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Responses of *Varroa*-resistant honey bees (*Apis mellifera* L.) to *Deformed wing virus*

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ABSTRACT

The negative impact of *Deformed wing virus* (DWV) on European honey bees *Apis mellifera* is magnified by *Varroa destructor* parasitism. This study compared the responses of two *Varroa*-resistant honey bee stocks, pure Russian honey bees (RHB) and out-crossed *Varroa* Sensitive Hygienic bees, Pol-line (POL) to DWV infection to that of *Varroa*- susceptible stock, Italian honey bees (IHB). Two-day-old larvae were fed with DWV lysate in different concentrations: undiluted DWV lysate (D1), D1:100, and D1:1000. The unfed larvae served as negative control. Combs containing test larvae were exposed to a common environment during their development using host colonies. Our results showed that only POL displayed variation in DWV levels when fed different DWV concentrations. POL fed highest concentration of DWV inoculum had the highest increase in DWV level than those fed low concentrations and unfed POL. This high increase in DWV level probably contributed to the decrease in the survival and median longevity (LT_{50}) of D1-fed POL. Weights of newly eclosed D1-fed POL were similar to those of the two controls and DWV-fed bees. However, within IHB, D1-fed bees showed significant reductions in weight, days of survival and LT_{50}. Regardless of the concentrations of DWV inoculum, the DWV levels were similarly low within RHB; adult bees had similar weights. Overall, larvae fed D1 had the highest rate of wing deformation. POL and RHB had numerically lower proportions of bees with deformed wings. This study suggests that RHB showed some degree of resistant to DWV as shown by no reduction on weight and numerically lower proportion of wing deformity when compared with the other bee stocks.
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Introduction

The health of honey bees, *Apis mellifera,* continues to decline due to a myriad of factors, including pathogens and parasitic mites. Among the many pathogens that infect honey bees, viruses are considered to be a major threat to honey bee colonies (Ball and Bailey, 1997; Potts et al., 2010). At least 24 viruses have been identified and characterized from the honey bees (Allen and Ball, 1996; de Miranda et al., 2011). Of them, the most common viruses are *Acute bee paralysis virus* (ABPV), *Black queen cell virus* (BQCV), *Chronic bee paralysis virus* (CBPV), *Deformed wing virus* (DWV), *Kashmir bee virus* (KBV), *Sacbrood virus* (SBV) and *Israeli acute paralysis virus* (IAPV). Infections by IAPV and DWV are thought to be associated with Colony Collapse Disorder (CCD) and the death of millions of colonies worldwide, respectively (Cox-Foster et al., 2007; Martin et al., 2012). DWV is the most common virus that has been detected in the European and Asian honey bees (*A. cerana*, *A. florea* and *A. dorsata*)(Allen and Ball, 1996; Tentcheva et al., 2004; Ellis and Munn, 2005; Berényi et al., 2006; Sanpa and Chantawannakul, 2009). It is a single-stranded, positive-sense RNA virus belonging to the family *Iflaviridae* (Lanzi et al., 2006). This virus infects all stages of honey bees (Bailey and Ball, 1991; Chen et al., 2006) and can be transmitted vertically and horizontally (Chen et al., 2005; Mockel et al., 2011).

In general, DWV infection shows the characteristic symptom of wing deformity in newly emerged bees and also death of individual bees and possibly colony collapse (Berényi et al., 2006; Highfield et al., 2009; Dainat et al., 2012a, 2012b). However, most DWV-infected bees do not show these visible symptoms (Bailey and Ball, 1991; Chen et al., 2005; Fievet et al., 2006; Lanzi et al., 2006; de Miranda and Fries, 2008). For these normal-looking infected bees, sucrose responsiveness and

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associative olfactory function may be impaired (Iqbal and Mueller, 2007). In addition to wing deformities, longevity of adults is significantly reduced when bees are fed DWV during their larval stage (Desai et al., 2012).

Mite parasitism remains the most serious problem for *A. mellifera* worldwide (Dietemann et al., 2012). While the mites deplete nutrients essential for bees' development, they also inject virus particles in the bee's haemolymph. DWV has been detected in bees especially those parasitized with *Varroa* mites and with deformed wings (Yang, 2004; Shen et al., 2005; Yue and Genersch, 2005). Hence, the impact of DWV infection on honey bees is magnified by mite infestations (Ball and Bailey, 1997). Both *Varroa* and *Tropilaelaps* are known vectors of DWV (Yue and Genersch, 2005; Dainat et al., 2009; de Miranda and Genersch, 2010; Khongphinitbunjong et al., 2015b). This feeding activity may also stimulate replication of naturally-occurring viruses (de Miranda and Genersch, 2010). Virus replication in the mites is linked to wing abnormalities in infested honey bees (Gisder et al., 2009). *Varroa* mites suppress immune response of honey bees, which may also permit the activation of latent viral infection (Shen et al., 2005; Khongphinitbunjong et al., 2015a). This reduction in the immunosuppression leads to the reduced life span of adult bees (Yang and Cox-foster, 2007). Honey bees vary in their ability to defend themselves against *V. destructor* infestation. Honey bee stocks resistant to *Varroa* have been developed in the United States and Europe (Spivak and Reuter, 2001; Büchler et al., 2010; Rinderer et al., 2010; Locke et al., 2012). The USDA Bee Lab in Baton Rouge, LA developed two *Varroa*-resistant bees, Russian honey bees (RHB) and *Varroa* Sensitive Hygienic (VSH) bees, and are commercially available. These stocks are known for their hygienic behavior towards *Varroa*-infested and freeze-killed brood (de Guzman et al., 2002; Danka et al., 2013; Kirrane et al., 2015). Pol-line, which is a VSH outcross, have useful mite resistance and beekeeping characteristics (Danka et al., 2012, 2016). In this study, we asked the question whether these varroa-resistant stocks also display resistance or tolerance to the infection of DWV. To answerthis question, the levels of DWV, weight of worker honey bees at emergence, proportion of bees with deformed wings, days of survival, and the longevity (LT_{50}) of adult bees that were fed DWV during their larval stages were compared.

Materials and methods

Preparation of Deformed wing virus lysate

DWV lysate was obtained by collecting worker honey bees with wing deformities from one colony that was highly infested with *Varroa* mites. Ten bees were then ground in 10 ml of phosphate buffered saline (PBS, pH 7) (Berényi et al., 2006). The supernatant was centrifuged at 5000 g for 10 min at 4 °C, and filtered with a 0.22 μm diameter filter. The serial lysate were diluted with PBS to obtain the concentrations (DWV: PBS) of 1:100 and 1:1000 and then frozen at −80 °C until used. The level of DWV and absence of other honey bee virus infections including ABPV, BQCV, KBV, and IAPV in the lysate were confirmed using qualitative RT-PCR as described by Chen et al. (2005).

Experimental design

The two *Varroa*-resistant stocks, VSH outcross Pol-line (POL, $n = 4$ colonies)) and Russian honey bees (RHB, n = 4 colonies) were compared to Italian honey bees (IHB, n = 4 colonies) known for their susceptibility to varroa mites (de Guzman et al., 2007). For each trial, queens (one colony per stock) were separately caged using push-in cages (12.5 cm × 25 cm) to obtain brood of uniform age. Gamma-irradiated combs were used for egg-laying since this method has been used to sanitize beekeeping equipment to control American foulbrood (AFB) and inactivate viruses (Sullivan et al., 1971; Hornitzky and Wills, 1993). After 24 h, all test combs were placed in a single host colony which had a low level of *Varroa* infestation in the brood cell (2.5 ± 0.29%). This procedure allowed uniform environmental exposure of all larvae of the three honey bee stocks. Four trials were employed.

DWV can be orally transmitted to developing larvae through trophallaxis between adult workers and larvae (DWV lysate) (Yue and Genersch, 2005; Iqbal and Mueller, 2007; Mockel et al., 2011). We used a feeding technique, which mimicked the oral acquisition of virus via larval food without the compounding effect of *Varroa* parasitism. When larvae were two days old, a test section of brood comprising 12 rows with 50 brood cells per row was created. Each row of larvae randomly received one of five treatments: a) without feeding (negative control), b) fed PBS only (negative control), c) fed DWV lysate without dilution (D1), d) fed DWV lysate in 100 dilution of PBS (D1:100), and e) fed DWV lysate in 1000 dilution of PBS (D1:1000). Each row of larvae was fed 2 μl of feed treatment lysate. Two rows were assigned for each treatment. Thereafter, the test brood frame (one for each stock) was incubated at 34 °C, 60% relative humidity (RH) for 2 h and returned to the host colony. Keeping the fed larvae in the incubator for 2- h allowed fed larvae to ingest the DWV lysate. Three host colonies consisting of three medium boxes (16.8 cm deep) were used. To prevent further brood removal by host bees, test frames were placed in an incubator when the test brood was capped (L6). At this time, the number of sealed brood was counted to determine brood removal. When the bees were about to emerge (19–20 days old), individual pupa was examined for the presence or absence of *Varroa* and placed in a 0.75 ml microcentrifuge tube for emergence in an incubator. Each microcentrifuge tube had a small hole in the cap for ventilation (Khongphinitbunjong et al., submitted for publication). This technique ensured that our test bees were *Varroa*-free, the few *Varroa*-infested pupae we found were excluded. A subsample of the brown-bodied pupae ($n = 8$ per colony per stock) was analyzed to determine the presence and level of honey bee viruses at this stage before they emerged as adults.

When the bees emerged, each bee was weighed and kept in with other bees of the same treatment in plastic cages to monitor longevity. Each cage was provided sugar syrup (1 water: 1 sucrose), water and had a small piece of bee comb as described by Kirrane et al. (2012). One cage was used per treatment per colony per stock (one colony represented one replicate). All cages were placed in an incubator at 34 °C and 60% RH. Dead bees were removed and the information recorded until the completion of the experiment.

Molecular analyses

A total of 144 pupae (IHB = 48, POL = 48, RHB = 48) were analyzed for this study. Total RNA was extracted from individual bees using the Maxwell® nucleotide purification system with the LEV simply RNA tissue kit (AS1280) (Promega, WI). The concentration (ng/μl) and purity (A260/280) of total RNA was determined using a spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE). Reverse transcription reactions for cDNA synthesis were performed using a QuantiTect Reverse Transcription Kit (Qiagen Inc., Valencia, CA). A spike in exogenous RNA was added into the RNA reaction to act as an external control (Alien Reference RNA RT-qPCR Detection Kit Agilent Technologies Inc., Santa Clara, CA). Double-Stranded cDNA was synthesized with 600 ng poly-A RNA template. The presence of honey bee viruses was determined by qPCR using CFX96TM Real-Time PCR (BioRad, Inc.) with virus specific primers (Table 1). Amplification was performed in 10 μl reaction volumes using SsoFast EvaGreen (BioRad), consisting of 4 μl SSo SYBR mix, 0.5 μl of 10 μM of each primer, 4 μl of nuclease free water and 1 μl cDNA. Reactions were run at 95 °C for 30 s, 40 cycles of 95 °C for 1 s, 59 °C for 5 s followed by a melt-curve dissociation analysis. All reactions included three technical replicates. The qPCR data were expressed as the threshold cycle (Ct) value and were determined by normalizing the Ct value of the geometric averaging of multiple reference genes (*β-actin* and *the exogenous Alien control*) to the target genes (ΔCt). To compare viral levels across treatments, the qPCR data were interpreted using the $2^{-\Delta\Delta Ct\ method}$ (Livak and Schnittgen, 2001; Chen et al., 2005;

Table 1
Sequences of specific virus oligonucleotide primers used for Real-Time Quantitative PCR.

Primer	Pathway/Target	Forward	Reverse	Reference/gene ID
β-actin	House keeping	TTGTATGCCAACACTGTCCTTT	TGGCGCGATGATCTTAATTT	Simone and Evans (2009) (GB17681)
DWV	Deformed wing virus (DWV)	GAGATTGAAGCGCATGAACA	TGAATTCAGTGTCGCCCATA	Boncristiani et al. (2013) (AY292384.1)
BQCV	Black queen cell virus (BQCV)	TTTAGAGCGAATTCGGAAACA	GGCGTACCGATAAAGATGGA	Boncristiani et al. (2013) (HQ655494.1)
IAPV	Israeli acute paralysis virus (IAPV)	GCGGAGAATATAAGGCTCAG	CTTGCAAGATAAGAAAGGGGG	Boncristiani et al. (2013) (EF219380.1)
KBV	Kashmir bee virus (KBV)	TGAACGTCGACCTATTGAAAAA	TCGATTTTCCATCAAATGAGC	Boncristiani et al., 2013 (AY275710.1)

Chaimanee et al., 2012; Boncristiani et al., 2013). The treatment group with the lowest viral level was used as the calibrator and the levels of viruses in all other groups were expressed as n-fold differences relative to the calibrator (Chen et al., 2005).

Statistical analyses

Before analyses, arcsine square-root transformation was used to transform data on the proportions of brood removed by the host colonies and bees with deformed wings. A square-root transformation was used to transform data on the weight of newly emerged bees and bee longevity (average number of days a bee survived) to approximate normality. The LT_{50} or median longevity (the day when 50% of the test bees died) for each cage was also calculated and compared using the NOTCH option in PROC BOXPLOT to determine differences in bee longevity among the different concentration of DWV inoculum within each honey bee stock (SAS version 9.4). The medians were considered significantly different when notches did not overlap. Only bees without DWV clinical symptoms were used for the analysis of adult weights. Bees that were alive at emergence with or without some form of wing abnormality were included in the survival and median longevity (LT_{50}) analyses. Data were subjected to a two-way analysis of variance (ANOVA) with honey bee stock and concentration of DWV inoculum fixed effects using SPSS (version 17). A survival analysis was done separately for each stock using PROC LIFETEST and differences in survival curves for each treatment level were determined with multiple comparison tests on the Wilcoxon Rank test with a Tukey adjustment. Levels of DWV were analyzed separately using a one-way analysis of variance (ANOVA) (SAS JMP). Where differences were found, means were compared using a Tukey-HSD with a 95% confidence.

Results

Proportion of brood fed different concentrations of DWV inoculum that were removed by host colonies

Overall, about 30% of the test brood (L2-L6) was removed by the host colonies regardless of the genetic background of the test brood ($F = 1.135$, $df = 2$, $\rho = 0.324$) or concentration of DWV inoculum fed to test larvae ($F = 0.037$, $df = 4$, $\rho = 0.99$). No two-way interaction was detected ($F = 0.148$, $df = 8$, $\rho = 0.99$).

Viral levels of pupae of three honey bee stocks fed different concentrations of DWV inoculum during their larval stage

Our qPCR results confirmed that there was a high level of DWV (Ct = 14.01 ± 0.01) in the lysate used to feed 2-day old larvae for this study. Low levels of ABPV (Ct = 36.84 ± 0.09), BQCV (Ct = 34.81 ± 0.05) and KBV (Ct = 36.19 ± 0.02) were also detected.

Fourteen days after feeding larvae DWV, analysis showed no significant effect of honey bee stock on the levels of DWV in pupae ($F = 2.60$, $df = 2$, $\rho = 0.074$). However, a significant interaction between honey bee stock and concentration of DWV inoculum was observed ($F = 2.48$, $df = 8$, $\rho = 0.017$). The levels of DWV varied among the bees fed different concentrations of DWV inoculum within POL ($F = 4.98$, $df = 4$, $\rho = 0.003$) (Fig. 1). POL bees fed D1 (undiluted DWV lysate) supported the highest increase in DWV level ($\approx 10^4$ fold) but was similar to that of bees fed PBS only (10^3 fold increase). The levels of DWV in POL fed low concentrations of DWV inoculum increased only 10× but was comparable to that of PBS-fed bees. Within IHB ($F = 2.62$, $df = 4$, $\rho = 0.052$) and RHB ($F = 5.66$, $df = 4$, $\rho = 0.148$), the levels of DWV among bees fed different concentrations of DWV inoculum were similar (about 100 fold increase for IHB and 10–100 fold increase for RHB).

Proportion of bees with deformed wings for three stocks fed different concentrations of DWV inoculum

For the proportion of bees that showed clinical symptoms, no interaction between honey bee stock and DWV concentration ($F = 0.19$, $df = 8$, $\rho = 0.991$) and honey bee stock effect ($F = 2.64$, $df = 2$, $\rho = 0.082$) were observed (Fig. 2). However, a significant effect of concentrations of DWV inoculum was detected ($F = 3.11$, $df = 4$, $\rho = 0.024$). The proportion of deformed bees numerically increased with an increasing concentration of DWV inoculum. Pupae fed D1 (undiluted lysate) as larvae eclosed with substantial wing deformity but was comparable to those of bees fed PBS and lower concentrations of DWV ($F = 3.19$, $df = 5$, $\rho = 0.009$). The lowest proportion of bees with deformed wings were observed in the control (unfed bees) but was similar to the rates recorded in bees fed PBS only and lower concentrations of DWV inoculum.

Weights of newly emerged worker bees of three honey bee stocks fed different concentrations of DWV inoculum during their larval stage

The weights of newly emerged bees (without wing deformity) were compared. ANOVA revealed a significant interaction between honey bee stock and concentration of DWV inoculum ($F = 5.67$, $df = 8$, $\rho < 0.0001$). Hence, honey bee stocks were analyzed separately. Overall, all test bees weighed over 110 mg. Within IHB, the unfed bees (control) were the heaviest (Fig. 3). However, these bees were as heavy as those bees fed PBS and D1:100. A significant weight reduction was observed in bees fed undiluted DWV lysate (D1) but was comparable to those fed D1:100. Weights of bees that were fed PBS and lower concentrations of DWV inoculum were similar. Within POL, bees fed D1:100 were the heaviest but was similar to those fed PBS, D1, and D1:1000. Unfed bees were the lightest but not different from those of PBS, D1 and D1:1000 bees. Weights of newly emerged RHB were similar regardless of treatment groups. Overall, newly emerged workers of IHB (117.04 ± 0.25 mg) and POL (117.23 ± 0.3 mg) were heavier than RHB (112.23 ± 0.25 mg) ($F = 110.02$, $df = 2$, $\rho < 0.0001$). Feeding a high concentration of DWV inoculum (D1 = 114.42 ± 0.36 mg) significantly reduced weights of newly emerged bees than the control groups (unfed = 116.1 ± 0.3, PBS = 115.6 ± 0. 35 mg) and lower concentrations of DWV inoculum (D1:100 = 115.33 ± 0.35, D1:1000 = 116.07 ± 0.35 mg) ($F = 3.55$, $df = 4$, $\rho = 0.007$).

Survival and median longevity of adult workers of three honey bee stocks fed different concentrations of DWV inoculum during their larval stage

Because a significant two-way interaction was detected ($F = 11.91$, $df = 8$, $\rho < 0.0001$), the bee survival (number of days until a bee died) of bees fed different concentrations of DWV inoculum was analyzed for

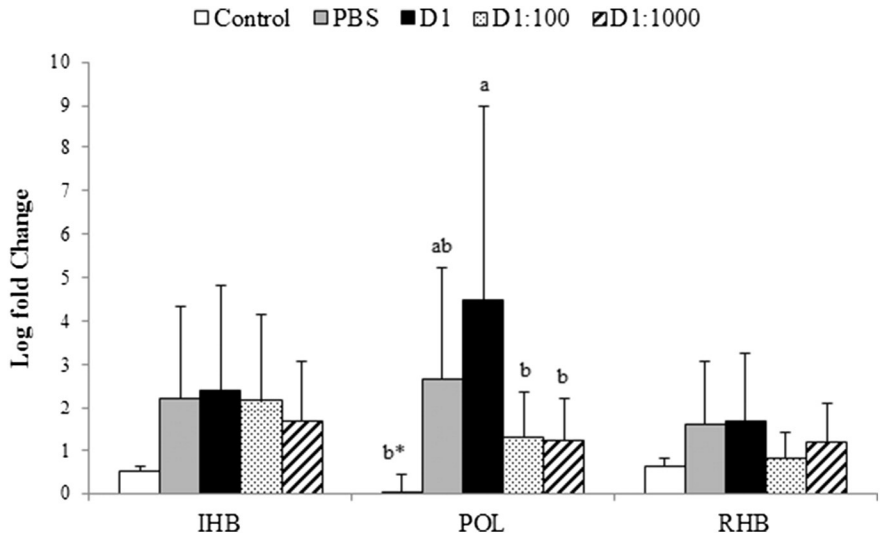

Fig. 1. Log fold change of DWV levels in pupae of three honey bee stocks fed different concentrations of DWV inoculum during their larval stage. For each honey bee stock, bars with different letters are significantly different ($P < 0.001$). IHB = Italian honey bees, POL = VSH outcross, RHB = Russian honey bees. PBS = larvae fed PBS only, D1 = larvae fed undiluted DWV lysate, D1:100 = larvae fed DWV lysate in 100 dilution of PBS, D 1:1000 = larvae fed DWV lysate in 1000 dilution of PBS. *Calibrator with the lowest DWV level.

each honey bee stock. Within IHB, the survival of adult bees varied among concentrations of DWV inoculum. Both control groups (unfed and those fed PBS) survived the longest ($F = 6.21$, $df = 4$, $\rho < 0.0001$) (Fig. 4). However, survival for bees fed PBS was similar to those bees fed D1:100 and D1:1000. IHB bees fed D1 showed the shortest survival but was comparable to those bees fed lower concentrations of DWV inoculum. Bees fed with PBS and lower concentrations of DWV inoculum had similar days of survival. Within POL, the unfed bees also survived longer but were not different from that of D1:1000, which was not different from PBS ($F = 7.23$, $df = 4$, $\rho = 0.0001$). Bees fed D1 and D1:100 had the lowest survival. In contrast, the unfed RHB worker bees lived the shortest followed by those fed higher concentrations of DWV inoculum and PBS as larvae ($F = 15.79$, $df = 4$, $\rho < 0.0001$). RHB workers fed D1:1000 and PBS comparably lived the longest.

The LT_{50} or the median longevity was also compared. Within IHB, the unfed control (13 days) had the longest median longevity than any of the fed groups (11 days) (Fig. 5a). Within POL, the unfed control (12 days) and D1:1000 (11 days) had similarly the longest median longevity. However, the median longevity of bees fed D1:1000 was not different from those fed PBS (10 days). POL bees fed high concentrations of DWV inoculum (D1) had the lowest median longevity of 8 days (Fig. 5b). In contrast, the unfed control (9 days) within RHB supported the shortest median longevity but was not different from the median longevity of bees fed D1 (10 days). Bees fed D1:100 (11 days) and PBS only (12 days) had longer median longevity than unfed bees but were comparable to that of bees fed D1. RHB bees fed D1:1000 had the longest median longevity of 13.5 days (Fig. 5c).

Discussion

The response of two *Varroa*-resistant stocks (RHB and POL) to DWV infection was compared to that of a *Varroa*-susceptible stock (IHB) without the compounding effect of *Varroa* parasitism. Our results demonstrated that honey bee stocks differ in their responses to DWV infection. Regardless of the concentration of DWV inoculum, IHB supported low increase (about 10^2 fold) in DWV levels. In spite of this low increase in DWV levels, however, IHB adult bees were negatively affected. Overall, newly emerged bees weighed over 110 mg regardless of honey bee stock or concentration of DWV inoculum, which was significantly higher than the 95 mg and 100 mg reported by de Jong et al. (1982)

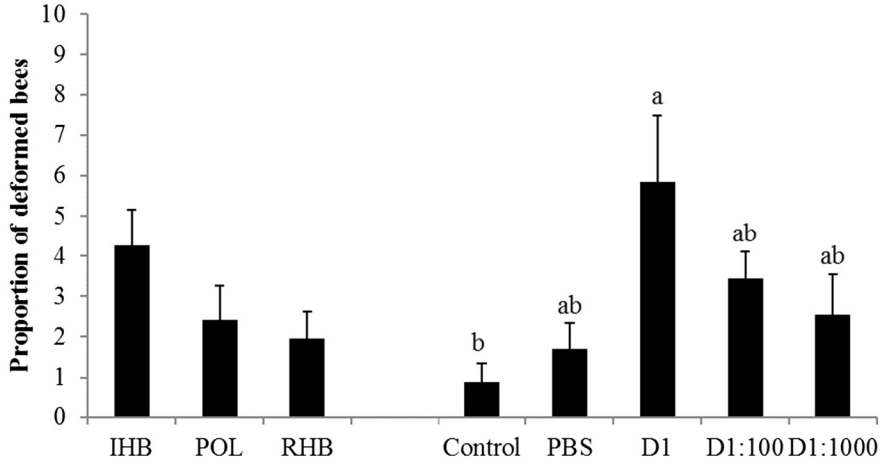

Fig. 2. Proportion of bees with deformed wings of three honey bee stocks fed different concentrations of DWV inoculum during their larval stage. For the effect of concentration of DWV inoculum, bars with different letters are significantly different ($P < 0.001$). Bars without letters indicate no significant difference among the three honey bee stocks ($P > 0.05$). IHB = Italian honey bees, POL = VSH outcross, RHB = Russian honey bees. PBS = larvae fed PBS only, D1 = larvae fed undiluted DWV lysate, D1:100 = larvae fed DWV lysate in 100 dilution of PBS, D 1:1000 = larvae fed DWV lysate in 1000 dilution of PBS.

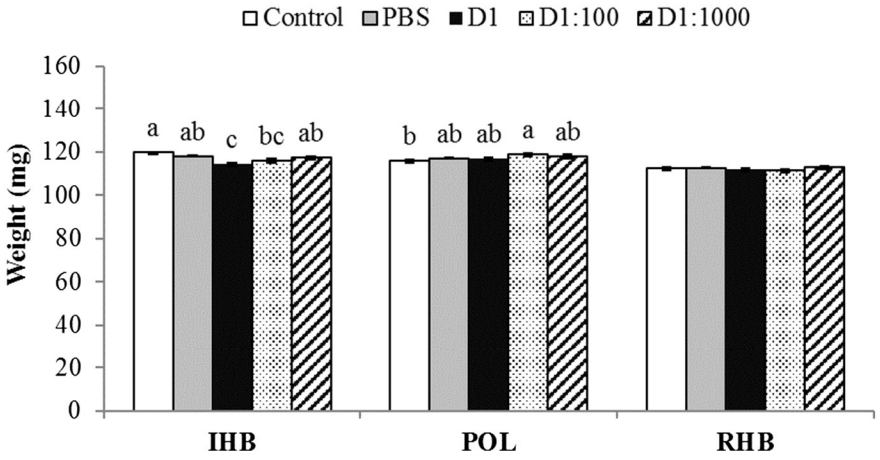

Fig. 3. Weights (mean ± SE) of newly emerged bees of three honey bee stocks fed different concentrations of DWV inoculum during their larval stage. For each stock, bars with different letters are significantly different ($P < 0.001$). Bars without letters indicate no significant difference among the concentrations of DWV inoculum ($P > 0.05$). IHB = Italian honey bees, POL = VSH outcross, RHB = Russian honey bees. PBS = larvae fed PBS only, D1 = larvae fed undiluted DWV lysate, D1:100 = larvae fed DWV lysate in 100 dilution of PBS, D 1:1000 = larvae fed DWV lysate in 1000 dilution of PBS.

and de Guzman et al. (2013), respectively. Nevertheless, there was a significant reduction in the weights of newly emerged adults, adult survival and median longevity (LT_{50}) in IHB fed high concentration of DWV inoculum (D1). Numerically, IHB also had the highest proportion of bees with deformed wings as compared to POL or RHB. Since DWV level only increased approximately 10^2 fold, it is possible that IHB was more susceptible to the virus infection through feeding and could express the characteristic symptoms more readily than the others.

For POL, D1-fed and control bees (both unfed and PBS only) were similarly heavy in spite of the high increase ($\approx 10^4$ fold) in DWV level in bees fed D1. However, the average days of survival and LT_{50} of adult POL were significantly reduced. These contrasting responses to DWV infection by POL may indicate that this stock is able to tolerate higher DWV levels during their earlier development (larvae to adult emergence). DWV infection is thought to be associated with impairments in sucrose responsiveness and associative olfactory learning (Iqbal and Mueller, 2007). Thus, it is possible that POL's uptake of sugar solution provided to them during the survival experiments was affected. We replenished sugar solution and water every day, however, we did not record the bees' daily food consumption.

The response of RHB to DWV infection was quite interesting. The amount of increase in DWV levels also was low (up to 10^2 fold) regardless of inoculum concentration, which may have resulted in the lack of variation in the weights of newly emerged RHB. Further, all RHB fed virus and PBS survived longer as compared to their unfed (control) bees. DWV is widely prominent even in *Varroa*-free colonies (Martin et al., 2012) and DWV infection via vertical transmission is less virulent than horizontally transmitted DWV (Desai et al., 2012). Our analysis showed that the unfed RHB and IHB had numerically higher DWV than that of POL likely due to the result of vertical transmission of the virus. Hence, the short survival and longevity of the unfed RHB (control) may be an artifact. Martin et al. (2012) identified about 10 DWV variants and also speculated that the presence of *Varroa* over time allows the selection of a DWV variant with the competitive advantage. It is possible that the variant or strain of DWV naturally found in the control RHB was different from the strain of the DWV lysate fed to the test larvae, and may also be different from those of control IHB and POL. However, we did not identify the DWV strain in either the lysate and the naturally occurring DWV in any of our control bees. Nevertheless, we obtained DWV lysate from 10 symptomatic bees from one highly infested colony. Hence, we expect that all test larvae received the same strain of DWV.

Earlier studies showed that DWV multiplies slowly during the immature stages (Bailey and Ball, 1991; Martin, 2001; Sumpter and Martin, 2004). In this study, DWV levels in pupae dramatically increased in POL bees fed high concentration of DWV inoculum (D1) but not in

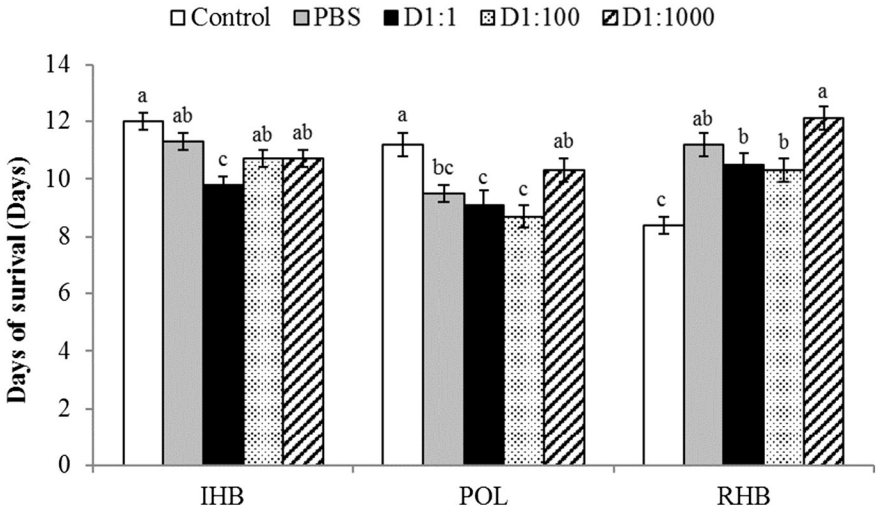

Fig. 4. Average days of survival of worker bees of three honey bee stocks fed different concentrations of DWV inoculum during their larval stage.

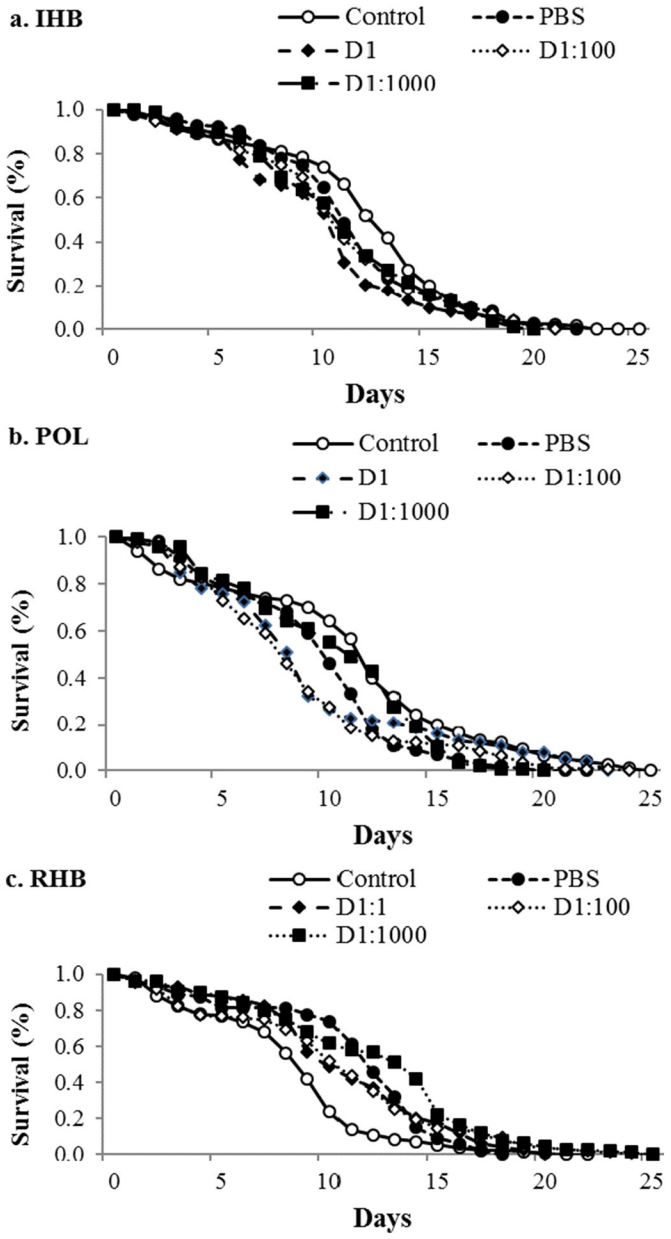

Fig. 5. Percentage of survival of worker bees of three honey bee stocks fed different concentrations of DWV inoculum during their larval stage. (a) IHB = Italian honey bees, (b) POL = VSH outcross, (c) RHB = Russian honey bees.

IHB and RHB. Thus, it is possible that DWV multiplication in the immature stages is perhaps influenced by the genotype of the bees. Our results also suggest that DWV can replicate without direct injection into the bees' haemolymph as claimed by Ryabov et al. (2014). Desai et al. (2012) also observed reduced survival in bees fed DWV-dsRNA as larvae. This study corroborates the findings of Desai et al. (2012). Perhaps DWV can continue to replicate or replicate faster during their adult stage. Unfortunately, we analyzed brown-bodied pupae instead of adult bees for the presence and levels of DWV. Nonetheless, we observed differences regarding adult bee survival and median longevity between D1-fed and unfed bees of POL and IHB.

It is possible that different stocks have different threshold levels in order for symptoms to manifest or significantly impact bees' health. In this study, we found that feeding high concentration of DWV inoculum resulted in a high proportion of bees with deformed wings. Mockel et al. (2011) also observed wing deformities in bees when pupae are artificially injected with high titers of DWV. However, for susceptible stocks, feeding even low concentrations of DWV inoculum probably increases the chance of infection and multiplication of DWV at localized tissues during the bees' development. In an earlier study, we found significant differential mRNA expression levels in 16 out of 24 genes among these three honey bee stocks (Khongphinitbunjong et al., 2015a). However, the virulence mechanisms and pathogenicity of different virus strains on different stocks of honey bees should be investigated further.

Conclusion

Effects of DWV on European honey bee has been investigated but this study represents the first demonstration that *Varroa*-resistant stocks also display some degree of resistance or tolerance to DWV. Honey bees fed the highest concentration of DWV inoculum had the highest rate of wing deformation regardless of honey bee stocks. Further, our results suggested that different honey bee stocks differed in their responses to DWV fed (on weight at emergence, longevity and level of DWV detected in infested bees). Thus, these results have significant implications for the breeding program of DWV tolerance, which may help protect honey bees from the serious impacts of DWV infection.

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