Divergent forms of endoplasmic reticulum stress trigger a robust unfolded protein response in honey bees

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ARTICLE INFO

Article history:
Received 24 July 2015
Received in revised form 4 December 2015
Accepted 12 December 2015
Available online 14 December 2015

Keywords:
Cellular stress response
Proteostasis
Endoplasmic reticulum
Unfolded protein response
IRE1
Xbp1
Honey bee

ABSTRACT

Honey bee colonies in the United States have suffered from an increased rate of die-off in recent years, stemming from a complex set of interacting stresses that remain poorly described. While we have some understanding of the physiological stress responses in the honey bee, our molecular understanding of honey bee cellular stress responses is incomplete. Thus, we sought to identify and began functional characterization of the components of the UPR in honey bees. The IRE1-dependent splicing of the mRNA for the transcription factor Xbp1, leading to translation of an isoform with more transactivation potential, represents the most conserved of the UPR pathways. Honey bees and other Apoidea possess unique features in the Xbp1 mRNA splice site, which we reasoned could have functional consequences for the IRE1 pathway. However, we find robust induction of target genes upon UPR stimulation. In addition, the IRE1 pathway activation, as assessed by splicing of Xbp1 mRNA upon UPR, is conserved. By providing foundational knowledge about the UPR in the honey bee and the relative sensitivity of this species to divergent stresses, this work stands to improve our understanding of the mechanistic underpinnings of honey bee health and disease.

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1. Introduction

The Western Honey Bee, Apis mellifera provides critical pollination services of paramount importance to humans in both agricultural (Eilers et al., 2011; Gallai et al., 2009; Klein et al., 2007) and ecological settings (Potts et al., 2010). Honey bee colonies in the United States and Europe have suffered from an increased rate of die-off in recent years. The phenomenon, called Colony Collapse Disorder, is characterized by an abrupt disappearance of adult worker bees from a honey bee colony, and likely stems from a complex set of interacting stresses that remain poorly described (Ratnieks and Carreck, 2010). Key stresses thought to be involved include nutritional stress due to loss of appropriate forage, chemical poisoning from pesticides, changes to normal living conditions brought about through large-scale beekeeping practices, and infection by insect parasites and pathogenic microbes (Oldroyd, 2007; Potts et al., 2010; vanEngelsdorp et al., 2009).

As no single cause for the recent increase in honey bee disease is evident, there is increased focus on the impact of interactions between various stressors. Many studies have found synergistic effects of pesticides and microbial infection with each other (Alaux et al., 2010; Aufauvre et al., 2012; Boncrystiani et al., 2012; Doublet et al., 2015; Pettis et al., 2013; Vidau et al., 2011) or other stresses such as nutritional stress (Di Pasquale et al., 2013; Huang, 2012). A critical first step in understanding these synergies involves defining specific common cellular processes that are impacted by multiple stressors and could therefore serve as links to cellular dysfunction, tissue pathology, disease, and mortality in honey bees. The various pathways that make up cellular stress responses provide logical and compelling processes to examine for such interactions. We have some appreciation of the physiological stress responses in the honey bee (Even et al., 2012). However, our molecular understanding of honey bee cellular stress responses is incomplete.

One critical cellular stress involves problems in proteostasis, which refers to the homeostasis of protein synthesis, folding, function, and degradation both within a cell and in an organism as a
whole (Taylor et al., 2014). A number of normal and pathologic conditions can lead to disruption of proteostasis, leading to a build-up of unfolded proteins in the cell and triggering a suite of responses designed to limit damage to the cell from problems in protein folding and return the cell to homeostasis (Taylor et al., 2014). Within individual cells, proteostasis is maintained by the responses of the proteostatic network, including the Heat Shock Response (HSR) (Morimoto, 2012; Vabulas et al., 2010), responding to proteostatic disruption in the cytoplasm, and the unfolded protein response (UPR), responding to proteostatic perturbation in the endoplasmic reticulum (ER) (Walter and Ron, 2011).

In other species, the UPR has been shown to influence cellular and organismal outcomes to exposures to the very environmental stressors suspected to play a part in recent honey bee losses, including microbial attack (Wang and Kaufman, 2012), chemical toxicity (Laflèche et al., 2013), nutritional stress (Lee and Ozcan, 2014), and physiological stress (Taylor et al., 2014). A few highly conserved pathways, characterized by unique receptors and signal transduction machinery, are responsible for sensing unfolded proteins in the ER (Fig. S1, reviewed in (Samali et al., 2010; Walter and Ron, 2011)). They include the IRE1 (inositol requiring enzyme 1)-dependent pathway, the PERK [double-stranded RNA-activated protein kinase (PKR)-like ER kinase] pathway, and the ATF6 (activating transcription factor 6) pathway. Activation of these pathways results in short-term responses designed to limit the influx of new proteins and activation of three bZIP transcription factors, X-box binding protein 1 (XBP1) by IRE1, Activating transcription factor 4 (ATF4) by PERK, and activating transcription factor 6 (ATF6) itself, which participate in a medium-term adaptive response through transcriptional upregulation of proteins involved in multiple ER processes.

The most highly conserved pathway of the UPR is the IRE1 pathway. IRE1 is a transmembrane receptor that contains both kinase and endonuclease activity that are important for its function. IRE1 is usually bound to the ER chaperone HSC70-3 and maintained in a monomeric, inactive form. Upon increase of unfolded proteins in the lumen of the ER, IRE1 is activated by loss of Hsc70-3 binding (as this molecule is sequestered by unfolded proteins) or by binding of unfolded proteins themselves. This leads to IRE1 dimerization, autophosphorylation of the kinase domain, further multimerization, and ultimately to activation of the endonuclease domain. The endonuclease participates in the activation of the UPR thru the non-canonical splicing of the mRNA encoding the bZIP transcription factor XBP1. In its unspliced form, the Xbp1 mRNA (Xbp1u) encodes a truncated protein (XBP1u) with low transactivation activity. Splicing removes a short sequence containing an in-frame stop codon, leading to the translation of the new transcript (Xbp1s), which encodes a longer form of XBP1 (XBP1s), with robust transactivation activity that upregulates the transcription of UPR target genes. In a recent paper (Hoks and Griffiths-Jones, 2011), the non-canonical intron structures in Xbp1 mRNA were predicted for 128 eukaryotes, including the honey bee. Interestingly, the honey bee Xbp1 mRNA possess a rather unique deviation from the CNGCNG site found at the 5′ splice site of the intron, instead having a CNACNG sequence. We hypothesized that this pronounced divergence in the Xbp1 mRNA non-canonical intron could have important consequences for honey bee UPR function and proteostasis. Thus, we began functional characterization of the UPR in this species, focusing on the IRE1 pathway, to answer this question and further our molecular understanding of honey bee cellular stress responses. We found that the core components of the UPR were conserved in the honey bee. In addition, we characterized a number of gene targets of the UPR that are robustly induced upon UPR activation in response to multiple known triggers. While the Xbp1 mRNA splice site has unique features in this species and other related bees, IRE1-dependent splicing of Xbp1 mRNA upon UPR stimulation is conserved.

2. Materials and methods

2.1. Honey bee tissue collection

Honey bees were collected from the landing board of outbred colonies in New York, New York consisting of a typical mix of A. mellifera subspecies found in North America. Only visibly healthy bees were collected and all source colonies were visually inspected for symptoms of common bacterial, fungal, and viral diseases of honey bees.

2.2. Ortholog screening of the honey bee genome

UPR pathway gene candidates from Drosophila melanogaster and Caenorhabditis elegans were used to find orthologs in the honey bee genome using the BLAST family of search functions (www.ncbi.nlm.nih.gov). Honey bee orthologs were identified by searching honey bee genome assemblies 4.5 directly using tblastn. In addition, the KEGG (Kyoto Encyclopedia of Genes and Genomes) database was used as a guide for comparing pathways between species (Kanehisa and Goto, 2000). The alignment of unconventional intron sites was created using INFERNAL 1.1.1 (Nawrocki and Eddy, 2013) cmalign command with an covariance model based on full alignment from (Hooks and Griffiths-Jones, 2011) and then manually adjusted in RALEE (Griffiths-Jones, 2005). Secondary RNA structures were drawn using VARNA (Darty et al., 2009).

2.3. Chemical treatments

For all caged experiments, honey bees were selected as above and kept in 177.4 mL (6 oz). Square-bottomed Drosophila Stock Bottles (Fisher Scientific, Pittsburg, PA) plugged with modified foam tube plugs (Jaece Industries, North Tonawanda, NY). Bees were maintained in incubators at 35 °C (unless otherwise stated) in the presence of PseudoQueen (Contech, Victoria, British Columbia, Canada) as a source of Queen Mandibular Pheromone (QMP) and used as per manufacturer’s instructions. Bees were fed 30% sucrose via a modified 1.5 ml screw-cap tube with or without the following chemicals at the doses indicated in the figure legends and text: 12–24 μM tunicamycin (Sigma, St. Louis, MO) (Chow et al., 2013; Kang et al., 2012) and 10–25 mM dithiothreitol (DTT) (Sigma, St. Louis, MO) (Caruso et al., 2008). The solute for tunicamycin is DMSO, so equivalent amounts of DMSO were added to the food of the control group in tunicamycin experiments. Water is the solute for DTT, so equivalent amounts of water were added to the food of the control group in DTT experiments. UPR stimulation experiments with tunicamycin were performed a minimum of four times and UPR stimulation experiments with DTT were also performed a minimum of four times. Survival experiments with Tunicamycin and DTT were each performed twice with similar results.

2.4. RNA isolation, reverse-transcription and quantitative PCR for gene expression analysis

Midguts, and sometimes abdomen tissue with all gut tissue removed, were placed into RNAlater (Invitrogen, San Diego, CA) for storage prior to gene expression analysis of individual workers. RNA was prepared from bees from the described populations by manually crushing the tissue of interest with a disposable pestle in Trizol Reagent (Invitrogen, San Diego, CA) and extracting the RNA as per the manufacturer’s instructions. RNA was subsequently DNase treated by using RQ1 RNase-Free DNase (Promega, Madison, WI).
and quantified. cDNA was synthesized using approximately 1 µg of RNA with the iScript cDNA Synthesis Kit (Biorad, Hercules, CA). Typically, 1 µl of cDNA was then used as a template for quantitative PCR to determine the levels of expression of genes of interest using the iQ SYBR Green Supermix (Biorad, Hercules, CA) in an iCycler thermo-cycler (Biorad, Hercules, CA). Primer sequences for transcripts of gene of interest are Table S1. Primer sequences using the iQ SYBR Green Supermix (Biorad, Hercules, CA) in an
level of that gene relative to
b

2.5. Xbp1 mRNA splicing
cDNA from above was used as template for a PCR using primers (Forward, 5′-ctgtgctgtgctctcctgg-3′ and Reverse 5′-tcagaggaagatgtgccagaa-3′) that spanned the predicted Xbp1 splice sites. PCR products were run on a 2.5% Agarose gel to separate spliced from unspliced Xbp1. DNA was purified from bands representing ampli-
cons from unspliced and spliced forms of the Xbp1 transcript using a Gel Extraction Kit (Qiagen, Hilden, Germany), cloned into pDrive (Qiagen, Hilden, Germany), and sequenced (Genewiz, South Plainfield, New Jersey).

2.6. Statistical analysis
For analysis, data was log10 transformed and compared using unpaired t-tests with Welch’s correction when values fit normal distributions or Mann–Whitney U nonparametric tests when they did not fit normal distributions. Normality was assessed using Shapiro–Wilk tests. When more than two groups were being compared, data was compared using one way ANOVA with Tukey’s multiple comparison test when values fit normal distributions or a Kruskall–Wallis test. For survival analysis, treated versus untreated groups were compared using the Gehan–Breslow–Wil coxon test.

3. Results
3.1. Hymenopteran species possess unique Xbp1 mRNA splice-site
Examination of the Xbp1 mRNA from honey bees and other Hymenopteran insects revealed that the unique CNACNG sequence observed in honey bees (Hooks and Griffiths-Jones, 2011) is con-
served in multiple species from the Apoidea superfamily including Apis florea, Bombus ignatius, Bombus terrestris, and Megachilidae rotundata. However, this deviation was not found in species representing other superfamilies of Hymenoptera, including the Vespoidea (Atta cephalotes) or Chalcidoidea (Nasonia vitripennis). In addition, as shown previously, this CNACNG sequence was not observed in select species representing other insect orders (Figs. 1 A, B and S2A, B). We reasoned that the divergence in the Xbp1 mRNA non-canonical intron could have important conse-
quences for honey bee UPR function and began functional character-
ization of the UPR in this species, focusing on the IRE1 pathway.

3.2. UPR pathway components are conserved in the honey bee
Analysis of the honey bee genome revealed that the core signal-
ing components of the IRE1, PERK, and ATF6 pathways are con-
served. As in Drosophila, A. mellifera possesses single copies of genes encoding IRE1, PERK, ATF6, the transcription factors XBP and ATF4, Elf2 and its regulator CaADD34, and apparent homologs encoding many of the proposed transcriptional targets of the path-
way, including Hsc70-3, the proposed inhibitory ligand for IRE1,
PERK, and ATF6 (Fig. S1 and Table S1). A core set of putative target genes were culled from lists of UPR target genes in Drosophila (Chow et al., 2013; Park et al., 2014), and include candidate genes from each of the following categories: chaperones (Hsc70-3, p58ipk, Gp93), disulfide bond generating enzymes (EroII, CaBP), and the ER-associated degradation (ERAD) pathway (Derlin1, Herp2, Hrd) (Fig. S1 and Table S1).

We examined the expression of the core components of the IRE1 pathway in a number of tissues from forager honey bees taken directly from the colony. We focused this analysis on head tissue (predominantly brain and sensory organ tissue), midgut, thorax tissue (predominantly flight muscle), and abdominal wall (predominantly fat body). Relative to β-actin, we found all components (the ligand, Hsc70-3, the receptor Ire1, and the transcription factor, Xbp1) were expressed in all tissues. In addition, we found that Ire1 and Hsc70-3 were more highly expressed in thorax and abdomen tissue than in the head and midgut, while Xbp1 was more highly expressed in head, thorax, and abdomen tissue than in the midgut (Fig. 2).

3.3. UPR gene induction after tunicamycin treatment in the honey bee
Before examining IRE1 pathway activation, we first character-
ized UPR activation dynamics in the honey bee, focusing on transcri-
ptional induction of putative target genes. We chose tunicamycin, a bacterially-derived compound that inhibits N-glycosylation of proteins in the ER, leading to a build-up of mis-
folded proteins (Samali et al., 2010). As previous findings dem-
strated robust UPR induction in the digestive tract of fruit flies after feeding 12 µM tunicamycin (Chow et al., 2013), we used a similar dose in our experiments. Each of the putative target genes, including chaperones (Hsc70-3, p58ipk, Gp93), disulfide bond generating enzymes (EroII, CaBP), and ERAD pathway components (Derlin1, Herp2, Hrd) was upregulated significantly in the honey bee digestive tract after 24 h of tunicamycin treatment relative to
bees consuming sugar syrup alone (Fig. 3A–C). Importantly, induction of a cytoplasmic chaperone (Hsc70-4) was not induced by tunicamycin (Fig. 3D) and the level of the reference gene (β-actin) was not altered by treatment (Fig. S3A). We observed similar levels of induction of these genes with 24 μM tunicamycin (data not shown). In addition, there was no apparent induction of these target genes at 4 or 6 h post tunicamycin treatment at either dose (data not shown). Finally, induction of these target genes was still observed if 12 μM tunicamycin treatment was continued to 48 h (data not shown).

3.4. Xbp1 mRNA splicing occurs in response to tunicamycin treatment

We next examined IRE1 pathway activation. Xbp1 mRNA splicing is a key step in the IRE1 pathway that is often monitored for evidence of pathway activation. Using the non-canonical intron structures predicted for the honey bee Xbp1 mRNA (Hooks and Griffiths-Jones, 2011; vanEngelsdorp et al., 2009) to design primers spanning the predicted intron, we were able to differentiate unspliced and spliced Xbp1 mRNA via PCR (Hooks and Griffiths-Jones, 2011; Samali et al., 2010). Honey bees fed only sugar water had a dominant PCR product ~150 bp at 24 h, while bees fed 12 μM tunicamycin for 24 h, had two abundant PCR products at ~150 and ~120 bp, representing unspliced and spliced Xbp1 mRNA species, respectively (Fig. 4A, band identities confirmed by purification and sequencing, sequences presented in Fig. S1B). We observed similar levels of splicing with 24 μM tunicamycin (data not shown) and found no increase in splicing in the midguts of honey bees at 4 and 6 h after tunicamycin treatment at either dose (data not shown). In addition to splicing after tunicamycin treatment, total Xbp1 mRNA was upregulated significantly in the honey bee digestive tract after 24 h of tunicamycin treatment relative to bees consuming sugar syrup alone (Fig. 4B), suggesting the existence of positive feedback in the pathway.

3.5. Perturbation of cellular redox state triggers the UPR in honey bees

In addition to modulators of protein glycosylation such as tunicamycin, chemicals that affect cellular redox, the secretory pathway, ERAD function, or Ca2+ homeostatic balance also lead to unfolded proteins in the ER and are commonly used to simulate environmental stresses in other organisms. Studies in these systems indicate that chemical induction of the UPR using pharmacological agents that disrupt ER function via disparate mechanisms have different temporal kinetics and different response characteristics (Shinjo et al., 2013). We wished to extend our understanding with regards to the breadth of ER perturbations that can activate the UPR in honey bees.

Using our previously validated target genes, we characterized an additional well-known UPR inducer already characterized in invertebrates, namely the cellular reducing agent, Dithiothreitol (DTT). We found that the target genes characterized above, including chaperones (Hsc70-3, p58Ick, Gp93), disulfide bond generating enzymes (Ero1l, CaBP), and the ERAD pathway component Herp1, were upregulated significantly in the honey bee digestive tract after as little as 8 h of 10 mM DTT treatment relative to bees fed sugar syrup alone (Fig. 5A–C). Interestingly, neither the other ERAD components Derlin and Hrd were not significantly increased by DTT treatment. As above, neither the relative expression of the cytoplasmic chaperone (Hsc70-4) (Fig. 5D) nor the level of the reference gene (β-actin) was altered by treatment (Fig. S3B). Examining Xbp1 mRNA levels and splicing after DTT treatment, we observed that bees fed 10 mM DTT for 8 h, had two abundant PCR products at ~150 and ~120 bp, representing unspliced and spliced Xbp1 mRNA species, while bees fed sugar syrup alone had a single dominant band at ~150 bp (Fig. 6A). Again, total Xbp1 mRNA was upregulated significantly in the honey bee midgut tissue after UPR induction, increasing after 8 h of DTT treatment relative to bees consuming sugar syrup alone (Fig. 6B).

3.6. Honey bee survival after acute UPR induction

We wondered if bee survival would be affected by ER stress at the doses used for survival assays in other species. After treatment with 12 μM tunicamycin, 45.2% bees survive for 7 days (significant relative to untreated, p < 0.001) (Fig. 7A). After treatment with 25 mM DTT, 0% bees survive beyond 3 days (significant relative to untreated, p < 0.001) (Fig. 7B).

4. Discussion

Honey bees possess an unusual sequence at the 5′ end of the Xbp1 mRNA non-canonical intron stem-loop recognized by IRE1,
prompting us to hypothesize alterations in function of this pathway and the UPR more generally. In the absence of any genomic or functional data for the UPR in this species, conclusions about its architecture and performance were not previously possible.

Here, we show that the signal transduction machinery and many putative target genes are conserved with no apparent reduction in functional categories. Our results also demonstrate robust UPR activation in the honey bee in response to multiple known activators. In addition, we show that the IRE1-dependent branch of activation of this pathway is conserved in function despite the unique deviation of the Xbp1 splice site. As the UPR has been well characterized in the fruit fly, *D. melanogaster* (reviewed in (Ryoo and Steller, 2007)), it might be expected that such a conserved pathway would be quite similar in the honey bee. However, genomic data in...
honey bees for other pathways and processes has uncovered a trend towards apparent simplicity, with fewer homologs found for genes encoding proteins of many functional classes compared with other insects examined (Elsik et al., 2014; Evans et al., 2006). In addition, differences in UPR structure and function in distant taxonomic groups are well appreciated (Hollien, 2013). Therefore, it is likely that more closely related species within the metazoa will have unique characteristics in this and other cell stress pathways that align with evolutionary pressures befitting their specific life histories.

Although we observe a vigorous activation of a set of UPR target genes in response to ER stress mediated by protein glycosylation inhibition (tunicamycin) and reductive stress (DTT), we note evidence of species-specific differences in activation kinetics and sensitivity to alterations in ER proteostasis caused by tunicamycin and DTT. Tunicamycin reduces protein glycosylation leading to a block in proper folding that results in ER stress. In fruit flies, increasing Xbp1 splicing is detectable by 5 h after 12 μM tunicamycin treatment (Chow et al., 2013). We found no difference in splicing in the midguts of honey bees 4 and 6 h after tunicamycin treatment at the same dose when compared to untreated bees. In addition, we did not observe any target gene induction at 6 h post tunicamycin feeding, while in Drosophila a subset of gene targets are highly induced by 8 h (Chow et al., 2013) and possibly earlier. Survival results in honey bees also demonstrate an apparent relative resistance to ER stress induced by protein glycosylation inhibition compared to fruit flies. After 12 μM tunicamycin treatment, 0% of flies survive for 7 days (Chow et al., 2013; Girardot et al., 2004; Kang et al., 2012), while in honey bees this same dose results in survival of 45% after 7 days. This apparent increased resistance is in contrast to that observed after ER stress caused by reduct imbalance after DTT treatment. We observe differences in splicing in the midguts of honey bees and target gene induction at 8 h post DTT feeding, which is similar to that observed in Drosophila (Ryoo et al., 2006). In addition, after 25 mM DTT treatment, 0% of bees survive for 3 days. By contrast, in fruit flies this same dose results in survival of nearly 100% for longer than 6 days (Park et al., 2011) and evidence exists that fruit flies survive at considerably higher doses (Clark et al., 2006).

While the mechanistic basis for differences in response to environmental stresses between species is incompletely understood, a few possible explanations can be offered for these observed differences. More generally, differences in the effective rate or dose of exposure after ingestion of the inducing agent may occur between species. For example, using our own data on food consumption volume of caged honey bees (unpublished observations) and published estimates of honey bee weight (Hrassnigg and Crailsheim, 2005) as well as food consumption volume (Deshpande et al., 2014) and weight (Mattila et al., 2009) data for the fruitfly, we were able to determine that the fruitfly would likely consume approximately 2-fold more mg/kg/day of tunicamycin or DTT than the honey bee at similar concentrations. This difference, while small, could contribute to the differences between species observed here. The honey bee digestive tract is structurally quite different from that of Drosophila (Buchon et al., 2013). In addition, because they are social organisms, the control of nutrient consumption is regulated at the level of the individual and the group (Crailsheim, 1988). For example, foragers may retain nectar in their foreguts for passing to nurses for colony use. The differential sensitivity to glycoprotein stress versus redox stress on overall ER dysfunction suggests that if such broad causes are involved, they operate in a manner specific for the inducing agent employed. To account for these trigger-specific differences observed, one mechanism could involve chemical metabolism. Honey bees have fewer xenobiotic detoxifying genes, except for an expansion of a few specific categories compared to other insects (Claudianos et al., 2006; Mao et al., 2009), which could help to process specific chemical ER stress inducers with different efficiencies. Honey bees may also be specifically resistant to ER stress mediated through disruption of glycoprotein synthesis. Recent evidence suggests that the hexosamine pathway is protective for ER stress caused by inhibition of protein glycosylation (Denzel et al., 2014; Wang et al., 2014). While the genes encoding the enzymes in this pathway are apparently similar between C. elegans, D melanogaster, and A. mellifera (Snow, unpublished), it is possible that differences in carbohydrate metabolism lead to differences in sensitivity to N-glycosylation inhibition in these species. Honey bees may also be more sensitive to alterations in redox homeostasis in the ER or at the cellular level. In support of this, honey bees are more sensitive to the oxidative stress caused by paraquat relative to fruit flies (Snow, unpublished).

While we do observe Xbp1 mRNA splicing after ER stress, the unique sequence of the honey bee Xbp1 mRNA non-canonical intron stem-loop recognized by IRE1 may still lead to functional alterations in this pathway. From our data it is not possible to determine whether this sequence difference is functionally relevant. Thus, further studies will be necessary to determine whether this stem loop sequence confers unique splicing properties on the honey bee Xbp1 mRNA. Here we demonstrate activity of the IRE1 pathway by measuring Xbp1 mRNA splicing. Although temporal correlation between Xbp1 splicing and activation of target gene expression is persuasive, we do not demonstrate a requirement for the IRE1 pathway in this induction. Two additional pathways, the PERK-dependent pathway and the ATF6-dependent pathway, both contribute to the UPR in other organisms to varying degrees (reviewed in (Walter and Ron, 2011)). Other than the descriptions of the honey bee homologs herein, these two pathways are uncharacterized in the honey bee. In addition, the significance of these
three pathways in mediating the UPR in the honey bee in response to routine and atypical proteostatic stressors is unknown. Future studies to map the functional characteristics of the complete UPR pathway and determine the relative importance of all three UPR pathways in UPR target gene induction in honey bees will be important.

Nutritional stress, chemical poisoning from pesticides, changes to normal living conditions, and infection by insect parasites and pathogenic microbes are all theorized to contribute to recent honey bee losses (Oldroyd, 2007; vanEngelsdorp et al., 2009). Based on the central role that the UPR plays in response to environmental insults in other organisms (Ryoo and Steller, 2007; Samali et al., 2010; Shen et al., 2001; Walter and Ron, 2011), the UPR may serve as a key molecular link mediating the individual and combined effects of environmental stresses on recent honey bee disease. For example, microbial infection often activates the UPR as microbes use host...
resources for their own replication and manipulate cellular pathways to enhance their own lifecycle (Wang and Kaufman, 2012). In mammals, UPR activation itself often signals the possibility for infection and UPR activation may be necessary to mount a full immune response (Cláudio et al., 2013). In the invertebrate model, C. elegans, UPR activation is essential for immune activation and infection survival in multiple contexts (Bischof et al., 2008; Lamitina and Chevet, 2012; Richardson et al., 2010). As honey bees are the target of a wide range of microbial assailants from multiple taxa, many of which may impact UPR function, it would be expected that microbial infection will lead to UPR activation in honey bees.

In addition, to microbial onslaught, exposure to chemical toxins alters UPR function. Naturally occurring and synthetic biologically active compounds can impact the UPR through diverse mechanisms (Cribb et al., 2005; Kitamura, 2013; Lafleur et al., 2013). For example, known biological compounds affect N-glycosylation, cellular redox, the secretory pathway, ERAD function, or Ca^2+ homeostasis and exposure to these chemicals leads to unfolded proteins in the ER, stimulating the UPR. As honey bees visit flowers from diverse plants, they come in contact with a wide variety of agricultural chemicals, including insecticides, herbicides, and fungicides. In addition, modern honey bee management has made use of an array of chemical pesticides designed to limit the pests of this beneficial insect (Johnson et al., 2013, 2010). While these chemicals have been tested for acute toxicity to honey bees, evidence suggests that chronic exposure, alone or in combination, can lead to alterations in cellular processes, tissue dysfunction, and changes in honey bee physiology and behavior. A recent study of effects of a sub-lethal dose of imidacloprid exposure on honey bee larva found that transcripts encoding proteins from the KEGG category that includes most of the transcriptional targets of the UPR were one of the most highly down-regulated groups (Derecka et al., 2013). Fumagillin is used extensively in honey bee hives to control the microsporidian parasite Nosema ceranae and its close relative, Nosema apis. Fumagillin has recently been found to have detrimental effects on honey bee midgut function at doses regularly used in honey bee management (Huang et al., 2013). Fumagillin acts as a potent inhibitor of METAP2, a regulator of EIF2-dependent translation and a target of the proteostasis network (Chatterjee et al., 1998). Further research to determine the role of the UPR and the proteostasis network more generally in responding to the environmental stresses described above will be critical for understanding the diseases of honey bees.

5. Conclusion

These results described here demonstrate robust UPR activation in the honey bee in response to multiple known triggers, establish a time course of activation, and provide a critical primary set of tools for measuring general activation of the UPR. In addition, we have developed an assay to measure specific activation of the IRE1-dependent pathway and demonstrate activation of this pathway in response to pharmacologically-mediated ER dysfunction. By providing foundational knowledge about the proteostasis network in the honey bee, this work stands to advance our understanding of cellular responses to stress in this critical species. Such knowledge will also further our understanding of proteostasis more broadly, by providing information on an additional species for comparison with existing models and contributing to our understanding of species-specific differences in this process.

Author contributions

BAJ and JWS conceived and designed the experiments. BAJ, MM and JWS performed honey bee experiments and analyzed the data. KBH contributed tools and performed analysis of sequence data. All authors contributed to the drafting and revision of the article.
Annex A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2015.12.004.

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