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Communal use of integumental wounds in honey bee (*Apis mellifera*) pupae multiply infested by the ectoparasitic mite *Varroa destructor*

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ABSTRACT. The ectoparasitic bee mite, *Varroa destructor*, is highly adapted to its natural and adopted honey bee hosts, *Apis cerana* and *Apis mellifera*. Adult females perforate the integument of bee pupae in such a way that they and their progeny can feed. We examined the wounds that founder females made, and usually found one, and rarely up to three, integumental wounds on pupae of *A. mellifera* multiply infested by *V. destructor*. The punctures were mainly on the 2nd abdominal sternite of the host. These perforations are used repeatedly as feeding sites by these hemolymph-sucking mites and by their progeny. The diameter of the wounds increased during pupal development. In brood cells containing 4-5 invading female mites and their progeny, healing of the wound is delayed, normally occurring just before the imaginal moult of the bee pupa. These wounds are subject to microbial infections, and they are relevant to the evolution of behavioral traits in these parasitic mites and their relations to host bees.

Key words: *Apis mellifera*, *Varroa destructor*, Pathogen invasion, Multiple brood infestation, Evolution of parasite-host relations, Integumental wounds

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INTRODUCTION

In colonies of European honey bees, *Apis mellifera carnica*, severely infested with the mite parasite, *Varroa destructor*, individual brood cells may be infested with up to 20 females of the ectoparasitic mite, *Varroa destructor* (Fuchs and Langenbach, 1989; Duay et al., 2002a). Invasion of a brood cell occurs during a short period of high attractivity of the L5 instar within several hours prior to capping of the cell (Aumeier et al., 2002). In the sealed cell, the parasites puncture the integument of the preimaginal host bee in order to suck hemolymph (Ifantidis and Rosenkranz, 1982). This perforation is repeatedly used by the mother mite and her progeny throughout pupal development (Donzé and Guerin, 1994). The small *Varroa*-made wounds are difficult to detect, but they can be visualized by vital staining with Trypan blue, using a recently described protocol (Kanbar and Engels, 2004).

Workers and drones emerging from heavily infested brood cells weigh much less than non-parasitized controls (De Jong et al., 1982; Duay et al., 2002a), due to severe hemolymph loss. Under Central European conditions, 2-5 female mites per brood cell are frequently observed in colonies with a high incidence of parasitism (Boot et al., 1997). It was yet unknown whether bee pupae multiply infested with *Varroa* females suffer increased puncturing of the cuticle corresponding to the number of mites that invaded the brood cell. We studied this question by staining with Trypan blue to detect the perforations of the bee integument. The number, ultrastructure and size of the wounds were determined in drone and worker pupae.

MATERIAL AND METHODS

Brood combs were removed from honey bee colonies (*Apis mellifera carnica*) in the apiary of the University of Tübingen. In order to obtain pupae with a high level of infestation with *Varroa destructor*, drone brood was sampled in spring and early summer and worker brood in autumn. Capped brood cells were opened and inspected for the presence of adult female mites. We examined 600 host bees from cells containing 1-5 *Varroa* females, including worker and drone pupae of all pupal stages. In addition, uninfested controls were inspected for wounds, but none were found.

The live pupae were vital stained in Ringer medium with 0.01% Trypan blue (Kanbar and Engels, 2004) in order to visualize the *Varroa*-made punctures in the integument of the host. These perforations were counted and their diameter measured under a stereomicroscope (Wild M8). After fixation, critical point drying, and sputtering, scanning electron microscope pictures were taken with a Stereoscan 250 Mk. II (Cambridge Instruments), as described earlier (Kanbar and Engels, 2003).

RESULTS

Number of wounds per pupal host

The integumental wounds were easily detected on the body of infested bee pupae by vital staining with Trypan blue. During the time that the colonies were most heavily infested, we inspected 20 drone and 20 worker individuals per level of brood cell infestation (one to five invading *Varroa* females). Only one perforation was found in most of the pupae. There was a slight increase in the number of wounds in each pupa in multiply infested brood cells (Figure 1). Of the 100 male and 100 worker host pupae, more than one wound was found in only 17 drones and 14 workers, and three punctures were found in only five bees.

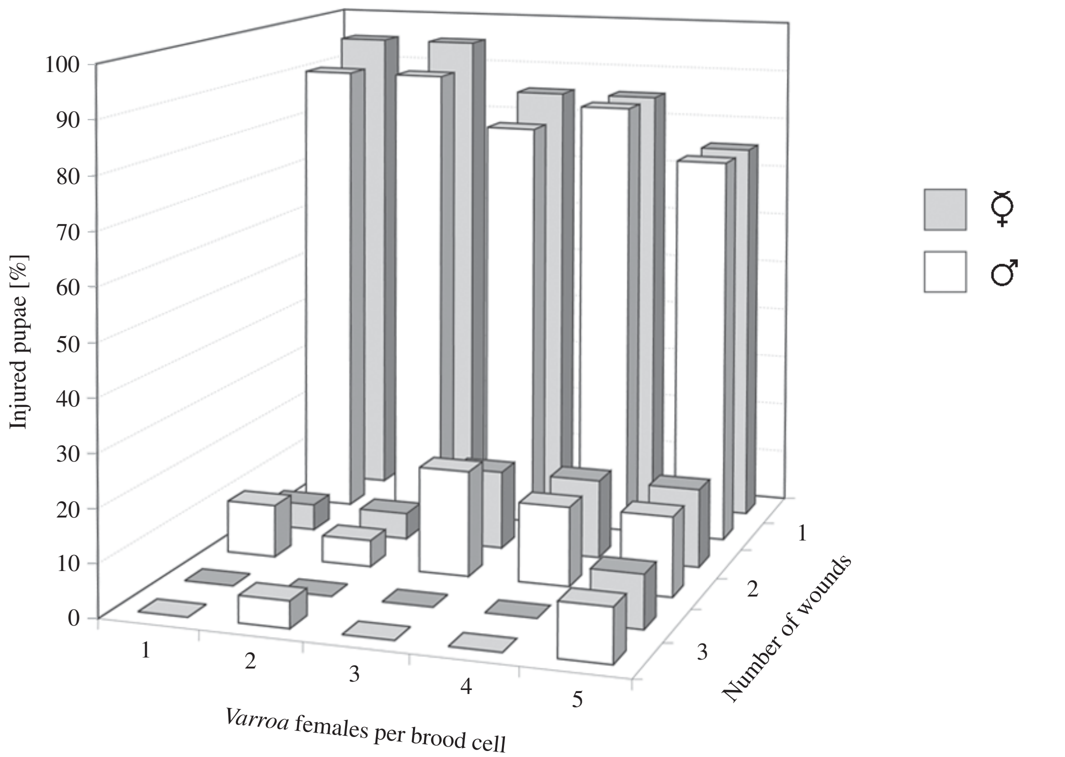

Figure 1. Frequency of *Varroa*-made wounds in pupae of the honey bee in relation to multiple brood cell infestation. Twenty drone and 20 worker pupae were investigated per level of individual cell infestation. Wounds were visualized by Trypan blue vital staining. ☿ = worker; ♂ = drone.

Aspect and position of wounds

In hosts infested with one mite, the perforation wounds were more or less round. In multiply infested pupae, however, the wounds often had an irregular shape (Figure 2). Sometimes, hemolymph was leaking out of the puncture (Figure 2a). In pupae infested with five *Varroa* females, deep holes were seen on the margin of the perforations (Figure 2b). In such pupae the wounds were usually large and oval (Figure 2c).

In all the drone pupae, the *Varroa*-made wounds were exclusively located on the anterior abdominal sternites, mostly in the middle of the 2nd segment. Only in worker pupae were some perforations found on the thorax, and these were in more lateral locations. Whenever there was more than one puncture, the localization was the same; usually the two (Figure 2d), and even three wounds, were found close together.

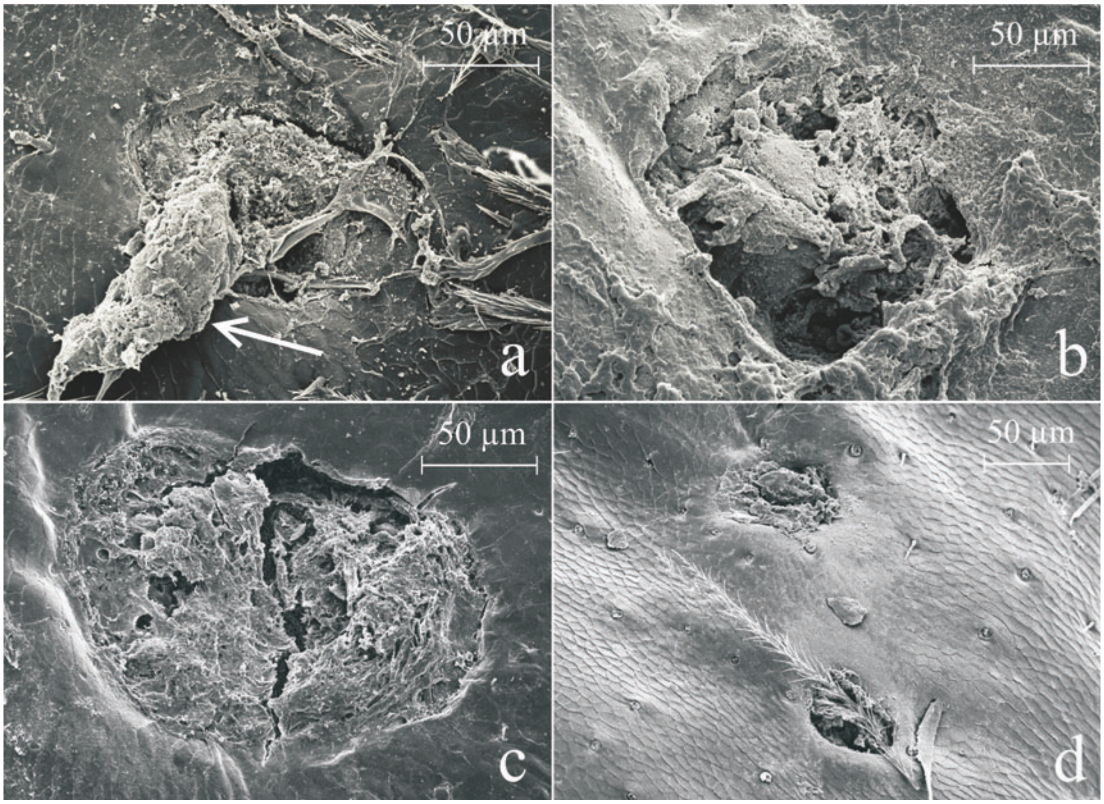

Figure 2. Scanning electron microscope photos of *Varroa*-made wounds in the integument of pupal honey bees. a = wound on drone pupa 21-22 days old, infested with three female mites. Hemolymph is leaking out of the perforation (arrow). b = wound on drone pupa 21-22 days old, infested with five female mites. Wound with deep holes around the margin. c = wound on worker pupa 20-21 days old, infested with four female mites. The large wound is only partly healed. d = wounds on drone pupa 21-22 days old, infested with two female mites, with two nearby perforations on the second abdominal sternite. Notice the developing hairs on the cuticular surface.

Wound size

Shortly after the pupal moult, the diameter of the round perforations was about 50 µm. During the subsequent development of the host, the wounds became enlarged. The maximum diameter was measured about three days prior to the adult eclosion of the bees (Figure 2). In some of the drone pupae the wounds then had (exceptionally) a diameter of over 300 µm. On average the wounds were found to be a little larger in drone pupae than in worker pupae. The diameter tended to increase with the level of infestation of the individual brood cell (Figure 3).

In multiply infested brood cells, the diameter of the two or even three wounds found on the same pupae varied. For instance, in one drone pupa three perforations were found, which measured only 70, 85 and 170 µm, and in one worker pupa the two wounds had diameters of 200 and 300 µm. These were the extremes found for very small and very large wound size.

Healing of the wounds

Towards the end of the pupal phase, the wounds increasingly scarred and healed. Complete closure of the integument was usually observed by the time of the imaginal moult only in pupae infested by a single *Varroa* mite. However, when there were large perforations, as found in multiply infested pupae (Figure 2), this process was evidently delayed. The large wounds were often still incompletely healed a few days prior to emergence of the adult bee (Figure 2b). This was in particular observed in drone pupae.

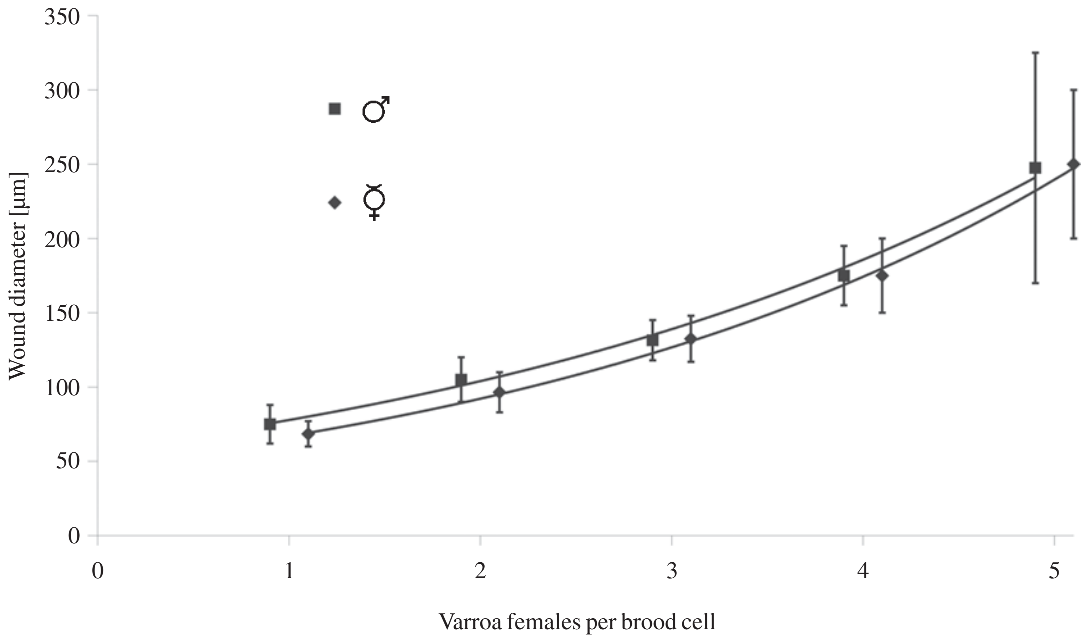

Figure 3. Size of *Varroa*-made abdominal wounds in drone and worker pupae of the honey bee in relation to the level of brood cell infestation. Bars indicate the range of the maximum diameter of the wounds about three days before adult bee eclosion. ♂ = drone; ☿ = worker.

DISCUSSION

The *Varroa* females punctured their host pupae, in particular drone brood, only at the 2nd abdominal sternite. In the rare cases of more than one perforation, they were quite close to each other. We assume that the relatively small area occupied by these feeding holes, compared to the relatively large body surface of the honey bee pupa, means that the mites are somehow attracted and oriented by specific local properties. Chemical cues may be involved. Both the local concentration and the constant approximation of the founder females to this specific area must be genetically fixed. This fidelity of the mite females to a single feeding area is most clearly expressed on drone pupae, which are the normal hosts for these mites in their original host honey bee species, *A. cerana* (Boot et al., 1997). Perhaps the chemical cues are not as concentrated in workers as they are in drones, resulting in more variation in the puncturing site.

The number of *Varroa*-made integumental wounds found on pupal honey bees was surprisingly not much higher in multiply infested hosts compared with single infestation of a brood cell. We mostly found only one and never detected more than three perforations per host bee, even in rare cases when 10 or more original female mites were present (data not shown). This could be due to selection pressure; a severely injured bee would not emerge, and the mites would be trapped within the brood cell. The female mites should not severely injure their host; this relatively uniform puncturing pattern was apparently genetically fixed during the evolution of this parasite-host relationship. Once established in the pupal integument, a common and repeated use of the same feeding sites occurs (Donzé and Guerin, 1994).

In multiply infested brood cells, the size of the punctures was found to increase considerably with ongoing pupal development. This was more pronounced in drone than in worker pupae. Because of the longer duration of the pupal phase in male bees, the use of the wounds by these hemolymph-sucking mites (Donzé and Guerin, 1997) is prolonged by three to four days. In addition, more nymphal mites will develop and can reach adulthood on infested drone brood; however, the number of progeny produced per female mite has been determined only for worker brood (Corrêa-Marques et al., 2003). The resulting damage to multiply infested brood is not only measurable as a dramatic weight loss of drones (Duay et al., 2002a), but also results in shorter life expectancy of the adult males (Duay, 2002) and in diminished reproductive performance by these drones (Rinderer et al., 1999; Duay et al., 2002b).

There is no information on the number, position and size of *Varroa*-made wounds in pupae of its original host, the Asian *Apis cerana*, in which the reproductive success of the mite is known to be significantly less than in the more recent host, *A. mellifera*, mainly because infestation is concentrated on drone brood in the native host (Boot et al., 1997). We can only speculate that the modalities of wounding *A. cerana* drone pupae will be similar to what we describe here for *A. mellifera*. Since the detection of various mito-haplotypes in Asian populations of *Varroa* (Anderson and Trueman, 2000), with considerable variation in the impact that they have on *A. mellifera* colonies (Anderson 2000), we have to consider the possibility that such variation in parasitic virulence may be caused by genetically determined specific behavioral traits of the female mites. This question has not yet been much studied (Garrido et al., 2001), in contrast to the clearly inherited hygienic behavior of worker bees in *A. mellifera*, which can reduce the extent of damage in *Varroa*-infested colonies (Spivak and Reuter, 2001).

The large wounds observed in drones suffering from a heavy parasitic load do not heal perfectly, as normally occurs towards the end of the pupal phase and before adult eclosion (Kanbar and Engels, 2003). This means that the remaining openings will allow pathogens to enter the host's body, which may contribute to the well-documented damage of many drones in honey bee colonies with severe symptoms of varroosis (De Jong, 1997). Not only does bacterial infection of bee larvae, i.e., by the foulbrood agents *Paenobacillus larvae* and *Melissococcus pluton* (Kanbar et al., 2002) have to be considered, but also quite a number of virus diseases known to occur world-wide in areas with massive occurrence of *Varroa* mites (Brødsgaard et al., 2000), as recently documented by PCR assays for Acute Bee Paralysis Virus (Bakonyi et al., 2002). We found that in pupal bees multiply infested by mites, the large and slowly healing wounds provide long-lasting open doors for pathogens. There is evidence of a correlation between the level of *Varroa* infestation and the risk of virus disease infection (Martin, 2001). These problems of combined mite and microbial infestations should receive more attention; they require laboratory studies on the molecular biology of bee virus transfer by *Varroa* mites (Shen et al., 2002). The frequent colony losses are not only of economic importance for the beekeeping industry, but also have a yet unestimated impact on the activity of honey bees as pollinators in agriculture and nature (Kevan and Imperatriz-Fonseca, 2002).

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