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HONEY BEE STOCK GENOTYPES DO NOT AFFECT THE LEVEL OF PHYSIOLOGICAL RESPONSES TO CHALKBROOD FUNGUS, *ASCOSPHAERA APIS*

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ABSTRACT

Breeding honey bees *(Apis mellifera)* for physiological resistance to diseases is a highly desirable and environmentally safe approach to increasing colony survival. Selection of desirable traits is a critical element of any breeding program. In this study we investigate whether honey bee stocks differ in the level of physiological resistance to chalkbrood disease caused by *Ascosphaera apis*, one of the major fungal pathogens of the honey bee. The level of brood resistance to systemic mycoses caused by the fungus was determined by the survival of larvae in *in vitro* bioassays.

Our results showed that there was no statistical difference in individual resistance to chalkbrood disease among the three honey bee stocks (Russian, Carniolan, VSH) tested in this study. Furthermore, activation of classical immunity and cellular stress responses did not influence the fate of infected individuals, indicating that activation of the individual physiological responses may not be the primary mechanism of protection against this fungal disease. Rather, behavioral responses by adult bees, as reported previously, are likely the primary mechanism providing resistance at the colony level.

Keywords: Honey bee, *Ascosphaera apis*, fungal pathogen, innate immunity, resistance.

INTRODUCTION

Honey bee (*Apis mellifera*) diseases affect not only the health of individual bees but also the strength and survival of the entire colony. Due in part to extreme polyandry, a complex genetic structure of bee colonies provides intra- and inter-colony variations in resistance to pathogens and parasites. The interaction of diverse genotypes may determine the rate of infection, disease progression and overall probability of colony survival. Genetic variations in honey bee populations have been historically exploited in selecting bees for resistance to virulent pathogens and parasites [Bamrick, 1964; Spivak, Reuter, 2001; Tarpy, 2002; Palmer, Oldroyd, 2003; Tarpy, Seeley, 2006; Evison et al., 2013]. Selected bee stocks (e.g., Russian, VSH, and Minnesota hygienic) have increased resistance to *Varroa* mites (*Varroa destructor*), a result of intensive artificial selection. Genetic variability in resistance to infectious diseases, such as American foulbrood (*Paenibacillus larvae*) and chalkbrood (*Ascosphaera apis*) is also reported [Bamrick, 1964; Spivak, Reuter, 2001; Invernizzi et al., 2009; Evison et al., 2013]. Researchers have long agreed that key mechanisms underlying honey bee survival at the colony level are mostly controlled by behavioral and social adaptations [Rothenbuhler, 1964; Gilliam et al., 1988; McKean et al., 2008; Arechavaleta-Velasco et al., 2012]. In an evolutionary context, honey bees acquired special adaptations to protect colonies against infectious diseases, likely using social immunity in place of energetically costly, individual immune responses [Armitage et al., 2003; Cremer et al., 2007; Swanson et al., 2009]. Some of these social adaptations are pathogen specific. For example, Simone-Finstrom and Spivak [Simone-Finstrom, Spivak, 2012] showed that honey bee colonies increased foraging of antimicrobial plant resin in response to *A. apis* infection, but not in response to any other entomopathogenic fungi or bacteria tested in the study. It has also been shown that genetic diversity of the honey bee colony correlates with disease resistance [Tarpy, 2002; Evans, Pettis, 2005]; i.e., the level of

colony hyperpolyandry directly correlates with the ability to withstand chalkbrood disease at the colony level.

Mechanisms mediating survival of honey bees at the individual level are less understood. Diverse mechanisms, including immune (both humoral and cellular) and stress responses are activated in responses to microbial pathogens in social insects [Evans et al., 2006; Even et al., 2012]. Significantly, these responses are highly specific to the interacting host-parasite genotypes [Gregorc, Bowen, 1999; Gregorc, Bowen, 2000; Corona, Robinson, 2006; Barribeau, Schmid-Hempel, 2013]. In addition to humoral factors, mostly regulated by the Toll and JNK signaling [Agaisse, Perrimon, 2004; Tanji et al., 2007; Salih, Brunet, 2008], oxidative stress associated with chronic fungal infection may activate heat shock responses (HSR) and unfolded protein responses (UPR) that prevent aggregation of damaged and misfolded proteins, and promote cell survival and recovery [Lamech, Haynes, 2015].

Improvement of the management of agricultural animals depends on development of disease-resistant breeds. However, new breeds need to maintain a high level of fitness, which is a very challenging task considering the potential trade-off between constitutively activated defenses and other basic physiological functions. Selection to boost resistance to diseases may increase demand on metabolic energy and potentially drain resources normally allocated to other physiological functions [Dubovskiy et al., 2013]. For example, artificial selection for allelic differences in master regulator genes (e.g., NF-kβ) can produce unpredictable pleotropic effects. Genetic homogeneity in immune function as a result of such selection may be disadvantageous in highly diverse abiotic and biotic environments [Lazzaro, Little, 2009].

Whether activation of immune defenses in social insects may negatively affect the physiology of behavior is still controversial. Early on, Mallon et al. [Mallon et al., 2003] and Alghamdi et al. [Alghamdi et al., 2008] showed that activation of innate immune responses impaired learning and ability to locate food in the honey bee. On the other hand, Harpur and colleagues [Harpur et al., 2014] concluded that there was no genetic tradeoff between individual immunity and social immune defenses in the honey bee, and no significant genetic correlation between hygienic behavior and average antimicrobial activity in the hemolymph of workers.

The objectives of this study were two-fold, to: 1) investigate whether honey bee stocks differ in their level of physiological resistance to chalkbrood, determined by survival analysis of larvae in *in vitro* bioassays; and 2) investigate blood antimicrobial activity by measuring zone of inhibition (ZOI) and expression of immune blood proteins in challenged vs. naïve bee larvae (SDS_PAGE, GeLC-MS/MS).

Evaluation of existing honey bee stocks for resistance to pathogens provides the basis for selection of improved disease-resistant honey bees. If no significant differences are found in physiological responses at the level of the individual insect, future selection for disease resistance should focus on colony-level mechanisms. In the absence of a negative correlation between social immunity (hygienic behavior) and humoral immunity in the honey bee [Harpur et al., 2014], we hypothesized that selection for behavioral hypersensitivity will not have a strong effect on the level of physiological responses to diseases in stocks of honey bees.

EXPERIMENTAL SECTION

Bee resources

Honey bee colonies were established in early spring of 2014 and maintained in USDA ARS Honey Bee Breeding, Genetics & Physiology Laboratory (Baton Rouge, LA) apiaries, as described in [Aronstein et al., 2016]. Briefly, three stocks (three colonies per stock) of bees were tested: Russian (selected for *Varroa* mite resistance in a closed population of honey bees sourced from Primorsky Krai, Far-East Russia), honey bees with the *Varroa* Sensitive Hygiene (VSH) trait (outcrossed to *A. m. ligustica*) and a commercial source of Carniolan (*A. m. carnica*) honey bees. Multiple colonies per stock were established to provide sufficient numbers of appropriate aged larvae at the time of experiments. Colonies were managed using routine beekeeping practices except that in-hive chemicals were not used. Colonies were examined for presence of diseases and parasites on a regular basis.

Fungal isolates and culture

A local *A. apis* strain (BBR) was purified in culture [Anderson, Gibson, 1998; Holloway et al., 2012; Aronstein et al., 2016] and confirmed by PCR [Murray et al., 2005]. Sequenced *A. apis* strains (ARSEF 7405 and 7406) were used as controls in identification [Qin et al., 2006]. Isolates were grown on YGPSA solid culture medium [Anderson, Gibson, 1998] with 100 µg/ml Ampicillin sodium salt and 6 µg/ml Streptomycin sulfate antibiotics and incubated at 33 ºC for 7 d, then at room temperature for 3 d. Ascospores were harvested from culture plates and stored at -20 ºC. A subset of spores was heat-treated at 70 ºC for 2 h. Viability of heat-treated spores was tested in culture by plating ~10^4 spores onto YGPSA culture medium and incubating at 33 ºC for 7-10 d to ensure no visible growth on culture plates. Heat-treated spores were stored at -20 ºC. Prior to larval inoculations, spores were tested for viability following the same protocol.

In vitro larval bioassay

All tests were conducted *in vitro* using the larval bioassay [Aronstein, Murray, 2010]. We collected

3-day-old larvae weighing between 18 - 38 mg from colonies that had the appropriate aged larvae at the time of the bioassay and placed them into 6-well cell culture plates lined with a circle of fine mesh fabric to keep larvae from sticking to the plate (5 larvae per well). Larvae from different colonies (n=1-3 per stock) were mixed among treatments. There were three treatment groups: larvae were initially fed 150 µl/well [Vandenberg, Shimanuki, 1987]: 1) larval diet only, 2) larval diet inoculated with heat-treated, non-viable *A. apis* spores (10^4 spores/larva), or 3) larval diet inoculated with viable *A. apis* spores (10^4 spores/larva), similar to LD_{50} for *A.apis* strains E and F used by Evison et al (2015). After 48 h post inoculation (hpi), larvae were moved to clean 6-well plates and fed as needed. Plates were incubated at 33 ºC and 97% RH for the duration of the experiment. Preliminary tests indicated that an initial dose of 10.000 spores/larva produced consistent infection with typical chalkbrood disease pathology [Aronstein, Murray, 2010] and larval mortality over 7 d [Aronstein et al., 2016]. Four replicate assays were conducted between May and September 2014. Each treatment consisted of 90 larvae from each stock, for a total of 270 larvae per treatment (N = 810 larvae total/assay).

Larval mortality test

Larval mortality, time to death and visible appearance of clinical signs of the disease were assessed for all treatment groups in the four independent assays. Mortality was recorded at 24 h intervals; dead larvae not showing clinical signs of the disease were incubated at 33 ºC on moistened blotting paper in petri dishes for 3-4 days to determine cause of death. Fungal growth indicated that larvae consumed fungal spores and potentially died due to fungal exposure. Larvae were rinsed in 1.5 ml microcentrifuge tubes containing 500 ul ice cold 0.9% saline, blotted on sterile paper towels, placed in new 1.5 ml tubes and stored at -20 ºC until further analysis. Larvae showing fungal growth at the time of assessment were scored as susceptible. The experiment was terminated 24 h after > 50% of the larvae pupated, 144 - 168 hpi. Remaining larvae and pupae from all treatment groups were scored as resistant and collected as above.

Statistical analyses were done using SAS/STAT® software 9.4 for Windows (SAS Institute Inc. Cary, NC, USA). All statistical significant differences inferred in this study were determined at the 5% confidence level. Data analysis of time of death and diet treatment on larval survival within the stocks was generated using SAS PROC LIFETEST, a non-parametric maximum likelihood estimate of survival using the Kaplan-Meier estimate. The Wilcoxon Test was used for multiple comparisons of tests of homogeneity of survival curves for time of death over treatment strata. Larvae that survived to the final observation were considered censored data. SAS PROC GLIMMIX was used to perform analysis of variance (ANOVA) to look at stock differences in larval mortality over time in susceptible individuals within the viable spore diet treatment and on survivorship of individuals, pooled across stocks, at specific times between diet treatments.

Larval hemolymph collection

Bioassays for collection of larval hemolymph were as described above (*In vitro larval bioassay*) using the same sources and stocks of bees, age of larvae, inoculation and feeding procedures. Sample sizes were reduced to 30 larvae from each stock per treatment. In addition, we subdivided larvae within the treatments into cohorts to be bled at 24 hpi and 48 hpi. Blood samples were collected by pooling blood from the five larvae in each well. Individual larvae were rinsed in phosphate buffered saline (PBS, pH 7.4) (Sigma-Aldrich, USA) and blotted on 2-mm thick blotting paper. Hemolymph was collected using a 1-ml syringe with a 26½ gauge needle (BD Bekton Dikinson, USA) by making a dorsal incision and collecting a drop of hemolymph (~ 10 µl/per larva) directly into a 1.5 ml microcentrifuge tube containing 5 µl of ice cold mixture of N-phenylthiourea (PTU) and aprotinin (Sigma Aldrich, USA) in PBS, pH 7.4 at a concentration of 0.1 µg/ml each [Randolt et al., 2008]. Samples were divided post collection for future analyses and stored at -20°C.

Zone of inhibition

Hemolymph samples collected from the above assays were used to visualize inhibitory properties of hemolymph against germinating *A. apis* spores and subsequent filamentous growth. In preparation for establishing test plates, *A. apis* spores were incubated overnight in YGPSA liquid medium [Anderson, Gibson, 1998] at 33 °C 125 rpm using Innova 40 Incubator Shaker (New Brunswick Scientific). An aliquot of *A. apis* spores (10^4 spores/25 µl) was spread onto YGPSA plates (60 x 15 mm petri dishes) using disposal cell spreaders and allowed to soak into the surface for 30 min. Supernatant from the undiluted hemolymph samples were applied to sterile paper discs (BD BBL™ blank paper discs, 6 mm) at 25 µl/disc. Discs were placed in the center of each plate; plates were incubated overnight at 33 °C then at room temperature for the duration of the test. Amphotericin B (AMB) (Sigma-Aldrich, USA), a fungicide, was used as a standard for inhibition at a dose of 6.25 µg/disc. Control plates containing paper discs with 25 µl of sterile water per disc were incubated the same way as treated-disc plates to determine time until first appearance of filamentous growth (n=3). A subset of plates was incubated at 33 °C to verify spore viability (n=3). All plates were viewed under a dissecting microscope at 20x magnification.

Plates were observed for *A. apis* growth at 24 h intervals and indications of inhibition by hemolymph samples were documented by measuring zone of inhibition. SAS PROC GLIMMIX was used to perform analysis of variance (ANOVA) to evaluate potential stock and bioassay-treatment group differences, as well as-, comparisons of time to fungal overgrowth of hemolymph soaked discs for the 24- and 48-hpi larval cohorts.

Hemolymph protein analysis using SDS-PAGE and GeLC-MS/MS

Total protein concentration in larval hemolymph was determined using the Quick Start Bradford Protein Assay Kit (BioRad, USA). A standard curve was developed using Quick Start Bovine Serum Albumin (BSA) Standard Set (BioRad, USA) with three replicates for each concentration (0.125-2.0 mg/ml) and included with every 96-well microplate of samples. Samples were diluted using PTU buffer (1:50 in 1X PBS) with an additional 1:100 concentration of Protease Inhibitor Cocktail (Sigma-Aldrich, USA) to limit proteolytic degradation during sample processing. Absorbency was measured at 595 nm in 96-well microplates using a BioTek ELX800 Microplate Reader (BioTek, Winooski, VT).

SDS-PAGE analysis was used to separate proteins based on size. Samples were prepared at a concentration of ~45 µg of total protein in 20 µl volumes per well using sterile MilliQ water and 10X reducing agent (Novex ®, NuPAGE). Samples were then incubated in a BioRad T100 Thermal Cycler for 2 min at 85 ºC and loaded into wells of Novex 10-20% Tricine precast 1-mm, 12- well protein gels (Novex ®, NuPAGE) to focus mostly on resolving low molecular weight proteins and peptides. Precision Plus Protein™ Dual Xtra Standard (BioRad, USA) was used for sizing proteins in the molecular weight range of 2-250 kDa. SDS-PAGE electrophoresis was performed at 150V constant in an XCell Sure Lock apparatus and run in a 1X Tricine SDS running buffer (Novex ®, NuPAGE). Gels were stained with SimplyBlue Safe Stain (Invitrogen, Cat. #LC6060) following distaining in sterile MilliQ water and images were recorded using Gel Doc™ XR+ Molecular Imaging System (Bio Rad, USA) and BioRad Image Lab software (v 4.1). Unique protein bands were excised from SDS-PAGE gels and analyzed by GeLC-MS/MS at Bioproximity, LLC (Chantilly, VA).

Bioinformatics

Peptide identification following GeLC-MS/MS was conducted using the OMSSA and X!Tandem search algorithms against the UniProt KB protein library. Functional groups were assigned using the UniProt KB database http://www.uniprot.org/ and NCBI tblastn and blastp searches with a cut off E-value in the range off (5e-7) to (2e-5) depending on length of alignments and % identity. After removing contaminating proteins (human keratins, trypsin and MRJPs), data were sorted by treatment groups (non-viable spores vs. viable spores).

RESULTS

Larval mortality test

There were no significant differences detected between stocks in mortality rates or numbers of chalkbrood-susceptible larvae (F=0.10; df=2, 6; P=0.90 and F=1.92; df=2, 6; P=0.23, respectively). There were no significant differences between stocks within each treatment (Table 1).

Table 1.

Testing homogeneity of survival curves for time of death over strata: pairwise comparisons of stock differences by treatment with larval diet, non-viable and viable *Ascosphaera apis* spores; Tukey-Kramer adjustment for multiple comparisons

Treatment		Chi-Square	p-Values
Larval Diet			
Carniolan	Russian	1.50	0.44
Carniolan	VSH	1.28	0.50
Russian	VSH	0.01	0.99
Non-viable Spores			
Carniolan	Russian	2.96	0.20
Carniolan	VSH	1.08	0.55
Russian	VSH	0.47	0.77
Viable Spores			
Carniolan	Russian	0.23	0.88
Carniolan	VSH	0.17	0.91
Russian	VSH	0.78	0.65

Relative to the data that we have, there was no evidence to treat stocks differently; therefore, we pooled data across stocks to compare treatment differences, giving us greater precision for comparing treatment groups. Analysis of pooled data showed that all treatments were significantly different (Table 2).

Table 2.

Testing homogeneity of survival curves for time of death over strata: pairwise comparisons of differences among treatments with larval diet, non-viable and viable *Ascosphaera apis* spores using data pooled across stocks; Tukey-Kramer adjustment for multiple comparisons

Treatments		Chi-Square	p-Values
Non-viable Spores	Larval Diet	25.9575	<.0001
Non-viable Spores	Viable Spores	557.8	<.0001

Larvae that were fed non-viable spores died at a significantly higher rate than those fed larval diet only (Figure 1). The instantaneous probability of death at any given time was similar (i.e., + 5%) for all treatments until about 96 hpi when the risk of dying increased significantly for larvae that were fed viable spores (Figure 1). At 96 hpi, differences were only due to treatment effect and not stock of bees (F=27.47; df=2, 19.36; P<0.0001) (Table 3).

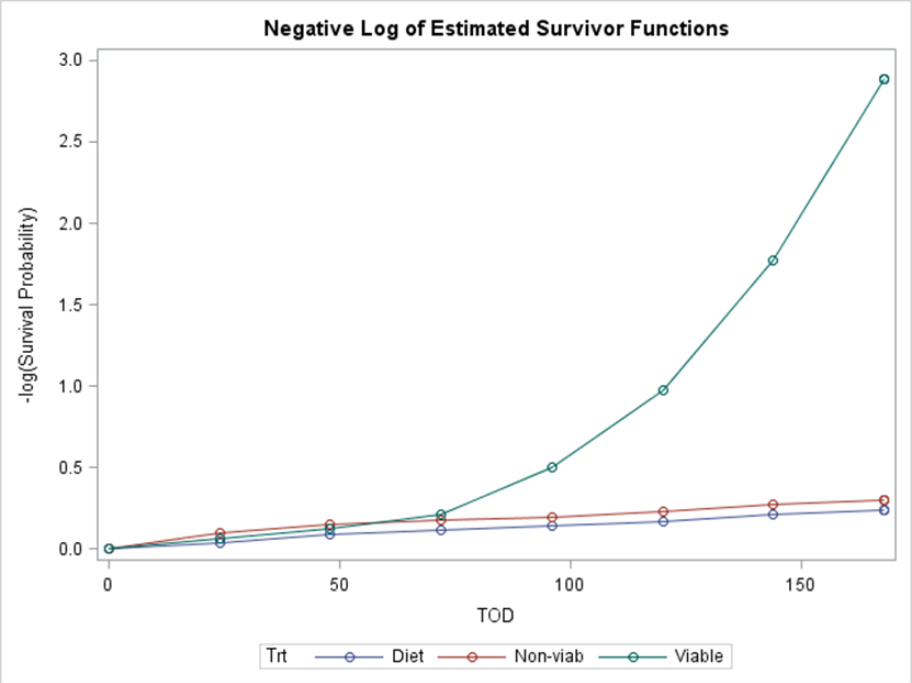

Figure 1. Instantaneous probability of larval death at 24 h intervals. Data were pooled across stocks and treatments. All treatments were significantly different. The difference between diet and non-viable spore treatments was not large but it was statistically significant because of the precision gained by pooling across stocks to compare treatment differences. At 96 hours post inoculation, mortality was significantly greater for larvae fed viable *Ascosphaera apis* spores than for larval diet with non-viable spores or larval diet only.

Table 3.

Survival comparisons for treatment by stock: T-grouping for treatment and stock least square means (LSM) frequencies of larval survival at 96 h PI. LSM with the same letter are not significantly different

Treatment	Estimate	
Larval Diet	86	A
Non-viable *A. apis* spores	80	A
Viable *A. apis* spores	60	B
Breed	Estimate	
VSH	76	A
Carni	76	A
Russian	75	A

There was no stock x diet treatment interaction (F=0.31; df=4, 18.84; P=0.87). Overall, 81% of larvae that were fed larval diet without inoculum survived to the final observation compared to 17% survival of larvae inoculated with viable spores (Table 4).

Table 4.

LSM frequencies of honey bee survival following treatments with larval diet, non-viable and viable *Ascosphaera apis* spores at 24 h intervals. Data pooled across stocks

Treatments	Time					
	24 h	48 h	72 h	96 h	120 h	144 h
Larval Diet	96	90	90	86	85	81
Non-viable Spores	91	86	85	80	77	75
Viable Spores	94	88	80	60	38	17

Zone of inhibition

There was a 24-48 h delay in first appearance of *A. apis* filamentous growth regardless of bioassay-treatment, compared to control plates. Colonies tended to appear and grow from the edges of plates with hemolymph and AMB soaked discs even though spores were spread over the entire surface of the medium. This was compared to a random appearance and evenly distributed growth observed on control plates. Often times a flattening of colony growth was observed as it approached a 3-4 mm zone around the disc where hemolymph or AMB leached into the medium. There was no statistical difference in fungal growth by bioassay-treatment group, stock or interaction of the two effects (F=1.20; df=3, 67.07; P=0. 32, F=2.89; df=2, 6.05; P=0.13 and F=0.55; df=4, 65.18; P=0.70, respectively). Also, there was no difference in time to fungal overgrowth of discs observed among bioassay-treatment groups or the interaction of time by bioassay-treatment (F=2.53; df=1, 65.18; P=0.12 and F=0.37; df=2, 65.18; P=0.69, respectively).

Hemolymph protein analysis and bioinformatics

SDS-PAGE analysis of blood protein samples collected at 24 and 48 hpi produced mostly uniform profiles both across honey bee stocks and treatment groups (Figure 2). Protein bands that differed in size or intensity were excised from the gel and analyzed using GeLC-MS/MS. Our preliminary analysis of hemolymph proteins expressed at 24 hpi showed significant representation of defense and stress-response proteins along with molecules involved in metabolic and neuronal activity suggesting a rapid response to changing biotic and abiotic conditions (Table 5). Reporting abundance of detected proteins was not possible since only selected bands and not entire lanes of proteins were analyzed by LC/MS.

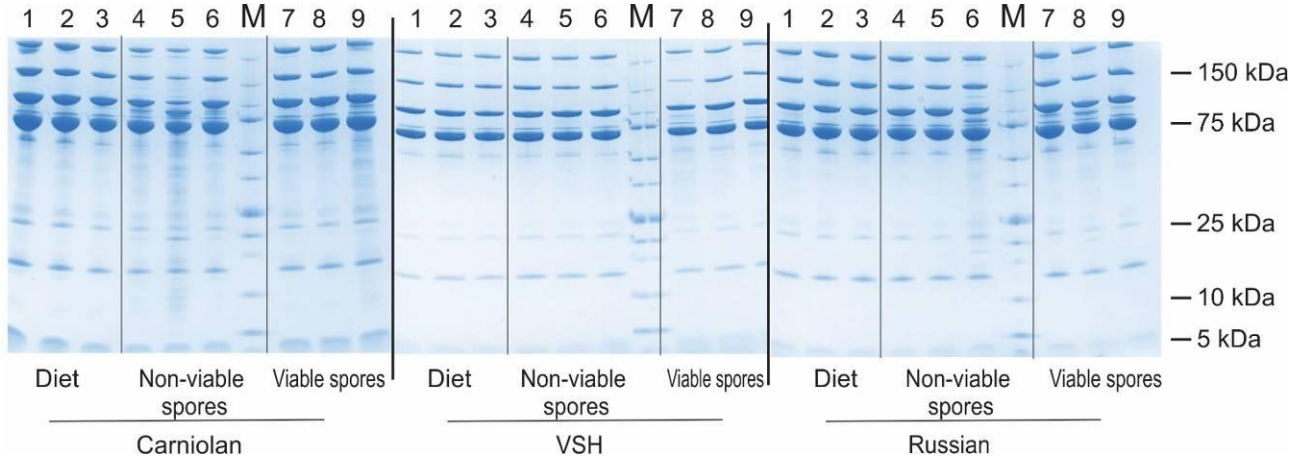

Figure 2. SDS-PAGE gel electrophoresis of total blood proteins collected from Russian, VSH and Carniolan honey bees 24 h post inoculation (PI) with non-viable or viable *Ascosphaera apis* spores or larval diet only.

Following GeLC-MS/MS analysis, 2.580 peptides were identified which associated to 1.047 proteins. Out of these, 269 proteins were identified as unique to hemolymph from larvae in a viable spore treatment group (Table_S1). We assigned these proteins to functional categories, such as immune and anti-fungal responses, stress responses, detoxification and apoptosis, neuronal activity, transcriptional and translational regulation (Table 5).

Table 5.

Functional groups of selected proteins identified in larval hemolymph samples collected 24 hours post inoculation with viable or non-viable *Ascosphaera apis* spores using liquid chromatography-mass spectrometry, GeLC-MS/MS

Functional groups/ UniProtKB Accession numbers	Protein Annotation	Non-viable spores	Viable spores
Immune and anti-fungal responses			
Q5J8R1	Defensin 1, Def-1	+	+
A0A088A2P7	methyltransferase-associated, DMAP1		+
A0A087ZTA5	Niemann-Pick disease type C2, NPC2	+	+
A0A088A045	Niemann-Pick disease type C2, NPC2 homologue	+	
A0A088AD69	NPC1*		+
A0A088AP33	colon cancer-associated protein, Mic1-like	+	+
A0A088AJM6	peptidoglycan recognition proteins, PGRP-S3-like	+	+
C7AHQ7	peptidoglycan recognition protein, PGRP-SA	+	+
A0A087ZUI4	peptidoglycan recognition protein, PGRP-LC	+	+
R4U544	Gram negative binding protein, GNBP-1	+	+
A0A088AL19	subtilisin-like serine protease, SP_S8		+
A0A088A984	Peptidase S1, SP_44	+	
Q86PH6	Transferrin, trf		+
A0A088AIY8	SIRTUIN6, Sirt6	+	+
A0A088A988	C-type lectin, Ctl 4		+
A0A088AAS7	chitinase-like, Idgf4-like		+
A0A087ZTX7	chitinase 3 -like	+	
A0A088A256	chitin deacetylase-like 4, Cda4	+	
A0A088AEY3	peptidase F1 family, Trypsin-SP		+
A0A088AUF9	trichohyalin-like		+
A0A088AF77	Ascaris inhibitor, Cathepsin G, chymotrypsin inhibitor	+	+
A0A088A8M7	LRR_DEATH domain containing protein	+	
A0A088AP33	colon cancer-associated protein Mic1-like	+	+
A0A087ZVC8	inhibitor for the elongation factor EF1A,tctp	+	+
Q5J8R1	vesicular glutamate transporter 3-like isoform X1	+	
H9KQJ7	Omega-conotoxin-like protein 1, OCLP1	+	+
A0A088A6Z2	Lysozyme 1, Lys-3	+	
A0A087ZTL6	PF11_0213-like	+	+
A0A087ZPT7	reticulocyte-binding protein 2 homolog a-like		+
A0A088AP33	WD40 containing protein, C18orf8-like		+
A0A088A0S4	Haem_peroxidase_animal, isoform 2; secreated,Pxn		+
Stress response, detoxification, apoptosis			
A0A087ZTX4	cytochrome P450, Cyt P450	+	+
E5RQ33	cytochrome P450, 315A1, Sad	+	
Q309A5	cytochrome P450, CYP6AS5	+	
A0A088ABV3	GST_C_like		+
A0A087ZVS9	GSTT1	+	
C1JYH6	heat shock protein,Hsp-90	+	+
A0A088AGJ8	heat shock protein, Hsp-70	+	+
A0A088ACF4	heat shock protein,Hsc70-3		+
A0A087ZQ27	heat shock protein Hsc70-4,		+
A0A087ZTY7	heat shock protein,Hsc70-5		+
A0A087ZND2	thioredoxin peroxidases, Tpx1	+	+
A0A088AD50	NADH-cytochrome b5 reductase		+
A0A087ZN39	Tryp_SP 53-like	+	+
A0A087ZSP7	Auxilin, Hsc70 co-chaperon		+

A0A088A462	Lon protease homolog, mitochondrial	+	+
A0A088ALI6	apoptosis 1 inhibitor-like		+
A0A088AUM2	programmed cell death protein 6		+
A0A087ZW99	cleft lip and palate transmembrane protein -like*	+	+
A0A088ASD3	Golgi organisation protein 11, tango11	+	
A0A088ACU2	FK506-binding protein 5	+	
A0A088AQG5	Insect allergen-related, IPR010629	+	
A0A087ZXS2	Insect allergen related, LOC727522	+	
A0A087ZXS3	Insect allergen related, LOC727344	+	
A0A088AFB3	Disintegrin-like Metalloproteinase	+	
A0A087ZVQ1	serine/threonine-protein kinase, Tao-1	+	
A0A087ZUH1	Calpain-like, Ca sensitive peptidase, CalpC	+	
A0A088A2W1	vanin-like protein 1-like, hydrolase		+
A0A088A8N0	trehalose transporter Tret1-like isoform X1		+
Neuronal activity, olfaction			
A0A087ZW85	allatostatin-cc-like, Ast-CC		+
A0A088AAX0	Manf, Neurotrophic factor		+
C0K3M9	Synaptotagmin I isoform A, sitI		+
A0A087ZTP7	GDNF/GAS1 domain protein		+
A0A088AS83	Sli, similar to slit		+
Q1W640	odorant binding protein, OBP14, PBP_GOBP_dom	+	+
A0A088A4K9	odorant binding protein, Obp14, PBP_GOBP_dom	+	+
A0A088A647	Ser/Thr protein kinase, S6kII	+	
B9VMQ7	Neuroligin 5	+	
Q8I6X7	Antennal-specific protein, Asp 3c	+	
Q6TLC7	GABA Transporter1B,GAT-1B		+
A0A087ZV49	Voltage-gated sodium Chanel, Para		+
Signal transduction			
A0A088A7M1	Small GTPase		+
A0A087ZN49	Small GTPase, RabX6		+
Exocytosis			
A0A088A9P5	syntaxin-12, Six7,		+
A0A088A105	syntaxin-18, Syx18*	+	
Transcriptional and translational regulation			
C9E0T0	Ago3, Argonaute Ast2 variant, PIWI sub- family	+	+
A0A087ZTK3	craniofacial development protein 1, BCNT-C domain, Yeti	+	
A0A087ZQZ5	Histone-lysine N-methyltransferase		+

* Genes encoding NPC1 lipid storage disorder protein, cleft lip and palate transmembrane protein and syntaxin-18 proteins (XP_006570445.1; XM_006570317.1; XP_006566739) were also found within the QTL (Amel 4.5 LG11) associated with chalkbrood resistance (Holloway et. al., 2012).

A number of key multifunctional proteins were detected in all stocks and treatment groups of bees, including Hex 110, Hex 70a, Hex 70b, Hex70c, ApoLp-III Vhdl and Vg (A7XZB1, A5YVK7, Q6J4Q1, A6YLP8, B0LUE8, A0A088AGQ0, A0A088ADL8), normally abundantly expressed in the hemolymph of worker larvae [Chan et al., 2006]. Other proteins involved in energy storage, metabolism and transport were identified in larval hemolymph, including Oscillin /A0A087ZPX4, Arginine kinase, ARGK /O61367, and trehalose transport, Tret1/A0A088A8N0.

Pattern recognition molecules known to activate classical NF-kβ immune signaling pathways (PGRP-S3-like /A0A088AJM6, PGRP-SA/ C7AHQ7, PGRP-LC /A0A087ZUI4, GNBP-1/ R4U544) and other immune related molecules (Def-1/Q5J8R1, RelA repressor Sirt6/ A0A088AIY8, NPC2/ A0A087ZTA5) were detected (Table 5). Some proteins were expressed only in the hemolymph of larvae inoculated with viable fungal spores, most notably transferrin (trf, Q86PH6), C-type lectin (Ctl 4, A0A088A988), subtilisin-like serine protease (SP_S8, A0A088AL19), inhibitor of growth protein 3 (ing3, A0A088A4I9), chitinase-like (Idgf4, A0A088AAS7) and IMD pathway modulator DMAP1(A0A088A2P7) (Table _S1).

A number of proteins identified in this study

have been previously described as disease bio-markers; some of them are known to be involved in oxidative stress responses: NCP2/A0A087ZTA5, PF11_0213-like/A0A087ZTL6, Tango11/A0A088ASD3, insect allergen-related proteins (A0A088AQG5, A0A087ZXS2, A0A087ZXS3), colon cancer-associated Mic1-like/ A0A088AP33, reticulocyte-binding protein-2/A0A087ZPT7 [Sahar et al., 2011; Korir et al., 2014], Cytochrome P450 (A0A087ZTX4) were identified in non-viable and viable spore treatment groups. Two other cytochromes (Cyt 315A1/ E5RQ33 and CYP6AS5/Q309A5) were found in the non-viable spore treatment only. The oxidant scavenging Thioredoxin peroxidase (Tpx1, A0A087ZND2) was found in bees exposed to both, active and inactivated fungal spores. Several heat shock proteins such as Hsc 70-3, HSC70-4, HSC-70-5, Hsp-90, Hsp-70 (A0A088ACF4, A0A087ZQ27, A0A087ZTY7, C1JYH6, A0A088AGJ8) and Glutathione-S-Transferases (GST_C and GstT1, A0A087ZVS9) were detected in both or one of the spore-treatment groups (Table 5).

The activation of HSPs interacting proteins (AmsGC beta-3/ Q5FAN0; chaperonin/A0A088ART2, FK506-binding protein 5) and other cellular stress biomarkers (A0A088A9C6; A0A088A8D4; Ndufb6/A0A088ATD6; A0A088ACU2) indicate activation of cellular stress responses to challenge by both non-viable and viable fungal spores [Gregorc, Bowen, 1999].

Similar to vertebrate stress responses, we found activation of honey bee neuro-hormonal signaling in response to fungal exposure. Proteins involved in neuronal activity, neurotransmission and olfaction were represented by odorant binding proteins (OBPs) 14 (A0A088A4K9 and Q1W640) in both treatment groups. GABA transporter, GAT-1B (Q6TLC7), voltage-gated sodium channel, para (A0A087ZV49), and neurotrophic factor supporting dopaminergic neurons, Manf (A0A088AAX0) were detected in the viable spore treatment group only.

Activation of Rab family Small GTPases (A0A087ZN49 and A0A088A7M1) and Rab GTPase activator (RabGAP9/A0A087ZZI8) in response to fungal challenge is consistent with their function in mounting cellular responses to intestinal infections in other eukaryotes [Nakano et al., 2001].

A number of contaminating proteins were also identified by GeLC-MS/MS, including MRJP1/ O18330, MRJP 2/ O77061, MRGP7/Q6IMJ9), possibly as contamination from larval diet or as water-soluble products of food digestion. In addition, several human keratins (KRT 1/P04264; KRT9/P35527, KRT 15/O77727) frequently found on the SDS-PAGE gels, were also detected.

DISCUSSION

Survival analysis of larval resistance to chalkbrood disease (as a function of differential physiological mechanisms) in three stocks of honey bees revealed no significant differences among the stocks with respect to larval mortality or time of death. However, an analysis of the combined data from all stocks found significant differences in rates of survival among all three treatment groups and at key times in *A. apis* pathogenesis. Inoculation with viable spores produced a significantly higher rate of mortality beginning 96 hpi. This was expected based on our previous observations; a dose of 10^4 spores produced a disease progression mimicking that observed in a natural infection. Jensen and colleagues [Jensen et al., 2009] observed similar results with 10^4 dosage of spores producing an LT_{50} at 96 hpi - 120 hpi; whereas, a 10^3 spore-dose led to greater variability and divergence from typically observed *A. apis* pathogenesis. However, a lower dose of the inoculum may be useful in future studies for teasing apart differences between individuals, patrilines or stocks.

Also, a single strain of *A. apis* was tested to challenge honey bee larvae in this study. It is possible that other strains of the fungus may produce differential responses among honey bee genotypes as has been previously reported for bumblebees infected with various strains of *Crithidian bombi* [Barribeau, Schmid-Hempel, 2013] and different patrilines of honey bees infected with several strains of chalkbrood [Evison et al., 2013]. Such interactions between host and pathogen genotypes may occur when the incidence of disease is sporadic and varying levels of pathogenicity and virulence are encountered by honey bee colonies in different localities. Immunity within colonies may then be more tailored to local selection pressures as opposed to being “globally optimized” [Sadd, Schmid-Hempel, 2009]. The contrast between our results comparing stocks of bees and those [Invernizzi et al., 2009; Evison et al., 2013] that compared patrilines within colonies is noteworthy.

Quality and concentration of total proteins in the hemolymph is critical for honey bee survival under stress conditions [Chan et al., 2006]. Honey bee hemolymph contains the most variety of hemolymph proteins in larval stages; variation in the composition of these proteins may hold the key to identification of disease biomarkers. A better understanding of the protein content of hemolymph may provide greater comprehension of the underlining molecular differences in susceptibility to diseases. We analyzed proteins in larval hemolymph samples and found storage proteins abundantly expressed in larval hemolymph across honey bee stocks and treatment groups. We also detected variation in expression of a wide variety of immune and stress related proteins in response to fungal exposure

(Table _S1), including pattern recognition molecules (e.g., PGRP-LC, PGRP-SA, GNBP-1) that activate classical NF-kβ dependent intracellular signaling pathways, Toll, IMD and effector molecules (e.g., Def1). Presence of proteases in hemolymph of infected larvae indicates activation of cascades commonly associated with host defenses, inflammation, tissue injury and wound repair (Table 5, Table_S1). In addition to classical immune related proteins, we identified molecules that are involved in other physiological functions (e.g., energy transport and catabolism, cell regeneration, repair of damaged proteins, apoptosis, epigenic gene regulation, etc.) that may provide an increased level of tolerance to individuals under stress. There is some evidence for activation of detoxification activity in response to *A. apis*. We found P450 monooxygenases expressed in response to inoculation with both viable and non-viable fungal spores, whereas Glutathione S-transferase (A0A088ABV3) was detected only in response to exposure to viable spores.

The signs of general stress responses included expression of heat shock proteins and apoptosis related molecules (Table 5). Furthermore, small Rab GTPases found in the hemolymph of infected larvae in this study were previously implicated in responses to pathogens presumably by regulating membrane trafficking and activation of endocytosis [Hultmark, 2003]), and were suggested to be useful biomarkers of oxidative stress responses [Takenaka et al., 2013]. The activation of the antioxidant proteins, GSTs family enzymes and Tpx1, thioredoxin peroxidases enzyme, described above, are all well-known responses to oxidative stress in damaged and apoptotic cells [Corona, Robinson, 2006; Gregorc, Ellis, 2011]

Based on our results, it is apparent that individual physiological responses in larvae did not provide any significant level of protection against *A. apis* infection for any of the honey bee stocks under our experimental protocols. Expression of diverse classes of proteins found in larval hemolymph related to antimicrobial defenses and cellular stress responses did not provide a level of protection that would have had an effect on the fate of infected animals, since the majority of larvae inoculated with viable fungal spores died, regardless of the stock. The spore dose tested in this study provided consistent infectivity throughout the experiment, providing a range of larval mortality during the test periods. Lesser spore inoculums tested in the preliminary study resulted in high variability in mortality data. While larval hemolymph delayed initial growth of fungal hyphae in ZOI assays, it did not have any noticeable, negative impact on growth of the mature *A. apis* mycelia. Potentially, stress related molecules other than those implicated in humoral immunity could be involved in the delayed growth of *A. apis* as part of a general response mechanism to adverse environments [Dubovskiy et al., 2013]. Even a short delay in spore germination and/or hyphal growth detected in ZOI assay may work in concert with the behavioral immunity to provide an increased level of resistance to pathogens.

The effect of non-viable spores on larval mortality was not as dramatic as that observed in the viable spore treatment, but still differed significantly from the larval diet treatment. This may be the result of a temporal avoidance behavior of contaminated food in larvae as a short term survival mechanism [Wright et al., 2010]. Based on casual observation, outside the scope of the study, larvae from the VSH stock appeared to avoid (non-viable and viable) spore-contaminated food until moved to a new dish after 48 hpi and fed with fresh larval diet, at which point they ate steadily and gained weight rapidly. This potentially indicates that honey bee larvae have the ability to detect fungal spores prior to consumption. However, avoidance of spore-contaminated food for 48 h resulted in smaller sized pupae which pupated later than those that consumed larval diet the entire time (K. Aronstein and D. Colby, personal observations). Evidence suggests that larvae and adult insects can ‘‘assess their environment based on cues related to mortality risks’’ [Meyling, Pell, 2006] including entompathogenic fungi [Villani et al., 1994]. This is particularly apparent in coevolved host-parasite relationships. In fact, there are many reports of behavioral resistance to fungal pathogens in social insects [Swanson et al., 2009], consistent with research showing that social immunity and other social adaptations are key mechanisms underlying honey bee resistance to diseases.

The most recent research indicates that individual immune responses in social insects may not play as critical a role as is postulated for solitary insects [Kraaijeveld, Wertheim, 2009; Yassine et al., 2012]. Even though AMPs are highly effective against bacteria, rapid up-regulation of AMPs in *Bombus terrestris* had no effect on the proliferation of *E.coli* in challenged bees [Erler et al., 2011]. Furthermore, the expression of most insect AMPs was not effective against entomopathogenic fungi [Glinski, Buczek, 2003] and induction of AMPs via IMD signaling in *Drosophila* during intestinal infection had a detrimental effect on the host [Berkey et al., 2009]. Importantly, rapid changes found in the expression of immune and other stress related molecules in the honey bee in response to fungal and bacterial pathogens did not provide a strong correlation with biologically-relevant levels of disease protection [Evans et al., 2006; Gätschenberger et al., 2013]. This led some researchers to hypothesize that responses to pathogens in social insects can be driven through alternative physiological pathways, other than classical immune signaling and in some cases could be under the

epigenetic control [Galbraith et al., 2015]. This underscores the fact that physiological resistance is most likely multifactorial, i.e., not entirely mediated by innate immune responses and may involve synergy of multiple processes, including stress responses, energy re-allocation, and tissue-specific adaptations, and can be profoundly affected by a variety of environmental factors [Lazzaro, Little, 2009; Dubovskiy et al., 2013; Otti et al., 2014].

Although the survival of VSH honey bees was not different from survival of other stocks, larval-mediated behavioral avoidance could be a basic survival mechanism during larval stages. Changes in larval behavior, such as avoidance and reduced feeding, if found, could affect disease susceptibility, even when the pathogen does not significantly alter individual physiological responses. Because larval behavioral plasticity and possible effects of stress on disease pathogenesis have been studied in solitary insects and vertebrate systems and are not well understood in social insects [Min, Condron, 2005], future research should address larval behavioral adaptation to microbial infections.

CONCLUSIONS

In this study we investigated potential differences in levels of resistance to the fungal pathogen, *A. apis* among honey bee stocks. All tested stocks are genetically diverse and two stocks, Russian and VSH, were selected for behavioral sensitivity to the *Varroa* parasite. Whether selection for behavioral traits would affect other physiological functions, such as innate physiological responses to chalkbrood pathogenesis, was unknown and needed investigation.

Our results provide evidence that there is no statistical difference in the level of resistance to chalkbrood disease (measured as the percentage of survival among inoculated larvae and time to death) among honey bee stocks tested in this study. Furthermore, activation of classical immunity and other physiological responses did not affect the fate of infected individual insects or differ among stocks; all infected larvae died showing clinical signs of the disease or if clinical signs were not observed at death, produced *A. apis* growth post incubation. Similarly, Evison et al (2015) [Evison et al., 2015] showed no correlation between constitutive expression of the honey bee AMP abaecin and resistance to *A.apis* fungus.

This research provides additional evidence that activation of classical immunity may not be the primary mechanism of protection against fungal diseases in honey bees. Rather, behavioral mechanisms may be more important. However, our conclusions do not dismiss the importance of physiological mechanisms in disease protection. These mechanisms could provide a basic level of protection that is evolutionarily conserved in this species.

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Author Contributions

KA and DC conceived and designed the experiments and conducted all experiments. DB and DC analyzed the data. DB consulted on interpretation of statistical analyses. KA, DC and DB wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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SUPPLEMENTAL DATA

Table_S1.

Proteins identified in larval blood samples collected 24 hours post inoculation with viable *Ascosphaera apis* spores using liquid chromatography-mass spectrometry, GeLC-MS/MS

UniProtKB Accession Number	Genomic location/ protein name	Description
A0A087T44		Uncharacterized
A0A087ZMS3	Dgk	diacylglycerol kinase 1
A0A087ZMT8	LOC100578243	*Apis mellifera* proteasome subunit alpha type-6-like
A0A087ZN49	RabX6	Small GTPase, Rab family
A0A087ZNC6	LOC551115	Uncharacterized
A0A087ZNL0	LOC105198936	Uncharacterized
A0A087ZNL5	LOC552129	Uncharacterized
A0A087ZNP4	LOC100867120	Uncharacterized
A0A087ZNX2	LOC409250	beta-ureidopropionase-like isoform 1
A0A087ZNZ9	Echs1	probable enoyl-CoA hydratase, mitochondrial
A0A087ZPA2	LOC105205951	*Solenopsis invicta* uncharacterized
A0A087ZPT7	LOC410070	reticulocyte-binding protein 2 homolog a-like isoform
A0A087ZPU2	LOC412829	arylsulfatase B-like isoform X2
A0A087ZPW8	LOC100578907	protein HOS4-like isoform X1
A0A087ZPX4	Oscillin	Glucosamine-6-phosphate isomerase, hydrolase
A0A087ZQ27	Hsc70-4	*Apis florea* heat shock 70 kDa protein cognate 4-like
A0A087ZQM5		phosphatidylserine synthase, isform X4
A0A087ZQZ5	LOC102654133	Histone-lysine N-methyltransferase
A0A087ZR35	pita	trichohyalin
A0A087ZRA2	actin	*Maconellicoccus hirsutus* clone WHMH3407 putative actin
A0A087ZRF3	LOC411381	*Apis mellifera* caspase-like
A0A087ZRN6	LOC411167	cysteine-tRNA ligase, cytoplasmic-like
A0A087ZS21	LOC552812, Ank_2	ankyrin repeat subunit A-like isoform 2
A0A087ZSG1	LOC727319	Uncharacterized
A0A087ZSK8	LOC410037	dnaJ homolog subfamily C member 7-like isoform 1
A0A087ZT96	LOC408831	*Apis mellifera* ribosome-releasing factor 2, mitochondrial
A0A087ZTJ5	LOC551203	Uncharacterized
A0A087ZTN7	LOC102655627	*Apis mellifera* glycine cleavage system H protein, mitochondrial-like
A0A087ZTP0	Ame.396, LOC102676004	*Apis dorsata* myosin heavy chain, non-muscle-like
A0A087ZTP7	LOC551647	GDNF/GAS1 domain protein
A0A087ZTR1	LOC724950	Uncharacterized
A0A087ZTT8	LOC100576169	Uncharacterized
A0A087ZTY5	Prpf3	U4/U6 small nuclear ribonucleoprotein
A0A087ZTY7	Hsc70-5	Heat shock 70 kDa protein cognate 5
A0A087ZU08	LOC409105	WAS/WASL-interacting protein family member 1-like
A0A087ZU40	LOC409305	*Apis mellifera* elongation factor Tu GTP-binding domain
A0A087ZU43	LOC551088	asparagine-tRNA ligase, cytoplasmic-like
A0A087ZU47	DNAH1	dynein, axonemal, heavy chain 1
A0A087ZUB3	LOC551180	aminopeptidase N-like transcript variant X1
A0A087ZUK9	LOC408950	malate dehydrogenase, mitochondrial-like isoform 1
A0A087ZUN6	JHBP1	*Apis mellifera* take-out-like carrier protein, JHBP-1
A0A087ZUP0	14-3-3epsilon	14-3-3 protein epsilon isoform X1
A0A087ZUP8	Fsn	F-box/SPRY domain-containing protein 1 isoform X3
A0A087ZUW4	LOC726382	Uncharacterized
A0A087ZV28	LOC727010	Uncharacterized
A0A087ZV38	cnk	multi-domain protein, RAS/MAPK cascade
A0A087ZV49	Para	Voltage-gated sodium chanel

A0A087ZVZ6	LOC725851	Actin-like_CS
A0A087ZW76		*Apis florea* FERM, RhoGEF and pleckstrin domain-containing protein 2-like
A0A087ZW90	LOC412154	thimet-like oligopeptidase-like isoform X2
A0A087ZWE5	sec5	Exocyst complex
A0A087ZWF7	Actn	*Apis mellifera* alpha actinin
A0A087ZWN2	LOC409077	aminopeptidase N-like isoform X2
A0A087ZX53	trx	Histone-lysine N-methyltransferase
A0A087ZXE7	Ame.17307	Uncharacterized
A0A087ZXF1	LOC102654315	*Apis mellifera* serine/threonine-protein kinase Nek5-like
A0A087ZXG0	LOC410484	*Apis mellifera* trehalase, transcript variant X3
A0A087ZXU0	Fer2LCH	ferritin heavy chain
A0A087ZYC2	LOC409303	E3 ubiquitin-protein ligase MIB2-like isoform X1
A0A087ZYG7		LRR_leucine-rich repeat-containing protein DDB_G0290503-like
A0A087ZYV5		*Apis mellifera* PIH1 domain containing 1 (PIH1D1)
A0A087ZYZ0	LOC410993	*Apis dorsata* valacyclovir hydrolase-like
A0A087ZZ76	Ank_2	ankyrin-2-like
A0A087ZZ79	LOC552249	Alpha-mannosidase
A0A087ZZI8	RabGAP9	Rab GTPase activator,
A0A087ZZK0	LOC552026	leucine-rich repeat-containing protein 16A-like isoform X2
A0A088A069	Ame.25716	Uncharacterized
A0A088A072	hop	tyrosine-protein kinase hopscotch, isoformX1
A0A088A084	LOC409444	AMP deaminase 2-like isoform X10
A0A088A0G4	LOC413451	reticulocyte-binding protein 2-like isoformX1
A0A088A0Q7	Nup62	Nucleoporin
A0A088A0S4	Pxn	Haem_peroxidase_animal, isoform 2; secreated,
A0A088A0U4	LOC412467	bifunctional purine biosynthesis protein PURH-like isoform X2
A0A088A114	cul-3	Culin_RING ligase
A0A088A1E0	LOC410330	phosphatidylserine synthase, isform X4
A0A088A1E9	LOC408353	proteasome subunit beta type-7-like
A0A088A1F1	DNAH7	dynein, axonemal, heavy chain 7
A0A088A1J5	LOC725689	golgin subfamily A member 4-like
A0A088A232	LOC100576947	Uncharacterized, similar to protein C16orf73
A0A088A2A9	Tsp	*Apis mellifera* thrombospondin
A0A088A2E2	VhaSFD	V-type ATPase
A0A088A2M2	LOC410685	*Apis mellifera* tyrosine-protein kinase-like otk-like
A0A088A2P7	DMAP1	methyltransferase-associated protein 1
A0A088A2P9	kon	ConA-like, lectin
A0A088A2R7	Ame.17170	ssDNA binding
A0A088A2T1	SP35	Sp-35, LC-SP domain,
A0A088A2W1		vanin-like protein 1-like, hydrolase
A0A088A2X8	Ame.20274, LOC100576384	*Apis mellifera* tyrosine-protein phosphatase 10D-like
A0A088A323		*Apis dorsata* transient receptor potential channel pyrexia-like
A0A088A367	Tg	Transglutaminase-like
A0A088A3I5	LOC409757	ecto-NOX disulfide-thiol exchanger 2-like isoform X6
A0A088A444	LOC100578239	*Apis mellifera* tetratricopeptide repeat protein 18-like
A0A088A462	LOC409459	Lon protease homolog, mitochondrial
A0A088A4B4	LOC100576872	cell cycle checkpoint control protein RAD9A-like
A0A088A4E8	Ame.14651	Uncharacterized
A0A088A4I9	Ing3	Inhibitor of growth protein 3
A0A088A4K0	LOC552736	phosphoglycerate mutase 2-like
A0A088A4Q7	lgs	BCL9_beta-catenin-bd_dom.
A0A088A4X9	PNGase	peptide-N(4)-(N-acetyl-beta-glucosaminyl) asparagine amidase
A0A088A579	dup	DNA replication factor Cdt1 isoform X2
A0A088A5X7	LOC409167	translation elongation factor 2-like isoform 1
A0A088A613	LOC412408	nuclear export mediator factor NEMF homolog isoformX1
A0A088A6C0	LOC100576853	Uncharacterized
A0A088A6Y6	Ame.15663	*Apis mellifera* canoe (cno), transcript variant X8
A0A088A777	LOC411653	sodium channel protein 60E-like isoform X1
A0A088A7F4		Lyase, cyclase family

A0A088A7G2	LOC409385	E3 ubiquitin-protein ligase HECW2-like
A0A088A7L1	Su(z)12	*Apis mellifera* polycomb protein Su(z)12
A0A088A7M1	LOC100577870	Small GTPase
A0A088A7S9	LOC551205	neutral alpha-glucosidase AB-like isoform 2
A0A088A7X7		*Apis dorsata* protein SON-like (LOC102674096)
A0A088A805	LOC726487	Histone H2B
A0A088A811	LOC100576257	Uncharacterized
A0A088A8D9	LOC412890	*Apis mellifera* A disintegrin and metalloproteinase with thrombospondin motifs 3-like
A0A088A8E3	LOC725662	Uncharacterized
A0A088A8F6		Uncharacterized
A0A088A8J2	LOC409620	*Apis mellifera* vacuolar protein sorting-associated protein 8 homolog
A0A088A8M6	LOC724948	Uncharacterized
A0A088A8N0	LOC724874	facilitated trehalose transporter Tret1-like isoform X1
A0A088A947	LOC102654422	*Apis dorsata* tyrosine-protein kinase transmembrane receptor ROR1-like (LOC102677061)
A0A088A988	CTL4	C-type lectin
A0A088A9L7	LOC724435	*Apis mellifera* helicase POLQ-like
A0A088A9P5	Syx7	syntaxin-12 isoform X1
A0A088A9Q0	LOC409162	retinoid-inducible serine carboxypeptidase-like isoform X3
A0A088AA38	LOC410420	isochorismatase domain-containing protein 2, mitochondrial-like isoform X3
A0A088AA49	LOC724591, HPS-3	Hermansky-Pudlak syndrome 3 protein homolog
A0A088AA70	LOC726292	uncharacterized protein LOC726292 isoform X1
A0A088AAL0	LOC411691	phosphotriesterase-related protein-like isoform X2
A0A088AAR4	JHBP-1	*Apis mellifera* take-out-like carrier protein
A0A088AAS7	LOC724987	*Apis mellifera* chitinase-like protein Idgf4-like
A0A088AAX0	Manf	mesencephalic astrocyte-derived neurotrophic factor homolog
A0A088AAY7	LOC409898	acyl-CoA-binding protein
A0A088AB73	LOC413542	*Apis mellifera* polyribonucleotide nucleotidyltransferase 1, mitochondrial-like
A0A088ABD2	LOC724485	mRNA splicing
A0A088ABF5	LOC410189	*Apis mellifera* 5'-nucleotidase domain-containing protein 3-like
A0A088ABV3	LOC102655694	GST_C_like
A0A088ABY1	ird1	phosphoinositide 3-kinase regulatory subunit 4 isoform X1
A0A088AC54		Helicase, ATP-binding, hydrolase activity
A0A088AC97	LOC410975	Uncharacterized
A0A088ACF4	Hsc70-3	*Apis mellifera* heat shock 70 kDa protein cognate 3
A0A088ACK6	LOC412256	Uncharacterized
A0A088ACM6	LOC551826	homeotic protein female sterile-like isoform X1
A0A088ACQ6	LOC725131	Uncharacterized
A0A088AD50	LOC100578628	NADH-cytochrome b5 reductase-like
A0A088AD69	LOC410166	Myelin gene regulatory factor,
A0A088AD69	LOC410166	Myelin gene regulatory factor,
A0A088AD76	LOC725656	uncharacterized protein DDB_G0288805-like isoform X1
A0A088ADA3	PITRM1	pitrilysin metalloproteinase 1,
A0A088ADC3	LOC727467	*Apis mellifera* ralA-binding protein 1-like, transcript variant X2
A0A088ADN5	LOC408818	hexokinase-1-like
A0A088ADW2	LOC552548	P13_4_kinase
A0A088AE95	LOC725892	transforming growth factor-beta-induced protein ig-h3-like isoform X5
A0A088AEE1	LOC410118	*Apis mellifera* uronosyl C5 epimerase, transcript variant X1
A0A088AEI6	LOC408396	Uncharacterized
A0A088AEP1		phosphatidylserine synthase, isform X4
A0A088AEP8	LOC413697	bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing)-like
A0A088AER3	DNAH3	dynein, axonemal, heavy chain 3
A0A088AEV7	LOC408516	phosphatidylethanolamine-binding protein homolog F40A3.3-like isoformX2
A0A088AEX8	LOC725586	*Apis mellifera* exonuclease 3'-5' domain-containing protein 2-like
A0A088AEY3	LOC725250	Peptidase F1 family, Trypsin-SP like
A0A088AEY5	LOC102656186	Peptidase_S1, trypsin_like

A0A088AF61	LOC411724	acetyltransferase
A0A088AF69	LOC408533	mitogen-activated protein kinase kinase kinase 15-like isoform X1
A0A088AF74	LOC725114	chymotrypsin inhibitor-like isoform 1
A0A088AFF5	LOC408519	*Apis mellifera* sterile20-like kinase-like protein, transcript variant X2
A0A088AFG1	LOC102655673	*Apis mellifera* mitochondrial import inner membrane translocase subunit Tim16-like
A0A088AFN7	LOC100577001	Alcohol dehydrogenase transcription factor, MADF_dom
A0A088AFP3		Uncharacterized
A0A088AFY2	LOC102654858	*Apis mellifera* zinc finger protein 62 homolog
A0A088AG02	LOC100577492	Uncharacterized
A0A088AG06	SF3A1	splicing factor 3A subunit 1 isoform X2
A0A088AG61	LOC100576361	Uncharacterized
A0A088AGE0	DNAH2	Dynein, heavy chain2
A0A088AGI8	LOC551968	aldose reductase-like isoform 1
A0A088AGZ8	SP36	Chymotrypsin -1, Tryp-SP
A0A088AHC8	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
A0A088AHG6		*Apis dorsata* 6-phosphofructokinase-like, LOC102681646
A0A088AHH9	RpIII128	DNA-directed RNA polymerase
A0A088AHJ0	LOC408291	3-ketoacyl-CoA thiolase, mitochondrial-like isoform X2
A0A088AHJ0	LOC408291	3-ketoacyl-CoA thiolase, mitochondrial-like isoform X2
A0A088AHR3	LOC410218	probable signal peptidase complex subunit 2-like
A0A088AHX8	Ame.7852	Uncharacterized
A0A088AI41	LOC100576233	Uncharacterized
A0A088AIL4	LOC412513	Uncharacterized
A0A088AIQ2	LOC725876	*Apis mellifera* probable helicase senataxin-like
A0A088AIV2		SAM-dependent_MTases.
A0A088AIY8	Sirt6	family of histone deacetylases, NFκB-dependant
A0A088AJ41	LOC413968	Actinin
A0A088AJE5	LOC412950	*Apis mellifera* nucleoporin GLE1 (LOC412950
A0A088AJF9	LOC724225	*Apis mellifera* myo-suppression receptor
A0A088AJG2	Ame.18921, LOC411894	*Apis mellifera* dynein beta chain, ciliary-like
A0A088AJK2	LOC551888	WASH complex subunit 7-like
A0A088AJM3	milt	kinesin-mediated axonal transport
A0A088AJN3	Ame.23594	Uncharacterized
A0A088AJN8	LOC100576760	Uncharacterized
A0A088AK81	LOC726524	Hydrolase activity, DNA binding
A0A088AKC2	LOC725618	myb-binding protein 1A-like
A0A088AKI9	Gmer	GDP-L-fucose
A0A088AKK7	LOC410019	uncharacterized protein KIAA1109-like isoform X3
A0A088AKK7	LOC410019	uncharacterized protein KIAA1109-like isoform X3
A0A088AKU0	LOC102654344	*Apis mellifera* uncharacterized LOC100577526
A0A088AKU3	SP40	Serine-type endopeptidase, Tryp_SPc
A0A088AL06	LOC409242	BAT2 domain containing, PRRC2C-like isoform
A0A088AL19	Ame.21769	Serine protease_S8, subtilisin-like
A0A088AL76	LOC724561	*Apis mellifera* DC-STAMP domain-containing protein 1-like
A0A088ALA0	LOC724850	BRO1 domain-containing protein BROX-like
A0A088ALI6	LOC726899	apoptosis 1 inhibitor-like
A0A088ALT6	LOC100577507	Uncharacterized
A0A088ALY6	Ame.3876 LOC100576326	Uncharacterized
A0A088AM00	LOC725681	Uncharacterized
A0A088AM00	LOC725681	Uncharacterized
A0A088AME4	LOC410533	ceramide-1-phosphate transfer protein-like isoform X4
A0A088AMR6	LOC409281	*Apis mellifera* protein sel-1 homolog 1-like
A0A088AN42	LOC411633	probable UDP-glucose 4-epimerase-like isoform X7
A0A088AN49	LOC552194	transmembrane protein 181-like isoform X2
A0A088ANF0	LOC411519	tubulin alpha-2/alpha-4 chain-like isoform X2
A0A088ANL0	LOC102654159	chaperone protein dnaJ 1, mitochondrial-like
A0A088ANT0		HAD-like _dom
A0A088ANW7	nemy	glutaminase activity, Ankyrin_dom

A0A088AP06	Prosalpha7	proteasome subunit alpha type-3; threonine type endopeptidase
A0A088AP18	LOC411294	Uncharacterized
A0A088APK9	LOC408874	Uncharacterized
A0A088APR3	LOC102654184	Uncharacterized
A0A088APU1	LOC551041	glycerol kinase-like isoform X4
A0A088APY3	LOC408742	fatty-acid amide hydrolase 2-A-like isoform X2
A0A088AQ25	LOC725699	Uncharacterized
A0A088AQ57	LOC726532	Uncharacterized
A0A088AQ60	beta4GalT7	*Apis mellifera* beta-4-galactosyl transferase 7
A0A088AQ74	LOC726210	Uncharacterized
A0A088AQB3		*Apis dorsata* zinc finger protein 395-like
A0A088AQJ6		Uncharacterized, C18orf63-like (LOC100742926)
A0A088AR17	LOC726928	Uncharacterized
A0A088AR64	LOC726582	*Apis mellifera* prominin-like protein-like
A0A088ARA6	LOC725200	ATPase
A0A088ARF5	Ame.25137	*Apis florea* zinc finger protein 93-like (LOC100872030)
A0A088ARK5	LOC724457	Uncharacterized
A0A088ART2	LOC409384	60 kDa chaperonin-like
A0A088AS83	sli	Similar to dSlit
A0A088AS84	axo	*Apis mellifera* axotactin (axo), transcript variant X11
A0A088ASD6	LOC726241	tudor domain-containing protein 7-like isoform X1
A0A088ASK2	Ame.17268	Uncharacterized
A0A088AST1	LOC100577515	Uncharacterized
A0A088ASV7	arm	Armadillo segment polarity, X3
A0A088AT34	LOC550789	selenocysteine Se-methyltransferase-like
A0A088ATD6	NDUFB6/B17	NADH:ubiquinone oxidoreductase
A0A088ATJ9	LOC102653626	Uncharacterized
A0A088AU05		*Apis florea* serine/threonine-protein kinase N-like (LOC100867837)
A0A088AU96	LOC408589	Uncharacterized
A0A088AUA4	LOC552091	group XV phospholipase A2-like isoform X1
A0A088AUB0	LOC551676	methylmalonic aciduria and homocystinuria type D homolog, mitochondrial-like isoform X3
A0A088AUF9	LOC724508	trichohyalin-like
A0A088AUH8	LOC412030	ras-related protein Rab-30-like isoform 1
A0A088AUM2	LOC408672	programmed cell death protein 6-like isoform 1
A0A088AUT9	LOC410793	Uncharacterized
A0A088AUV7	LOC408656	RNA exonuclease 1 homolog isoform X2
A0A088AUY4	LOC550804	transketolase isoform 1
A0A088AV09	LOC551492	NADH dehydrogenase [ubiquinone] complex I, assembly factor 7 homolog isoform X1
A0A088AV23	LOC726461	Uncharacterized
A0A088AV29	LOC727363	Uncharacterized
A0A088AV87	LOC100577248	*Apis mellifera* Pcf11, cleavage and polyadenylation factor subunit
A0A088AV90	LOC409221	dentin sialophosphoprotein-like isoform X1
A0A088AVA4	LOC724775	tudor domain-containing protein 3-like isoform X1
C0K3M9	sytI	Synaptotagmin I isoform A
C9E0T0	Ago3	Argonaute Ast2 variant
Q5FAN0	AmsGC beta-3	Soluble guanylyl cyclase beta-3
Q6TLC7	GAT-1B	GABA Transporter1B
Q86PH6	trf	Transferrin/iron transfer
R4U544	gnbp-1	Gram-negative protein binding-1

УРОВЕНЬ ФИЗИОЛОГИЧЕСКОЙ РЕАКЦИИ НА ГРИБОК ИЗВЕСТКОВОГО РАСПЛОДА *ASCOSPHAERA APIS* НЕ ЗАВИСИТ ОТ ГЕНОТИПОВ ЛИНИЙ МЕДОНОСНОЙ ПЧЕЛЫ

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АННОТАЦИЯ

Селекция медоносных пчел (*Apis mellifera*) на увеличение физиологической устойчивости к болезням является весьма желательным и экологически безопасным подходом для увеличения выживаемости семей. Отбор желательных признаков является важнейшим элементом любой программы по разведению.

В этой работе мы изучаем, зависит ли уровень физиологической реакции на грибок известкового расплода *Ascosphaera apis*, одного из главных грибковых патогенов пчел, от генотипа линий медоносной пчелы. Уровень устойчивости расплода к системным микозам, вызываемых грибками, определялся по выживаемости личинок под воздействием патогена *in vitro*. Результаты наших исследований не обнаружили статистически значимых различий в индивидуальной устойчивости к аскосферозу среди трех линий пчел (Русская, Краинская, Устойчивая К Варроатозу).

Кроме того, активация классического иммунитета и клеточных реакций на стресс не влияет на выживание инфицированных особей, что свидетельствует о том, что активация отдельных физиологических реакций, возможно, не является основным механизмом защиты против грибковых заболеваний. Поведенческие реакции взрослых особей пчел, как публиковалось ранее, вероятнее всего, являются первичными механизмами, обеспечивающими устойчивость к аскосферозу на уровне пчелиной семьи.

Ключевые слова: медоносная пчела, *Ascosphaera apis*, грибковый патоген, естественный иммунитет, устойчивость