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ORIGINAL PAPER

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Ultrastructure and bacterial infection of wounds in honey bee (*Apis mellifera*) pupae punctured by *Varroa* mites

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Abstract The damage to western honey bee, *Apis mellifera*, colonies caused by the originally Asian ectoparasitic mite *Varroa destructor* is mainly a consequence of the infestation of host bee pupae. In the capped brood cell, female mites puncture the host's integument at preferred sites in order to suck haemolymph. Due to repeated feeding by the mother mite and her progeny, these perforations are kept open until shortly before the imaginal moult of the bee. Thereafter scarring takes place, thus preventing microbial infection after the adult bee has emerged from the protected environment of the sealed brood cell. However, colonies of various bacteria were found in the open wounds of about 15–30% of all inspected host pupae with an abundance depended on the level of host brood cell infestation by the mite. The small punctures of the pupal integument are difficult to detect but, by vital staining with trypan blue, the wounds can be visualised. The ultrastructure of the pupal wounds, the bacterial colonies and the scarring process are documented by a series of scanning electron micrographs.

Introduction

Ectoparasitic mites of the genus *Varroa* are known from Asian honey bees, of which nine extant *Apis* species are recognised (Koeniger and Koeniger 2000). All life stages of *Varroa* mites feed exclusively on bee haemolymph after perforating the host's integument with their chelicerae (Smirnow 1979; Donzé and Guerin 1994). The so-called western honey bee, *Apis mellifera*, with 24 subspecies distributed over Europe, Africa and the Near East (Ruttner 1988), has been repeatedly infested with *Varroa destructor* during the last century. This occurred through contacts with the closely related *Apis cerana* as a consequence of the worldwide transport of bee colonies and apicultural projects in developing countries (Matheson 1993). Today varroatosis is the main problem for beekeeping with *A. mellifera* colonies (De Jong 1997). *V. destructor* is a taxon recently separated from *Varroa jacobsoni* Oud. (Anderson and Trueman 2000).

The life cycle of *Varroa* mites can be divided into a phoretic phase, with females only attached to adult bees, and a reproductive phase which is spent in capped bee brood cells containing a last instar, which female mites invade shortly before they are sealed (Ifantidis and Rosenkranz 1988). In the still open brood cell, honey bee workers deposit a large portion of larval food into which the invading *Varroa* female submerges (Ifantidis 1988). After capping of the cell, these provisions are eaten by the bee larva within about 5 h. The parasite is thereby freed and immediately begins to suck bee haemolymph (Steiner et al. 1994). The post-feeding stage of the L5 larva and also the prepupa are fed on by *Varroa* females which puncture the host's still soft cuticle at various sites (Garrido et al. 2001; G. Kanbar and W. Engels unpublished data). A final feeding perforation is then made during the moult of the prepupa, which lasts only about 1 h (Donzé and Guerin 1994), evidently before the sclerotisation of the pupal cuticle. In drone pupae, nearly all of the punctures are located on the sternite of the second abdominal segment, but in worker pupae about a quarter are found on the mesothorax. The feeding site is used by the female mite and her progeny throughout the pupal phase (G. Kanbar and W. Engels unpublished data). The first egg is not inseminated and develops into a male (Rehm and Ritter 1989). Copulations with the adult female mites take place in the sealed brood cell prior to emergence of the imaginal host bee.

Varroa wounds in the host can be visualised by vital staining with trypan blue (G. Kanbar and W. Engels, unpublished data). Due to repeated feeding of the adult and nymphal mites, the healing of the perforation is prevented until scarring occurs prior to the imaginal moult. Various microbial organisms may be transferred

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through the punctures, although evidence for vector transfer by the *Varroa* mite is scarce (Wiegers 1988; Brødsgaard et al. 2000). In the open wound, which always contains bee haemolymph, colonies of bacteria were frequently detected. One type was identified as the European foulbrood agent *Melissococcus pluton* (Kanbar et al. 2002). According to recent observations, colony collapse, as usually occurs in the European honey bee under severe varroatosis, is mostly accompanied by various, now cosmopolitan, virus infections (Brødsgaard et al. 2000).

The aim of our present study was to follow the fate of the long-lasting pupal wounds, mainly by SEM analyses. The structure of the punctures, bacterial infection of the wound and its closure in the course of the host bee's pupal-adult moult were documented for the first time. For future studies on the pathways of viral as well as bacterial transfer, detailed knowledge of the nutritional relations between the *Varroa* mite and the host bee, including that on punctures used as haemolymph feeding sites, is required.

Materials and methods

Honey bees and mites

Colonies of Carniolan type *A. mellifera,* kept in the apiary of the University of Tübingen, were used. All of the experimental hives were left without control of varroatosis during the summer season in order to have large numbers of mites for the study. In addition, combs which contained a capped drone used for trapping female mites were sampled from many other colonies. In the winter, bee brood was collected from colonies kept in an indoor flight room. The mites infesting our apiary were identified as *V. destructor* according to molecular genetic determination of their mitochondrial haplotype (C. Garrido et al., in preparation). *Varroa*-infested bee brood of all stages of pupal development was sampled by inspecting sealed comb cells. The age of the brood after oviposition was estimated to the nearest day according to external differentiation and pigmentation.

Staining and scanning electron microscopy of pupal integumental wounds

Varroa-made punctures of host bees were visualised and located by staining with trypan blue, by which damaged epidermal cells around the wound are coloured (G. Kanbar and W. Engels, unpublished data). The same is true for the final perforation made by the female mite during the host's pupal moult. Because unstained perforations are difficult to detect, especially in early pupal stages, the trypan blue treatment was often applied to localise wounds which were subsequently studied by scanning electron microscopy (SEM). The appropriate technique was developed by varying the fixation procedure. Bee pupae were fixed in cacodylate-buffered (pH 7.2) glutaraldehyde (1.0 mM) for 2 h, followed by 2 h under vacuum for better penetration and 0.5 h rinsing in buffer. After stepwise dehydration in ethanol, critical point treatment under CO_2 was applied. The dried pupae were mounted, sputtered with gold-palladium, and the wounds studied using SEM (Cambridge Instruments Stereoscan 250 Mk. II).

Results

During the initial feeding of a *Varroa* mite in a previously capped brood cell, the host larva was punctured at

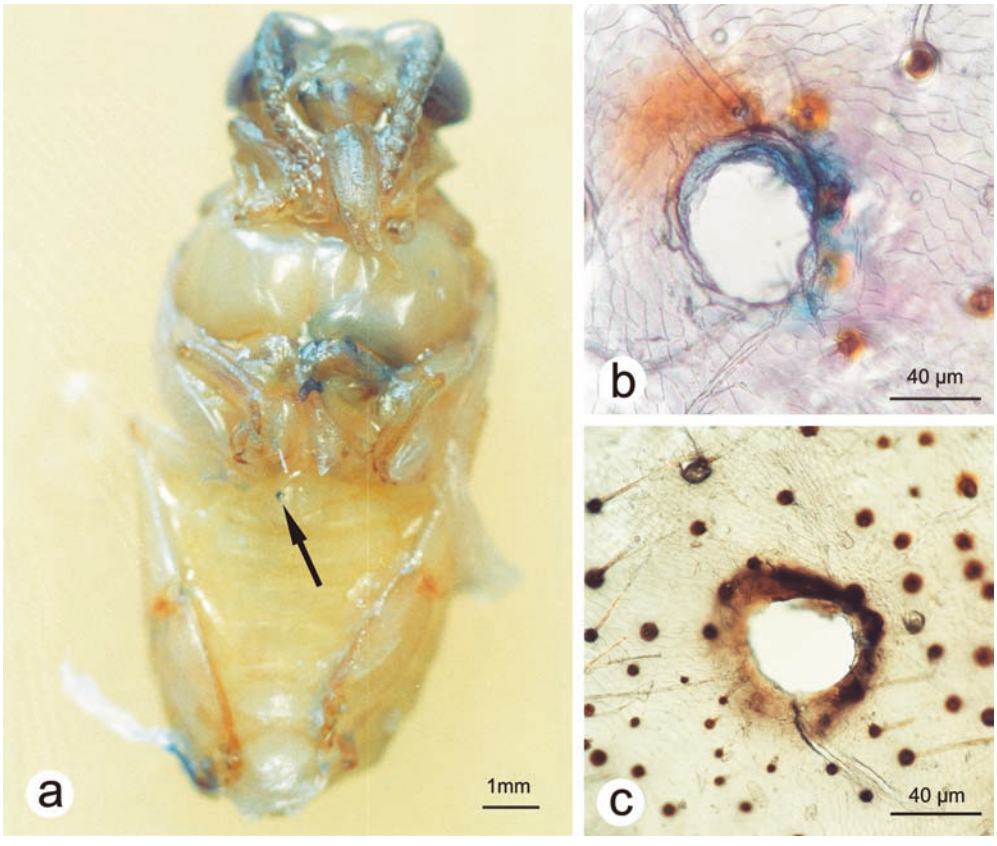

Fig. 1 a Pupa of a 16- to 17-day-old drone honey bee with a small trypan blue stained puncture (*arrow*) on the ventral side of the second abdominal segment. **b** Detail shows that the trypan blue dye was taken up by damaged epidermal cells in the margin of the wound. **c** Integumental perforation in a 19–20 day old drone pupa: wound with the beginning of scarring, cuticle already brownish

various positions. These small short-term feeding sites were difficult to localise and may close rapidly because the larval integument, covered by a thin cuticle, is very elastic. Only in the prepupa was the number of perforations reduced, usually to two. These normally occurred on the sixth abdominal segment and were repeatedly used by the feeding female mite. The final perforation made by the female mite during the host's pupal moult was usually on the ventral side of the second abdominal segment of the host bee (Fig. 1a) and was kept open for several days (Fig. 1b), until the end of the pupal phase. During this period, the diameter of the wound was enlarged from 40–60 μm to80–120 μm, and was surrounded with more stainable cells (Fig. 1c). Only about a quarter of the worker pupae have these permanent feeding sites located on the thorax (Figs. 2c, 3a). Other puncture locations only occurred at rates under 2–3%.

The closure of the wound was initiated by the accumulation of haemocytes in the centre, not by the ingression of epidermal cells (Fig. 4), which was only observed later. However, sometimes the wound was kept open even in pharate adults, especially in drones (Fig. 4a), by haemolymph sucking nymphal mites (Fig. 4b). These inserted their chelicerae and palps into small holes in the already scarring integumental

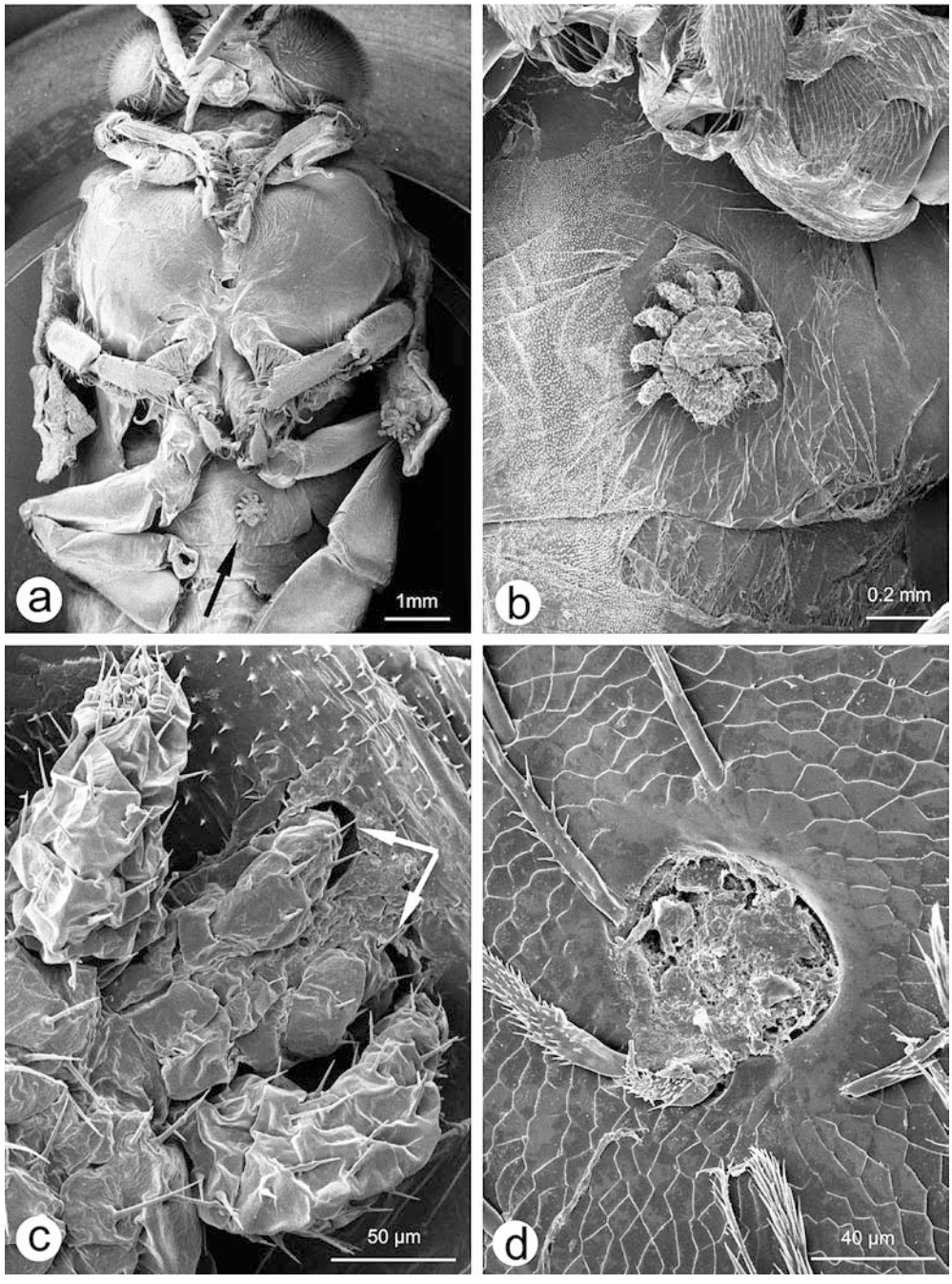

Fig. 2 **a** Scarred wound (*arrow*) on the sternite of the second abdominal segment of a pharate adult worker. **b** Detail of **a** shows that the first step in wound closure is an aggregation of haemocytes between small holes that are kept open. **c** The wound on the left side of the thorax of a 16- to 17-day-old worker pupa, with a colony of bacteria (*arrow*). (**d** Detail of **c**: the bacterial cluster identified as *Melissococcus pluton*. **e** Partially scarred wound (*arrow*) on the sternite of the second abdominal segment of a 17- to 18-day-old drone pupa. **f** Detail of **e** showing the infestation of this wound with a colony of as yet unidentified bacteria

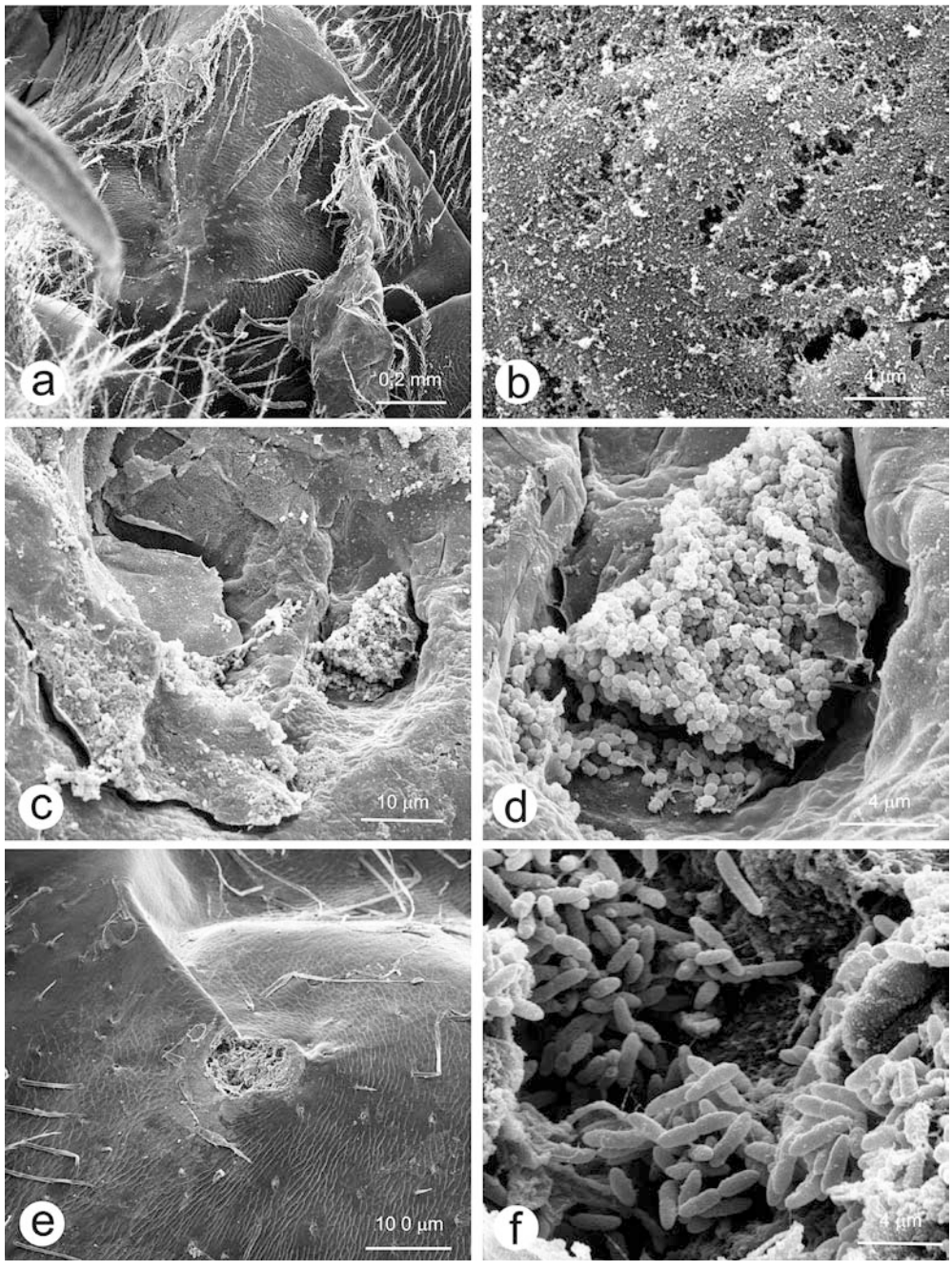

Fig. 3 a Perforation on the left side of the thorax (*arrow*) of a 16- to 17-day-old worker pupa. **b** This wound is a deep puncture reaching through the cuticle into the haemocoel. **c** After partial removal of the cuticle, three more or less scarred wounds (*arrows* **d–f**) are visible on the second abdominal segment of a drone pupa about 21–22 days old with the covering cuticle partially removed. **d** Several small holes are left in one of the wounds, but only one hole in the second **e** and also in the third **f** wound

perforation (Fig. 4c). Under such conditions, closure of the wound remained incomplete until shortly before eclosion of the imaginal drone (Fig. 4d). Usually some days prior to emergence of the bee, the wound, now with a reduced diameter, was more or less scarred (Fig. 2a), although some small holes were often still visible (Fig. 2b).

Large colonies of bacteria often develop in the thoracic wounds of worker pupae (Fig. 2c) as well as in the abdominal punctures of drone pupae (Fig. 2e). Various kinds were seen in the perforations inspected. Some of the globular forms were identified as *M. pluton* (Fig. 2d), and as yet undetermined elongate types were also found (Fig. 2f). In worker pupae, the perforations were sometimes found in a lateral position on the thorax (Fig. 3a). These wounds look like deep holes extending into the underlying muscles (Fig. 3b).

Especially in the case of multiple infestation of the same brood cell, several wounds were found, usually near to one another on the sternite on the preferred feeding site on the second abdominal segment (Fig. 3c). Even three punctures (Fig. 3d–f) can scar normally by the stage of the pharate imago. Thus the process of wound healing is part of the formation of the imaginal integument at the end of metamorphosis, including the secretion and sclerotisation of the adult cuticle.

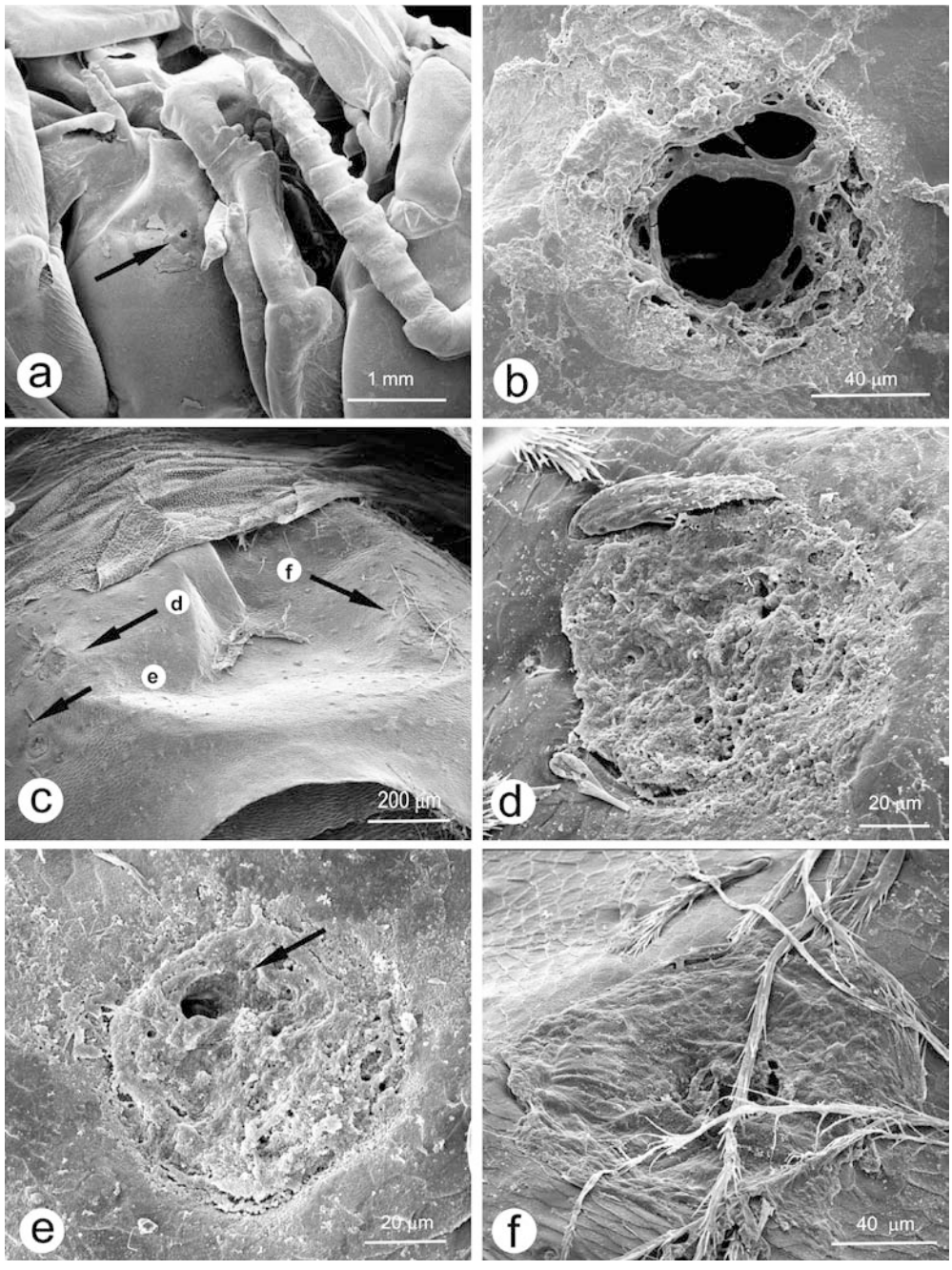

Fig. 4 **a** Pharate adult drone with a *Varroa* deutonymph sucking haemolymph in a wound on the sternite of the second abdominal segment (*arrow*), the typical position of the puncture performed by the female mite during the pupal moult. **b** Detail of **a** showing the feeding male deutonymph. **c** The palps of the deutonymph are inserted into small, still unscarred holes (*arrows*) of the wound. **d** Another nearly scarred wound with an irregular arrangement of the epidermal cells, with the same position of the perforation as **c** and also on a pharate drone

Discussion

The western honey bee, *A. mellifera*, is a relatively new host for *Varroa* mites (De Jong 1997). But the original host species, *A. cerana*, is evidently so closely related with *A. mellifera* (Ruttner 1988) that the interspecific change of hosts has occurred several times without problems for the mites (Kovac and Crailsheim 1987). However, there are consequences for the infested *A. mellifera* colonies (Büchler 1992). The main difference in the behaviour of the parasite is the massive invasion of worker brood cells by female mites with the resulting danger to the host (Murilhas 2002), a trait never observed in colonies of the original host (Koeniger et al. 1981; Tewarson et al. 1992). Although no details are known on feeding site selection by reproductive *Varroa* females in sealed drone brood cells of *A. cerana*, the highly specific localisation of the punctures, in particular on drone pupae of *A. mellifera,* is presumably the same in both host species. We interpreted the low number of perforations and their precise location to be a trait of the female mite to limit host exploitation (Kanbar and Engels, unpublished data). Preferred feeding sites on the anterior abdominal seg-

ments of insect hosts are also known from mites of other taxa (Baker 1991).

The possibility of keeping the regularly used feeding perforation open is a precondition for the nutrition of the parasite's nymphal progeny. On the other hand, it is a maternal investment, only used by the female mite during the short period of the prepupal-pupal moult, and lasting no longer than about an hour (Donzé and Guerin 1994). Since ca. 15–30% of the pupal wounds investigated by us turned out to contain bacterial colonies, but no corresponding rate of mortality could be observed, such secondary infections are apparently are not fatal to the host. Our observations indicate that early wound healing should be possible during the pupal phase, but this is normally prevented by repeated sucking of the haemolymph by which small holes persist even into the final scarring process. Before the imaginal bee emerges from its brood cell, which remains sealed during the entire period of host metamorphosis and parasite reproduction, all wounds are closed. Therefore, the closure of the cuticle is finished before the adult bee enters the unprotected environment of the nest and field when open *Varroa*-made wounds would allow many pathogens to enter.

Whether the closure of the pupal wounds depends not only on the aggregation of haemocytes but also on an immune response of the host bee remains an open question. The induced expression of apidaecin genes as a specific response to bacterial and fungal infestation of preimaginal bee stages (Casteels et al. 1989) and the involvement of the *Apis dorsal* protein in the respective signal cascade (Fan 2001) have not as yet been studied in the context of wound healing of *Varroa*-made integumental perforations in pupae.

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