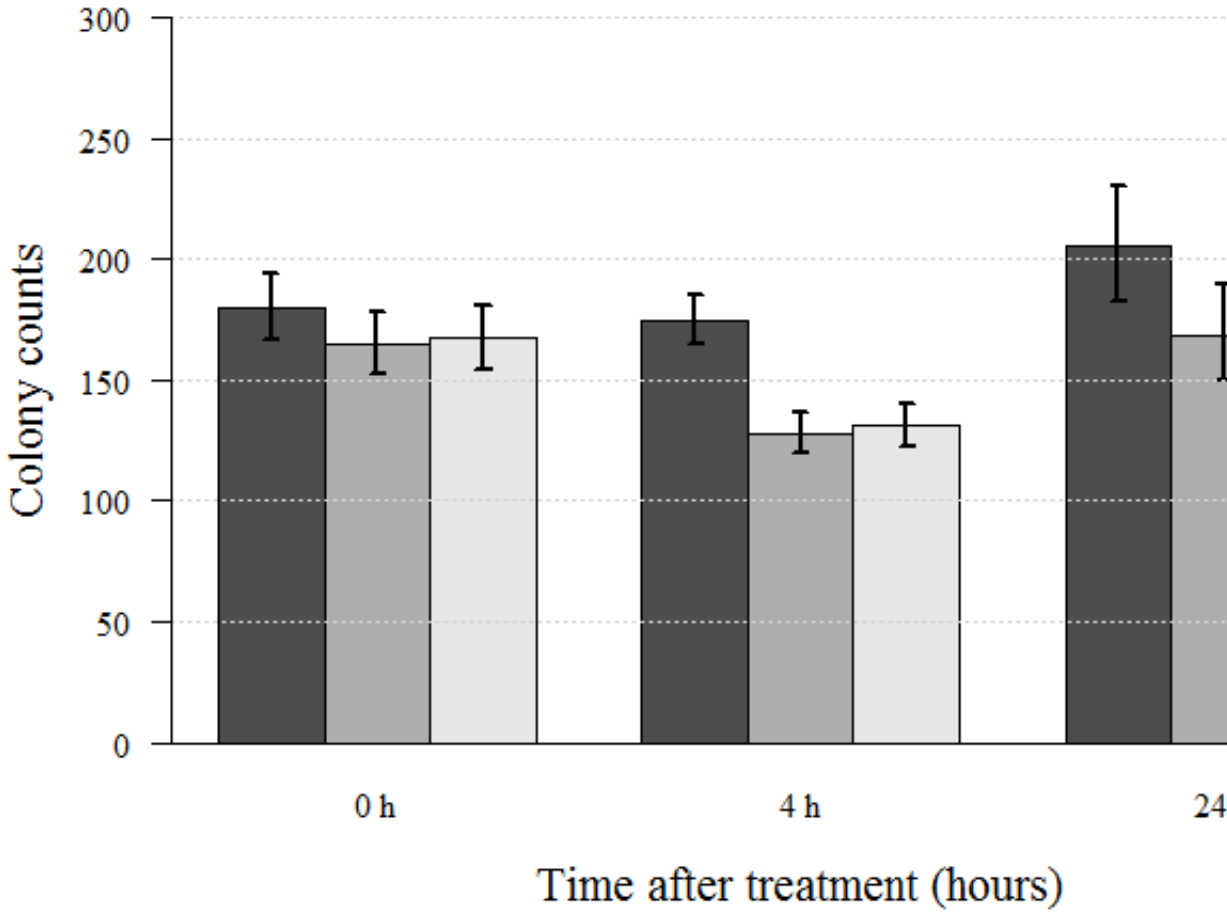
944

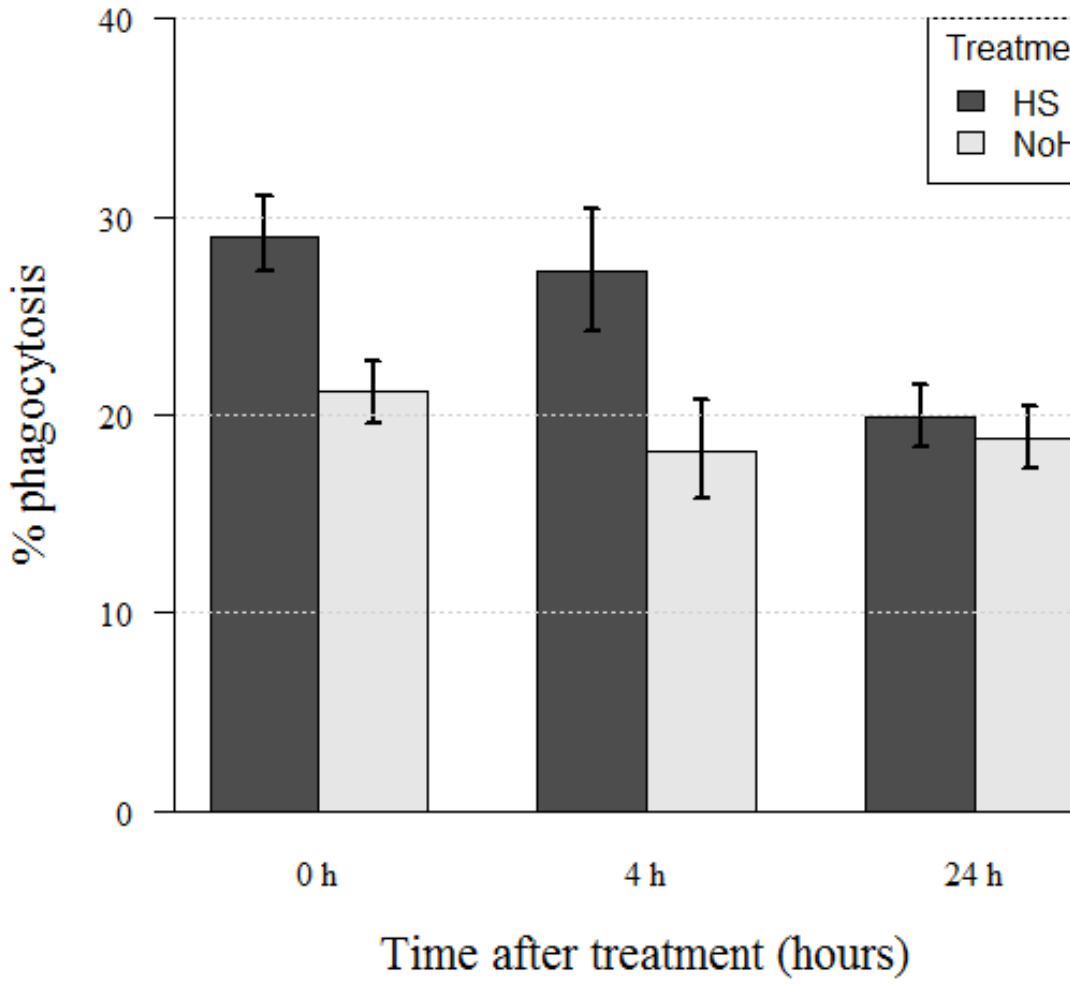

100
90
80
70
60
50
40
30
20
10
0
% mortality
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time post dipping (days)
No F
3x10^4
3x10^5
3x10^6
3x10^7

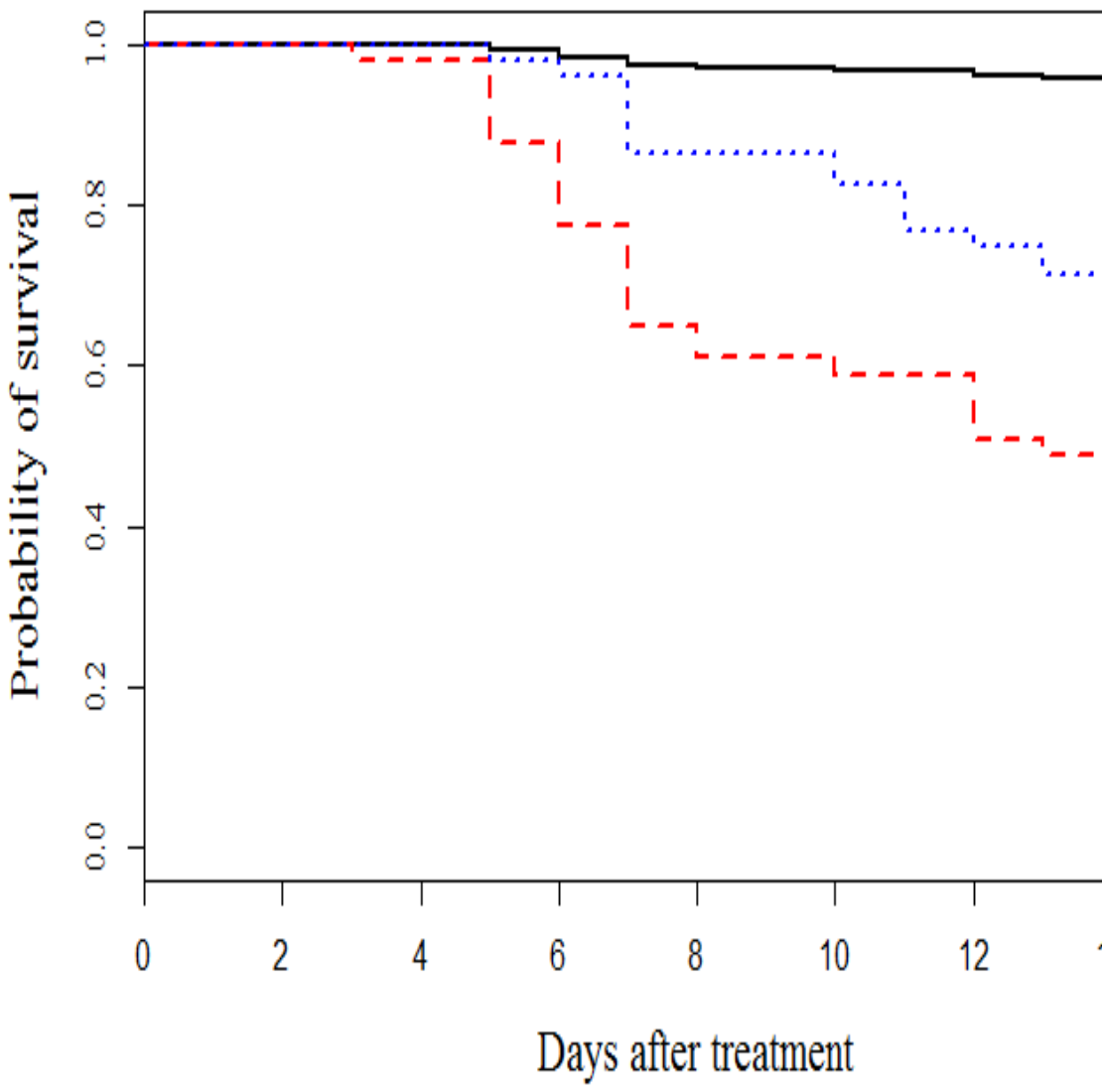


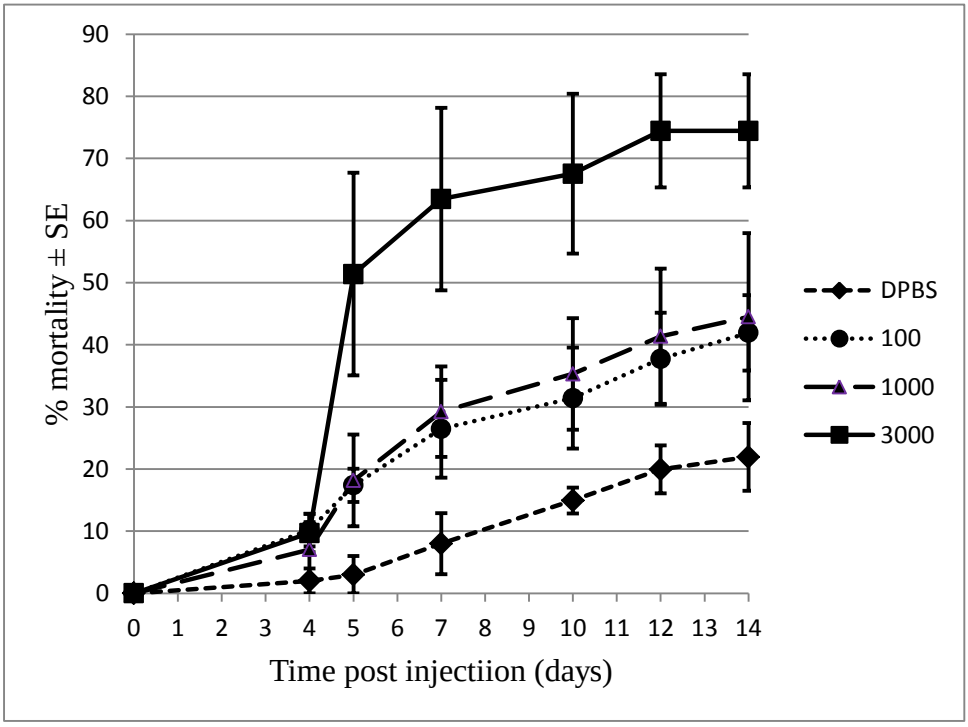


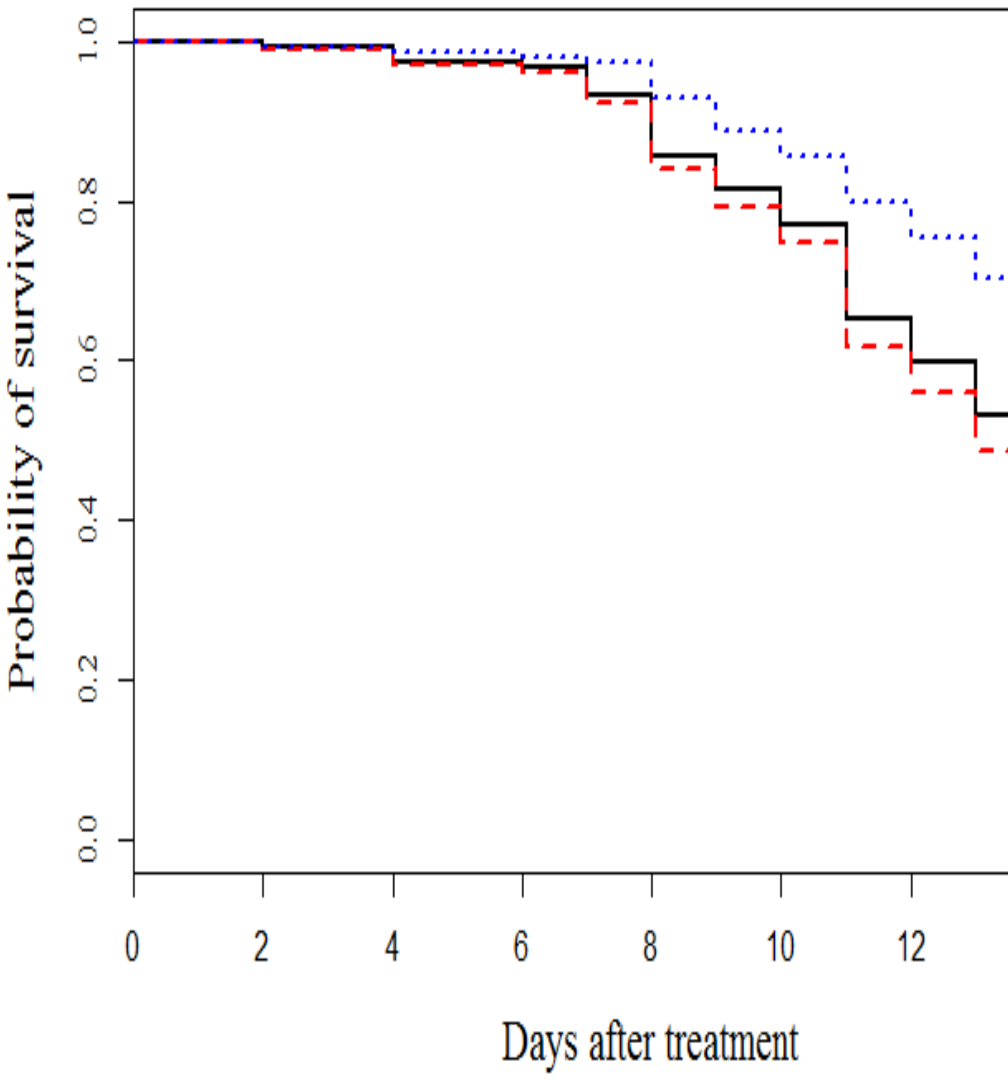

HSP 90
HSP 70
HSP 60
No HS
HS
No HS
HS
No HS
HS
Haemocytes
9 min exposure
30 sec exposure
overnight exposure
No HS
HS
No HS
HS
No HS
HS
Fat body
1 min exposure
1 min exposure
overnight exposure

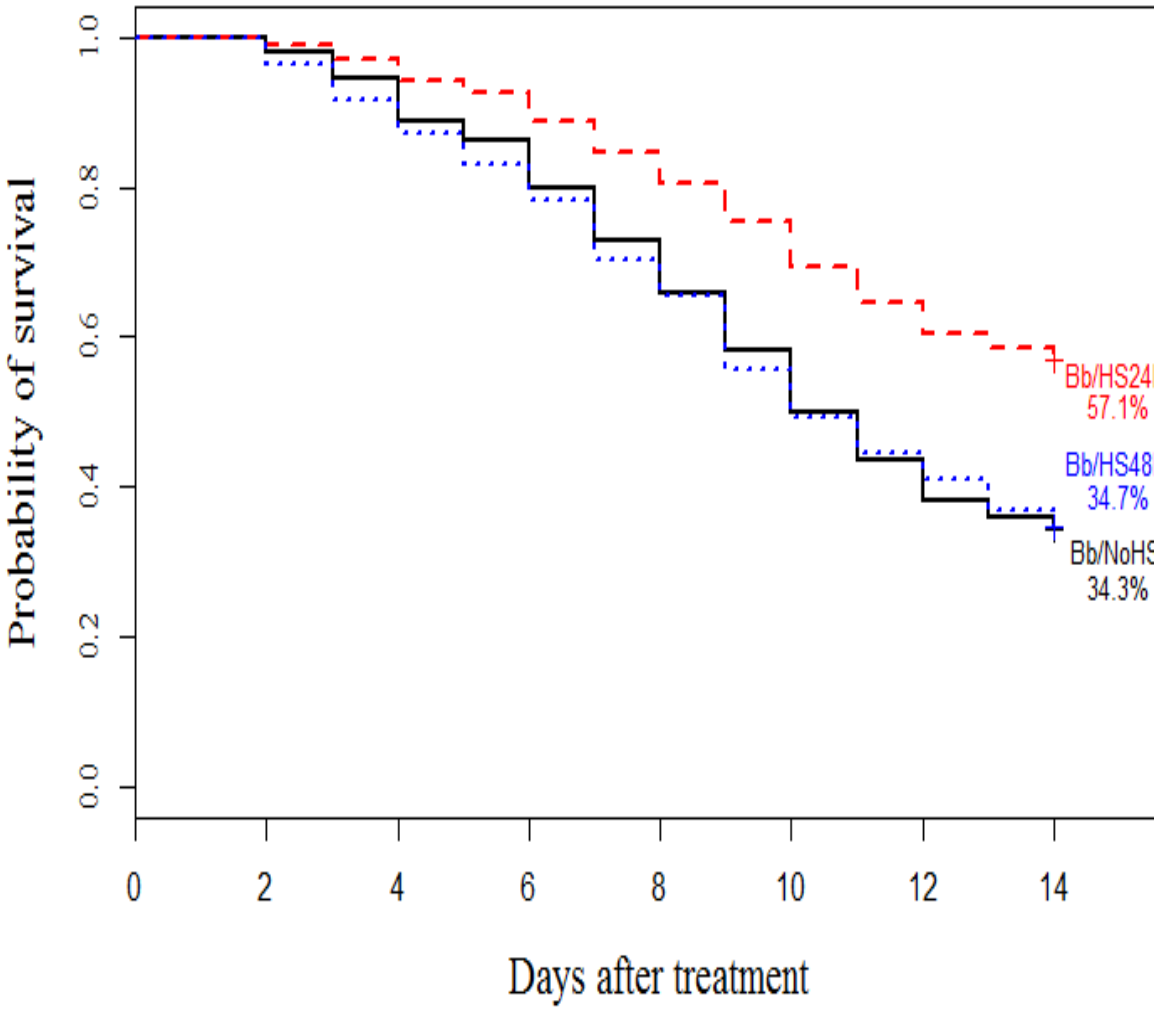


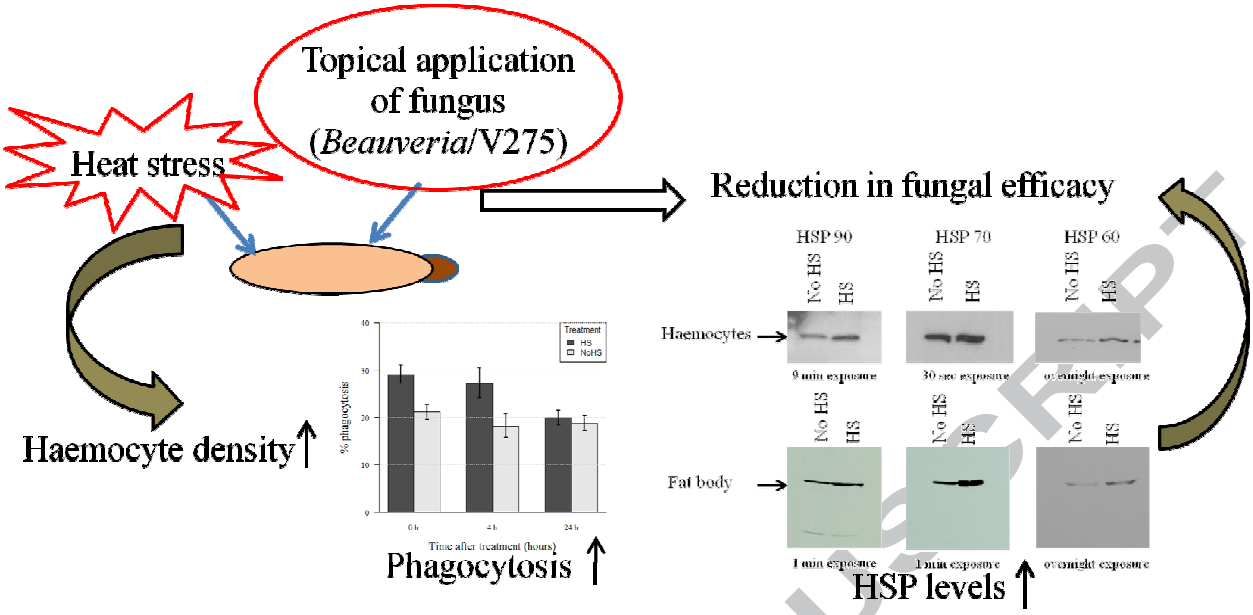





90
80
70
60
50
40
30
20
10
0
% mortality ± SE
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time post injectiion (days)
DPBS
100
1000
3000







945 Highlights

946

947 • *Mamestra brassicae* larvae were exposed to heat stress (HS) and fungal infection
948 • The HS altered larval physiology and reduced the efficacy of the fungi
949 • Exposure to HS increased HSP 90, 70 and 60 levels in haemocytes and fat body
950 • The HS also resulted in increases in haemocyte density and phagocytosis
951 • HS-mediated induction of immune responses may contribute to increased survival
952 after fungal infection

953

954