Effect of oral infection with Kashmir bee virus and Israeli acute paralysis virus on bumblebee *(Bombus terrestris)* reproductive success

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Abstract

Israeli acute paralysis virus (IAPV) together with Acute bee paralysis virus (ABPV) and Kashmir bee virus (KBV) constitute a complex of closely related dicistroviruses. They are infamous for their high mortality after injection in honeybees. These viruses have also been reported in non-*Apis* hymenopteran pollinators such as bumblebees, which got infected with IAPV when placed in the same greenhouse with IAPV infected honeybee hives. Here we orally infected *Bombus terrestris* workers with different doses of either IAPV or KBV viral particles. The success of the infection was established by analysis of the bumblebees after the impact studies: 50 days after infection. Doses of 0.5×10^7 and 1×10^7 virus particles per bee were infectious over this period, for IAPV and KBV respectively, while a dose of 0.5×10^6 IAPV particles per bee was not infectious. The impact of virus infection was studied in micro-colonies consisting of 5 bumblebees, one of which becomes a pseudo-queen which proceeds to lay unfertilized (drone) eggs. The impact parameters studied were: the establishment of a laying pseudo-queen, the timing of egg-laying, the number of drones produced, the weight of these drones and worker mortality. In this setup KBV infection resulted in a significant slower colony startup and offspring production, while only the latter can be reported for IAPV. Neither virus increased worker mortality, at the oral doses used. We recommend further studies on how these viruses transmit between different pollinator species. It is also vital to understand how viral prevalence can affect wild bee populations because disturbance of the natural host-virus association may deteriorate the already critically endangered status of many bumblebee species.

48 Keywords: Israeli acute paralysis virus; Kashmir bee virus; *Dicistroviridae*;
49 bumblebees; multi-host pathogens
50

1. Introduction

The Apoidea, encompassing different families of bees, perform a valuable pollination service (Garibaldi et al., 2013). With up to 80% of the plant species being dependent on insect pollination, in particular by bees (Potts et al., 2010). This results in an estimated value of 9.5% of the total economic value of crops that are directly used for human food (Gallai et al., 2009; Potts et al., 2010). Because of a lack of abundance/presence of wild bees, managed bees are used to pollinate crops (Allsopp et al., 2008).

Because different bee species have a similar foraging behavior (gathering pollen and nectar), with overlapping flower networks, sympatric distributions and direct interactions between species or their stored resources, it is very likely that they are exposed to each other's parasites and pathogens. Indeed, parasite networks between bee species are complex and comprise a mixture of multi-host parasites (e.g. *Apicystis bombi* (Maharramov et al., 2013), *Nosema ceranae* (Graystock et al., 2013a), deformed wing virus (DWV) (Fürst et al., 2014)), as well as multi-parasite hosts (Rigaud et al., 2010). However, with the exception of honeybees (*Apis* spp.), little is known about the parasites and pathogens of pollinators, even less about the extent to which they cross-infect different pollinators, and almost nothing about the damage of such cross-infections to different hosts.

Here we focus on the effects of interspecific transmission of bee viruses. Most of what is known about bee viruses relates to the European honeybee (*Apis mellifera*) and its sister species (primarily the Asian hive bee; *A. cerana*), largely through the pioneering work of Bailey and Ball (1991) during the second half of the twentieth century. The evidence increasingly suggests a large degree of commonality of honeybee viruses among the *Apis* species (Ai et al., 2012; Choe et al., 2012; Kojima

et al., 2011; Yañez et al., 2012; Zhang et al., 2012), usually with similar symptoms. Many honeybee viruses have also been detected in other Hymenopteran pollinators, predators and scavengers, initially mostly through incidental observations (Anderson, 1991; Bailey and Ball, 1991) and more recently also through dedicated research (Celle et al., 2008; Evison et al., 2012; Fürst et al., 2014; Genersch et al., 2006; Li et al., 2011; Peng et al., 2011; Singh et al., 2010; Yañez et al., 2012). Bee viruses have also been detected in non-Hymenopteran hosts associated with honeybees (Celle et al., 2008; Dainat et al., 2009; Eyer et al., 2008; Gisder et al., 2009). Honeybees may also be hosts or vectors of certain aphid viruses (Runckel et al., 2011), through the collection of honeydew, or possibly even plant viruses (Li et al., 2014), which could also be transmitted on to other pollinators, through their overlapping contact network with honeybees.

Because of their wide foraging range, large diversity of floral resources visited, long foraging seasons and extensive accumulation of stored pollen and nectar, honeybees are likely to be major factors in any pathogen transmission network involving other (Hymenopteran) pollinators. The worldwide trade in honeybees and bee products coupled with the increasing pathogen prevalence and loads in honeybee colonies, due to a variety of biological and environmental stressors (Genersch et al., 2010a; vanEngelsdorp and Meixner, 2010), could therefore have potentially serious consequences for local wild bee populations (Fürst et al., 2014; McCallum and Dobson, 1995; Meeus et al., 2011).

However, the above mentioned arguments have so far been largely speculative. Other than detecting honeybee pathogens in other insects, and thus establishing possible transmission routes (*e.g.* (Evison et al., 2012; Li et al., 2011; Peng et al., 2011; Singh et al., 2010), there has been little research as to whether these viruses are actually

infectious or, more importantly, cause damage to species other than honeybees. The only recorded exceptions so far are the association of DWV with wing deformities found naturally in both wild and commercially reared bumblebees (Genersch et al., 2006), the reduced survival of bumblebees orally inoculated with DWV (Fürst et al., 2014) and the rapid mortality of bumblebees injected with low doses of Israeli acute paralysis virus (IAPV; Niu et al., 2014). Studies of the effects of interspecific transfer of pollinator viruses are especially important for bumblebees, since bumblebee diversity is diminishing rapidly in many regions of the world (Biesmeijer et al., 2006; Cameron et al., 2011; Potts et al., 2010).

This study concerns the pathogenic effects on bumblebees (*Bombus terrestris* or the buff-tailed bumblebee) of two dicistroviruses: IAPV and Kashmir bee virus (KBV), which together with Acute bee paralysis virus (ABPV) form a complex of closely related viruses (de Miranda et al., 2010). These three viruses share a similar pathology, all being rapidly lethal after injection in honeybees. In honeybee colonies, they are normally present in low titer as persistent infections. But under certain environmental stresses, such as for example *Varroa destructor* infestation, they can undergo re-emergence toward an overt infection-type that can contribute to colony failure (Ribière et al., 2008). Injection of low numbers of IAPV particles in bumblebees also resulted in rapid mortality (Niu et al., 2014). However, the most likely natural virus transmission route for bumblebees is oral. We therefore infected newborn bumblebee workers orally with IAPV or KBV and assessed the effects of this on the performance of bumblebee micro-colonies, a standardized method for studying colony development and reproduction.

2. Materials and methods

2.1. Bumblebees source

All bumblebee (*Bombus terrestris*) workers were obtained from a continuous mass rearing program (Biobest, Westerlo, Belgium) and were maintained on commercial sugar water (BIOGLUC, Biobest) and honeybee-collected pollen (Soc. Coop. Apihurdes, Pinofranqueado-C'aceres, Spain) as energy and protein source, respectively. The insects were kept under standardized laboratory conditions with 29 – 31 °C, 60–65 % relative humidity, and continuous darkness.

2.2. Bumblebee fitness parameters

We used micro-colonies to quantify the effects of virus infection on colony development and bumblebee fitness, as well as worker mortality. The micro-colonies were established by introducing 5 newborn (maximum one day old) workers in an artificial 15×15×10 cm nest box. In this set-up, one worker becomes dominant, i.e. a pseudo-queen, within 2 days and starts laying unfertilized eggs that develop into drones. The remaining workers take care of the brood. The number and mass of the (drone) offspring is a measure of colony fitness. Colony development follows a well-defined pattern and timing under these controlled conditions when receiving the same diet *ad libitum*. Development is measured by the time until the first oviposition, the occurrence of the first developed larvae and the first pupae. Any deviation from this pattern and timing is indicative of alterations in the reproductive capacity of the pseudo-queen or in larval development. The micro-colonies were kept under standardized rearing conditions, as reported above.

2.3. Virus and control extracts

For each extract, fifty white-eyed pupae from a healthy honeybee colony were injected with previously purified IAPV or KBV and incubated at 30^{o}C for 4 days following the protocols of the virus chapter of the BeeBook (de Miranda et al., 2013). The control extract was prepared from uninjected pupae incubated for the same length of time. The pupae were homogenized in 10 mM phosphate buffer (pH 7.0) 0.02% diethyl dithiocarbamate, clarified with chloroform and centrifuged at 8000g for 15 minutes (de Miranda et al., 2013). The particle concentration of each virus extract was determined using transmission electron microscopy (TEM). Undiluted and 10-fold diluted viral stock solutions were analyzed at the CODA-CERVA (Uccle, Belgium). They were negatively stained according to the protocol described by Mast and Demeestere (2009). Zones of "wet staining" could be identified on each grid where the particles were evenly spread over the grid with limited competition for binding sites and little overlap of particles. TEM specimens were examined using a Tecnai Spirit microscope (FEI, Eindhoven, The Netherlands) operating at 120 kV, at a spot size of 1. An entire grid surface 1537 nm by 1537 nm was analyzed with a 30.000x magnification under parallel beam conditions. The IAPV extract contained 1 x 10^{6} viral particles/µl and the KBV extract 2 x 10^{6} viral particles/µl, while the control extract was largely devoid of virus particles. The IAPV and KBV extracts had <0.1% and <0.01% contamination, respectively with other common honeybee viruses, as determined by RT-qPCR using specific assays for ABPV, Chronic bee paralysis virus, DWV, Varroa destructor virus-1 (VDV-1), slow bee paralysis virus (SBPV), sacbrood virus (SBV), black queen cell virus (BQCV), Lake Sinai virus-1 and -2 (Locke et al., 2012). The control extract had similar background levels of the same viruses (mostly SBV and BQCV) as the IAPV and KBV extracts.

2.4 Experimental design and infection

There were three treatment groups in this experiment; control, IAPV infection and KBV infection, each with ten micro-colonies. Five newborn workers were added to each micro-colony and kept under standard rearing conditions for one day. They were then deprived of pollen and sugar water for 3 hours. The starved bees were then placed in a feeding box (a cylinder of 1 dm diameter) containing a 30-µl droplet containing 5 µl experimental extract plus 25 µl of 50% sugar water solution (BIOGLUC, Biobest). Therefore each bee in the IAPV treatment received 0.5×10^7 IAPV particles while in the KBV treatment group each bee received 1×10^7 KBV particles. Additionally, 10 workers (2 micro-colonies) were fed 5 µl of a 10-fold dilution of the IAPV extract (*i.e.* 0.5×10^6 particles/bee) to assess if we could still infect workers with this lower dose. After inoculation, the bees were returned to their micro-colony where they immediately received *ad libitum* sugar water and after three days also pollen *ad libitum*.

2.4. Virus detection

Bumblebees were dissected and the gut was grounded individually in 300 µl of RLT buffer (Qiagen, Venlo, Netherlands) supplemented with 3 µl β-mercapto-ethanol. RNA was extracted with the Qiagen RNeasy Mini Kit following manufacturer's instructions, eluting the RNA in 30 µl of RNase free water. We used reverse transcriptase multiplex-ligation probe dependent amplification (RT-MLPA) technology to determine the virus infection status of our samples. This technology, called BeeDoctor (De Smet et al., 2012), detects 6 targets simultaneously and covers 10 common "honeybee" viruses: Black queen cell virus (BQCV); the acute bee paralysis virus complex including ABPV, KBV and IAPV; the DWV-complex

including DWV, VDV-1 and Kakugo virus (KV); SBPV; SBV; and chronic bee paralysis virus (CBPV). Since the BeeDoctor does not distinguish between IAPV and KBV, all samples were also analyzed by RT-PCR using primers specific for either IAPV (CGATGAACAACGGAAGGTTT and ATCGGCTAAGGGGTTTGTTT (Cox-Foster et al., 2007) or KBV (GCCGTACAACGACGACTACA, and CGTCATTTTAACCGCTGCTT). The viral identity of both amplicons was confirmed by Sanger sequencing (LGC Genomics, Berlin, Germany). A two-step RT-PCR protocol was used for this. The cDNA was synthesized with SuperScript-II Reverse Transcriptase (Invitrogen, Merelbeke, Belgium) according to the manufacturer's guidelines with 0.8 µM virus-specific reverse primers. One microliter of cDNA was added to a final 25 µl PCR reaction mixture containing 2.5 µl 10x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 µM primers and 1.25 U Recombinant Taq DNA Polymerase (Invitrogen). The PCR reactions were run in a Sensoquest Labcycler for 2 min at 94 °C followed by 30 amplification cycles of (30 s denaturation at 94 °C; 30 s annealing at 56 °C; 45 s extension at 72 °C) followed by 3 min final extension at 72 °C.

2.5. Statistics

Statistical analysis of the data was conducted in SPSS v21.0 (SPSS Inc., Chicago, Il.). The normal distribution was confirmed by the Kolmogorov-Smirnov test ($P = 0.05$). The non-normal distributed dependent variable (time until oviposition) was divided into regular and delayed oviposition. A χ^2 Goodness of Fit test was used to determine if virus treatment resulted in significant deviation from the control treatment. The number and mass of drones produced in micro-colonies with a regular time until oviposition were analyzed by one-way analysis of variance (ANOVA) and the mean ±

standard error were separated with a post hoc Tukey test ($\alpha = 0.05$). The numbers of drones produced by all micro-colonies, including both regular and delayed oviposition, were analyzed by a non-parametric Whitney U test.

3. Results

3.1. Infection status

The pseudo-queen of a micro-colony, the one that lays the eggs, has the highest impact on the performance of her micro-colony. Therefore we tested the virus infection status of the pseudo-queens after following micro-colony development for 50 days. Six out of 10 IAPV-treated pseudo-queens and 9 out of 10 KBV-treated pseudo-queens tested positive for infection with an ABPV-KBV-IAPV complex virus, using the BeeDoctor RT-MLPA technology, while none of the other viruses covered by BeeDoctor (De Smet et al., 2012) were detected. IAPV- KBV-specific RT-PCR reactions, followed by sequencing of the RT-PCR products, confirmed that IAPV treatment resulted only in IAPV infections and the KBV treatment only in KBV infections. The control pseudo-queens as well as and bumblebees receiving a ten fold dilution of the IAPV stock (n = 10) were entirely free of any virus covered by the BeeDoctor.

3.2. Impact of virus infection on bumblebee colony development

Infection with either IAPV or KBV did not result in any major increase in mortality of the bumblebee workers. The IAPV treatment resulted in 6 dead workers out of 50 workers by day 50; the KBV treatment only had 1 dead worker, and the control treatment had 3 dead workers out of 50.

Bumblebee micro-colonies develop very predictably under standard, uniform nutritional conditions, with oviposition starting 7-8 days after introducing the bees into their micro-colony, with usually no more than 1 day variation in oviposition between colonies (Meeus et al., 2013). However, in these experiments the micro-colonies were deprived from pollen for 3 days, which delayed oviposition to a mean of 11 days in the control group, and also increased the variation in oviposition time around this mean. Consequently, the time until oviposition in these 30 experimental and control micro-colonies did not show a normal distribution (One-Sample Kolmogorov-Smirnov Test, P = 0.00014). The control group had an interquartile (IQR) of 1, everything lower than Q1 -1.5 x IQR = 8.8, and everything higher than Q3 +1.5 x IQR = 12.5 is an outlier. Based on this we saw two groups: those with 9, 10, 11 or 12 days until oviposition ("regular colonies") and those with oviposition starting at day 13 or later ("delayed colonies"). There were 2 out of 10 colonies with delayed oviposition in the control group; 4 out of 10 in the IAPV-treated group and 6 out of 10 in the KBV-treated group (Table 1a). The difference between the KBV-treated colonies and control colonies is significant, as determined by a χ^2 Goodness of Fit Test. KBV treatment also resulted in significantly more micro-colonies with no drone production at all compared to control samples; this effect did not occur for IAPV treatment (Table 1b).

The delay in oviposition will further influence the total number of drones produced by these colonies. Therefore we only used the colonies with a "regular" oviposition time (10-12 days after start-up of the experiment) to compare drone production between treatments. The ANOVA indicated a significant difference in numbers of drones produced between the treatments ($F_{(2,15)}$ = 4.127; P = 0.036). Using the post hoc Tukey test, to determine which treatment caused the effect, we saw that both

274 treatments (KBV and IAPV) produced fewer drones than the control colonies, with a
275 probability of 0.07 (Fig 1). These comparisons excluded the micro-colonies with
276 delayed oviposition time, which reduces the statistical power of the comparisons.
277 When we compare all IAPV-treated micro-colonies that produce drones, irrespective
278 of oviposition time, to similar micro-colonies from the control group, than we see a
279 significant drop in drone production in IAPV-treated colonies (N = 18; Mann Whitney
280 U test: z = 17.5; P = 0.04). Furthermore, drone production in all virus-treated colonies
281 combined (i.e. both KBV and IAPV) was significantly reduced when compared with
282 the control colonies ($F_{(1,16)}$ = 8.828; P = 0.009) (Fig 1).
283 The same analyses applied to drone mass for all drone-producing micro-colonies,
284 revealed a lower mean mass of the drones in virus-treated colonies compared to
285 control colonies, although this difference was not significant (F(2,18) = 1.801; P =
286 0.194) and $F_{(1,19)}$ = 1.782; P = 0.198).
287
288 **4. Discussion**
289 There is extensive historical literature on the effects of ABPV and KBV on honeybees
290 (for reviews see Ribière et al. (2008) and de Miranda et al. (2010)). Both viruses have
291 been implicated in *Varroa*-associated colony losses (de Miranda et al., 2010; Ribière
292 et al., 2008). More recent European data links ABPV with honeybee winter mortality
293 (Genersch et al., 2010b; Siede et al., 2008). IAPV, which was only recently described
294 as a separate virus (Maori et al., 2007), has also been implicated as a marker for
295 Colony Collapse Disorder (CCD) in North America (Cox-Foster et al., 2007),
296 although this was re-assessed in subsequent, more comprehensive studies
297 (vanEngelsdorp et al., 2009). Instead mortalities have been linked to KBV and ABPV
298 infections (Cornman et al., 2012) and overall pathogen load as an indicator of

299 compromised honeybee health (Ravoet et al., 2013). Despite the acute virulence of
300 these viruses in honeybees and their ability to infect other hymenopteran species,
301 including bumblebees (Bailey and Gibbs, 1964; Singh et al., 2010), few systematic
302 host-range studies have been conducted for any of these viruses. Moreover, no study
303 to date has investigated their impact on such alternative hosts. Using the buff-tailed
304 bumblebee, a generalist forager in the Palearctic region, we demonstrate that oral
305 feeding of 0.5×10^7 and 1×10^7 viral particles per bee of either IAPV or KBV,
306 respectively, results in an active infection and fitness loss. Lower doses of IAPV (0.5×10^6
307 IAPV particles/bee) did not result in a detectable infection. Thus, our oral
308 administration dose is close to the minimum required for inducing an infection, and
309 may not have been sufficient to affect worker mortality. This may also explain the
310 slightly reduced virulence of IAPV compared to KBV in these experiments, since the
311 KBV infectious dose was twice that of IAPV. Experiments elsewhere showed that
312 oral infection of *B. terrestris* workers with 10^9 genome copies of a different honeybee
313 virus, DWV, reduced the mean survival of *B. terrestris* workers by 6 days (Fürst et
314 al., 2014).
315 With KBV-infected bumblebees, the time until oviposition was delayed and fewer
316 colonies initiated drone production than with uninfected bumblebees. We speculate
317 that the exclusion of pollen in the first 3 days of the experiment exacerbated these
318 effects, as pathogenic effects are often context dependent, with low nutritional status
319 being an important stressor for pathogen infections (Brown et al., 2003). In colonies
320 without delayed ovipostion, drone production was also impaired. We can thus
321 conclude that under the experimental conditions KBV infection reduces *B. terrestris*
322 fitness.

For IAPV the situation is less obvious. IAPV-infected bumblebees showed deviations in time until oviposition and drone production, but these were not significant. However, when we only analyze micro-colonies with drone production, we see that IAPV-infected colonies produce significantly fewer drones than non-infected colonies. We can therefore conclude that IAPV impacts *B. terrestris* fitness as well. The lower virulence of IAPV in these experiments, relative to KBV, may be partly due to the lower IAPV infectious dose used (half that of KBV).

Here we report fitness impact of KBV and IAPV, and Fürst et al. (2014) showed lower survival after DWV infection (Fürst et al., 2014) in bumblebees. The time is now to clarify what this could mean for critically endangered bumblebee populations (Biesmeijer et al., 2006; Cameron et al., 2011; Potts et al., 2010). Could anthropogenic movement of bees disturb the natural multi-host pathogen association by spilling over pathogens? And how severe is this stressor compared to other factors such as pesticide use and land use change? Two potential reservoirs of pathogens from which pathogens can potentially infect wild pollinators are: domesticated honeybees, notorious for their viral infection loads, and commercially bred bumblebees escaping greenhouses (Murray et al., 2013) can carry viruses (Graystock et al., 2013b). For now the threats toward wild pollinators is unknown. A critical factor in the overall risk-determination is the pathogen's infectivity (the capacity to initiate an infection), virulence (the capacity to cause damage) in the wild pollinator and host tolerance, genetics and condition (Casadevall and Pirofski, 1999; Casadevall and Pirofski, 2001), in relation to the amount and concentration of virus produced by the domesticated or bred bees. It is therefore important to know if the oral doses applied here are realistic in their ecological context. This study shows that the infectivity of IAPV and KBV in bumblebees is relatively low (high oral doses are

required to start an infection) and of the same order of magnitude as their oral infectivity in honeybees (Bailey and Ball, 1991; de Miranda et al., 2013). The other factors important for risk assessment are the exposure rates and probabilities, either through direct contact (bumblebees feeding at honeybee hives) or through flower networks. The results of Fürst et al. (2014) and Singh et al. (2010) have shown that this exposure can be high for those bumblebee colonies in the immediate vicinity of honeybee colonies, but that for bee viruses most of this risk is related to the primary contact with honeybee colonies, with currently little evidence for independent secondary proliferation within the bumblebee community itself.

As a final point, healthy domesticated honeybee hives and bred bumblebee colonies are desirable. It has been proposed that relatively clean commercial bumblebees may actually dilute the natural occurrence of *Crithidia bombi* (Whitehorn et al., 2013). It is clear that studies on viral dynamics within and between different pollinators communities are needed to better understand the risks associated with allopatric and sympatric transport of bees to determine if these transports could deteriorate the endangered status of wild bees.

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529 **Legend of figure**
530 Fig. 1.
531 The mean number of drones produced (±SE) and their mean mass (±SE) for Israeli
532 acute paralysis virus- and Kashmir bee virus-infected bumblebee micro-colonies
533 versus their control. Dicistroviruses represents the pooled data of both IAPV and
534 KBV infection.
535

Tables

Table 1: The number of micro-colonies with a regular and delayed time until oviposition (a), and with a without drone production (b).

a)	The number of micro-colonies (mean oviposition day)			
	regular oviposition	delayed oviposition		χ^2
Control	8 (10.5)	2 (16.5)	Expected	
IAPV	6 (10.5)	4 (14)	Observed	$\chi^2 = 2.5$, df = 1, $P = 0.11$
KBV	4 (10.5)	6 (16.3)	Observed	$\chi^2 = 10$, df = 1, $P = 0.002$

b)	The number of micro-colonies			
	with drone production	without drone production		χ^2
Control	9	1	Expected	
IAPV	9	1	Observed	$\chi^2 = 0$ df = 1, $P = 1$
KBV	5	5	Observed	$\chi^2 = 17.778$, df = 1, $P < 0.001$

Figure1
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