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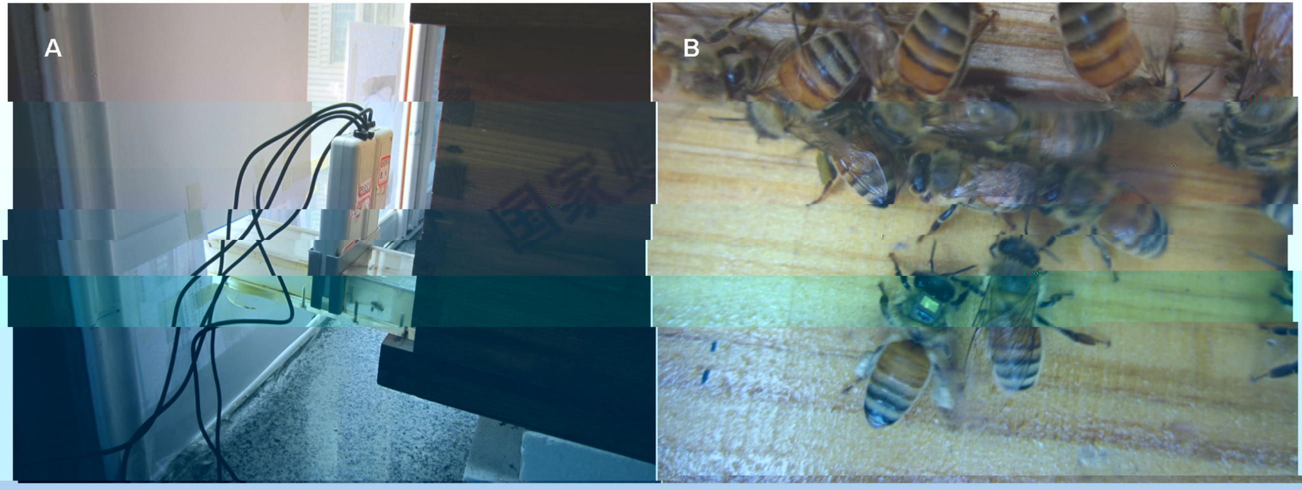
Honey bee health is mainly affected by *Varroa destructor*

Honey bees (*Apis mellifera* L.) play a vital role in global food production and economy [1]. However, honey bee colony losses in recent years have had a devastating effect on the agricultural industry and ecosystems that rely on honey bees for pollination. Colony Collapse Disorder (CCD) is a poorly understood phenomenon in which workers abruptly disappear and do not return to the hive but leave behind live queen and brood in the colony. This occurred during the winter of 2006-2007 in the US and colony losses reported in recent years in other parts of the world [2,3], poses a particular threat to apiculture and agriculture worldwide. A metagenomic survey of microflora in CCD hives showed that Israeli acute paralysis virus (IAPV), a virus that had not been previously reported in the US, displayed a strong correlation with CCD. IAPV was detected in 86% of CCD-affected colonies but in less than 5% in healthy colonies [4]. As a result, the disease mechanisms of IAPV infection in honey bees have been the subject of extensive research since then.

IAPV is a member of the genus *Aparavirus*, a new class of viruses in the *Dicistroviridae* family [5]. IAPV was first reported in 2004 in Israel where IAPV infected honey bees were found with symptoms of shivering wings, paralysis, and then death outside of the hives. The severe bee mortality caused by IAPV led to heavy losses in Israeli apiculture [6]. Since its first detection, IAPV infection has been reported in many other parts of the world [7-10]. A new study showed that *Varroa destructor* [11], an ectoparasite of the honey bee, has been catastrophic for the beekeeping industry and is an effective vector of IAPV [12]. The number of copies of IAPV in honey bees was positively correlated with the density of *Varroa* mites and the time period of *Varroa* feeding. The association of IAPV and *Varroa* mites could cause increased damage to honey

immobilized in small glass vials individually by chilling on ice for about 3 minutes. Sixty honey bees from each group were



(A) Two RFID readers were placed at the customized tunnel entrance of a nucleus hive with 3 frames. (B) A honey bee with the RFID tag glued to its thorax carrying pollen on its hind legs returns to the hive.

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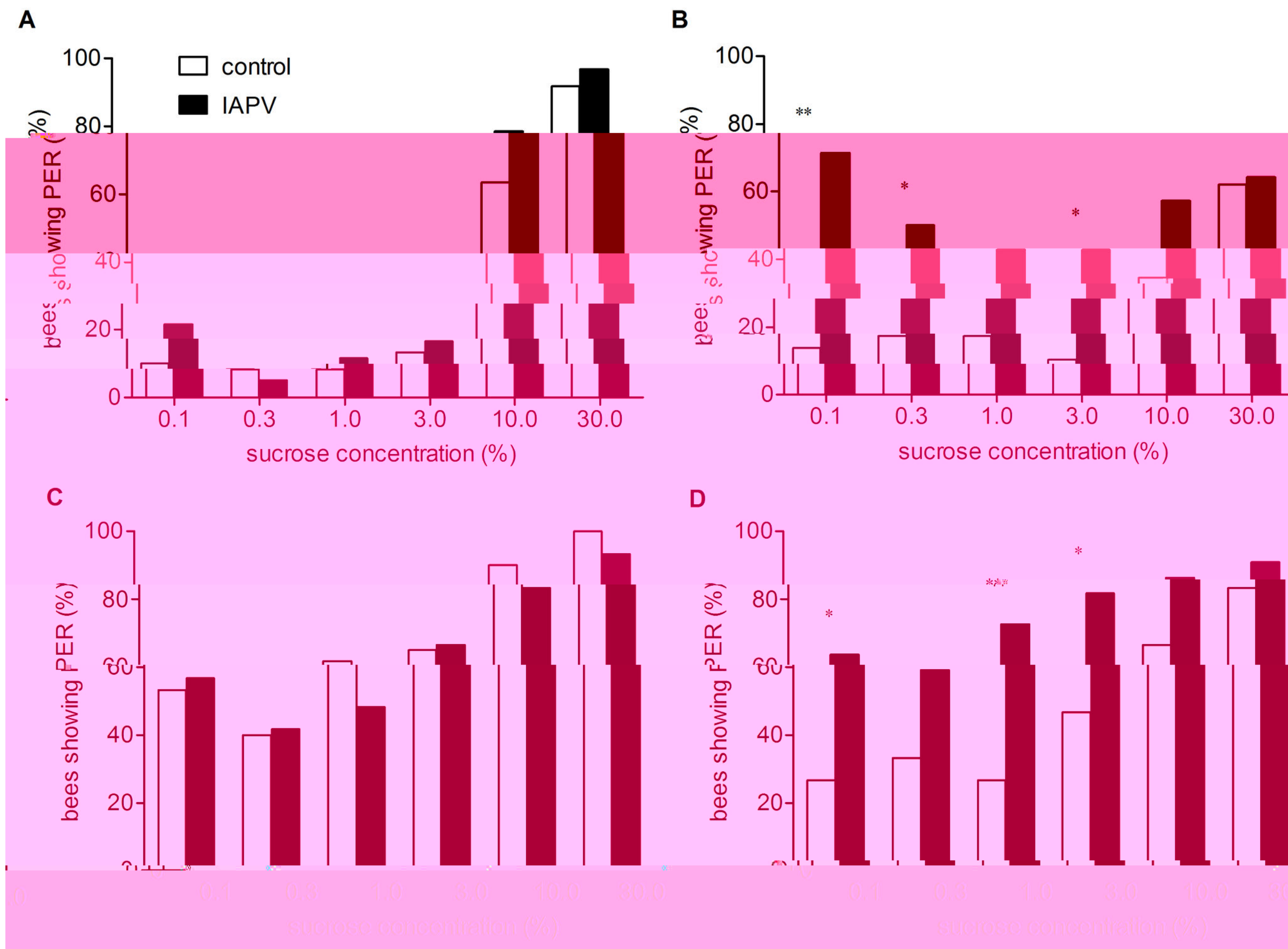
in the homing experiment. The mic3-TAG passive 13.56 MHz tags stored a unique 64-bit number (approximately 2.4 mg, 2.0 x 1.6 mm) and readers (Microsensys 2k6 HEAD) were bought from Microsensys [32]. The ID numbers of tags were input into software running on a computer by a USB-Pen prior to being glued to the thoraxes of honey bees. A 30 cm long customized tunnel was linked to the entrance of a nucleus hive, and two readers were attached to the top of the tunnel (Figure 1). The software used for storing tag ID numbers and exporting data from the readers was designed by Sebastian Streit ("Beegroup ID2DB" © Beegroup, Sebastian Streit, 2003) [31].

As the methods described above, honey bees were captured from the nucleus hives and were then immobilized on ice before injection. One group of 20 pollen foragers was injected with 1ul of IAPV with a 1: 200 dilution in PBS and another group of 20 pollen foragers was injected with 1 ul of PBS as a control. Each pollen forager was then equipped with an RFID tag on the thorax, using shellac glue, to identify the honey bee. Subsequently, the honey bees were restored in cages at room temperature (24-26 °C) and fed ad libitum with 50% (wt/wt) sucrose solution and water for an hour. After that, the honey bees were then placed in a black box and transported 500 meters away from the hive, at which point we then released these honey bees. Honey bees that could not take off within five minutes were discarded from the study [32]. The two readers recorded the entering or leaving of pollen foragers each day after the start of the experiment [32]. We did not stop observing returning honey bees until the last honey bee didn't return to the hive for each individual trial. The homing experiment was carried out in three different nucleus colonies and each homing experiment was repeated three times for each nucleus colony.

The percentage of honey bees showing PER was calculated by the number of honey bees showing PER divided by the total number of honey bees used for data collection each day after the treatment. Differences in responsiveness to sucrose solution between honey bees injected with IAPV and the control group was analyzed by the Fisher exact test. Comparison of survival rates between the two groups of honey bees was analyzed by independent samples t-test (SPSS Statistics 13.0, SPSS Inc., Chicago, IL, USA). Homing ability was assessed based on the number of bees not returning to the hive each day after the treatment. The data regarding homing ability was not normally distributed. Therefore, a non-parametrical Kruskal-Wallis test followed by Dunn's multiple comparison test was used to analyze the difference of homing ability between the two groups of honey bees using the Graphpad Prism 5 (GraphPad Software Inc, San Diego, CA, USA). Differences were considered significant at $p<0.05$ for all statistic tests and all tests were two-tailed.

The honey bees infected with 1 ul of IAPV (1: 500 dilution) containing approximately 44 copies of IAPV showed similar sucrose responsiveness at days 0, 1 and 2 after injection in comparison to the sham-injected group of honey bees (Figure 2A). However, significant differences in responsiveness to the low sucrose concentrations between the two groups were found at day 3 after injection (Fisher exact test, $p<0.05$). These honey bees were found to be more responsive to low sucrose concentrations than that of the sham-injected group of honey



Sucrose responsiveness of honey bees injected with IAPV with 1: 500 dilution and PBS-injected honey bees were tested at days 0 (A) and 3 (B) after injection. The number of honeybees tested at day 0 post-injection is 60 for each group; the number of honeybees tested at day 3 for IAPV-injected group is 14 and 29 for the PBS-injected group (*=p<0.05, **=p<0.01); sucrose responsiveness of honey bees injected with IAPV with 1: 200 dilution and PBS-injected honey bees tested at days 0 (C) and 2 (D) after injection. The number of honey bees tested at day 0 post-injection is 60 for each group; the number of honey bees tested at day 2 for IAPV-injected group is 22 and 30 for the PBS-injected group (*=p<0.05, **=p<0.01).

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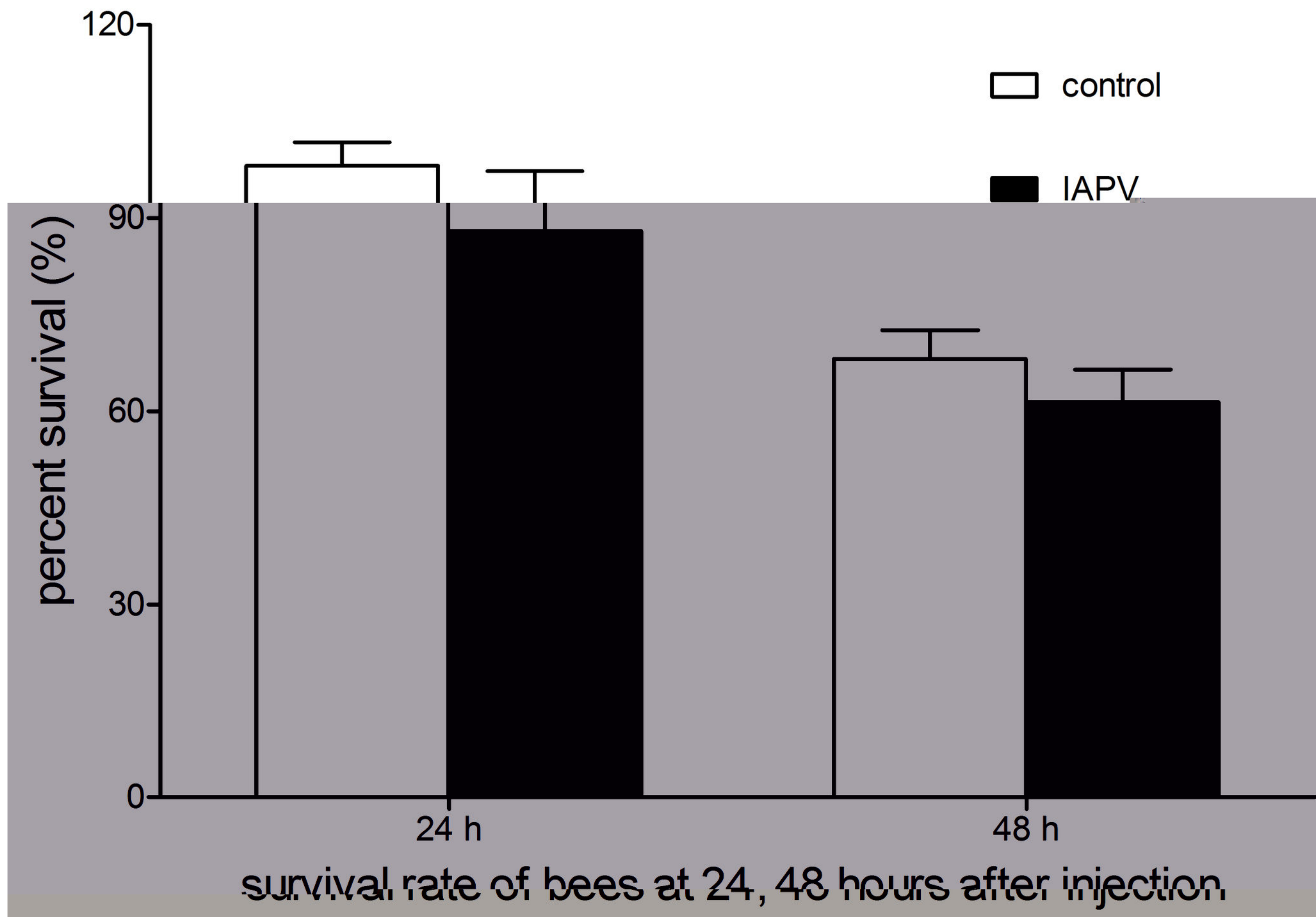
bees, and they showed no difference in responsiveness to the high sucrose water (Figure 2B).

There was no difference regarding sucrose responsiveness between honey bees infected with 1 ul of IAPV (1: 200 dilution) containing approximately 110 copies of IAPV and honey bees injected with PBS at days 0 and 1 after injection (Figure 2C). However, foragers infected with IAPV exhibited significantly higher responsiveness to the low sucrose water than that of foragers injected with PBS at day 2 after injection (Fisher exact test, p<0.05), and they showed no difference in responsiveness to the high sucrose water (Figure 2D).

There was no significant difference between honey bees injected with IAPV and the control group in survival rates (Figure 3). The proportion of live honey bees to dead honey bees was 98.1% and 88.0% for the control group and IAPV-infected honey bees at 24 hours after injection (independent samples t-test, t=2.025, df=3.933, p>0.05), and the proportion at 48 hours after injection was 68.1% and 61.4% for the two groups (independent samples t-test, t=2.012, df=6, p>0.05).

The exact number of copies of IAPV in heads of honey bees was shown in Figure 4 by relating the Ct values to the standard curve (Figure S1). There were about 48 copies of IAPV in the



Comparison of survival rates between honey bees injected with IAPV and honey bees of the control groups at 24, 48 hours after injection. The data are expressed as mean ± SD of four independent experiments.
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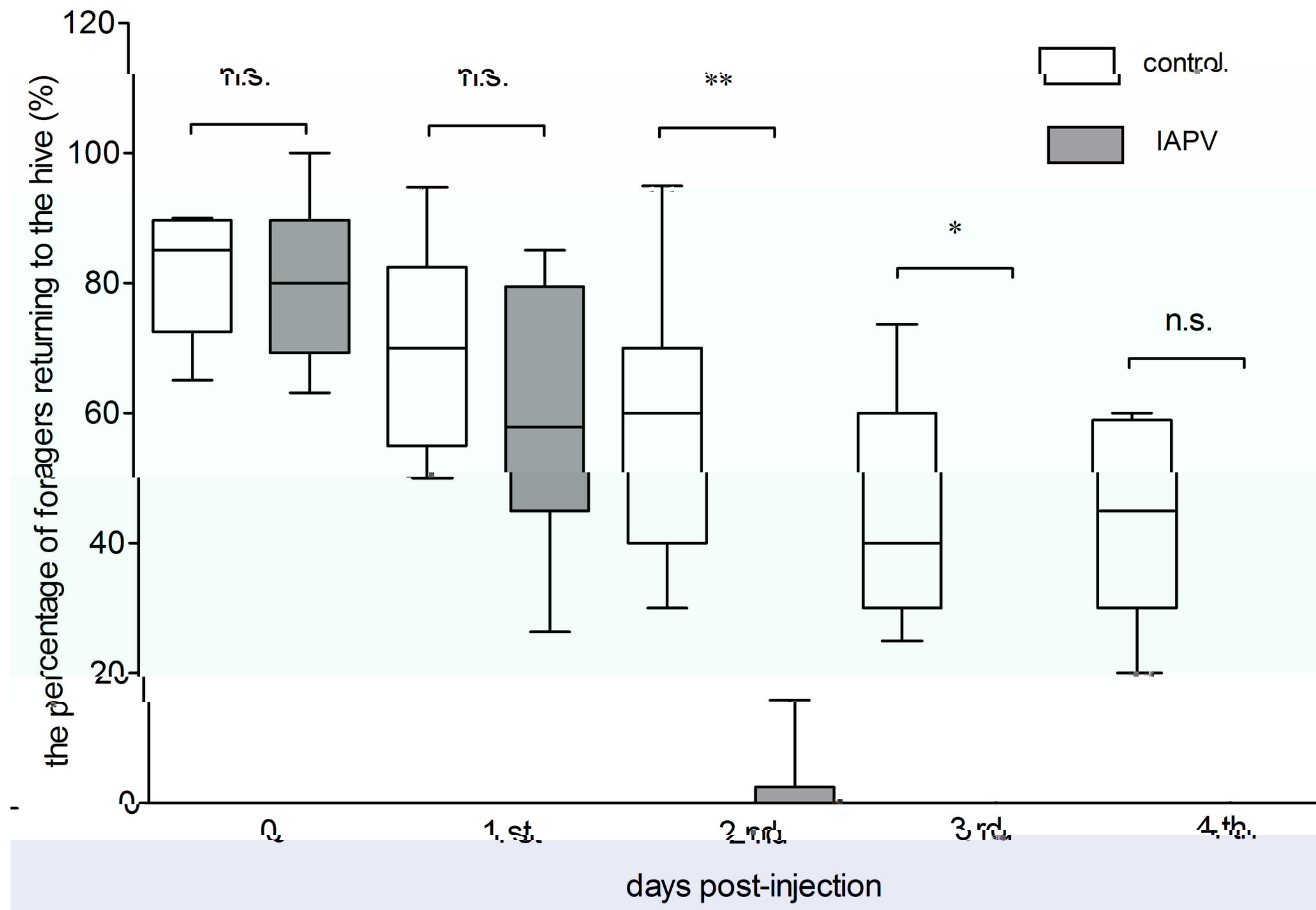
heads of honey bees at day 0 post-injection with IAPV; 6.9×10^6 IAPV copies were detected in the heads of honey bees at day 1 post-injection, and the number of IAPV copies in the heads of honey bees at day 2 post-injection was 1.2×10^7. IAPV was not detected in the heads of honey bees injected with PBS. The number of copies of IAPV increased dramatically at day 1 post-injection, however, the IAPV load exhibited no obvious increase in heads of honey bees at day 2 post-injection compared to day 1 post-injection.

The number of honey bees equipped with tags for control animals and IAPV-infected honey bees was 180, respectively. Only 4 honey bees (1.1%) failed to take off within 5 minutes and these honey bees were excluded from analysis. The homing ability of foragers infected with IAPV was depressed significantly in comparison to foragers injected with PBS (Figure 5). There was no significant difference (Kruskal-Wallis test followed by Dunn's multiple comparison test, $p>0.05$) between the two groups in the percentage of foragers returning to the hive at days 0, 1 and 4 post-injection. However, significant differences were found at 2 ($p<0.01$) and 3 ($p<0.05$) days post-injection between the two groups. The percentage of returning foragers injected with PBS returning at days 0, 1, 2 and 3 post-injection was 81.0%, 68.9%, 58.7% and 46.0%. For the IAPV injection group, the percentage was 79.6%, 59.2% , 2.3% and 0%, respectively.

At day 2 post-injection, there were around 12 foragers injected with PBS returning to the hive compared with almost no IAPV-injected foragers returning to the hive. About 10 foragers were departing and returning to the hive 3 and 4 days after being injected with PBS, however, there were no foragers departing and returning at day 3 or day 4 after being injected with IAPV. Similar results were obtained from three independent experiments and the data provided clear evidence that a viral infection of IAPV in the heads may make honey bees lose their way back to the hive.

Previous studies showed that DWV impaired the sucrose responsiveness of pollen foragers [21]. Our results demonstrated that IAPV also affected the sucrose responsiveness of pollen foragers, causing the honey bees to be more responsive to low concentrations of sugar water as a result of that metabolic stress, compared to honey bees without IAPV infection. IAPV and DWV are both common honey bee viruses and may affect behaviors of honey bees in the same way. However, the underlying disease mechanisms of the virus infections warrants further investigation. Additionally, our study of honey bees infected with different concentrations of viral particles showed similar changing patterns towards different



The abscissa represents days after foragers were injected with PBS and IAPV respectively. The ordinate represents the percentage of foragers departing and returning to the hive per day. *=p<0.05, **=p<0.01, n.s.= not significant. Error bars show SEM.

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infested foragers, which was explained as a common response to pathogens responsible for honey bee diseases [29,30]. Bortolotti et al. reported that honey bees treated with sub-lethal doses of imidacloprid did not return to the hive and showed decreased foraging activity compared to that of the control [49]. Henry et al. also reported that the homing ability of forager honey bees was impaired by thiamethoxam intoxication using RFID systems [28]. Here, we provided additional evidence that IAPV infection could impair the homing ability of honey bee foragers as well. Because there was no significant difference between IAPV-infected honey bees and honey bees of the control groups in survival rates after 24 and 48 hours post-injection, we can exclude the possibility that the honey bees that were not returning to the hive in the evening at day 2 post-injection (approximately after 48 hours injection) were primarily due to death caused by mechanism damage. In fact, honey bees that were infected with IAPV at day 2 post-injection departed from the hive in the morning and some honey bees also foraged normally during the daylight hours, however, they did not return to the hive in the evening and they may have gotten lost and died later in the field before returning to the hive. There were also no dead honey bees found in and outside the hives during the following days. PBS treated honey bees, however, foraged every day, albeit, with the gradual reduction in the number of honey bees returning to the hive during the following days even eighteen days after injection.

A limited number of IAPV copies were detected in the heads of foragers injected with IAPV at day 0 post-injection, and the number of IAPV copies increased rapidly in the heads of foragers on day 1 post-injection. However, the data also show little difference regarding the number of IAPV copies between day 1 post-injection and day 2 post-injection in the heads of foragers injected with IAPV. We concluded that IAPV may interfere with normal nervous system functions in the brains of honey bees and cause foragers to lose their way back to the hives based on the homing behaviors of IAPV-injected foragers and the number of IAPV copies detected in the heads of honey bees. In addition, detection of the viral infection in the heads of honey bees from commercial apiaries was rare, based on our preliminary experiments. Previous studies revealed that the detection of DWV in honey bees' heads is rare and represents an overt DWV infection which is also a significant indicator for

colony loss associated with DWV infection [44,50,51]. Given the fact that there were about $6.9x10^6$ and $1.2x10^7$ IAPV copies detected in the heads of honey bees at days 1 and day 2 post-injection, respectively, we propose that the high levels of IAPV replication might lead to severe disease in infected honey bees, which lost their navigational abilities and were unable to return to the hives.

An impressive result was found during a homing experiment carried out on a rainy day. There were 14 and 13 foragers for IAPV-injected and PBS-injected control group at day 1 post-injection respectively. All the IAPV-injected foragers that foraged on that day did not return to the hive in the evening. However, only 5 PBS-injected foragers foraged on that day, and 4 of those foragers returned to the hive in the evening. Therefore, 12 PBS-injected foragers foraged outside while the number of IAPV-injected foragers was 0 at day 2 post-injection (a sunny day). Foragers infected with IAPV foraged more actively in adverse weather conditions than the sham-injected foragers did, however, they did not return to the hive. A similar phenomenon was also found in honey bees infected by *Nosema* sp., which was interpreted, in part, as compensation for foraging yield because of their shortened lifespan [30]. Regardless, forager honey bees infected with IAPV at day 2 post-injection showed a trend of foraging earlier in the morning compared to that of the control group. Previous studies showed that foragers with lower response thresholds collect lower concentrations of nectar than those with higher response thresholds [52,53], which can be used to explain why foragers infected with IAPV departed the colony earlier in the day and also foraged on the rainy day.

Our studies clearly showed that both sucrose responsiveness and homing ability of forager honey bees were affected by IAPV which was injected into the hemolymph of honey bees using a 5 ul microsyringe. The wound and stress caused by the needle might simulate the process of a mite's bite. So, it is conceivable that the stress caused by the needle might have some subtle effects on the honey bees. We can’t rule out the possibility that microsyringes used to administer injections might impose additional stress on the honey bees, but it is an effective method to infect the viruses in honey bees [21]. Sucrose responsiveness reflects the division of foraging labor of honey bees [54] and homing involves spatial memory and navigation of honey bees [26,27]. Viral infection in heads may cause disorders in foraging roles of honey bees, with honey bees foraging abnormally, and may enable the virus to interfere with brain functions that are responsible for navigation, orientation and spatial memory in the honey bees. After foraging, IAPV-infected honey bees initiating homing flight from the foraging site to the hive may lose their way back to the hive due to loss in spatial memory. Our results provided first evidence that viral infection in the heads of honey bees could impair the homing ability of forager honey bees. This study is in line with previous studies that sublethal dosages of insecticides (imidacloprid, thiamethoxam et al.) could affect homing ability and foraging activity of honey bees [28,33,49,55]. Colony losses reported worldwide in recent years can, therefore, be triggered in part by multiple stressors including insecticides and viruses.

(TIF)

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Conceived and designed the experiments: SKS SWZ YPC. Performed the experiments: ZGL WFL LMY. Analyzed the data: SKS YPC ZGL LW SLC. Contributed reagents/materials/ analysis tools: SWZ SLC LGS SKS. Wrote the manuscript: ZGL YPC AS SKS.

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