Insect science

Insect Science (2019) 26, 831–842, DOI 10.1111/1744-7917.12593

ORIGINAL ARTICLE

Comparative susceptibility and immune responses of Asian and European honey bees to the American foulbrood pathogen, *Paenibacillus larvae*

Sasiprapa Krongdang[1,2], Jay D. Evans[3], Yanping Chen[3], Wannapha Mookhploy[1] and Panuwan Chantawannakul[1,4]

[1]*Bee Protection Laboratory, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand;* [2]*Interdisciplinary Program in Biotechnology, Graduate School, Chiang Mai University, Chiang Mai, Thailand;* [3]*USDA-ARS, Bee Research Laboratory, Beltsville, Maryland, USA and* [4]*Environmental Science Research Center, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand*

Abstract American foulbrood (AFB) disease is caused by *Paenibacillus larvae*. Currently, this pathogen is widespread in the European honey bee—*Apis mellifera*. However, little is known about infectivity and pathogenicity of *P. larvae* in the Asiatic cavity-nesting honey bees, *Apis cerana*. Moreover, comparative knowledge of *P. larvae* infectivity and pathogenicity between both honey bee species is scarce. In this study, we examined susceptibility, larval mortality, survival rate and expression of genes encoding antimicrobial peptides (AMPs) including *defensin*, *apidaecin*, *abaecin*, and *hymenoptaecin* in *A. mellifera* and *A. cerana* when infected with *P. larvae*. Our results showed similar effects of *P. larvae* on the survival rate and patterns of AMP gene expression in both honey bee species when bee larvae are infected with spores at the median lethal concentration (LC_{50}) for *A. mellifera*. All AMPs of infected bee larvae showed significant upregulation compared with noninfected bee larvae in both honey bee species. However, larvae of *A. cerana* were more susceptible than *A. mellifera* when the same larval ages and spore concentration of *P. larvae* were used. It also appears that *A. cerana* showed higher levels of AMP expression than *A. mellifera*. This research provides the first evidence of survival rate, LC_{50} and immune response profiles of Asian honey bees, *A. cerana*, when infected by *P. larvae* in comparison with the European honey bee, *A. mellifera.*

Key words *Apis cerana*; *Apis mellifera*; honey bee; immune gene; *Paenibacillus larvae*; susceptibility

Introduction

Honey bees are the most economically valuable pollinators in the world (Gallai *et al.*, 2009). Nevertheless, honey bees are attacked by numerous parasites and pathogens, such as ectoparasitic mites, microsporidia, fungi, viruses, and bacteria (Chen *et al.*, 2004, 2009; Evans & Schwarz, 2011; McMenamin & Genersch, 2015; Chantawannakul *et al.*, 2016; Tehel *et al.*, 2016). One of the most destructive diseases affecting honey bee larvae is American foulbrood (AFB) disease caused by a Gram-positive, spore-forming bacterial pathogen, *Paenibacillus larvae* (Ebeling *et al.*, 2016). AFB is a serious concern in beekeeping worldwide (Genersch, 2010). *P. larvae* is generally transmitted within hives by contaminated larval food, and across hives by foraging and robbing activities and transmission by beekeepers (Genersch, 2010). This pathogen is highly contagious and rapidly spreads among colonies to apiaries (vanEngelsdorp & Meixner, 2010).

Correspondence: Panuwan Chantawannakul, Bee Protection Laboratory, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand. Tel: +66 05 3943346; fax: +66 053892259; email: panuwan@gmail.com

Currently, infectivity and pathogenicity of *P. larvae* remain an active field of study (Ebeling *et al.*, 2016). Many studies have addressed pathogenic processes (Garcia-Gonzalez & Genersch, 2013; Poppinga & Genersch, 2015) and genotypic diversity of the pathogen that could affect infectivity and pathogenicity (Krongdang *et al.*, 2017). Genersch *et al.* (2005) demonstrated that virulence of *P. larvae* in *A. mellifera* worker larvae varied across bacterial genotypes. Furthermore, sexes of honey bee hosts affect the lethal infection threshold of *P. larvae* because drones appeared to be more resistant to *P. larvae* infections when compared with worker honey bees (Behrens *et al.*, 2010).

Honey bees defend themselves against pathogenic infections in several ways. At the colony level, honey bees possess behavioral defense strategies including hygienic behavior, grooming, removal of dead bees, and collecting antimicrobial tree resins (propolis) (Wilson *et al.*, 2015). Likewise, at the individual level, honey bees enlist both cellular and humoral innate immune responses against bee pathogens (Evans *et al.*, 2006).

Innate immunity is one mechanism that plays essential roles in both insects and mammals (De Gregorio *et al.*, 2002). As in other insects, one aspect of innate immunity in honey bees is the production of antimicrobial peptides (AMPs) through three signaling pathways (Toll, Imd, and Jak/STAT) (Evans *et al.*, 2006). In general, AMPs are expressed in response to pathogens and parasites. Fungal and Gram-positive bacterial infections activate primarily the Toll pathway, whereas Gram-negative bacterial infections stimulate the Imd pathway (Hoffmann & Reichhart, 2002). *P. larvae*, the AFB pathogen, stimulated transcript levels of those AMPs such as *defensin*, *apidaecin*, *abaecin*, and *hymenoptaecin* regulated by the Toll pathway in honey bee—*A. mellifera* (Evans, 2004; Chan *et al.*, 2009; Cornman *et al.*, 2013).

AFB is widespread in colonies across several European countries and the United States (vanEngelsdorp & Meixner, 2010). In contrast, honey bees infected by *P. larvae* were found to be less frequent in southeast Asia (Chantawannakul *et al.*, 2016). Currently, *A. mellifera* occurs throughout most of the natural distribution area of *A. cerana* in Asia (Yañez *et al.*, 2015). This has led to interspecific pathogen transmission from *A. mellifera* to *A. cerana* (Forsgren *et al.*, 2015; Lin *et al.*, 2016) or vice versa some pathogens also can be spread from native *A. cerana* to *A. mellifera* (Rath, 1999; Paxton *et al.*, 2007; Locke *et al.*, 2012). However, little is known about *P. larvae* incidence in Asiatic cavity-nesting honey bees; *Apis cerana* (Chen *et al.*, 2000). This might be due to colony-level resistance mechanisms in Asian honey bees (Chantawannakul *et al.*, 2016). *A. cerana* shows a great ability to resist the ectoparasitic mite; *Varroa destructor*, a vector of Deformed wing virus (DWV) (Li *et al.*, 2012). Moreover, Lin *et al.* (2016) reported that *A. cerana* displayed significantly faster hygienic behavior than *A. mellifera*, in order to detect and remove unwanted brood in the experiments. This suggests a mechanism of eliminating pathogenic agents in colonies.

Interspecific transmission of pathogens between different host systems is currently viewed as one of the most essential sources of biodiversity loss and honey bee colony losses in particular (Forsgren *et al.*, 2015). Many studies have reported interspecific transmission of pathogens in different honey bees such as *Nosema* spp. (Chaimanee & Chantawannakul, 2015), viruses (Li *et al.*, 2012), *V. destructor* (Hamiduzzaman *et al.*, 2015), and *Melissococcus plutonius* (Forsgren *et al.*, 2015). Therefore, it could help to better understand how pathogens and parasites contribute within and between species. Hence, more research on pathogens from Asian honey bee species is warranted to prevent disease-induced losses in these bees. It is also imperative to identify possible threats from interspecific transmission of pathogens. Our particular focus is on the impact of *P. larvae* infection on survival rate and immune responses of Asian honey bees. Therefore, in this study, we designed comparative experiments to measure susceptibility and immune responses of the two different honey bee species, *A. mellifera* and *A. cerana*.

Materials and methods

Honey bee larvae samples

The experiment was set up in experimental apiaries of the Bee Protection Laboratory (BeeP) located at Chiang Mai University, Suthep district, Chiang Mai, Thailand, during 2014–2016. All first-instar honey bee larvae were obtained from 6 colonies of *A. mellifera* and 20 colonies of *A. cerana indica* that were housed in wooden hives.

Cultivation of P. larvae and endospore harvesting

P. larvae strain LMG9820, classified as ERIC I, was purchased from the BCCM Belgian Coordinated Collections of Microorganisms (BCCM, Belgium). The bacteria were isolated on THClYGP agar contained (per liter), 15 g yeast extract (Difco), 1 g pyruvic acid (Sigma), 200 mL 0.1 mol/L Tris-HCl, pH 7.0, 20 g agar, 40 mL 10% glucose with 3 μg/mL of nalidixic acid for 7–10 d after which cells were stored in glycerol stock and storage at –20 °C.

To prepare a spore suspension, one full loop of *P. larvae* after cultivated for 7–9 d was picked into 1 mL of sterilized distilled water in a sterile microcentrifuge tube and mixed. The tube was centrifuged at 13 000 × *g*, 4 °C for 15 min and washed twice with sterilized distilled water. The supernatant was discarded and the pellet was resuspended in 200 μL of sterile water and mixed. To reduce contamination of vegetative cells, the suspension was put in a water bath for 15 min at 80 °C (Alippi & Aguilar, 1998). Endospores were counted manually using heamacytometer and diluted concentration to 1 × 10^8 spores/mL as a stock spore inoculant for further *in vitro* bioassay experiment.

In vitro rearing and inoculation of larvae

To obtain first instar worker larvae of *A. mellifera*, the queen was allowed to lay eggs over empty brood cells with an excluder cage that placed into a hive with a healthy colony. The queen was released from the excluder cage after laying eggs for 24–72 h (Evans & Wheeler, 1999; Crailsheim *et al.*, 2013). Afterward, the cage was placed into the middle of the hive, so that the nurse bees were able to easily take care of the queen and the larvae (Vandenberg & Shimanuki, 1987; Crailsheim *et al.*, 2013). On the fourth day after caging the queen, the frames which were full with newly hatched larvae (first instar larvae stage) were removed from the hive and brought to the laboratory for larval grafting (Fourrier *et al.*, 2015; Mao *et al.*, 2015). To obtain first instar worker larvae of *A. cerana indica*, the first instar larvae were directly retrieved from brood cells of the combs and were then grafted with the same procedures.

The same artificial diet compositions were used for feeding both honey bee larvae. This diet was adapted from Rembold *et al.* (1974) and Fourrier *et al.* (2015) with modifications in the ratios of the nutrients, resulting in a diet with 30% total solids, as recommended by Vandenberg and Shimanuki (1987). The artificial diet was prepared as follows: sugar (50% w/w) and yeast extract (1% w/w) were dissolved in sterile water, filtered through a Millipore membrane (0.22 μm), and then added with 50% (w/w) of royal jelly. Before each experiment, the diet was freshly prepared.

In vitro bioassay of P. larvae inoculation

To evaluate virulence of *P. larvae* on larval growth, for each group, newly hatched larvae were inoculated with a final concentration of 100, 500, 1000, and 2000 spores/mL from pathogenic isolates of *P. larvae* mixed directly into their artificial diet (Brødsgaard *et al.*, 1998) in the first day, while control larvae were fed with normal larval diet throughout the larval stages. Experimental larvae (approximately 12–20 larvae) were grafted into a sterilized Petri dish (Ø 6 cm) containing 1000 μL of diet in each plate. Larvae were randomly dispatched directly from the comb into the plate (Crailsheim *et al.*, 2013). The artificial diet was refreshed every 24 h (Brødsgaard *et al.*, 1998). Plates of larvae were kept in an incubator at 34 ± 1 °C and 96% RH for *A. mellifera* and 70% RH for *A. cerana* larvae (Chen *et al.*, 2000). Three replicates were done for each trial. Mortality was recorded in control and treatment trials every day until all larvae had died (Fourrier *et al.*, 2015). In this case, the Abbott method (Abbott, 1987) was used to correct natural mortality. After that, mortality results were compared with Kaplan-Meier survival analysis (Rich *et al.*, 2010) and the Probit analysis (Finney, 1971) using SPSS version 17.0 for window (SPSS, Inc.). Based on the relationship between the concentrations of *P. larvae* spore concentration/larval mortality (see Tables S1 and S2), the median lethal concentration (LC_{50}), the 95% confidence interval, the slope of the regression and Pearson goodness-of-fit were calculated. This analysis was chosen since the result of the bioassay was a binomial response (survivor or death of exposed larvae).

Once LC_{50} values were obtained for *A. mellifera* and *A. cerana*, *in vitro* larval-rearing experiments and pathogen infection were set up to measure immune gene expression. There were two experimental groups consisting of a treatment group in which the first-instar larvae inoculated with an exposure level of *P. larvae*'s LC_{50} (n = 120) and a control group (without *P. larvae*; n = 120). All trials were incubated at of 0, 6, 12, 24, 36, and 48 h. At each time-point of incubation, treatment (n = 10) and control larval (n = 10) samples were randomly collected and immediately put on dry ice mixed with 95% ethanol to clean surfaces and then the samples were directly frozen at –80 °C.

RNA extraction and cDNA synthesis

Each honey bee sample was manually homogenized using pestles. Total RNA was extracted from individual larval samples using TRIZOL® (Invitrogen, Carlsbad, CA, USA) according to manufacturer's recommendations. RNA quantity was determined with a BioDrop Duo spectrophotometer. First-strand cDNA synthesis was performed on 300 ng of total RNA using the Tetro cDNA synthesis kit (Bioline, Alexandria, NSW 10 μmol/L of oligo(dT)18 and 40 μmol/L of Random hexamer were mixed together) according to the manufacturer's instructions.

Table 1 Primers used for qPCR amplification in this study for *Paenibacillus larvae* inoculation experiment.

Amplification target	Primer	Sequence 5′–3′	References
Housekeeping			
Ribosomal protein S5 (RPS5)	AmRPS5.F	AATTATTTGGTCGCTGGAATTG	Evans & Pettis, 2005
	AmRPS5.R	TAACGTCCAGCAGAATGTGGTA	
β-actin	Actin.F	TTGTATGCCAACACTGTCCTTT	Simone *et al.*, 2009
	Actin.R	TGGCGCGATGATCTTAATTT	
Immune-related			
Antibacterial peptide abaecin	Abaecin.F	CAGCATTCGCATACGTACCA	Evans, 2006
	Abaecin.R	GACCAGGAAACGTTGGAAAC	
Antibacterial peptide defensin	Defensin.F	TGCGCTGCTAACTGTCTCAG	Evans, 2006
	Defensin.R	AATGGCACTTAACCGAAACG	
Antibacterial peptide apidaecin	ApidNT.F	TTTTGCCTTAGCAATTCTTGTTG	Simone *et al.*, 2009
	ApidNT.R	GTAGGTCGAGTAGGCGGATCT	
Antibacterial peptide hymenoptaecin	Hymenopt.F	CTCTTCTGTGCCGTTGCATA	Evans, 2006
	Hymenopt.R	GCGTCTCCTGTCATTCCATT	
P. larvae			
Spore germination protein	PlGermSA.F	CCATTTGCTTCAGGGAAGAG	Evans & Pettis, 2005
	PlGermSA.R	CAAGCCAGCGTATGCTGTAA	
Flagella biosynthesis protein	PlFLiP.F	TGCAGTCCAGCCGTACATTA	Evans & Pettis, 2005
	PlFLiP.R	ATATCATGACCGGAGGCAAC	

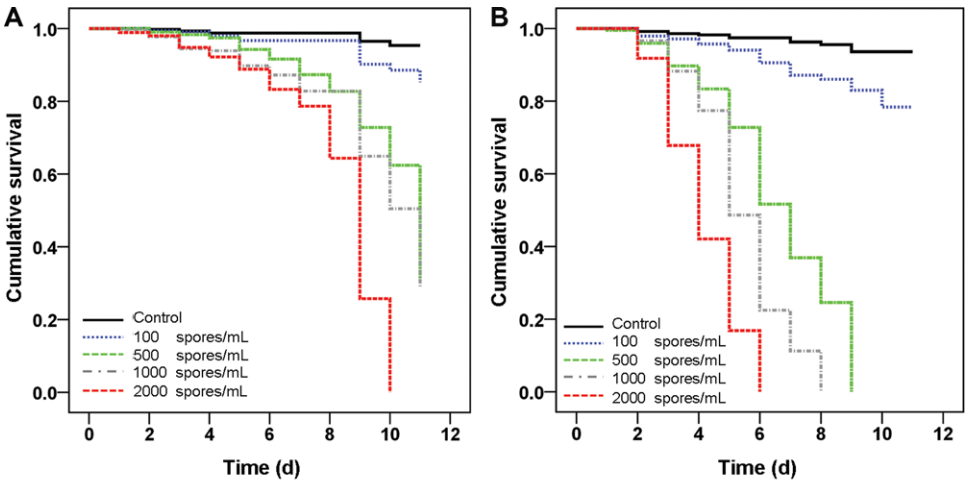
Quantitative real-time PCR parameters

qPCR was performed in a 20 μL reaction mixture consisting of SensiFAST SYBR® No-ROX Kit master mix (Bioline, Alexandria, NSW), 0.4 μmol/L of each primer, and 1 μL (~100 ng) of cDNA template. The oligonucleotide primers for qPCR in this experiment are summarized in Table 1. The reaction was carried out in 96-well plates using a BioRad iQ™ 5 (Bio-Rad Crop., Hercules, CA, USA). Amplification was performed with the following temperature profile: 95 °C for 30 s followed by 50 cycles of 95 °C for 5 s, 60 °C for 30 s. Fluorescence was measured at the elongation step and negative controls (without DNA) were included in each reaction run. Specificity of the reaction was confirmed by analysis of the melting curve of the final qPCR amplification (from 65 to 95 °C in 0.5 °C/5 s increments).

The expression differential of each target was calculated according to the $\Delta\Delta Ct$ method. The geometric mean of ribosomal protein subunit 5 (RPS5) and β-actin (Evans, 2006) was used as reference genes. The mean value and standard deviations of each target (Table 1) were normalized using the Ct value corresponding to the geometric mean following the formula: ΔCt = (average Ct_{target}) − (average Ct_{geomean}). The group that had the lowest value was chosen as a calibrator and equaled to 1. The ΔCt value of each group was subtracted by the ΔCt value of the calibrator to yield $\Delta\Delta Ct$. Concentrations of target transcripts among different experimental treatment groups relative to the concentration of geometric mean in honey bee larvae were determined by the following equation: $2^{-\Delta\Delta Ct}$ and expressed as log fold change, with the calibrator being the lowest observed value (Schmittgen & Livak, 2008; Abbo *et al.*, 2017). In our pilot studies, when a reaction showed no transcript detection, we usually assigned a Ct value of 42 cycles as the lowest detected, as it seems preferable to conclude that the transcript level was below detection limits rather than absent (Simone *et al.*, 2009; Cornman *et al.*, 2013; Chaimanee *et al.*, 2016).

Normalization of the real-time data and statistical analysis

Normality and homogeneity of variances of the data were checked using SPSS v17. The variation in pathogen transcript levels between different groups was analyzed by one-way ANOVA, where the means were compared by a Tukey-HSD test. Antimicrobial peptide transcripts were compared amongst each treatment and control group by Mann–Whitney U test using SPSS v17. P values below 0.05 were considered significant. Data are presented for overall mean transcript levels across all trials. Gene expression was calculated as $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001) and in every sample was normalized to two endogenous controls (RPS5 and β-actin).



Honey bee species	Comparisons of survival curve (Log rank test)		
	χ^2	df	*P*-value
(A) ***A. mellifera***	175.63	4	<0.0001
(B) ***A. cerana***	392.64	4	<0.0001

Fig. 1 Kaplan–Meier survival curves of honey bee larvae after inoculation with various spore numbers of *Paenibacillus larvae* (100, 500, 1000, and 2000 spores/mL diet) and controls in (A) *A. mellifera* and (B) *A. cerana*.

Results

Survival, larval mortality of honey bee larvae during exposed to P. larvae and LC_{50} analysis

Comparing survival curves was of particular interest for the experimental trials. Across five survival curves each for *A. mellifera* (Fig. 1A), and *A. cerana* (Fig. 1B), the probability of surviving of differed significantly across treatment groups (Kaplan–Meier Log-Rank test, $\chi^2 = 175.83$; $P < 0.0001$; $\chi^2 = 392.64$; $P < 0.0001$, for *A. mellifera* and *A. cerana*, respectively). The curve was determined by survival until 11 d in both species. With the same concentration of *P. larvae*, *A. cerana* larvae inoculated with *P. larvae* died more rapidly compared to *A. mellifera*.

For *A. mellifera*, results showed that survival rates of larvae infected with *P. larvae* inoculated at different concentrations were lower than in control larvae at the same time point (80.4% cumulative survival in the control group). In treatment groups, larvae inoculated with *P. larvae* at the endpoint of 11 d with tested concentration 100, 500, 1000, and 2000 spore/mL, showed survival of 60.47%, 12.50%, 8.70%, and 0%, respectively. Survival rates at different time points were obtained and compared between curves in the trials. All cumulative survival curves among treated and control larvae of *A. mellifera* showed significant differences in larval mortality (all P values; $P \leq 0.0125$; Table S3), except 500 versus 1000 spores/mL ($\chi^2 = 2.48$; $P = 0.1150$).

For *A. cerana*, the cumulative survival of control group (71.12%) was lower than that of control groups of *A. mellifera* larvae (80.4%). Additionally, the results showed that survival rates of larvae infected with *P. larvae* inoculated at different concentrations were lower than in control larvae the same time point. In treatment groups, larvae inoculated with *P. larvae* with varied concentrations of 100, 500, 1000, and 2000 spores/mL at day 7 showed cumulative survival of 53.33%, 8.89%, 4.44%, and 0%, respectively. All cumulative survival curves among treated and control larvae of *A. cerana* showed significant differences in larvae mortality (all P values; $P \leq 0.0047$; Table S4). The highest rate of mortality was recorded at days 9 and 5 of treatments for *A. mellifera* and *A. cerana*, respectively (Fig. 1). However, at the end point with a tested concentration of 2000 spores/mL, all infected larvae were dead within 11 d and 7 d postinoculation, for *A. mellifera* and *A. cerana*, respectively.

The total mortality of honey bee larvae of *A. mellifera* and *A. cerana* treated with *P. larvae* was calculated across all five concentrations, 0 (control), 100, 500, 1000, and 2000 spores/mL. The effects of *P. larvae* progressively increased with the time duration of the treatment groups. Peak mortality was observed in the ninth and fifth day of treatment. In the control groups, larvae also showed more than 10% cumulative mortality during the observation

Table 2 Results of the Probit analysis based on the mortality of honey bees larvae after exposure to *Paenibacillus larvae*.

Statistical parameters	Honey bee species	
	Apis mellifera[†]	*Apis cerana*[‡]
Probit parameters		
Regression equation	$y = 1.520 \log x - 3.828$	$y = 1.889 \log x - 4.879$
Slope ± SEM	1.520 ± 0.153	1.889 ± 0.168
95% confidence interval	1.220	1.561
	1.820	2.218
Intercept ± SEM	−3.828 ± 0.420	−4.879 ± 0.462
95% CI	−4.248	−5.341
	−3.408	−4.417
LC_{50}[§]	329.752	382.120
95% Confidence limit of dose	257.148	313.763
	408.146	456.167
Pearson goodness-of-fit		
χ^2	2.776	3.539
Degree of freedom	2	2
P value	0.250	0.170

[†]Observed at 9 d postinoculation due to the highest mortality rate.
[‡]Observed at 5 d postinoculation due to the highest mortality rate.
[§]Probit probability = 0.50.

period, therefore, corrected mortality was applied in both *A. mellifera* and *A. cerana* (Tables S1 and S2).

The mortality rates and Probit analysis are shown in Table 2 and Figure S1. The result showed regression equations for both honey bee species; $y = 1.520 \log x - 3.828$ (*A. mellifera*: $\chi^2 = 2.776$, $P = 0.250$) and $y = 1.889 \log x - 4.879$ (*A. cerana*: $\chi^2 = 3.539$, $P = 0.170$). Results of *in vitro* bioassays showed the estimate of *P. larvae*'s LC_{50} and the 95% confidence interval, *A. mellifera* = 329.752 (257.148, 408.146), *A. cerana* = 382.120 (313.763, 456.167), the slope ± standard deviation was found to be 1.520 ± 0.153, and 1.889 ± 0.168 for *A. mellifera* and *A. cerana*, respectively. Comparison of LC_{50} of two species was done, the result showed no significant difference between *A. mellifera* and *A. cerana* ($\chi^2 = 8.803$; $P = 0.117$; Fig. S1).

P. larvae transcript levels in A. mellifera and A. cerana larval bioassays

To confirm the LC_{50} dose for *P. larvae*, the temporal dynamics of the transcripts in larvae of both honey bee species were analyzed using the qPCR technique for 0, 6, 12, 24, 36, and 48 h as shown in Figure 2. Two transcripts were used to assess pathogen levels, a spore germination protein (PlGermSA) and the flagella biosynthesis protein (PlFLiP). The results showed that there were no pathogen transcript-level signals that occurred in any

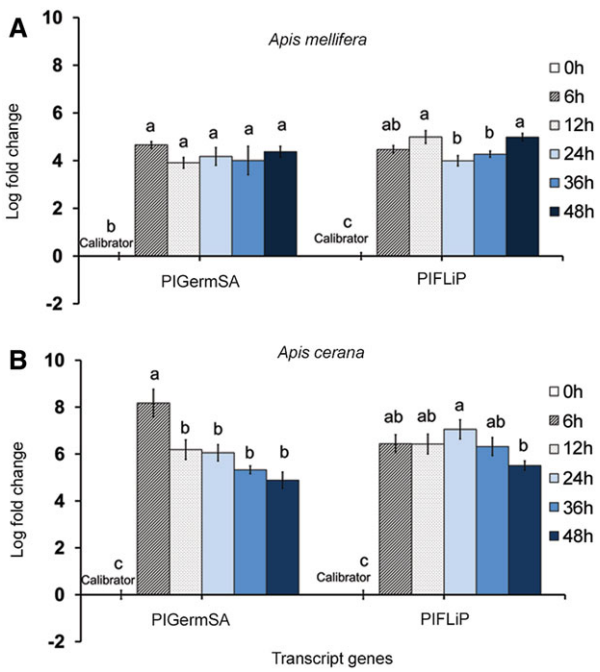


Fig. 2 Expression levels for larvae exposed to *Paenibacillus larvae* for the two transcript genes (PlGermSA and PlFLiP) after inoculation at 6, 12, 24, 36, and 48 h postinoculation (mean ± SEM) of (A) *A. mellifera* and (B) *A. cerana.* Vertical bars with different letters indicated significantly higher transcript levels of *P. larvae* by a Tukey–HSD test at $P < 0.05$.

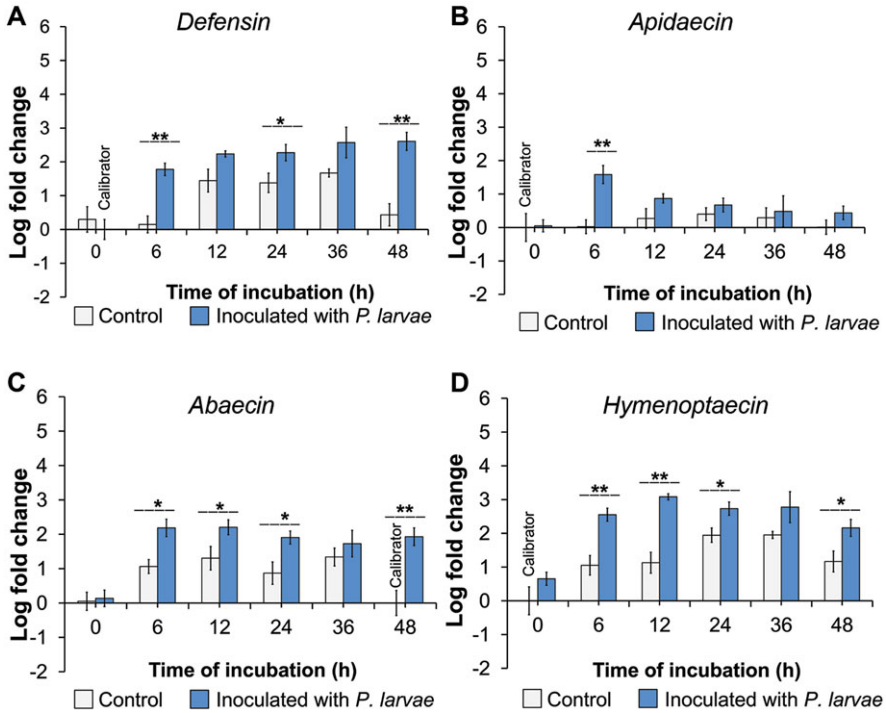


Fig. 3 Quantitative analysis of immune transcripts of *A. mellifera* larvae at time intervals after incubation with an estimated median lethal concentration (LC_{50}) of *Paenibacillus larvae*. Relative fold changes were obtained using the $\Delta\Delta Ct$ method. Gene expression in every pilot has been normalized to two endogenous controls (RPS5 and β-actin). Calibrators were showed in the bar with the lowest expression level. Vertical bars represent means ± SEM. For each group, bars with Asterisks (*) are significant differences between control and inoculated with *P. larvae* (*$P < 0.05$; **$P < 0.01$; Mann–Whitney *U*).

control groups. The signals were also not expressed in any trials at an initial time (0 h) of incubation. *P. larvae* transcripts were firstly detected in larvae 6 h postinoculation.

In addition, pathogen gene expression levels were higher in *A. cerana* larvae, than in *A. mellifera* larvae. The PlGermSA and PlFLiP transcript levels of *P. larvae* from *A. mellifera* larvae were significantly increased more than 4 folds at 6, 12, 24, 36, and 48 h (ANOVA, PlGermSA, $F = 30.456$; $P < 0.0001$; PlFLiP, $F = 59.90$; $P < 0.0001$; Fig. 2A). Meanwhile, the two transcript levels of *P. larvae* from *A. cerana* larvae were significantly increased more than 6 fold at 6, 12, 24, 36, and 48 h (ANOVA, PlGermSA, $F = 40.03$; $P < 0.0001$; PlFLiP, $F = 32.08$; $P < 0.0001$; Fig. 2B).

Effects of P. larvae inoculation on antimicrobial peptide gene expressions in A. mellifera and A. cerana

The mRNA levels of four AMP genes were determined from honey bee larvae fed with *P. larvae* (LC_{50} value) after 6, 12, 24, 36, and 48 h infection. The expression levels of antimicrobial peptides were compared among different treatment groups at the initial time of incubation (0 h). The results demonstrated that gene expression patterns of all immune transcripts in both *A. mellifera* and *A. cerana* larvae showed similar trends between experimental trials at each time point. In *A. mellifera* larvae, mRNA levels of *defensin* were significantly increased in when compared to control larvae at 6, 24, and 48 h of inoculation (Mann–Whitney *U*, $P = 0.003$; $P = 0.037$; $P = 0.004$, for incubation time respectively; Fig. 3A). The expression of *apidaecin* did not change significantly in treated larvae when compared to control larvae. However, at 6 h after inoculation, *apidaecin* expression showed a significant increase after 6 h incubation time (Fig. 3B). Transcript levels of *abaecin* were particularly high in response to infection at 6, 12, 24, and 48 h after inoculation when compared to the controls (Mann–Whitney *U*, $P = 0.014$; $P = 0.037$; $P = 0.037$; $P = 0.004$, for incubation time, respectively; Fig. 3C). *Hymenoptaecin* gene expression was also significantly higher in larvae inoculated with *P. larvae* at 6, 12, 24, and 48 h after inoculation (Mann–Whitney *U*, $P = 0.007$; $P = 0.004$; $P = 0.037$; $P = 0.038$, for incubation time respectively; Fig. 3D). For *A. cerana*, *defensin* gene expression was significantly higher

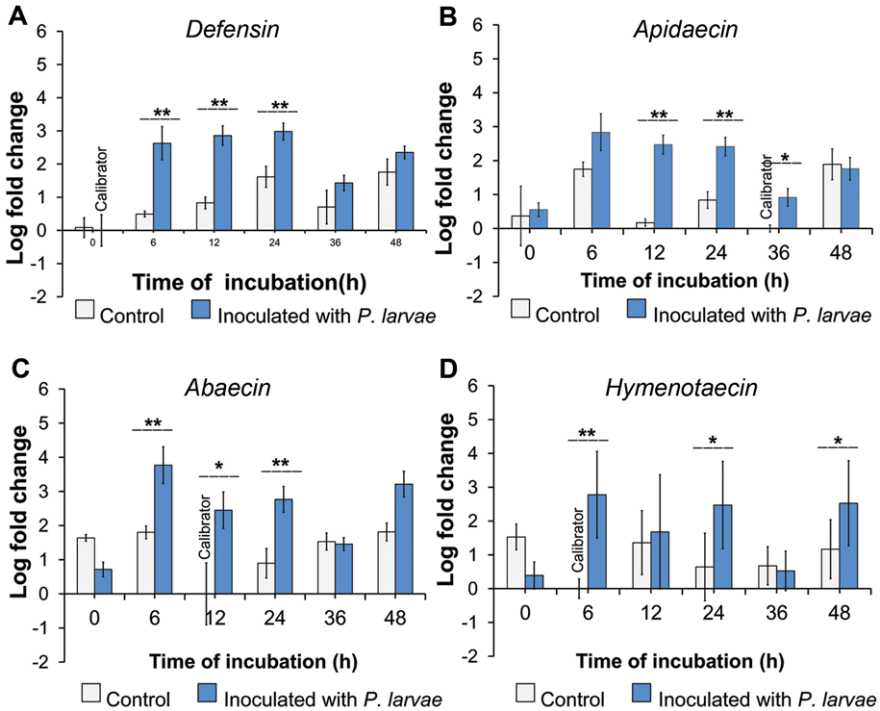


Fig. 4 Quantitative analysis of immune transcripts of *A. cerana* larvae at time intervals after incubated with estimated median lethal concentration (LC_{50}) of *Paenibacillus larvae*. Relative fold changes were obtained using the $\Delta\Delta Ct$ method. Gene expression in every pilot has been normalized to two endogenous controls (RPS5 and β-actin). Calibrators were showed in the bar with the lowest expression level. Vertical bars represent means ± SEM. For each group, bars with asterisks (*) are significantly different between control and inoculated larvae (*$P < 0.05$; **$P < 0.01$; Mann–Whitney *U*).

in treated larvae as compared to control larvae at 6, 12, and 24 h of incubation (Mann–Whitney *U*, $P = 0.004$; $P = 0.001$; $P = 0.010$, for incubation time respectively; Fig. 4A). *Apidaecin* was also upregulated in response to *P. larvae* inoculation at 6, 24, and 36 h of incubation (Mann–Whitney *U*, $P = 0.001$; $P = 0.010$; $P = 0.017$, for incubation time respectively; Fig. 4B). The gene encoding *abaecin* displayed a significant increase at 6, 12, and 24 h of incubation (Mann–Whitney *U*, $P = 0.010$; $P = 0.039$; $P = 0.010$, for incubation time respectively; Fig. 4C). *Hymenoptaecin* transcript was upregulated significantly at 6, 24, and 48 h after inoculation (Mann–Whitney *U*, $P = 0.002$; $P = 0.038$; $P = 0.018$, for incubation time respectively; Fig. 4D). It was noticeable that expression of all immune genes in *A. cerana* larvae was much higher than that in *A. mellifera* larvae.

Discussion

A. mellifera larvae are vulnerable to *P. larvae* (Lindström & Fries, 2005; de Graaf *et al.*, 2006; Lindström, 2008); however, *P. larvae* infection is rare in the Asian honey bee *A. cerana* (Chen *et al.*, 2000). The pathological effects of *P. larvae* and host susceptibility were previously documented in *A. mellifera* (Lindström *et al.*, 2008; Behrens *et al.*, 2010). In this study, we compared the susceptibility and AMP transcriptional expression for honey bees exposed to *P. larvae* in both honey bee species, *A. mellifera* and *A. cerana*. The first instar larvae were performed for all experiments because this larval age is the most susceptible to infection with *P. larvae* (Brødsgaard *et al.*, 1998; Evans, 2004). The cumulative survival of infected *A. cerana* was lower than that *A. mellifera*, thus we can infer that individual *A. cerana* larvae are more susceptible to *P. larvae* than *A. mellifera* larvae. Additionally, when pathogen infections were confirmed in both species of larvae, prevalence of multiple pathogen infection was found in *A. cerana* more than *A. mellifera*. Our findings indicate that more susceptible host traits lead to more greater infection levels. Therefore, the role of multiple infections may impact epidemiological or evolutionary processes in host species (Fels *et al.*, 2008). However, Chen *et al.* (2000) suggested the larval food of *A. cerana* may have some

effective substances that inhibit the growth of vegetative cells in the larval midgut, leading to AFB resistance in larvae of *A. cerana*. Therefore, alternative factors might explain the susceptibility of honey bee larvae including royal jelly, components of which probably induce honey bee resistance to pathogens (Zhang *et al.*, 2014). The major protein components of both species had different molecular weights, isoelectric points, and immunological characteristics (Takenaka & Takenaka, 1996). Many studies have shown the effect of royalisin against Gram-negative bacteria and some Gram-positive (Ilyasov *et al.*, 2012) including the agent causing AFB (Bíliková *et al.*, 2001; Bachanová *et al.*, 2002; Yoshiyama & Kimura, 2010; Bílikova *et al.*, 2015). In this study, we used royal jelly collected from *A. mellifera* therefore this might affect the AFB resistance of *A. cerana* as it may be somewhat different when compared in nature.

Our result is similar to the previous finding that *A. mellifera* larvae challenged with *P. larvae* showed higher levels of expressions of genes encoding AMPs (Evans, 2004; Evans *et al.*, 2006; Chan *et al.*, 2009; Cornman *et al.*, 2013). However, the immune response in *A. cerana* was not previously reported, therefore our results are the first report to show that AMP transcripts were increased in *A. cerana* after *P. larvae* artificial infection. This also highlights that *A. cerana* had a higher level of constitutive expression of AMPs encoding genes than that of *A. mellifera*. This observation corroborated the findings of Xu *et al.* (2009) who reported that *A. cerana* could produce higher expression levels of immune genes than *A. mellifera* due to the higher diversity of AMPs observed in *A. cerana* (Xu *et al.*, 2009; Danihlík *et al.*, 2015). However, more resistance to infection in individuals may be determined by immune trait selection in honey bees (Evans *et al.*, 2006; Decanini *et al.*, 2007). Pathogen levels and genetic variability across intraspecies/interspecies of host species might affect immune responses to bacterial infection. Yoshiyama and Kimura (2010) could not observe transcript levels of *abaecin* and *defensin2* in *A. cerana japonica* even though the larvae were challenged with numerous of *P. larvae* (100 spores/μL).

Evans and Pettis (2005) suggested that higher immune response may not be worthwhile for the larvae because there was a developmental cost to defend themselves against pathogens and parasites that would decrease the production at the colony level. In addition, increased immune responses might have physiological costs that induced increasing metabolic rate in larval and the effect would lead to decreased antimicrobial activity for pathogen defense (Ardia *et al.*, 2012). Further research is needed to examine whether the increased metabolic activity has a direct influence on AMP activation. Chen *et al.* (2000) also suggest that the resistance of *A. cerana* larvae apparently was not totally linked to their innate immune proficiency toward the invasion of *P. larvae*. Instead, a rapid hygienic response and the larval age stages were contributing to the resistance of *A. cerana*.

Further investigation is needed to clarify the impact of immune responses at individual and colony levels on resistance mechanisms of honey bees and other social insects to disease infection. Immune gene expression can be used as a tool for breeding strategies to select honey bee traits for beekeeping industry in the future.

Acknowledgments

S.K. was supported by The Program of Strategic Scholarships for Frontier Research Network for the Joint Ph.D. Program Thai Doctoral degree from the Office of the Higher Education Commission, Thailand (Grant No. CHE-SW215/2551). This research is supported by Chiang Mai University fund and National Research Council of Thailand (NRCT). We also thank to the Graduate School of Chiang Mai University.

Disclosure

The authors report no conflicts of interest to be declared.

References

Abbo, P.M., Kawasaki, J.K., Hamilton, M., Cook, S.C., DeGrandi-Hoffman, G., Li, W.F. *et al*. (2017) Effects of imidacloprid and *Varroa destructor* on survival and health of European honey bees, *Apis mellifera*. *Insect Science*, 24, 467–477.

Abbott, W.S. (1987) A method of computing the effectiveness of an insecticide. *Journal of the American Mosquito Control Association*, 3, 302–303.

Alippi, A.M. and Aguilar, O.M. (1998) Characterization of isolates of *Paenibacillus larvae* subsp. *larvae* from diverse geographical origin by the polymerase chain reaction and BOX primers. *Journal of Invertebrate Pathology*, 72, 21–27.

Ardia, D.R., Gantz, J.E., Brent, Schneider, B.C. and Strebel, S. (2012) Costs of immunity in insects: an induced immune response increases metabolic rate and decreases antimicrobial activity. *Functional Ecology*, 26, 732–739.

Bachanová, K., Klaudiny, J., Kopernicky, J. and Simúth, J. (2002) Identification of honeybee peptide active against *Paenibacillus larvae larvae* through bacterial growth-inhibition assay on polyacrylamide gel. *Apidologie*, 33, 259–269.

Behrens, D., Forsgren, E., Fries, I. and Moritz, R.F.A. (2010) Lethal infection thresholds of *Paenibacillus larvae* for honeybee drone and worker larvae (*Apis mellifera*). *Environmental Microbiology*, 12, 2838–2845.

Bílikova, K., Huang, S., Lin, I.P., Šimuth, J. and Peng, C. (2015) Structure and antimicrobial activity relationship of royalisin, an antimicrobial peptide from royal jelly of *Apis mellifera*. *Peptides*, 68, 190–196.

Bíliková, K., Wu, G. and Šimúth, J. (2001) Isolation of a peptide fraction from honeybee royal jelly as a potential antifoulbrood factor. *Apidologie*, 32, 275–283.

Brødsgaard, C.J., Ritter, W. and Hansen, H. (1998) Response of in vitro reared honey bee larvae to various doses of *Paenibacillus larvae larvae* spores. *Apidologie*, 29 569–578.

Chaimanee, V. and Chantawannakul, P. (2015) Infectivity of *Nosema ceranae* isolates from different hosts and immune response in honey bees *Apis mellifera* and *Apis cerana*. *Journal of Apicultural Research*, 54, 200–206.

Chaimanee, V., Evans, J.D., Chen, Y., Jackson, C. and Pettis, J.S. (2016) Sperm viability and gene expression in honey bee queens (*Apis mellifera*) following exposure to the neonicotinoid insecticide imidacloprid and the organophosphate acaricide coumaphos. *Journal of Insect Physiology*, 89, 1–8.

Chan, Q., Melathopoulos, A., Pernal, S. and Foster, L. (2009) The innate immune and systemic response in honey bees to a bacterial pathogen, *Paenibacillus larvae. BMC Genomics*, 10, 387.

Chantawannakul, P., de Guzman, L.I., Li, J. and Williams, G.R. (2016) Parasites, pathogens, and pests of honeybees in Asia. *Apidologie*, 47, 301–324.

Chen, Y., Evans, J.D., Zhou, L., Boncristiani, H., Kimura, K., Xiao, T. *et al*. (2009) Asymmetrical coexistence of *Nosema ceranae* and *Nosema apis* in honey bees. *Journal of Invertebrate Pathology*, 101, 204–209.

Chen, Y., Zhao, Y., Hammond, J., Hsu, H., Evans, J. and Feldlaufer, M. (2004) Multiple virus infections in the honey bee and genome divergence of honey bee viruses. *Journal of Invertebrate Pathology*, 87, 84–93.

Chen, Y.W., Wang, C., James, A. and Ho, K. (2000) Susceptibility of the Asian honey bee, *Apis cerana*, to American foulbrood, *Paenibacillus larvae larvae*. *Journal of Apicultural Research*, 39, 169–175.

Cornman, R.S., Lopez, D. and Evans, J.D. (2013) Transcriptional response of honey bee larvae infected with the bacterial pathogen *Paenibacillus larvae*. *PLoS ONE*, 8, e65424.

Crailsheim, K., Brodschneider, R., Aupinel, P., Behrens, D., Genersch, E., Vollmann, J. *et al*. (2013) Standard methods for artificial rearing of *Apis mellifera* larvae. *Journal of Apicultural Research*, 52, 1–15.

Danihlík, J., Aronstein, K. and Petřivalský, M. (2015) Antimicrobial peptides: a key component of honey bee innate immunity: physiology, biochemistry, and chemical ecology. *Journal of Apicultural Research*, 54, 123–136.

de Graaf, D.C., De Vos, P., Heyndrickx, M., van Trappen, S., Peiren, N., Jacobs, F.J. *et al*. (2006) Identification of *Paenibacillus larvae* to the subspecies level: an obstacle for AFB diagnosis. *Journal of Invertebrate Pathology*, 91, 155–123.

De Gregorio, E., Spellman, P.T., Tzou, P., Rubin, G.M. and Lemaitre, B. (2002) The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*. *The EMBO Journal*, 21, 2568–2579.

Decanini, L.I., Collins, A.M. and Evans, J.D. (2007) Variation and heritability in immune gene expression by diseased honeybees. *Journal of Heredity*, 98, 195–201.

Ebeling, J., Knispel, H., Hertlein, G., Fünfhaus, A. and Genersch, E. (2016) Biology of *Paenibacillus larvae*, a deadly pathogen of honey bee larvae. *Applied Microbiology and Biotechnology*, 100, 7387–7395.

Evans, J.D. (2004) Transcriptional immune responses by honey bee larvae during invasion by the bacterial pathogen, *Paenibacillus larvae*. *Journal of Invertebrate Pathology*, 85, 105–111.

Evans, J.D. (2006) Beepath: an ordered quantitative-PCR array for exploring honey bee immunity and disease. *Journal of Invertebrate Pathology*, 93, 135–139.

Evans, J.D., Aronstein, K., Chen, Y.P., Hetru, C., Imler, J.L., Jiang, H. *et al*. (2006) Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect Molecular Biology*, 15, 645–656.

Evans, J.D. and Pettis, J.S. (2005) Colony-level impacts of immune responsiveness in honey bees, *Apis mellifera*. *Evolution*, 59, 2270–2274.

Evans, J.D. and Schwarz, R.S. (2011) Bees brought to their knees: microbes affecting honey bee health. *Trends in Microbiology*, 19, 614–620.

Evans, J.D. and Wheeler, D.E. (1999) Differential gene expression between developing queens and workers in the honey bee, *Apis mellifera*. *Proceedings of the National Academy of Sciences USA*, 96, 5575–5580.

Fels, D., Vignon, M. and Kaltz, O. (2008) Ecological and genetic determinants of multiple infection and aggregation in a microbial host–parasite system. *Parasitology*, 135, 1373–1383.

Finney, D.J. (1971) Probit analysis. *Journal of Pharmaceutical Sciences*, 60, 1432–1432.

Forsgren, E., Wei, S., Guiling, D., Zhiguang, L., van Tran, T., Tang, P.T. *et al*. (2015) Preliminary observations on possible pathogen spill-over from *Apis mellifera* to *Apis cerana*. *Apidologie*, 46, 265–275.

Fourrier, J., Deschamps, M., Droin, L., Alaux, C., Fortini, D., Beslay, D. *et al*. (2015) Larval exposure to the juvenile hormone analog pyriproxyfen disrupts acceptance of and social

behavior performance in adult honeybees. *PLoS ONE*, 10, e0132985.

Gallai, N., Salles, J.M., Settele, J. and Vaissière, B.E. (2009) Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological Economics*, 68, 810–821.

Garcia-Gonzalez, E. and Genersch, E. (2013) Honey bee larval peritrophic matrix degradation during infection with *Paenibacillus larvae*, the aetiological agent of American foulbrood of honey bees, is a key step in pathogenesis. *Environmental Microbiology*, 15, 2894–2901.

Genersch, E. (2010) American foulbrood in honeybees and its causative agent, *Paenibacillus larvae*. *Journal of Invertebrate Pathology*, 103, S10–S19.

Genersch, E., Ashiralieva, A. and Fries, I. (2005) Strain-and genotype-specific differences in virulence of *Paenibacillus larvae* subsp. *larvae*, a bacterial pathogen causing American foulbrood disease in honeybees. *Applied and Environmental Microbiology*, 71, 7551–7555.

Hamiduzzaman, M.M., Guzman-Novoa, E., Goodwin, P.H., Reyes-Quintana, M., Koleoglu, G., Correa-Benítez, A. *et al*. (2015) Differential responses of Africanized and European honey bees (*Apis mellifera*) to viral replication following mechanical transmission or *Varroa destructor* parasitism. *Journal of Invertebrate Pathology*, 126, 12–20.

Hoffmann, J.A. and Reichhart, J.M. (2002) *Drosophila* innate immunity: an evolutionary perspective. *Nature Immunology*, 3, 121–126.

Ilyasov, R., Gaifullina, L., Saltykova, E., Poskryakov, A. and Nikolenko, A. (2012) Review of the expression of antimicrobial peptide defensin in honey bees *Apis mellifera* L. *Journal of Apicultural Science*, 56, 1–9.

Krongdang, S., Evans, J.D., Pettis, J.S. and Chantawannakul, P. (2017) Multilocus sequence typing, biochemical and antibiotic resistance characterizations reveal diversity of North American strains of the honey bee pathogen *Paenibacillus larvae*. *PLoS ONE*, 12, e0176831.

Li, J., Qin, H., Wu, J., Sadd, B.M., Wang, X., Evans, J.D. *et al*. (2012) The prevalence of parasites and pathogens in Asian honeybees *Apis cerana* in China. *PLoS ONE*, 7, e47955.

Lin, Z., Page, P., Li, L., Qin, Y., Zhang, Y., Hu, F. *et al*. (2016) Go east for better honey bee health: *Apis cerana* is faster at hygienic behavior than *A. mellifera*. *PLoS ONE*, 11, e0162647.

Lindström, A. (2008) Distribution of *Paenibacillus larvae* spores among adult honey bees (*Apis mellifera*) and the relationship with clinical symptoms of American foulbrood. *Microbial Ecology*, 56, 253–259.

Lindström, A., Korpela, S. and Fries, I. (2008) The distribution of *Paenibacillus larvae* spores in adult bees and honey and larval mortality, following the addition of American foulbrood diseased brood or spore-contaminated honey in honey bee (*Apis mellifera*) colonies. *Journal of Invertebrate Pathology*, 99, 82–86.

Lindström, A. and Fries, I. (2005) Sampling of adult bees for detection of American foulbrood (*Paenibacillus larvae* subsp. *larvae*) spores in honey bee (*Apis mellifera*) colonies. *Journal of Apicultural Research*, 44, 82–86.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods*, 25, 402–408.

Locke, B., Conte, Y.L., Crauser, D. and Fries, I. (2012) Host adaptations reduce the reproductive success of *Varroa destructor* in two distinct European honey bee populations. *Ecology and Evolution*, 2, 1144–1150.

Mao, W., Schuler, M.A. and Berenbaum, M.R. (2015) A dietary phytochemical alters caste-associated gene expression in honey bees. *Science Advances*, 1, e1500795.

McMenamin, A.J. and Genersch, E. (2015) Honey bee colony losses and associated viruses. *Current Opinion in Insect Science*, 8, 121–129.

Paxton, R.J., Klee, J., Korpela, S. and Fries, I. (2007) *Nosema ceranae* has infected *Apis mellifera* in Europe since at least 1998 and may be more virulent than *Nosema apis*. *Apidologie*, 38, 558–565.

Poppinga, L. and Genersch, E. (2015) Molecular pathogenesis of American foulbrood: how *Paenibacillus larvae* kills honey bee larvae. *Current Opinion in Insect Science*, 10, 29–36.

Rath, W. (1999) Co-adaptation of *Apis cerana* Fabr. and *Varroa jacobsoni* Oud. *Apidologie*, 30, 97–110.

Rembold, H., Lackner, B. and Geistbeck, I. (1974) The chemical basis of honeybee, *Apis mellifera*, caste formation. Partial purification of queen bee determinator from royal jelly. *Journal of Insect Physiology*, 20, 307–314.

Rich, J.T., Neely, J.G., Paniello, R.C., Voelker, C.C.J., Nussenbaum, B. and Wang, E.W. (2010) A practical guide to understanding Kaplan-Meier curves. *Otolaryngology-Head and Neck Surgery*, 143, 331–336.

Schmittgen, T.D. and Livak, K.J. (2008) Analyzing real-time PCR data by the comparative C_T method. *Nature Protocols*, 3, 1101–1108.

Simone, M., Evans, J.D. and Spivak, M. (2009) Resin collection and social immunity in honey bees. *Evolution*, 63, 3016–3022.

Takenaka, T. and Takenaka, Y. (1996) Royal jelly from *Apis cerana japonica* and *Apis mellifera. Bioscience*, *Biotechnology and Biochemistry*, 60, 518–520.

Tehel, A., Brown, M.J. and Paxton, R.J. (2016) Impact of managed honey bee viruses on wild bees. *Current Opinion in Virology*, 19, 16–22.

Vandenberg, J.D. and Shimanuki, H. (1987) Technique for rearing worker honeybees in the laboratory. *Journal of Apicultural Research*, 26, 90–97.

vanEngelsdorp, D. and Meixner, M.D. (2010) A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. *Journal of Invertebrate Pathology*, 103, S80–S95.

Wilson, M.B., Brinkman, D., Spivak, M., Gardner, G. and Cohen, J.D. (2015) Regional variation in composition and antimicrobial activity of US propolis against *Paenibacillus larvae* and *Ascosphaera apis*. *Journal of Invertebrate Pathology*, 124, 44–50.

Xu, P., Shi, M. and Chen, X.X. (2009) Antimicrobial peptide evolution in the Asiatic honey bee *Apis cerana*. *PLoS ONE*, 4, e4239.

Yañez, O., Zheng, H., Su, X., Hu, F., Neumann, P. and Dietemann, V. (2015) Potential for virus transfer between the honey bees *Apis mellifera* and *A. cerana*. *Journal of Apicultural Research*, 54, 179–191.

Yoshiyama, M. and Kimura, K. (2010) Characterization of antimicrobial peptide genes from Japanese honeybee *Apis cerana japonica* (Hymenoptera: Apidae). *Applied Entomology and Zoology*, 45, 609–614.

Zhang, Y., Zhang, G.Z., Huang, X. and Han, R.C. (2014) Proteomic analysis of *Apis cerana* and *Apis mellifera* larvae fed with heterospecific royal jelly and by CSBV challenge. *PLoS ONE*, 9, e102663.

Manuscript received September 19, 2017
Final version received November 29, 2017
Accepted March 1, 2018

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Comparison of Probit regression plot of two honey bee species after postinoculation (p.i.) with *P. larvae* (A) *A. mellifera* after days 9 p.i. and (B) *A. cerana* at days 5 p.i. (*A. mellifera* $R^2 = 0.9890$, *A. cerana* $R^2 = 0.9980$) ($\chi^2 = 8.803$; df = 5; $P = 0.117$).

Table S1. Percentage of dead larvae/days of treatment of *A. mellifera* when inoculation with *P. larvae* at different doses.

Table S2. Percentage of dead larvae/days of treatment of *A. cerana* when inoculation with *P. larvae* at different doses.

Table S3. Statistical analysis of spore concentration that compared for each pairs used *in vitro* bioassay for *A. mellifera* larvae.

Table S4. Statistical analysis of spore concentration that compared for each pairs used *in vitro* bioassay for *A. cerana* larvae.