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# Sublethal fluvalinate negatively affect the development and flight capacity of honeybee (*Apis mellifera* L.) workers

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ABSTRACT

Fluvalinate has been heavily used to control the pest *Varroa destructor* and residues in honeybee colony causing long-term exposure threat for bees. But, little is known about the lifetime trips and homing ability of worker bees under fluvalinate stresses during the development period. In this study, honeybees from 2-day-old larvae to 7-day-old adults were continuously fed with different concentrations of fluvalinate (0, 0.5, 5 and 50 mg/kg) and the effects of fluvalinate on the development of larvae were examined. And then, all the treated bees were reintroduced into the original source colony and were monitored, and the homing ability of 20 days old bees at 1000 and 2000 m away from the beehive were tested using the radio frequency identification (RFID). We found that fluvalinate significantly activates the superoxide dismutase (SOD) activities of larvae and 5 mg/kg fluvalinate reduced the homing rate of workers at 2000 m away from colony. 50 mg/kg fluvalinate reduced proportion of capped worker cells, activated Cytochrome P450 (CYP450) activity of larvae, affected the foraging times, influenced the homing rate and homing time of one trip at 2000 m away from colony. Our results showed that the larvae can activate the activities of SOD and detoxification enzymes in detoxification of fluvalinate and reduce the influence on honeybees. But, when the concentration is higher than 5 mg/kg fluvalinate, it is difficult for bees to detoxify fluvalinate completely, which affect the homing rate. The results reflect the potential risk for honeybees in the development stage continuously exposed to fluvalinate.

## 1. Introduction

Honeybee is a social insect closely related to human and nature. It can not only provide us with nutritious bee products, but also play a significant role in agricultural production and in maintaining ecological balance through bee pollination (Shi et al., 2020a). However, the number of western honeybee colonies has continually declined in many countries. The exact reason for the loss remains unclear. But bee disease and pesticide residues in colonies are one of the main reasons (Henry et al., 2012; Raine and Gill, 2015; Sánchez-Bayo et al., 2016).

The mite *Varroa destructor* has been posing devastating effects on beekeeping in recent decades, which suck the fat body and hemolymph from honeybee brood, causing reduced birth weight and flight performance of freshly worker bees (Duay et al., 2002; Bowen-Walker and Gunn, 2010; Dooremalen et al., 2013). Acaricides are used for *Varroa* control and fluvalinate is one of the most important varroacides, which has been used to control *varroa* mites worldwide. However, the extensively repeated using of fluvalinate in colonies causes the increase of mite resistance and reduces the efficacy of the acaricide, and higher doses of fluvalinate would be applied in the colonies, which will result in high pesticide residues in honeybee colonies and toxic effects against bees. Mullin et al. (2010) found that the residue of fluvalinate in honey, pollen, wax and bees ranged from 0.001 to 6.376, 0.001–2.67, 0.002–204, and 0.001–5.860 mg/kg separately, and the pesticide accumulates in the honey, pollen, brood comb, and so on (Bogdanov et al., 1998). The fluvalinate residues would reach 40–60 mg/kg when Apistan (fluvalinate) strips were used in colony for 6 months of continuous use (Bogdanov et al., 1998). Residues of fluvalinate within the beehives affect the health of bees. Previous studies showed that sublethal fluvalinate doses could induce decreased survival rate of queen bees (Berry

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et al., 2013; Wu et al., 2014), together with the reduced body weight and lower expression level of hexamerin gene (*hex*), vitellogenin gene (*Vg*) (Haarmann et al., 2002; Liao et al., 2018b), and disturb the development of immature drones (Rinderer et al., 1999). Honeybee workers chronically received field-level fluvalinate treatment exhibited improved legacy mortality compared with the non-treatment (Medici et al., 2012; Berry et al., 2013), while there was a lack of behavioral effects of sublethal fluvalinate on adult worker bees (Decourtye et al., 2005). Therefore, after we performed the early processing of bee larvae treated with sublethal fluvalinate doses, we further investigated differences of lifetime flighting behaviors of adult bees among four groups (0, 0.5, 5 and 50 mg/kg) after treatment. Meanwhile, we put insights into the variations of antioxidant and detoxification enzymes of worker bee larvae with the increasing doses of fluvalinate.

Antioxidant enzymes have many important functions and superoxide dismutase (SODs) rearrange superoxide to oxygen and hydrogen peroxide which can resist and repair the damaged cells in time (Corona and Robinson, 2006). Cytochrome P450 (CYP450), glutathione-s-transferase (GST) and carboxylic acid esterase (CarE) are important detoxification enzymes in honeybees which mediate xenobiotic detoxification of acaricides in the honeybee (Mao et al., 2011; Berenbaum and Johnson, 2015). Previous studies have shown that sublethal doses of pesticides can activate the activities of SOD, P450 and other enzymes of honeybees and affect the foraging flights (Wang et al., 2017; Dai et al., 2018; Colin et al., 2019; Shi et al., 2019, 2020a, 2020bbib_Shi_et_al_2019bib_Shi_et_al_2020a,2020b). However, there is still knowledge gap for effects of fluvalinate on the antioxidant and detoxification enzymes of worker larvae in larvae stage.

In this study, three concentrations (0.5, 5 and 50 mg/kg) of fluvalinate were set to study the effects of fluvalinate on development of larvae and foraging behaviors of adult worker bees while a control (0 mg/kg) group was also set in this study. The proportion of capped cells, the weight of larvae, the SOD and detoxifying enzyme activities of larvae, birth weight and emergence rate of newly emerging worker bees were completely documented. And then, the emerged bees in each group continuously fed with different concentrations of fluvalinate-sugar solution and pollen for 7 days and reintroduced into the original source colony (Liao et al., 2018a). The flight behaviors of each bee from 7-day--old to deadline were monitored and the homing ability of 20-day-old worker bees were tested at the places where are 1000 and 2000 m away from colony using the radio frequency identification (RFID) technology. This study added new evidence that fluvalinate residue in the food of honeybee inhibits the development of larvae and flight capacity of worker bees.

## 2. Materials and methods

### 2.1. Insect and fluvalinate preparation

The Western honeybee colonies were used and were kept at Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, China (28.77° N, 115.83° E). The fluvalinate was purchased from American JK Co., Ltd. In order to obtain larvae with the same age, the queen was restricted on empty comb for laying eggs for 12 h, The number of eggs laid by the queen in 12 h and subsequent hatching larvae is limited, so we set 4 groups containing 3 level of fluvalinate groups and 1 control group. The concentrations of fluvalinate need to be within the residue of fluvalinate in colonies (Bogdanov et al., 1998; Mullin et al., 2010). So,the compound was dissolved in acetone and diluted with 50 % sucrose water to concentrations of 0.5, 5 and 50 mg/kg (acetone accounted for 0.025 % by volume) according to the $LC_{50}$ of fluvalinate on honeybees (Dai et al., 2007), the residue of fluvalinate in colonies (Bogdanov et al., 1998; Mullin et al., 2010) and the references (Decourtye et al., 2005; Dai et al., 2018; Liao et al., 2018b) , 50 % sucrose water containing 0.025 % acetone (*v/v*) as a control (CK) (Decourtye et al., 2005; Dai et al., 2018; Liao et al., 2018b).This experiment was repeated three times by using three different colonies.

### 2.2. The development of larvae experiments

An empty frame was artificially divided into three parallel regions which were inserted into the comb foundations, then put into the beehive for comb building (Shi et al., 2020b), so as to eliminate the influence of natural residue of bee colony. And then, the queen bee was restricted on the empty comb for laying eggs for 12 h. 4 days later, the experimental comb containing 2-day-old larvae was taken out from the colony and larvae were divided into 4 groups which were fed with 2 μL prepared syrup containing different concentrations of fluvalinate (0, 0.5, 5 and 50 mg/kg) respectively using a pipette. After that,the comb was put back into colony. Each bee larva was fed continuously for 4 day with 2 μL fluvalinate solution respectively until it was capped, and proportion of capped worker cells were counted according to the following formula.

As larvae were hatched from the eggs laid by queen in 12 h and divided into 4 groups respectively, the capped day of the larvae is about the same time.On day 6 of larvae, the larvae are being capped. When the larvae are capped, we randomly obtained larvae from the capped worker cell of each group at the same time using tweezers. 20 larvae in capped worker cells were collected from each group and the larvae were individually weighed. And then, the larvae were stored in - 80 °C refrigerator for the determination of detoxification enzyme activity and superoxide dismutase. The detoxification enzyme activity of GST (glutathione-S-transferase), CarE (carboxylesterase), P450 (Cytochrome P450) and SOD (Superoxide dismutase activity) were tested according to the instructions of the kit for GST, CarE, P450 and SOD respectively.

After that, the comb containing capped larvae were put into the original colony for developing. 2 days before the bees emerging, the treated frames were taken out and put into an incubator for emerging and was maintained at 34 °C, 70±5 % relative humidity. When the worker bees emerged on the following days, the birth weight of each bee was measured and the emergence rate of worker bees in each treatment was calculated.

### 2.3. The lifetime trips experiments

The honeybee larvae were treated as described above and the newly emerged worker bees were hatched in an incubator. Newly emerged worker bees was fed adequate fluvalinate-sugar solution and pollen for 7 days.

Two empty hives were selected to setup the radio frequency identification (RFID) and a one-way tunnel for bees (one in and one out) at the entrance of the hive was artificially designed and were equipped with antennae connected to an RFID reader so that bees come and go could be sensed by antennae and documented by the computer through a network cable (Colin et al., 2019; Shi et al., 2020a). The electronic tags of RFID were named and given an ID number by computer.

A new colony including 4-frame with bees and a queen which were both from the same colony with the treated bees was put into the above empty hives with RFID device ahead a week to adapt bees to flight (Shi et al., 2020a). Then, 7 days old bees from different treated groups were glued with a named tag and introduced into the colony with RFID device. The lifetime trips of bees were monitored.

### 2.4. The homing ability experiments

The honeybee larvae and the worker bees in the first 7 days after emerging were treated with fluvalinate in the same way as described above. After that, the treated bees with various doses of fluvalinate were marked with different colors and were reintroduced into the treated source colony. 13 days later, 20 marked bees of 20 days old in each group were caught with tweezers and were labeled with RFID tags (Liao et al., 2018a; Shi et al., 2019; Colin et al., 2019). Locations precisely 1000 and 2000 m away from the beehives were identified using a global

positioning system (GPS) and tagged bees were released from each (Liao et al., 2018a). Their return to their hive was recorded by the RFID antenna located at the hive entrance (Liao et al., 2018a; Shi et al., 2019; Colin et al., 2019; Shi et al., 2020a).

### 2.5. Statistical analysis

The larvae weight, birth weight of newly emerged bees, enzyme activity, age at onset of foraging (bees leaved the hive for 15 min were regarded as a foraging activity (Perry et al.,2015), the average foraging trips, homing rate and homing time were analyzed by One-way ANOVA. The data of the proportion of capped cells and emergence rate was conducted arcsine substitution to perform the One-way ANOVA analysis. When the $P$ values were <0.05, we used the ANOVA test followed with Fisher's LSD test to determine whether there were any differences among different groups. Finally, the survival among different groups was analyzed based on the Log Rank (Mantel-Cox) test of Kaplan-Meier method in SPSS17.0 software (Kleinbaum et al., 2012).

## 3. Results

### 3.1. Fluvalinate exposure affected the development of larvae

The proportion of capped worker cells in 50 mg/kg group was significantly lower than the 0 mg/kg group and 0.5 mg/kg group ($F_{3,8} = 4.023$, $P = 0.041$ ) , but there was no significant differences between 50 mg/kg group and 5 mg/kg group or among the first three experimental groups (LSD test: $P > 0.05$; Table 1). There was no significant difference in larvae weight among four groups (0, 0.5,5, and 50 mg/kg) ( $F_{3,236} = 0.428$, $P = 0.733$ ) , and differences of the emergence rate and birth weight among the four groups were not significant (Emergence rate: $F_{3,8} = 3.357$, $P = 0.051$; Birth weight: $F_{3,236} = 5.939$, $P = 0.154$; Table 2).

Data in the table are mean ± SE (standard error). a and b are different letters used to represent significant differences ($P < 0.05$, Fisher's LSD test) following the data in the same column. The same for tbl2Tables 2 and 3tbl3.

With the increase of fluvalinate concentration, the SOD activity of larvae increased gradually, and there were significant differences among the four experimental groups ( $F_{3,8} = 28.962$ , $P < 0.001$; Fig. 1). The specific activity of CYP450 of larvae in 50 mg/kg group was the highest, which was significantly higher than the other three experimental groups ($F_{3,8} = 63.299$, $P < 0.001$; Fig. 2), but there was no significant difference in the specific activity of CYP450 among the first three experimental groups (LSD: $P > 0.05$). No significant differences were found in the specific activity of CarE and GST among the four experimental groups ( GST:$F_{3,8} = 0.832$ , $P = 0.513$; CarE $F_{3,8} = 1.293$, $P = 0.342$; Fig. 2 ) .

### 3.2. The foraging trips of worker bees were influenced by fluvalinate

It can be seen from Fig. 3 that the first onset of flights rate of the four experimental groups reached the peak on days 9. Within one week after being put into the colony, worker bees in all groups basically carried out the first onset of flights. The difference of the first nesting rate among the four groups was not significant (Pearson's Chi square test, $P > 0.05$). However, the foraging flights of worker bees in 50 mg/kg group was 23.9 times, which was significantly lower than that of the first three experimental groups ($F_{3,396} = 6.437$, $P < 0.001$; Fig. 4), but there was no significant difference among the first three experimental groups ($P > 0.05$).

**Table 1**
Effects of fluvalinate on the growth and development of *Apis mellifera* larvae.

| Concentration of fluvalinate (mg/kg) | Proportion of capped worker cells (%) | larvae weight (mg) | Sample size |
|---|---|---|---|
| 0 (CK) | 87.15±1.18 a | 154.15±3.37a | 60 |
| 0.5 | 86.56±2.42 a | 153.30±3.90 a | 60 |
| 5 | 84.27±1.13 ab | 151.78±5.10 a | 60 |
| 50 | 80.66±3.43 b | 151.20±4.65 a | 60 |

**Table 2**
Effects of fluvalinate on emerged rates and birth weight of *Apis mellifera* work bees.

| Concentration of fluvalinate (mg/kg) | Birth weight (mg) | Emergence rate (%) |
|---|---|---|
| 0 (CK) | 124.72±5.35 a | 95.91±0.57 a |
| 0.5 | 124.43±4.96 a | 97.53±1.26 a |
| 5 | 123.96±5.62 a | 96.44±1.37 a |
| 50 | 124.21±5.24 a | 96.34±1.24 a |

**Table 3**
Effects of fluvalinate on lifespan of worker bees *Apis mellifera*.

| Concentration of fluvalinate (mg/kg) | Mean ± SE ( day ) | Median | Sample size |
|---|---|---|---|
| 0(CK) | 25.936±1.146a | 25 | 100 |
| 0.5 | 26.750±1.199a | 25 | 100 |
| 5 | 25.900±1.013a | 24 | 100 |
| 50 | 25.430±1.046a | 23 | 100 |

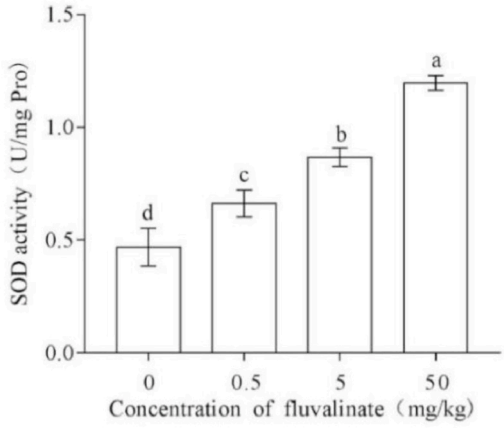


**Fig. 1.** Effects of fluvalinate on the superoxide dismutase (SOD) activities of *Apis mellifera* larvae. Each bar corresponds to a single group represented as the mean ± SE of its biological replicates. Different letters above bars mean significant difference (Fisher's LSD test, F3,8 = 28.962 , P < 0.001).

### 3.3. The homing rate and homing time of worker bees were influenced by fluvalinate

Both homing rate and homing time of worker bees at 1000 m were no significant difference among the four groups ($P > 0.05$). However, the results were completely different when the bees were released at 2000 m away from the colonies. Homing rates of workers in 5 mg/kg and 50 mg/kg were significantly lower than those in 0.5 mg/kg and 0 mg/kg ( $F_{3,20} = 6.437$, $P = 0.003$ ) , while there was no significant differences between 5 mg/kg and 50 mg/kg group as well as between 0.5 mg/kg and 0 mg/kg($P > 0.05$). Moreover, the homing time of worker bees in 50 mg/kg group was significantly longer than those in 0.5 mg/kg and 0 mg/kg groups ( $F_{3,260} = 6.437$, $P = 0.003$; Fig. 5 ) .

### 3.4. The lifespan of worker bees was not shortened by fluvalinate

Results showed that there was no significant difference among the four groups on lifespan ( $X^2 = 2.596$, $P = 0.107$; Table 3, Fig. 6 ) . The average lifespan of the four groups was about 25.43–26.75 days.

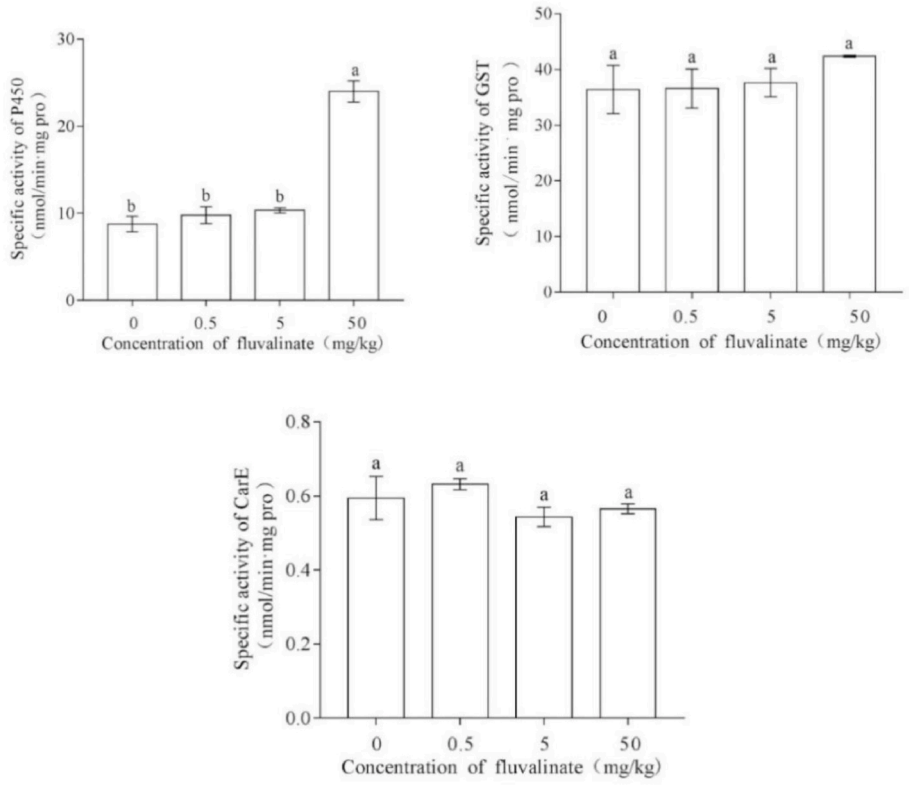


**Fig. 2.** Effects of fluvalinate on the detoxifying enzyme activities of larvae *Apis mellifera*. Each bar corresponds to a single group represented as the mean ± SE of its biological replicates. Different letters above bars mean significant difference. For P450 test: $F_{3,8} = 63.299$, $P < 0.001$; for GST test:$F_{3,8} = 0.832$ , $P = 0.513$; for CarE test: $F_{3,8} = 1.293$, $P = 0.342$).

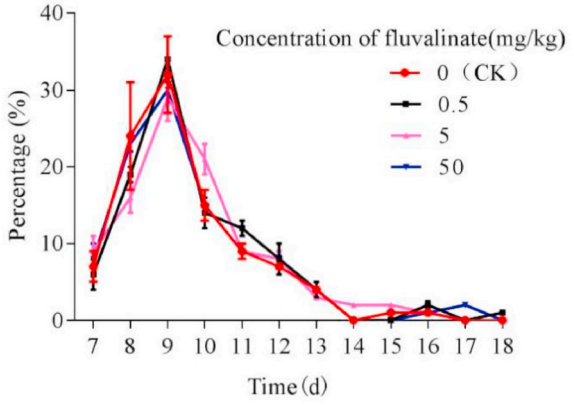


**Fig. 3.** Effects of fluvalinate on the first onset of flight of *Apis mellifera* worker bees. Y axis shows the percentage of the first onset of flights of worker bees treated with different concentrations of fluvalinate, whereas X axis shows the age of worker bees treated with fluvalinate. The colors mean different groups. There is no significant difference among the four groups (Pearson's Chi square test, $P > 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

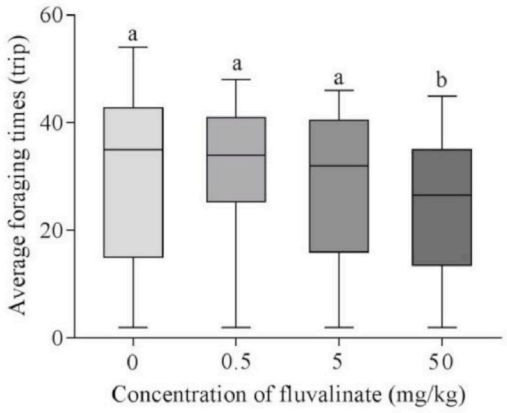


**Fig. 4.** Effects of fluvalinate on the average foraging times of *Apis mellifera* worker bees. Y axis shows the average foraging times of worker bees treated with different concentrations of fluvalinate, whereas X axis shows the treated groups. For all panels, boxplots show median, quartiles, and range. Different letters above bars mean significant difference.

## 4. Discussion

This study aimed to study the effect of fluvalinate exposure on development of larvae and flight behavior of worker bees in actual rearing conditions. In addition, it would also let us understand its medium and long-term effects on honeybees.

Our results showed that the capping rate of larvae in 50 mg/kg group was significantly reduced compared with other groups which means that the residue of fluvalinate over 50 mg/kg in food would influences the survival of honeybee larvae significantly. However, low doses of fluvalinate did not affect the survival ability of the larvae, the bees might reduce the harm of fluvalinate through their own immune system. Results show that the content of SOD in the larvae body increased significantly with the increase of fluvalinate concentration. SOD is an antioxidant enzyme which perform a variety of important functions (Corona and Robinson, 2006). Christen et al. (2019) find that the genes encoding enzymes involved in oxidative phosphorylation was altered. Moreover, the concentrations of fluvalinate over 50 mg/kg also significantly activated the P450 activity of larvae, indicating that bees can

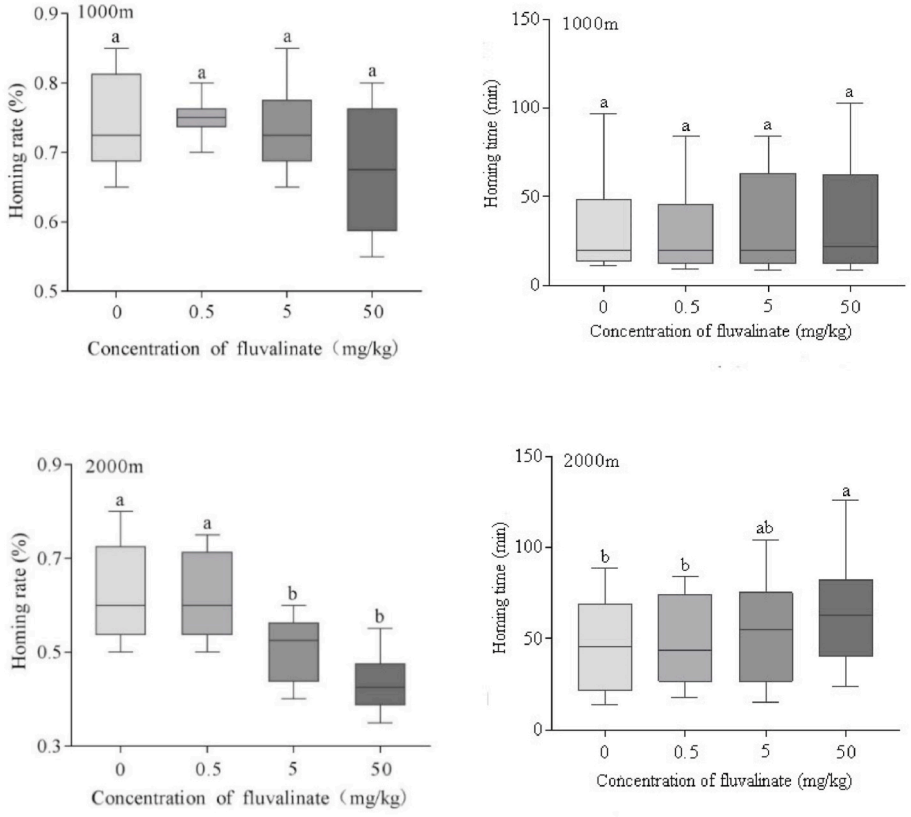


**Fig. 5.** Effects of fluvalinate on homing rates of worker bees for *Apis mellifera.* Y axis shows the homing rate and homing time of worker bees treated with different concentrations of fluvalinate from 1000 m to 2000 m away the colonies, whereas X axis shows the treated groups. For all panels, boxplots show median, quartiles, and range. Different letters above bars in each fig mean significant difference.

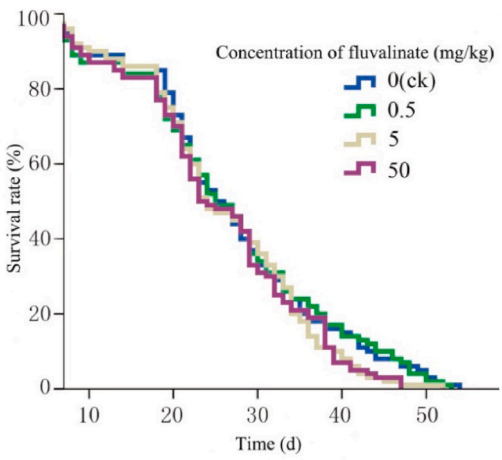


**Fig. 6.** Effects of fluvalinate on survival of worker bees for *Apis mellifera* worker bees, using the Kaplan-estimator of the survival function. Y axis shows the survival rate of worker bees treated with different concentrations of fluvalinate, whereas X axis shows the days after bees were treated with lambda-cyhalothrin. The colors mean different groups. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

activate more detoxification enzymes to detoxify, thus reducing the harm. Colin and Belzunces (1992) found that P450 was involved in the detoxification of pyrethroids in honeybees and P450s is mainly responsible for detoxification of tau-fluvalinate in honeybees (Johnson et al., 2006). In addition, fluvalinate did not affect the weight of larvae, emergence rate of worker bees and the birth weight, which was similar to that reported by Dai et al. (2018). It may be that food intake of larvae was not affected by fluvalinate. The capped larvae in cells do not need any food and its development and survival would be not significantly affected.

The effect of fluvalinate on foraging behavior of worker bee is unknown. Monitored bees on day 9 reached the peak of first onset of flight similarly with previous studies (He, 2011). He (2011) found that the peak period of the first flight of worker bees *Apis mellifera* was 7–11 days old. In addition, in this study, 7 days old bees treated with fluvalinate solutions were introduced into the colony with RFID device. Bees need 1–2 days to adapt to this environment. So, the first onset of flights rate of the four experimental groups reached the peak on days 9. But there was no observed precocious foraging phenomenon of fluvalinate on bees compared with neonicotinoid insecticides (Colin et al., 2019; Shi et al., 2020a). This may be the biological characteristics of bees and fluvalinate will not affect the division of labor of honeybees. However, the average foraging times of worker bees in 50 mg/kg group was significantly reduced compared with other groups, meaning that the excessive intake of fluvalinate by honeybees in the early stage will affect their enthusiasm for foraging. Meanwhile, the homing rate of worker bees in 5 mg/kg and 50 mg/kg groups at 2000 m away from the colony were significantly reduced comparing with control group, means that the worker bees will lose when they are foraging food from a long distance in the absence of food source. The main reason for the decline of flight ability is that fluvalinate affects the structure of sodium channel on honeybee in various degree which influence the flight function or ventilator function. In addition, the homing time of bees in 50 mg/kg group was significantly higher than that of other groups, which means that the concentration of fluvalinate over 50 mg/kg will affect the flight capability or the memory and the mechanism is worthy of further study. This may also be one of the reasons for honeybee colony collapse disorder (CCD) phenomenon, as the honeybees losing happened in winter

when it is absence of nectar and pollen while those honeybee colonies were treated with acaricide for the control of mites in autumn. It's very interesting that there was no significant difference among the four groups, it could be that honeybees can improve some part of physiological functions when they are fed with nutrients in later life (Yang et al., 2021).

## 5. Conclusions

Fluvalinate residue had no significant effect on the larvae weight, birth weight of worker bees, emergence rate of capped larvae, first onset of flights, and the homing behavior of worker bees, and the bees at 1000 m away from the colony. But, the SOD activities of larvae were activated when fluvalinate is over 0.5 mg/kg while the P450 activities of larvae and the foraging trips of worker bees were affected when fluvalinate is over 50 mg/kg. Meanwhile, the homing rate of worker bees at 2000 m away from the colony was decreased significantly when the residue of fluvalinate was higher than 5 mg/kg while the foraging times, homing time of one trip and the homing rate at 2000 m away from the colony were significantly affected when the residue is over 50 mg/kg. These data reflect the potential risk for honeybees continuously exposed to fluvalinate, and the present study contributes to the understanding of honeybee lost. Results provide a warning for beekeeping and fluvalinate using in honeybee colonies. However, there are several limitations of this study. For example, bees are usually exposed to synergistic factors, such as the mixture toxicities of pesticides on bees, the fluvalinate is used when colony has Varroa destructor mite which causes honeybees threatened by both bee mites and insecticides at the same time. We will examine the effect of fluvalinate residues on the colonies and its individuals with other factors.

*Author contribution statement*

In this work, X Wu conceived this research and wrote the manuscript, X Wu and Z Zeng designed the experiments. C Liao carried out the laboratory work and performed the experimental analysis. W Yan, X He and L Zhang contribute to the laboratory work. All authors read and approved the final manuscript.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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