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NOTES

Validation of hygienic *Apis mellifera* L. colonies against *Varroa destructor* Anderson and Trueman infestation

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Varroa destructor is a major bee parasitic mite causing huge losses to *Apis mellifera* colonies worldwide. Apart from various chemical based strategies, hygienic behaviour is an important ecological *Varroa* management strategy. This trait plays an important role in imparting the colony resistance against the *V. destructor*. Here, we assessed the colony level hygienic behaviour of 100 colonies using pin-killed brood method and from these 100 colonies, ten colonies (7 hygienic and 3 non-hygienic) were validated against *V. destructor* infestation for two seasons, autumn and spring. The worker larval brood near capping stage was inoculated with *Varroa* mite. In total, 21 inoculations were made in every test colony and replicated thrice. The observations were recorded at every 2 h interval till complete removal of mite. During the autumn season, in the 7 hygienic colonies, the mean of *Varroa* mite inoculated brood cells emptied after 2, 4 and 6 h was 1.36±0.11, 3.17±0.10 and 5.66±0.68%, and while in the non-hygienic colonies, it was 0±0.00, 0.52±0.10 and 2.11±0.53%, respectively. After 24 h a mean of 93.43±2.43% of brood cells were emptied in the hygienic colonies, while in the non-hygienic colonies, it was only 61.90±4.59%. During the spring season, in the hygienic colonies, mean mite inoculated brood cells emptied after 2, 4 and 6 h were 3.62±1.24, 6.57±0.73 and 7.25±0.47%, respectively while in the non-hygienic colonies the mean was 0±0.00%, 1.57±0.00 and 2.11±0.53%. After 24 h, it was 96.83±1.86% and 77.25±0.53% in the hygienic and non-hygienic colonies, respectively. In the autumn season, the hygienic colonies on an average took 28 h, whereas non-hygienic colonies took 50.67 h to achieve 100% uncapping and cleaning of cells. On the contrary, the hygienic colonies on an average took 25.71 h, whereas non-hygienic colonies took 47.36 h to achieve the same in the spring season. Hence, the hygienic behaviour can contribute to the colony's resistance towards *V. destructor* mite inoculation in capped brood cells and result in reduced use of chemicals into the honey bee colonies.

Keywords: European\Western honey bee, Bee colony, Brood cells, Mite resistance

The Asian hive bee, *Apis cerana* Fab. is the natural host of *Varroa* mite. Damage to Asian honey bee colonies is rarely experienced since a stable host-parasite relationship has been developed over a long evolutionary period[1]. Such a relationship is lacking in the European or Western honey bee. The infestation of *Apis mellifera* by *Varroa destructor* has been reported to originate nearly half a century ago[2] when the *A. mellifera* colonies were brought into contact with *A. cerana*. *V. destructor* is an obligate ectoparasite and feeds on the haemolymph of adult bees and the brood[3]. For reproduction, it chooses only the capped worker and drone brood of *A. mellifera*, and only the drone brood of *A. cerana*[4]. If timely and proper mites control measures are not taken[5], the mortality of *A. mellifera* colonies due to *V. destructor* can reach up to 100% within 2-5 years. Besides, high mite populations were also observed to be associated with increased incidences of viral infections, lower weight at hatching, and shortened life span of the adult bees[6] as well as deformed wing and shortened abdomen.

Hygienic behaviour in *A. mellifera* is a mechanism of resistance to American foulbrood[7], chalk brood[8] and *V. destructor*[9]. The hygienic honey bee workers have the ability to detect diseased brood, uncap the wax covering over the brood cells and remove infected larvae or pupae. Afterwards, it has been demonstrated that hygienic bees detect and remove pupae infested with the parasitic *Varroa* mites. It has the potential to limit the population growth of *Varroa* in three ways: Firstly, the immature mites are killed when the pupa is removed, which decreases the average number of offspring per reproducing mite, second, the phoretic period of adult female mites is extended that survive the removal of the pupae, and consequently the mortality of the adult mites increases if they are damaged by the adult bees through grooming when they escape through the opened cell[10]. The honey bees enemies and diseases negatively affect colony growth and productivity, resulting in economic losses[11]. Therefore, keeping colonies having higher degree of hygienic behaviour is recommended as a natural method of minimizing the incidence of pests and diseases. In the present study, we assessed the hygienic behaviour as defense against *V. destructor* infestation in capped brood cells, so that colonies expressing resistance to the mite population can be selected for honey bees breeding experiments.

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Materials and Methods

Mapping of hygienic colonies

The studies were conducted at the *Apis mellifera* Apiary at Entomological Research Farm, Department of Entomology, Punjab Agricultural University, Ludhiana. The hygienic behaviour of 100 colonies was assessed by pin-killed brood method and the experiment was replicated thrice to account for the variability in sub-families (patrilines) with respect to their hygienic behaviour. The percentage of brood removal in each colony was recorded after 24 h and the colony that removed a mean of 80% or more of the dead brood, was considered hygienic and <80% was considered as non-hygienic[12]. Based on the hygienic response of the evaluated 100 colonies expressed within the first 24 h of the brood pricking, 10 colonies (7 the most hygienic and 3 the most non-hygienic) were validated against *V. destructor* infestation for two seasons; autumn, 2016 and spring, 2017.

Assessing hygienic colonies for *Varroa* infestation

V. destructor adult mites were collected from infested worker brood and drone brood using Varroa fork and from adult bees using 'sugar roll method'. The mites were used in inoculating worker larval brood near capping. In total, 21 inoculations (three groups of seven brood cells each) were made in every test colony, thrice in succession. The observations were recorded at every 2 h interval till the complete removal of mite infested brood. The time period between inoculation and uncapping and brood removal was also recorded.

Statistical analysis

The data were analyzed using ANOVA for finding the significance of difference among the colonies for removal of inoculated *V. destructor* mite from the brood cells and were separated by least significant difference (LSD) at p=0.05 level[13]. The data on mean percentage of mite inoculated brood cells emptied at various intervals were transformed using arc sine √percentage transformation.

Results and Discussion

Emptying of *Varroa destructor* inoculated brood cells

Autumn season, 2016

In the seven hygienic colonies, the mean of *Varroa* mite inoculated brood cells emptied after 2, 4 and 6 h was 1.36±0.11, 3.17±0.10 and 5.66±0.68%, respectively, while, in the non-hygienic colonies it was 0±0.00, 0.52±0.10 and 2.11±0.53%. After 20 h of inoculation, in four of the hygienic colonies the percentage of inoculated brood cells emptied ranged between 85.71±2.75-88.90±1.59% and in the non-hygienic colonies it was in the range of 42.86±0.00-58.71±1.59%. After 24 h, in the selected hygienic colonies, a mean of 93.43±2.43% of brood cells removal was recorded, while in the non-hygienic colonies, it was only 61.90±4.59%. After 30 h of inoculation, six hygienic colonies have achieved 100±0.00% emptiness of inoculated brood cells and in non-hygienic colonies it was in the range of 71.83±0.00 - 85.71±0.00%. After 44 h all the hygienic colonies have reached 100±0.00%% removal of inoculated brood cells and in non-hygienic colonies a mean of 94.7±1.84%% was observed (Table 1).

Spring season, 2017

During spring season, in the seven hygienic colonies, the mean of *Varroa* mite inoculated brood cells emptied after 2, 4 and 6 h was 3.62±1.24, 6.57±0.73 and 7.25±0.47%, respectively while in the non-hygienic colonies the mean was 0±0.00%, 1.57±0.00 and 2.11±0.53%. In the hygienic colonies, the percentage of inoculated brood cells emptied ranged between 82.52±1.59 - 95.24±2.75% after 20 h of inoculation and in the non- hygienic colonies at this hour, the brood cells emptied were in the range of 71.43±0.00 76.19±2.75%. After 24 h of inoculated brood cells, in the selected hygienic colonies, a mean of 96.83±1.86% of brood cells were emptied, while in the non-hygienic colonies, it was 77.25±0.52%. After 30 h of inoculation, six hygienic colonies have achieved 100±0.00% emptiness of inoculated brood cells and in non-hygienic colonies it was in the range of 80.95±1.59 - 85.71±0.00%. After 44 h all the hygienic colonies have reached 100±0.00% removal of inoculated brood cells and in non-hygienic colonies it was a mean of 95.24±1.24%. The non-hygienic colonies recorded 100±0.00% removal of inoculated brood cells after 52 h of inoculation (Table 1).

In the hygienic colonies the overall mean of *Varroa* mite inoculated brood cells emptied after 20 24 and 30 h (end of day one) was 85.49 ± 2.73, 95.13 ± 1.71 and 99.55 ± 0.23% and in non-hygienic colonies it was 61.65 ± 11.94, 69.58 ± 7.70 and 81.75 ± 1.33%. On day two, 44 and 46 h after inoculation, the brood cells emptied in hygienic colonies was 99.89 ± 0.12 and 100 ± 0.00% and

Table1 — Mean percentage of *Varroa destructor* inoculated brood cells emptied at various time intervals during autumn 2016 and spring 2017

COL NO.	Autumn 2016							Spring 2017							Pooled mean
	6 h	20 h	24 h	28 h	44 h	48 h	Overall Mean	6 h	20 h	24 h	28 h	44 h	48 h	Overall Mean	
1H	9.52 ±0.00 (7.96)	77.77 ±3.18 (61.95)	88.89 ±1.59 (70.59)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	79.36 (70.07)	6.35 ±1.59 (14.39)	85.71 ±0.00 (67.76)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	82.01 (73.67)	80.69 ±1.33 (71.87)
2H	4.76 ±0.00 (12.60)	85.71 ±2.75 (67.95)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	81.75 (73.40)	6.35 ±1.59 (14.39)	87.30 ±1.59 (69.17)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	82.27 (73.90)	82.01 ±0.27 (73.65)
3H	6.35 ±1.59 (14.39)	87.30 ±1.59 (69.17)	93.65 ±1.59 (75.58)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	81.22 (71.50)	9.52 ±0.00 (17.96)	90.48 ±0.00 (72.00)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	83.33 (74.97)	82.27 ±1.06 (73.24)
4H	4.76 ±0.00 (12.60)	76.19 ±0.00 (60.77)	85.71 ±0.00 (67.76)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	77.78 (68.50)	6.35 ±1.59 (14.39)	82.54 ±1.59 (65.32)	88.89 ±1.59 (70.59)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	79.63 (70.03)	78.70 ±0.93 (73.24)
5H	4.76 ±0.00 (12.60)	76.19 ±0.00 (60.77)	85.71 ±0.00 (67.76)	95.24 ±0.00 (77.37)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	76.98 (66.40)	6.35 ±1.59 (14.39)	84.12 ±0.00 (66.54)	90.48 ±1.59 (72.00)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	80.16 (70.47)	78.57 ±0.09 (68.44)
6H	4.76 ±0.00 (12.60)	88.89 ±1.59 (70.59)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	82.27 (73.84)	7.93 ±1.59 (16.18)	92.07 ±1.59 (73.79)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	83.33 (74.97)	82.80 ±0.53 (74.40)
7H	4.76 ±0.00 (12.60)	87.30 ±1.59 (69.17)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	82.01 (73.60)	7.93 ±1.59 (16.18)	95.24 ±2.75 (79.78)	98.41 ±1.59 (85.76)	98.41 ±1.59 (85.76)	98.41 ±1.59 (85.76)	100.00 ±0.00 (89.96)	83.07 (73.87)	82.53 ±0.53 (73.74)
1NH	3.17 ±1.59 (8.40)	42.86 ±0.00 (40.88)	53.97 ±1.59 (47.26)	68.26 ±1.59 (55.70)	92.07 ±1.59 (73.79)	95.24 ±0.00 (77.37)	59.26 (50.56)	3.17 ±1.59 (8.40)	73.02 ±1.59 (58.70)	76.19 ±0.00 (60.77)	82.54 ±1.59 (65.32)	92.07 ±1.59 (73.79)	100.00 ±0.00 (89.96)	71.16 (59.49)	65.21 ±5.97 (55.03)
2NH	1.59 ±1.59 (4.20)	47.62 ±0.00 (43.62)	61.90 ±0.00 (51.86)	84.12 ±1.59 (66.54)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	65.87 (57.69)	1.59 ±1.59 (4.20)	71.43 ±0.00 (57.67)	77.78 ±1.59 (61.88)	77.78 ±1.59 (61.88)	100.00 ±0.00 (89.96)	100.00 ±0.00 (89.96)	71.43 (60.92)	68.65 ±2.79 (59.31)
3NH	1.59 ±1.59 (4.20)	58.73 ±1.59 (50.01)	69.84 ±1.59 (56.68)	85.71 ±0.00 (67.76)	95.24 ±0.00 (77.37)	100.00 ±0.00 (89.96)	68.52 (57.66)	1.59 ±1.59 (4.20)	76.19 ±2.75 (60.84)	77.78 ±1.59 (61.88)	85.71 ±0.00 (67.76)	95.24 ±0.00 (77.37)	100.00 ±0.00 (89.96)	72.75 (60.33)	70.63 ±2.12 (59.00)
Mean	4.60 (11.21)	72.86 (59.49)	83.97 (70.74)	93.33 (80.72)	98.73 (87.09)	99.52 (88.70)		5.71 (12.47)	83.81 (67.16)	90.95 (77.27)	94.44 (82.05)	98.57 (86.67)	100.00 (89.96)		
Pooled mean (Autumn & Spring)	5.16 (11.84)	78.33 (63.32)	87.46 (74.01)	93.89 (81.38)	98.65 (86.88)	99.76 (89.33)	-	-	-	-	-	-	-		
Seasons (Autumn & Spring)	75.50 (66.3)	78.91 (69.26)	-	-	-	-	-	-	-	-	-	-	-		

[Data values represent mean of 3 replications±$S.E._m$. H, Hygienic NH, Non-hygienic H, Hours. Figures in parentheses are the means of arc sine √percentage transformation]

LSD (p=0.05) for	Autumn	Spring	Pooled (Autumn & Spring)
Colony	(1.37)	(1.97)	(1.20)
Time	(1.06)	(1.52)	(0.93)
Colony x Time	(3.35)	(4.82)	(2.93)
Season	-	-	(0.54)
Colony x Season	-	-	(1.69)
Time x Season	-	-	(1.31)
Colony x Time x Season	-	-	(4.15)

95.77 ± 0.00 and 97.09 ± 1.32% in non-hygienic colonies (Fig. 1).

Time interval to attain 100% uncapping and cleaning of inoculated brood cells

In the autumn season the hygienic colonies on an average took 28 h whereas non-hygienic colonies took 50.67 h to achieve 100% uncapping and cleaning of cells. On the other hand, the hygienic colonies on an average took 25.71 h whereas non-hygienic colonies took 47.36 h to achieve the same in spring season. Fig. 1 depicts that the hygienic colonies overall took 28 h for100% removal of mite from the capped brood

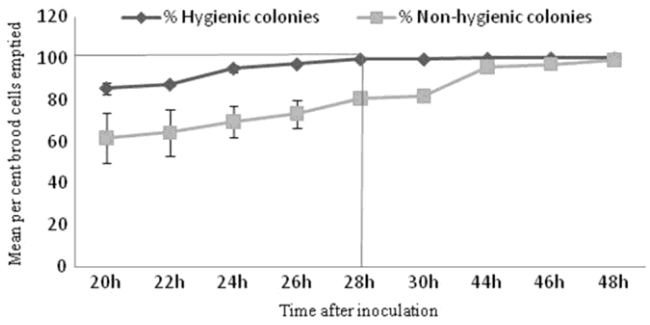

Fig. 1 — Overall mean per cent brood cells emptied in hygienic and non-hygienic colonies after various time intervals of *Varroa* inoculation

cells and the non-hygienic colonies took 48 h for 100% uncapping and cleaning of cells.

It was observed that during spring season, the process of uncapping and cleaning of *Varroa* inoculated brood cells was rapid than that recorded in autumn season. This may be due to the season, wherein in spring season there is brood build up activity and more number of brood cells are required for egg laying and storage and the population of the colonies is strong as compared to autumn season.

Results of the present study are in conformation with the earlier studies. In European or western honey bees, some colonies of *A. mellifera carnica* Pollmann detected, uncapped, and removed mite-infested pupae[9]. Colonies of *A. m. ligustica* Spinola specifically bred for hygienic behaviour removed significantly more infested pupae than non hygienic colonies[10]. Carniolan bees in Germany removed 26-30% of *Varroa*-infested worker brood[14]. Africanized colonies removed a significantly greater proportion of brood infested with the parasitic mite *Varroa jacobsoni* Oudemans[15]. There was a significant negative correlation between hygienic behavior and the numbers of *Varroa* in the colonies[16,17]. Hygienic bees removed the majority of mite infested brood at least 60 h after the cell is sealed, which is after the mite has initiated oviposition[5]. Removing the pupa at that time ensures destruction of any mite progeny. Removal of brood cells invaded by mites interrupts the reproductive cycle of *V. destructor* and prolongs its phoretic phase or kills the parasite[18]. During good nectar flow bees remove dead brood faster, thereby preparing cells for nectar collection[19]. Since hygienic behaviour is mainly exhibited by workers that are younger than three weeks[20-22] and different balance of young and old bees in the colony may affect expression of the trait at the colony level. This may, explain the lower cleaning rate which was observed during autumn as compared to the spring season[23]. A perusal of literature reveals that the increased hygienic response of Russian Honey Bee (RHB) to brood infested with *V. destructor* as well as removal of phoretic mites are probably major contributors in their resistance against mite parasitism[24]. Studies on African bees also supported that hygienic behaviour to be one of the driving forces in defence against pests and diseases[25,26]. Our study too have shown that the hygienic colonies were quick in removing the brood cells inoculated with the *Varroa* mite and exhibited defensive mechanism against the mite as compared to the non-hygienic colonies. Another study confirmed that *A. mellifera scutellata* bees are able to remove introduced mites and the brood cells were recapped in about 26% of the artificially infested brood cells[27]. Hygienic behaviour specifically targeting *Varroa* infested capped brood cells (VSH-*Varroa* sensitive hygiene) has been confirmed as a major trait in reducing mite population growth in European and African bee populations[28]. Selection for hygienic behaviour is being used by beekeepers to help reduce their mite treatment regime, and the *Varroa* sensitive Hygiene line that targets the removal of mite infested brood is undergoing further selection in Hawaii to make it suitable for *Varroa* mite management[29]. Also, the colonies headed by new queens reported low level of *Varroa* infestation as compared to bees headed by old queens and colonies led by new queens removed 84.67% of artificially introduced mites[30]. The colonies expressing high hygienic behaviour was negatively correlated with phoretic mite counts and mite infestation levels in brood[31].

Conclusion

Validation of hygienic behaviour in *Apis mellifera* against *Varroa destructor* revealed that the bee colonies mapped as hygienic removed significantly more *Varroa* infested brood. The hygienic colonies cleaned the colony from mite infestation within 28 h completely while the non-hygienic colonies took 48 h to clean. It shows that *A. mellifera* colonies with better hygienic behviour were effective in the mite management. Thus, the breeding bees carrying high hygienic behaviour trait would be an eco-friendly and economical strategy avoiding the usual application of chemical acaricides for mite management.

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Conflict of Interests

Authors declare no conflict of interests.

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