INTRODUCTION

Honeybees and bumblebees are the two most important bee species used in modern apiculture and agriculture. They host many viruses. When looking at the honeybee, there have been around 23 viruses reported and many of these viruses only persist as covert and asymptomatic infections which show no detectable impact at both the individual and colony level (McMenamin & Genersch, 2015). However, under certain conditions, for example the presence of the ectoparasitic honeybee mite Varroa destructor, the covert infections of some viruses turn into overt ones with observable symptoms (Martin et al., 2012; McMahon et al., 2015; Singh et al., 2010). The symptoms of overt infections vary from virus to virus, including deformed wings, discoloration, hair losses, bloated abdomen, paralysis, decreased locomotion, impaired cognition and both brood and adult mortality (Lanzi et al., 2006; McMenamin & Genersch, 2015; Singh et al., 2010). Some symptoms can be easily recognized by the behaviour and appearance of the infected bees, while others can only be confirmed through meticulous and continuous observations or by molecular techniques.

In bumblebees (Bombus spp.) a subset of honeybee-infecting viruses has been reported, such as deformed wing virus (DWV), Israeli acute paralysis virus (IAPV), acute bee paralysis virus (ABPV), Kashmir bee virus (KBV), slow bee paralysis virus (SBPV), sacbrood virus (SBV), black queen cell virus (BQCV) and Lake Sinai virus (LSV) (Genersch et al., 2006; Levitt et al., 2013; Parmentier et al., 2016; Peng et al., 2011). However, only DWV, KBV and IAPV have been reported to cause clinical symptoms (Genersch et al., 2006; Meeus et al., 2014). DWV caused crippled wings in bumblebee (Bombus terrestris and Bombus pascuorum) workers even without Varroa (Fürst et al., 2014; Genersch et al., 2006). Interestingly, viral tissue tropism was different between DWV-infected (asymptomatic) and clinically diseased (symptomatic) honeybees and bumblebees, indicating that overt infection may also be associated with virus tissue spreading (Genersch et al., 2006).

IAPV is positive single-stranded RNA virus in the family Dicistroviridae with a global distribution range (de Miranda et al., 2010; Maori et al., 2007; Palacios et al., 2008). The nomenclature of this virus is largely based on its symptoms that are similar to ABPV. Both viruses can induce abnormal
body trembling (Galbraith et al., 2015; Li et al., 2013; Maori et al., 2009). In bumblebees, injection of a low dose of IAPV particles into healthy workers could induce rapid mortality (Niu et al., 2014a). Orally delivered IAPV also increased mortality in adults, but a much higher starting dose was needed (Piot et al., 2015). Low oral viral doses did not induce mortality but had effects on reproduction of bumblebees (Meeus et al., 2014). Aside from the above mortality and sub-lethal effects, the symptoms associated with IAPV have not been well characterized and thus hinder our ability to observe the progression of IAPV infection in these important pollinators, especially when considering that virus-induced paralysis symptoms are normally the signal of overt infection and death. Besides, the initial observation of IAPV symptoms are from honeybees; symptom analysis in bumblebees would enhance our knowledge of this virus (de Miranda et al., 2010; DeGrandi-Hoffman & Chen, 2015; Maori et al., 2007, 2009; Piot et al., 2015). Therefore, in this study, we report progressive viral symptoms in workers of *B. terrestris*, which is one of the most numerous bumblebee species in Europe (Velthuis & van Doorn, 2006). Furthermore, we investigated the relationships between IAPV symptoms and tissue tropism. Finally, a major insect immune response against viral infections, the siRNA pathway, was followed to infer if its impairment is correlated with viral symptoms. We aim to provide a better understanding of viral tissue dynamics and host immune responses and the possible relationships with symptomatic infection in this important pollinator.

**RESULTS**

**Symptoms and mortality associated with IAPV infections in bumblebees**

IAPV injection (*n*=25) caused a rapid mortality and all workers died within 5 days (Fig. 1). In the control group of PBS injection (*n*=25), all workers survived by the end of the recording, which was 15 days post-injection (p.i.) (Fig. 1). We found some bees from the IAPV-injected group presented progressive symptoms and defined them as SP+ workers. The initial symptom as observed at 1 day p.i. was crippled/immobilized forelegs, and this resulted in impaired mobility (Movie S1, available in the online Supplementary Material). At this stage, bees moved slowly and did not fly or buzz when perturbed by gentle shaking of the micro-colony. Also, when compared with the workers injected with IAPV that did not show symptoms (SP−) and workers injected with PBS, SP+ bees visited the food source (sugar water feeder) less frequently and showed a ‘quiet’ behaviour. They passed the sugar water feeder slowly and seemed to ‘ignore’ it. An additional observation was that the normal healthy workers expelled the workers with crippled/immobilized forelegs from the main group. When we isolated the workers with crippled/immobilized forelegs and kept them alone in a new micro-colony with nutrition, they exhibited intense abdomen shaking and body trembling within 12 to 24 h. At that time, they were not capable of standing or walking anymore. Finally, they all died in the following 2 to 3 h. To verify that the above reported symptoms before death are induced by IAPV, we included another two control groups in which we induced stress by starvation (*n*=10) and physical injury (*n*=10) (bees injected with PBS using a blunted needle) (Fig. 1). Results showed that the starved workers also died quickly and the survival rate dropped to only 10% by the 2nd day post-treatment. However, no symptoms of crippled/immobilized forelegs, paralysis or trembling were observed from this group. Of the starved workers, which were also inactive and gathered around in a group, none were expelled by the others. Interestingly, bees seemed to handle physical injury better than starvation, as 70% of the bees survived after the injection of PBS with a blunted needle. Bees in this group showed no symptoms of crippled/immobilized forelegs, paralysis or trembling.

**IAPV relative normalized quantities (RNQs) in tissues of SP+ and SP− workers**

To explore the relationships between IAPV infection and paralysis symptoms, we determined the viral RNQs in 500 ng RNA of each tissue sample and in association with two factors: tissue (brain, midgut, ovary and fat body) and symptom (SP+ and SP−) at 3 days p.i. We used peptidyl-prolyl isomerase A (*PPIA*) and 60S ribosomal protein L23 (*RPL23*) as internal reference genes in the quantitative (q) PCR assay. The reference genes stability M value and cv value calculated by qbase were all under the threshold (M<1.0 and cv<0.5) for inter-tissue comparison (Helleman et al., 2007). Thus, the normalization of qPCR results among tissues was reliable. IAPV was detected in all tissues examined, but the viral RNQs varied (Fig. 2).
Specifically, the viral RNQs in the fat bodies were significantly higher (ANOVA: F=70.7380, P<0.001, P value was calculated with post-hoc Tukey HSD test) than in the other tissues. Unfortunately, no effect of the factor symptom (SP+ vs SP−) on the viral RNQs (ANOVA: F=0.0012) or interaction between the factors tissue and symptom (ANOVA: F=0.0604) could be reported.

**IAPV localization in brains by fluorescence in situ hybridization (FISH) microscopy**

FISH was utilized to localize IAPV-infected brain regions and retrieve differences between SP+ workers (n=4) and SP− workers (n=7). A summary of the FISH assay can be found in Table S1. IAPV signal (red) could be detected in various brain regions including the somata in the mushroom bodies (MBs), antennal lobes (ALs) and optic lobes (OLs) (Fig. 3a, b, e, f). No IAPV signal could be detected in the no virus control samples (n=5) stained with or without IAPV probes (Fig. 3c, d, g, h). We compared the viral distribution in MBs, ALs and OLs between SP+ and SP− samples. In MBs, the Kenyon cells (KCs) were found to be a main viral reservoir (Fig. 4a–f). KCs are classified into two types based on their localizations within the brains: KCs I are located inside of the mushroom body calyces (mbc) while the KCs II are mainly at the periphery of the calyces. For SP+ workers, IAPV signal could be observed in both types of KCs in the MBs (Fig. 4a–c). Similar viral distribution could also be found in the samples of SP− workers (Fig. 4d–f). ALs are the main olfactory centre responsible for receiving and processing olfactory signals. We recorded a strong IAPV signal in the ALs of both SP− and SP+ workers (Fig. 5a–f). Finally, we also localized the IAPV genome in the OLs, which are the main visual processing centre of bees. In both SP+ and SP− brains, IAPV signal was mainly found in the lamina monopolar cells (Fig. 6a–f), which are responsible for receiving visual signals from the retina and transferring them to the second optic lobe, the medulla.

**DISCUSSION**

**Symptoms after infection with IAPV**

IAPV symptoms reported in honeybees, such as body trembling, were also observed in the current study with *B. terrestris* workers. This symptom was exhibited in the last couple of hours before death. Other symptoms described in the honeybee, such as discoloration and hair losses on thorax and abdomen, were not observed in our experiment setup. We observed a yet unreported symptom, namely crippled/immobilized forelegs in the IAPV-infected workers after 1 to 2 days of viral injection. Our results indicated that this early symptom was induced by IAPV and not by other stresses such as injury or starvation. Although confounding infection of other viruses cannot be guaranteed, the 10,000 times dilution of the IAPV purified stock significantly lowered the chance of confounding infection.

In 1974, Bailey and his colleague reported a similar front-leg symptom in honeybees infected with SBPV 1 or 2 days before death (Bailey & Woods, 1974). Unfortunately, we cannot compare the current front-leg symptoms to the previous one.
in detail since no visual material of Bailey’s observation was preserved. Interestingly, IAPV and SBPV are genetically unrelated and belong to different families. Another paralysis-related virus is ABPV, which is genetically closely related to IAPV but not reported to be associated with the front-leg symptoms. This evidence demonstrates that genetically unrelated viruses could be symptomatically related (IAPV and SBPV), while genetically related viruses could be symptomatically unrelated (IAPV/ABPV and KBV).

Another interesting observation is that we found the SP+ bees were expelled from the main group by the others. A similar behaviour was noticed in ABPV- and CBPV-infected honeybees (Bailey et al., 1963). Although, it remains to be determined if this behaviour is to avoid transmission of the virus through the entire colony, it is known that the contacts of networks are important for parasite transmission dynamics within bumblebee nests, with bee contact rate being a significant predictor of *Crithidia bombi* transmission (Otterstatter & Thomson, 2007). The forelegs are able to provide food-related information to the nerve system of worker bees and are an important sensor organ (de Brito Sanchez et al., 2014; Mommaerts et al., 2013). This could explain our observations that bees with crippled/immobilized forelegs seem to ‘ignore’ the sugar water feeder.

We performed a paired sampling, meaning that workers showing obvious symptoms (SP+) and no symptoms (SP−) were sampled at exactly the same time after injection. This setup allowed us to see if dynamics in tissue tropism could explain the appearance of symptoms. According to our results, it was clear that IAPV accumulated in all four tissues tested (midgut, fat body, brain and ovary), and no significant differences in viral RNQs were observed between SP+ and SP− bees. Thus, the appearance of symptoms after IAPV infection cannot be explained by a specific viral tissue accumulation.

IAPV-infected honeybees have a decreased homing ability (Li et al., 2013). In the present study, the SP+ workers of *B. terrestris* also showed a decreased sensation and mobility, which could be explained by the viral presence in the brain, although the qPCR data did not reveal any differences between workers with and without symptoms (SP+ vs SP−). However, it still remains possible that brain regions with various functions are differentially infected, which may lead to paralysis symptoms. Therefore, we performed FISH to localize IAPV genomes in *B. terrestris* brains. FISH is a powerful method to determine DNA or RNA molecules in multiple types of samples. In honeybees, *in situ* hybridization has been applied to detect viruses such as DWV, IAPV and Kakugo virus (KV) and bacterial diseases in both adult
and larval tissues (Chen et al., 2014; Fujiyuki et al., 2009; Martinson et al., 2012; Möckel et al., 2011; Shah et al., 2009; Yue et al., 2008). In the present study, we localized IAPV in different parts of the brains of B. terrestris workers using paraffin-embedded tissue sections. Our results clearly demonstrated the presence of IAPV in the MBs, antennal and optic neuropils. Therefore, it is reasonable to speculate that the sensory experience such as vision, taste and smell of the bees is affected. Unfortunately, we did not find any specific distribution of IAPV in SP+ brains compared with SP− brains, indicating that the viral accumulation in the functional regions of the brain does not definitely lead to clinical symptoms. However, we cannot draw a definitive conclusion on this, as in our setup the FISH can only determine presence and absence of the virus. For more detailed information on viral distribution in brains, further analysis, for instance 3D optic imaging, is needed.

**Host immune response after IAPV infection**

The innate immune response plays a major role in controlling and eliminating invading pathogens in insects (Kingsolver et al., 2013). It has been described that the insect host recruits the small interfering RNA (siRNA) pathway when challenged with virus (Deddouche et al., 2008; Niu et al., 2016). Here Dicer-2 produces virus-specific siRNAs that are then loaded into the RNA-induced silencing complex (RISC) which is capable of cutting multiple viral RNAs (Brutscher et al., 2015; Niu et al., 2014b; Sabin et al., 2010). In the present study, we checked the expression of Dicer-2, a key gene in the siRNA pathway in different tissues of SP+ and SP− B. terrestris workers. Differences in Dicer-2 expression within the same tissue could inform us how individual workers cope with the viral infection. Our results demonstrated that Dicer-2 transcription increased after IAPV infection in all tissues tested, but there was no difference between Dicer-2 expression in the same tissue. For more detailed information on viral distribution in brains, further analysis, for instance 3D optic imaging, is needed.

**Fig. 4.** Localization of IAPV in mushroom bodies (MBs). (a–c) MBs of three IAPV-infected workers with symptoms (SP+) stained with IAPV probe; (d–f) MBs of three IAPV-infected workers without symptoms (SP−) stained with IAPV probe; (g) MBs of PBS-injected worker stained with IAPV probe. IAPV signal (red) was found in both types of Kenyon cells (KCs I and KCs II), nuclei are in blue. KCs I, class I Kenyon cells; KCs II, class II Kenyon cells. Arrowheads indicate the position of the IAPV signal. Bars, 100 µm.

**Fig. 5.** Localization of IAPV in antennal lobes (ALs). (a–c) ALs of three IAPV-infected workers with symptoms (SP+) stained with IAPV probe; (d–f) ALs of three brains from IAPV-infected workers without symptoms (SP−) stained with IAPV probe; (g) ALs of PBS-injected worker stained without IAPV probe; (h) ALs of PBS-injected workers stained with IAPV probe. IAPV signals are shown in red and nuclei are shown in blue. Arrowheads indicate the position of the IAPV signal. Bars, 100 µm.
abdomen, heads and remnants (Niu et al., 2014a). In this study we used M value <1.0 and CV value <0.5 for the quality check over different tissues. The specific M and CV values for the fat bodies are M=0.587 and CV=0.204, and thus above the within-tissue criteria of M<0.5 and CV<0.2 (Hellemans et al., 2007). For tissues with high IAPV titers, the use of exogenous internal references is therefore needed to be conclusive.

In natural conditions, the insect midgut epithelium is the first barrier against viral invasion (Hakim et al., 2010). So a sensitive immune response in the midgut could be beneficial to the host. However, in our experimental setup, IAPV was able to infect multiple tissues because viral particles were injected into the haemocoel. Therefore, the differences of viral titres we observed between midgut and fat body can very probably be explained by the differences of tissue-specific immunity against viral infection and/or viral uptake. We should also keep in mind that the insect innate immune system comprises several other immune responses, such as Jak/STAT, Toll, Imd and autophagy. The function of these immune responses upon viral infection is not fully understood. Thus, further study focusing on multiple immune responses, especially at the tissue level would be helpful to reveal more details of host–virus interactions.

**METHODS**

**Insects and viruses.** Callow workers were collected from virus-free colonies of *B. terrestris* (Biobest, Westerlo Belgium), and kept in micro-colonies supplied with sugar water under standardized conditions in an incubator (Panasonic) at 30°C and 60% relative humidity with continuous darkness (Mommaerts et al., 2010). The IAPV inoculum used in this study was produced by propagating virus reference isolates in 50 white-eyed honeybee pupae and preparing a chloroform-clarified extract in 10 mM phosphate buffer (pH 7.0)/0.02% diethyl dithiocarbamate as described previously (Niu et al., 2014a). This IAPV inoculum had <0.1% contamination of other common honeybee viruses, such as ABPV, KBV, CBPV, DWV, SBPV, SBV and BQCV, determined by reverse-transcriptionase (RT)-qPCR assay (Locke et al., 2012). The particle concentration of this IAPV stock was determined by electron microscopy as 1×10^6 particles µl^-1 and this was diluted to 1×10^7 particles µl^-1 by PBS (pH7.4) for virus injection.

**Symptom observation and survival analysis.** Five-day-old *B. terrestris* workers (n=25) were injected with 5 µl (500 particles) of IAPV inoculum with a nanoinjector (Eppendorf) for mortality analysis. Workers of the same age and injected with 5 µl PBS (pH7.4) were employed as control (n=25). In addition, we included two extra controls: (i) physical injury (n=10), workers injected with PBS by a blunted needle and (ii) starvation (n=10), workers kept without any nutrition. The controls allowed us to record symptoms of a dying bee. We scored specific symptoms of paralysis and trembling, and when these appeared, those individuals were isolated. Additionally, we scored for abnormalities in worker behaviour, such as foraging, feeding and the response of the workers to a gentle shaking (challenge) of the micro-colony where normal workers would fly up immediately (Mommaerts et al., 2010).

**Sample selection and tissues dissection.** To test the tissue tropism and infection level of IAPV, 60 workers (5 days old) were injected with the same amount of viral solution (5 µl, 500 particles) and subdivided into three micro-colonies (20 bees per colony). Workers that started showing IAPV-related symptoms were collected and labelled as SP+ bees. When detecting and selecting a SP+ bee, another bee infected with virus but without showing IAPV-related symptoms was also collected and labelled as SP–. SP– and SP+ from the same time point were collected from the same micro-colony. Twenty workers injected with 5 µl PBS (pH7.4) served as negative control. All bees sampled were sacrificed immediately after collecting. Dissections were performed in PBS (pH7.4) under a binocular microscope to remove the brain, midgut, fat body and ovary. Briefly, each worker was fixed on a wax dish by steel insect pins and the abdomen was opened by using a fine scissor. Then ovary, midgut and fat body were removed. To isolate the brain, the head was cut off from the body and fixed firmly in a wax dish with sterile PBS (pH7.4) to keep the structure of the tissues. Forceps and fine sharp needles were used to remove the antennae and open the front mask covering the compound eyes. Then brains were isolated carefully. Scissors, forceps, pins and other tools were cleaned during dissection by spraying with 70% ethanol and RNASE AWAY surface decontaminant (Molecular Bio Products) to prevent potential cross-contaminations. Freshly dissected tissues were transferred into either RLT buffer (Qiagen) or fixative for RNA extraction or tissue fixation, respectively.

**RNA isolation, cDNA synthesis and qPCR.** Tissues were homogenized and lysed in RLT buffer (300–500 µl) and total RNA was extracted following the instructions of the RNeasy mini kit (Qiagen). The remaining genomic DNA was removed by using a TURBO DNA-free kit (Ambion). The integrity and quantity of the purified RNA were checked by 1% agarose gel electrophoresis and a Nanodrop spectrophotometer. Afterwards, 500 ng total RNA from each sample was used to synthesize the cDNA by SuperScript II Reverse Transcriptase (Invitrogen) using oligo(dT) primers. The qPCR was performed on a
amplification factor was calculated for each pair of qPCR primers from a 10-fold dilution series of a cDNA mixture of each tissue used. The qPCR primers used in this study and their amplification factors are shown in Table S2.

To check the IAPV RNQs in tissues, SP+ (n=5), SP− (n=5) and PBS-injected workers (n=5) were collected at 3 days p.i. The cDNA samples at 3 days p.i. used for IAPV RNQ analysis were also used for Dicer-2 expression in this late infection stage. In addition, IAPV-injected workers (n=5) and PBS-injected workers (n=5) were also collected at 1 day p.i. to check the Dicer-2 expression in this early infection stage.

**FISH** and microscopy. FISH was performed as described previously with several modifications (Müller et al., 2009; Möckel et al., 2011; Yue et al., 2008). SP+ (n=10), SP− (n=10) and PBS-injected (n=6) bees were collected at 3 days p.i. from three micro-colonies. Dissected brains were immediately fixed in freshly prepared 4 % formaldehyde (in PBS) at 4 °C overnight. After fixation, tissues were washed in PBS (pH7.9) (10 min, 3 times) and 75 % ethanol (30 min, 3 times) and then stored in 100 % ethanol at 4 °C until embedding. Prior to embedding, samples were firstly cleared in xylene (20 min, 3 times) and then soaked in liquid paraffin at 65–70°C (40 min, 3 times). Next, samples were embedded into paraffin and 3–5 μm sections were then cut with an electronic rotary microtome using sharp steel blades. Sections were then quickly straightened on warm sterile water (46–50°C) and mounted on advanced adhesive StarFrost slides (Knittel Glass). All the slides were stored at 4°C until hybridization.

For the specific detection of IAPV RNA via FISH, a 5'-Cy5-labelled oligonucleotide (5'-Cy5-CTTAGCGATGAAGTATCCTGAGCC) (Eurofinsdna) was used as probe (sequence designed according to Genbank accession no. EU 436423.1). Slides were first incubated at 65–70°C for 1 h and then deparaffinized in xylene (5 min, 3 times) and a series of ethanol (100 % for 5 min, 2 times; 90 % for 5 min, 70 % for 5 min; 50 % for 5 min) followed by brief dipping in sterile water for 2 min. To get the slides prepared for hybridization, pre-treatments were performed by incubating the sections in MES (Acros Organics) at 95–99°C for 10 min followed by proteinase K (200μg ml⁻¹) treatment for 5 min at 37°C. To make hybridization solution, 100ng of the probe was diluted in 20 μl hybridization buffer [20 % (v/v) formamide, 0.9 M NaCl, 20 mM Tris-HCl (pH7.9), 0.01 % (w/v) SDS]. Each slide was applied with 20 μl hybridization solution. Hybridization was performed at 46°C in a plastic humid chamber overnight. Subsequently, sections were washed in PBS (pH7.9) three times (5 min at room temperature, 5 min at 46°C and 5 min at room temperature). Finally, nuclei were counter-stained with DAPI, and sections were mounted with Fluoroshield reagent (Sigma). Fluorescence microscopy was performed using a confocal laser scanning microscope (Nikon). Images were analysed by NIS-Elements v. 4.2 and ImageJ software.

**Data analysis.** Statistics of IAPV RNQs and immune gene expressions were processed by Excel and SPSS 23. Independent Student’s t-test and two-way ANOVA with post-hoc Tukey HSD test were used for either two or multiple samples mean comparison.

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REFERENCES


