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Preliminary monitoring on contrasted defensive tactics used by Iranian honey bee *Apis mellifera meda* against invader Yellow-legged hornet predator (*Vespa velutina*)

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Abstract

The aim of this study was to describe how Iranian honey bees *Apis mellifera meda* are able to defend their colonies against skirmish Asian hornet predator *Vespa velutina* in Iran; Identification differential reaction and defensive tactics that operate at the colony level during predation, and are Iranian honey bees *Apis mellifera meda* able to kill the hornet *Vespa velutina* and effective struggle or retreat under attack hornets? The present study analyzed hornet predatory behavior and collective response of colonies under attack. The results showed that Iranian honey bee *A. mellifera meda* in Iran exhibit an inefficient and unorganized defense against Asian hornet *Vespa velutina*. In some colonies, when attacked, the numbers of guards at the hive entrance increases rapidly to attack, engulf, and kill invading hornets. Balling behavior is reported here for the first time under natural conditions in *A. m. meda* against *V. velutina* in the Savojbolagh district of Iran.

Keywords: *Apis mellifera meda*, defensive tactics, yellow-legged hornet, *Vespa velutina*

1. Introduction

The yellow-legged or Asian hornet *V. velutina* was first confirmed sighting of invasive Asian hornet in Savojbolagh apiary in the Alborz province of Iran in the year 2016. The invasive Asian hornet *Vespa velutina var. nigrithorax* has been confirmed for the first time in Iran. It was found in southern Savojbolagh apiary in the Alborz province and has spread widely in Iran since then. Here, we present the first report of these species in Iran. Unfortunately, it so far no studies exist regarding the defense behavior of Iranian honeybee *Apis mellifera meda* against attack Asian predatory hornet *Vespa velutina* in Iran. The Asian Hornet *Vespa velutina* is a species of wasp that originates in Asia. It is a highly aggressive predator of native insects including honey bees. The first report of the presence of *Vespa velutina* in Iran was in 2016 in Iran, near the city of Savojbolagh apiary in Alborz province. Moreover, it is difficult to distinguish damage from *V. velutina* from other factors that threaten *A. mellifera meda* colonies Iranian honeybee, such as parasites, viruses, insecticides, pesticides and problem of absence or inadequate rainfall (climate change), extreme temperatures and drought in the world may be the major causes for hornet spread very rapidly over a large part of the countries in the worldwide [1, 3, 10, 11, 32]. Although *V. velutina* contributes to the loss of honeybee colonies. Little is known about its biology and behavior both in the native and in the invaded area. Though the invasive yellow-legged hornet, *Vespa velutina*, which first invaded Iran in 2016 and this hornet was first encountered in Savojbolagh apiary in Alborz province in Iran in 2017 spread out across 2 Iran districts within 3 years. At the very least, guards can recruit other workers to exit the colony in an agitated state [2, 17]. The hornets often attack honeybee hives to steal the larvae and some species can easily destroy a bee colony. In the evolutionary arms race between prey and predator, honeybees have evolved various defense mechanisms against hornet attacks. Many species of hornets (Vespinae) are serious enemies of honeybees [18]. Unfortunately, so far no studies exist regarding the defense behavior of Iranian honeybee *Apis mellifera meda* against attack hornets of *Vespa orientalis*, *Vespa crabro* European hornet and Asian predatory hornet *Vespa velutina* in Iran. Many studies have described several coordinated and massive defense tactics performed by the honeybee colonies when attacked by hornets. These studies were conducted in Asia on *A. cerana* and *Vespa mandarinia* [27, 28, 30], in Cyprus with *Apis mellifera cypria* and *Vespa orientalis* [21, 22, 23].

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However, research on the thermo-balling phenomenon revealed that heat alone is not sufficient to kill an engulfed hornet; a high concentration of CO₂ and relatively high humidity interact with temperature to kill *V. mandarinia* inside balls of *A. cerana* workers^[28]. In Cyprus, the defensive behavior of the native honeybee *A. mellifera cypria* against its natural predator, the oriental hornet *Vespa orientalis*, also involves a balling behavior^[22], but since its lethal thermal limit is higher than that of *Vespa mandarinia*. This action limits the functioning of the predator's respiratory system and, combined with the increase of temperature and CO₂ concentration in the insect haemolymph, causes the death of the insect. Honeybee colonies can also use defensive strategies that do not require physical contact with their enemies, including intimidation behaviors or physical barriers. Examples include colony aggregation on the beehive platform in a manner called "bee-carpet behavior"^[24], emitting an alarm sound defined as hissing^[22]. It is believed that such behavioral differentiations result from co-evolution and adaptation to environmental pressures and variable predation tactics among species^[5, 23]. Although some studies show the influence of genetic^[1, 10, 14] and neurophysiological^[32] factors on the development and evolution of such anti-predator behaviors, more studies are required to better understand these processes. This lacks the defensive abilities of *A. cerana* such as heat-balling^[20] nor does it possess defensive behaviors such as increased guard bees and changed flying behavior including reduced foraging when *V. velutina* is present^[30]. A recent study suggests that *V. velutina* may be more inclined to prey upon *A. mellifera* colonies with the lowest demonstrated defensive behaviors^[12]. Invasive species are now recognized as a major cause of native biodiversity loss worldwide^[15]. Wasps are major invertebrate enemies of honeybees, invading hives to steal honey, pollen, larvae and adults to provide sugar and protein for themselves and their offspring^[17]. The observed predation is relatively continuous. In that respect, comparison of such behaviors with those observed in other species of *Apis* and *Vespa* should provide useful insights to understanding this evolutionary arms race. The objective of this study is to better understand how Iranian honeybee *A. mellifera meda* defends its colonies against the yellow-legged hornet predator *V. velutina*, by addressing the following questions: (i) how do the two species, *A. mellifera meda* and *V. velutina*, interact at the entrance of the colony? (ii) Can Iranian honeybees *Apis mellifera meda* develop an effective balling behavior against the *Vespa velutina* predator? (iii) Provoked an increase of the number of the number of honeybees on the flight board and also are honeybees able to kill the hornet, and if so, how? We took advantage of the experimental apparatus that was used to video monitor the predation behavior of *V. velutina* at the hive entrance.

2. Materials and Methods

2.1 Study area

This study was conducted for identification differential of defense reaction and tactic of *A. mellifera meda* colonies Iranian honeybee under attack yellow-legged hornet *Vespa velutina* from 2017 to 2018 in the Savojbolagh apiary, located between (35° 50' 8" N and 51° 0' 37" E) in the Alborz province of Iran.

2.2 Experimental layout

This study describes the tactics used by Iranian honeybees *Apis mellifera meda* to defend their colonies against predatory hornet *Vespa velutina* attacks. We use simulated hornet attacks and a combination of video recordings and image analysis to reveal, for the first time, contrasted intra-subspecies defensive tactics that operate at the colony level during predation. Predator-prey relationships between sympatric species allow the evolution of defense behaviors, such as honeybee colonies defending their nests against predatory wasps.

2.3 Treatments

We investigated the predator-prey relationship between the Iranian honeybee race *Apis mellifera meda* and the Asian predatory hornet *Vespa velutina* by evaluating the effectiveness of attack and defense behaviors, which have coevolved in these sympatric species, as well as the actual damage and disturbance caused to the colonies under attack. *Vespa velutina* and *Apis mellifera meda* Iranian honey bee race being diurnal, video recording was programmed with a digital recording software (Numeriscope, Viewpoint, Iran) to begin at sunrise and to stop at sunset on the two hives. Recording began on the 10/06/2017 (before the first observation of a hunting hornet) and ended on the 10/12/2017. Two video cameras (Canon LEGRIA HF R506) connected to a computer (for video storage) was fixed on a mast, 1.50 m above the ground, at a distance of about 0.80 m from the hive and also two temperature sensor was set up on front of the hive entrance and beside hive (Fig. 1). In such a position, the camera does not disturb honeybees and hornets. Each colony was recorded for two 15 minute sessions per day using a Canon LEGRIA HF R506 video camera placed 80 cm from the hive's flight board. This work took place in the middle of each month between May 2017 and December 2018 at 10:00 A.M to 18:00 P.M and indirectly by analyzing brief (up to 600 frames/sec) video sequences recorded with Canon LEGRIA HF R506 video camera. Recordings were taken during the hottest part of the day (between 10:00 A.M and 18:00 p.m) when the wasps were most active. Two sample points at 08:00 am (05/10/2017 and 10/10/2017) were not included in the analyses because of little visibility on videos. A total of 600 h of video footage was recorded (279 h in 2017 and 321 h in 2018) and two colonies were observed for the same duration (8 h). Subsequently, two operators independently screened the video recordings using a slow motion system (VLC software v2.2.0) and the agonistic behaviors observed were used to establish an ethogram as described below. In a single-frame analysis, video images were transferred to a digitizer and measured using interactive software (Video pad). The ethogram was supplemented with further "attack" and "defense" behaviors not observed by us but reported in the literature for similar species, or in this foreigner hornet *V. velutina* facing different antagonists. This approach allowed us to evaluate the repertoire of agonistic behavior between Iranian honeybee *Apis mellifera meda* against Asian hornet *V. velutina* in a wider context. The frequency (Number of events per unit of time) was reported for all the observed attack and defense behaviors.

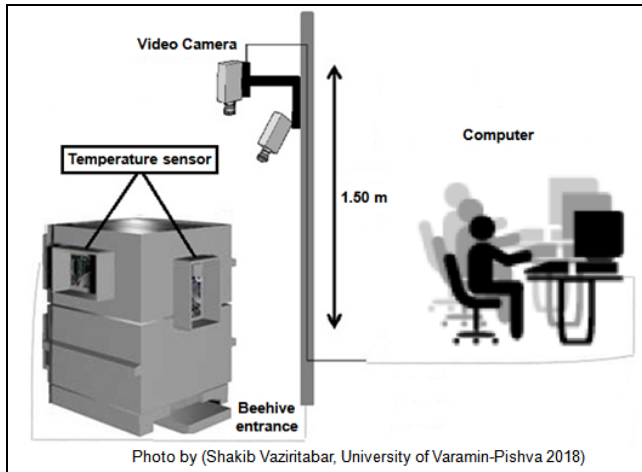


Fig 1: Position of the video cameras above and the front beehive entrance (honeybee passageway) in an experimental apiary located between (35° 50' 8" N and 51° 0' 37" E) in the Alborz province of Iran.

2.4 Observations taken

The 15-min video clips taken at the nest entrance in each colony were used to evaluate the disturbance caused by wasps on the foraging activity of the honeybees. We compared the frequency of pollen foragers entering the hive 5 min after wasp *attack* ("attack context") with the frequency at random times before the attack "control context" over a fixed 2-min interval. The comparisons were carried out for 27 agonistic events observed in 2017 to account for any interference that prevented us counting the number of pollen foragers, e.g. continuation of balling, successive attacks, or other bees blocking the view of the video camera (Fig. 1).

2.5 Statistical analysis

The disturbance of foraging activity was measured by comparing the number of pollen foragers in the attack context to the number of pollen foragers in the control context using the Wilcoxon signed rank test (paired comparisons). A chi-squared test was used to measure the proportional difference in support events (individual agonistic support and balling) between the threats and fight categories. To reduce the chance of a type I error, continuity correction was used for the chi-squared tests because the sample size was less than 250. The Wilcoxon rank sum test (unpaired comparisons) was used to compare the number of supporters in the threat and fight categories (excluding balling). We also tested for correlation (non-parametric Spearman correlation) between the number of supporters and the duration of attacks. The same test was also applied for testing possible correlations among environmental temperature, honeybee ball core temperatures and the number of bees forming the balls.

3. Results and Discussion

3.1 Observations of the natural interactions between honey bees and *V. velutina*

We observed interactions between Iranian honeybees and Yellow-legged hornets under natural conditions in both March–April 2017 and September–October 2018, and qualitatively described the general trends of both species' behavior.

3.2 Bee ball formation and balling temperature

Dead Yellow-legged hornet *Vespa velutina* Asian hornet,

(captured directly in the field and rapidly killed at high temperature) were tethered with a fine fishing-line to the tip of a 5 mm diameter, 35 mm long electronic sensor hanging from a 50 cm thin long stick and connected to a thermometer by means of an electric wire (Fig. 2). The hornets were suspended close to the beehive entrance (About 5 cm away) and were soon covered with bees. We recorded the temperature variation inside the hive using two 2 mm micro-probes connected to a highly accurate ($\pm 0.1^\circ\text{C}$) digital thermometer (YCT RS-232 thermometer) Papachristoforou *et al.* [23].

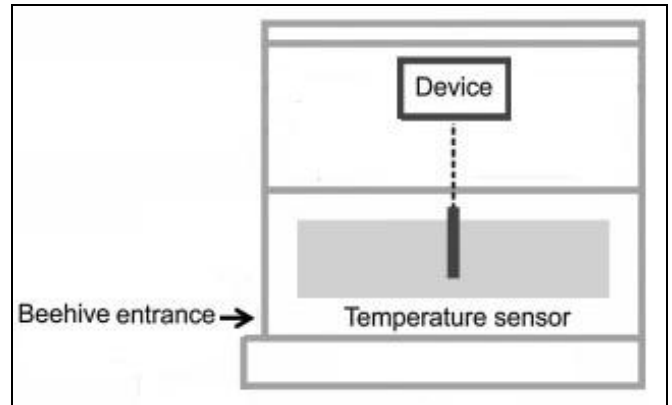


Fig 2: Shown thermo-grams and the evaluated temperatures of a social attack (thermal cluster) of bees on a wasp *Vespa velutina* that had intruded into the colony to rob the honey.

We moved the camera from section to section of the grid and recorded data for each marked bee. The first probe was attached to the hornet's thorax and the second about 15 cm from the hornet. The rise in temperature in the core of the bee ball, whenever it formed after presentation, as well as environmental temperature, was checked every 30 secs for 15 min. In all experiments the dead hornets were replaced after every three presentations. When the honey bees detect these pheromones, one hundred twenty-one or so honey bees will gather near the entrance of the nest, apparently to draw the hornet further into the hive. As the hornet enters the nest, a large mob of about hundred honeybees surround the hornet, completely covering it and preventing it from moving, and begin quickly vibrating their flight muscles. This has the effect of raising the temperature of the honeybee mass to 47°C . Though the honeybees can tolerate such a temperature, it is fatal to the intruder, which can handle a maximum temperature of about 45°C , and is effectively baked to death by the large mass of vibrating bees. The wasps died at 45.7°C , but the Asian honeybees survived temperatures up to 48.7°C and the European bees survived up to 45.5°C . Iranian honey bees *Apis mellifera* meda, have been showed to kill Asian hornet *Vespa velutina* by 'thermo-balling' and finally when killed hornets were presented at the beehive entrance, guards and other honeybees engulfed them in balls of 47.5 ± 8.4 workers within 10 min, but in another experiment 284 bees were counted in the ball. This accounts for the great variability in response to a possible threat from different colonies. A similar variability was recorded in the temperatures reached in bee balls formed by different colonies in the experiment sites with dead hornets mounted on a thermal sensor (Fig. 3). On average, at the end of 15 min the maximum temperature recorded at the core of the bee balls was $47.5 \pm 8.4^\circ\text{C}$ ($n = 284$).

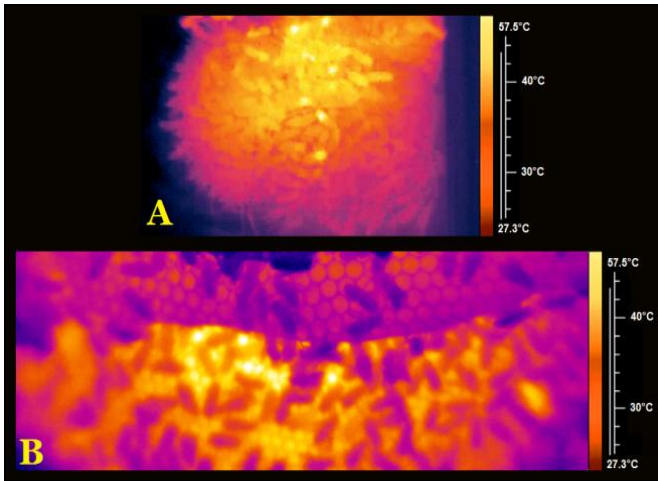


Fig 3: Illustrations A and B are shown that *Apis mellifera* meda to kill hornets by ‘thermo-balling’ in which they surrounded a hornet to from within the temperature increased to a lethal level and unusual thermal defended by honey bees against mass attack by *V. velutina* hornet. Hornets asphyxiated by *Apis mellifera* meda. (Photograph by Shakib Vaziritabar, 2017).

Nearly 56% of colonies could reach temperatures of over 47.5 °C, while the other 57.5% could not. The highest temperature measured in the honeybee ball core was 47.5°C in a single colony. The environmental temperature during the experiment averaged 27.35 ± 5.2 °C, while the difference between the environment and bee balls temperatures averaged 24.20 ± 10.6 (range = 1–40). The number of worker bees forming the ball around the hornet was strongly correlated to the maximum temperature reached by each colony (Spearman correlation test, $n = 50$, $r = 0.86$, $P < 0.001$). A strong correlation was also found between the maximum temperature each colony reached and the environmental temperature (Spearman correlation test, $n = 75$, $r = 0.87$, $P < 0.001$). The temperature inside defensive honeybee balls increased rapidly. After the first 8–10 min, the temperature remained stable for about 15 min and then decreased slightly, but it never returned to its initial value.

3.3 *Vespa velutina* death under elevated temperature conditions

When hornets were placed inside an incubator, 87% of observed *Vespa velutina* hornets died when the temperature reached 47°C and also only one *Vespa velutina* hornet was still alive after 1 h at 47.5°C (Table 1).

Table 1: Test to indicate if the highest temperature recorded inside the bee-ball is sufficient to kill the hornets.

Test	No hornets	Dead at 47°C	Dead at the end of the test	Alive at the end of the test
1	10	5	4	1
2	9	3	6	0
3	8	7	1	0
4	7	2	5	0
5	6	2	4	0

Note: The table gives the number of hornets tested at each experiment in the incubator, the number of hornets dead at 47°C, the number the hornet dead at the end of the test, the number of hornets alive at the end of the test and the test duration.

3.4 Behavior of *Vespa velutina* attack

We observed 65 attacks at the hive entrance in 279 h of video footage, specifically 55 attacks in 2017 (279 h) and 30 in 2018 (321 h) representing ~0.18 attacks per hour. The agonistic events most commonly supported by nest mates either individually or by balling were those involving physical contact (fights) rather than warning behavior (threats). Accordingly, we observed a statistically significant difference between the number of supported threats and the number of supported fights as shown in Fig 4, (chi-squared= 53.07, $df = 1$, $P < 0.001$). There was also a positive correlation between the number of supporters and the duration of attack ($S = 22658$, $P < 0.001$, $\rho = 2.53$). Agonistic support was observed only at the hive entrance, not at ground level. The hornet did not react to bees after they landed. We did not notice any reaction of the hornet to bees moving on the surface or performing body shaking behavior. Also, bees flying out from the nest passed the hornet from behind and rarely elicited any position changes or catching reaction of the hovering hornet.

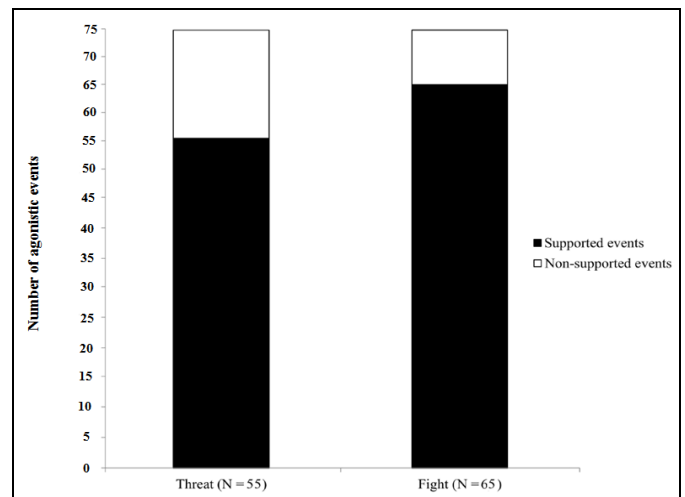


Fig 4: Number of supported and unsupported events classed as threats (Agonistic interaction without physical contact) and fights (Agonistic interaction with physical contact). The difference between the two groups was highly significant (Chi-squared test, $P < 0.001$). N = number of agonistic events observed in 60 colonies.

3.5 Honey bee response outside the hive

In response to *V. velutina* the presence around the hive, colonies exhibited three different behavioral patterns. (1) In 40% of observed colonies, a large number of honeybees gathered on the flight board and on the vertical walls near the entrance to form a cluster or a bee-carpet (Fig. 5). (2) In 22% of observed colonies, honeybees on the flight board exhibited a bee-carpet and a coordinated behavior, with individuals clinging together in groups and following the hornet’s movements by turning their body in its direction. We did not observe any shimmering in front of the hive entrance, but we did observe frequent hissing. (3) The remaining colonies (38%) did not exhibit any coordinated behavior; instead, the honeybees dispersed on the flight board and on the front wall of the hive. During our observations under natural conditions, honeybees rarely abandoned the bee-carpet formation to attack the hornet.



Fig 5: Left illustration is showing a honey bee colony under *V. velutina* predation pressure. The foraging activity has totally stopped and a large number of honeybees the bee-hive flight board to form a bee-carpet (Photos by Shakib Vaziritabar, University of Varamin-Pishva in Iran, 2017).

3.6 Social aggression

Wasps attacking bees or invading their nest to rob their honey are a nuisance for them, especially, when the wasp colonies reach their maximum strength in late summer and early autumn. Several bees introduced into an observation hive with fine thermocouples glued on the thorax were attacked by the hive bees and eventually engulfed in balls. Ball temperature increased up to 45°C. Esch^[8] had assumed that it was the attacking bees that caused this temperature increase. Our body temperature measurements show that probably both the attacking and the attacked bees contributed to the temperature increase (Fig. 6). The observation that defense by heat may also be directed against members of the own species (Fig. 6) shows that it is a general strategy in social defense of insect enemies. The bees we observed inside aggressive clusters (see, Fig. 6) additionally support the ‘temperature dependence of identification’ hypothesis. Both their behavior and their high body temperature resembled that of thoroughly examined foragers Stabentheiner *et al.*^[30]. It seems not conceivable that they heated up even higher than the clinging guards to ‘fight back’ by heat. Rather, we suggest that they did not know what was happening to them and went into a standard behavioral programme typical for intense examination, including intense thoracic heating to improve identification.

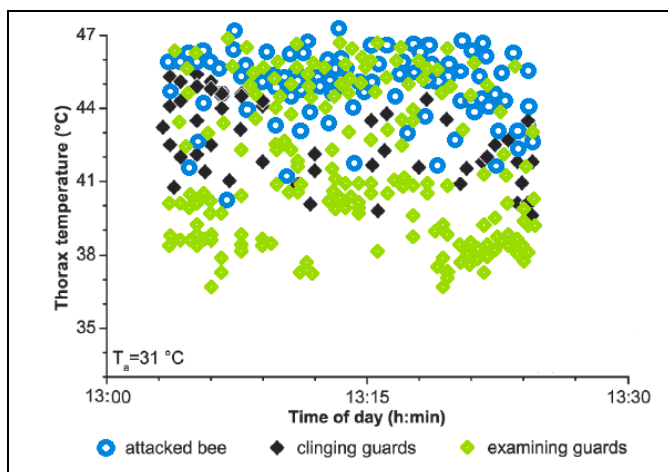


Fig 6: Body surface temperatures of Iranian honeybees *Apis mellifera* meda during thermal attacks (balling) against Yellow-legged hornet *Vespa velutina* (Graph by Shakib Vaziritabar, University of Varamin-Pishva in Iran, 2017).

By the use of real-time infrared thermography, we observed that both the thermal and the locomotor behavior differ between different cluster parts. Intensive heat production occurs only in the core bees clinging to the combated insects (Fig. 6), which applies the heat directly to the target. The

absence of movements reduces convection and contributes to an efficient use of the heat.

3.7 Seasonal abundance of *V. velutina* population

The total numbers of trapped hornet during 2017 were, (40638.4 individuals/trap) higher with about 4.3 times than those trapped during 2018, (9695.7 individual/trap). Gradual increase in hornet population was occurred in the following weeks by the first week of August to reach its climax (5845.3 and 1389 hornet/trap) in the second week of October of both 2017 and 2018 seasons, respectively. On the other hand, the numbers of trapped hornets were noticeably declined from the 3 rd. and 1 St. Week of November during the previous seasons respectively, (Fig. 7). The highest mean numbers of hornet individual were recorded during October, for the two studied seasons where represented 45.6% and 70.2% of the total trapped hornets, (Fig. 7).

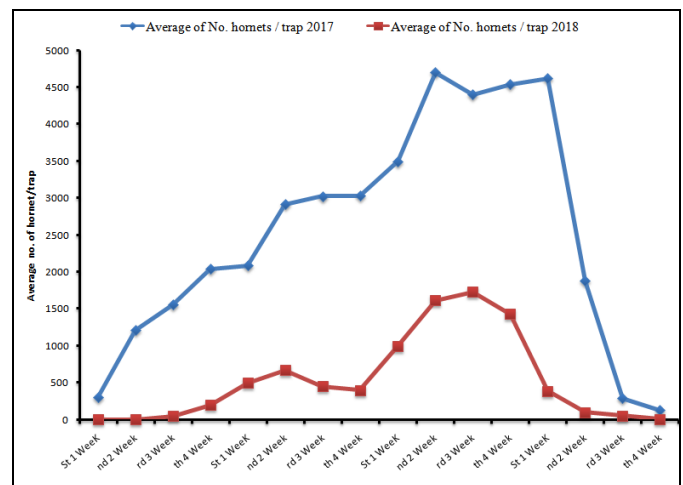


Fig 7: Weekly average number of *V. velutina* hornets/trap during active of 2017 and 2018.

4. Conclusion

The present study concluded that the colonies showed Bee-carpet behavior as a colony activity is influenced by predictors other than experimental exposure to hornets. In this case, all predictors had a significant influence. This strategy usually involves reducing colony activity then coordinating a tight group of workers on the flight board or on the vertical walls near the entrance, forming a bee-carpet. The same strategy has been observed in response to natural predators such as *V. crabro* and *V. orientalis*, though it is an efficient defense against natural predators Papachristoforou *et al.*^[24]. Fewer bees were involved in the bee-carpet in colonies tested in September and October, when predation pressure was higher, compared to those tested in August; when predation pressure was lower Monceau *et al.*^[13, 14]. Similarly, colonies tested in 2018 (Data from apiaries exposed to hornets for a long time) had fewer guards on the flight board than those tested in 2017 at the same of year (data from apiaries exposed to hornets for a shorter period). These opposing effects of long/medium-term and short-term predation may be due to different factors. The decreased response to predation pressure could be related to a specific strategy in which colonies retreat into the hive to defend their nest^[23]. The low colony activity recorded in September and October could also be linked to lower food availability; forager activity may be lower at this time of the year due to flowering phenology. It could also be linked to a natural decrease in the bee

population size in autumn or to a weakening of the colony after continuous predation by hornets in the preceding months. However, to distinguish between these different possible reasons for the apparent reduced defensive response over time, it would be interesting to know the strength and survival status of the colonies after several months of hornet predation. In Iran, defensive balling behavior occurred only in response to experimentally simulated *V. velutina* attacks. Only 9.5% of the hornets tested were killed by balling within the 5 min of simulated attacks while about 22.2% died of a sting, a defensive strategy that is absent among sympatric honeybees and hornets. Nevertheless, the honeybees were only able to kill the hornet when it was artificially introduced inside the hive over a long period. We demonstrated that *A. mellifera* in Iran produced a maximum temperature that could be sufficient to kill the hornet. However, while incubator experiments indicate that the temperature could be lethal, other possible reasons in natural conditions cannot be excluded, like the production of CO₂ Sugahara and Sakamoto [28] or limits to the function of the hornet's respiratory system Papachristoforou *et al.* [23]. Furthermore, the time required to kill the new predator at high temperature is more than 30 min in the incubator. Furthermore, factors other than temperature, or in combination with temperature, should be investigated further in the future. Indeed, colonies of the same strength and that exhibited similar temperatures inside the ball differed in their ability to kill hornets. Many studies Rothenbuhler [26]; Collins *et al.* [4]; Moritz *et al.* [16]; Guzman-Novoa and Page [9] have demonstrated the importance of genetics in honeybee aggressiveness and defensiveness, with some strains being more aggressive than others Ruttner [25] and Breed *et al.* [1]. Direct attacks on hornets by honeybees are also ineffective because of *V. velutina*'s highly effective predatory behaviors. In contrast to other well described predatory habits observed in other *Vespa* species [21], *V. velutina* only attempted to land on the honeybee hive flight board and enter the hive after the colony had become too weak to react; instead, they tended to maintain stationary flight and catch foraging honeybees at safe distance. Future studies should focus on the progressive weakening of colonies in response to predation pressure from *V. velutina* to better understand if this weakening is the result of reduced flow of food from the environment. It may either result from an indirect reduction in overall foraging behavior in response to predation risk or from a direct reduction in the number of available foragers because of wasp predation.

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