Salivary secretions from the honeybee mite, *Varroa destructor:* effects on insect haemocytes and preliminary biochemical characterization

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SUMMARY

Introduction. The ectoparasitic honey bee mite *Varroa destructor* feeds on the haemolymph of the honey bee, *Apis mellifera*, through a single puncture wound that does not heal but remains open for several days. It was hypothesized that factors in the varroa saliva are responsible for this aberrant wound healing. **Methods.** An *in vitro* procedure was developed for collecting salivary gland secretions from *V. destructor*. Mites were incubated on balls of cotton wool soaked in a tissue culture medium (TC-100), and then induced to spit by topical application of an ethanolic pilocarpine solution. **Results.** Elution of secretions from balls of cotton wool, followed by electrophoretic analysis by SDS-PAGE and electroblotting indicated the presence of at least 15 distinct protein bands, with molecular weights ranging from 130 kDa to <17 kDa. Serial titration of *V. destructor* salivary secretions in TC-100 followed by an 18-h incubation with haemocytes from the caterpillar, *Lacanobia oleracea*, indicated that the secretions damage the haemocytes and suppresses their ability to extend pseudopods and form aggregates. **Conclusion.** We suggest that these secretions facilitate the ability of *V. destructor* to feed repeatedly off their bee hosts by suppressing haemocyte-mediated wound healing and plugging responses in the host.

Key words: *Varroa destructor*, honey bee, saliva, salivary gland secretions, spit, haemocyte, haemocyte aggregation.

INTRODUCTION

The ectoparasitic mite, *Varroa destructor*, is a common parasite of the European honey bee, *Apis mellifera*. Originally, *V. destructor* exclusively parasitized the Eastern honey bee, *Apis cerana*. However, during the first half of the last century, parasitization spread to *A. mellifera* (Rosenkranz *et al.* 2010). Male and female mites live on the outside of their hosts and the adult females can be found within sealed drone or worker cells (where they undergo the reproductive stage of their life cycle), or attached to adult bees (see review by Rosenkranz *et al.* 2010). The adult female mites feed off the haemolymph (blood) of both larval and adult bees (Rosenkranz *et al.* 2010). In addition to weakening its host by extracting haemolymph, *V. destructor* transmits a number of pathogens to the bee hosts, notably deformed-wing virus (de Miranda and Genersch, 2010). As a result of this, and the emergence of acaricide-resistant strains of *V. destructor* (Martin, 2004), this ectoparasite represents the greatest threat to apiculture, seriously undermining the economic importance of *A. mellifera,* both as a pollinator of plants and producer of honey (Klein *et al.* 2007). Clearly, there is an urgent need to better understand all aspects of the biology of *V. destructor* and its interaction with host bees so that better control methods may be devised.

One aspect of *V. destructor* biology, which is relatively poorly understood, is its feeding behaviour. To feed, *V. destructor* pierces the cuticle and body wall of its bee host and then extracts haemolymph. The individual *V. destructor* that created this feeding site and any other *V. destructor* resident within the brood cell repeatedly revisit this feeding site which remains open for many days (Kanbar and Engels, 2003, 2005). Usually, when the cuticle and underlying epidermis of an insect is damaged, a number of immune and wound-healing responses are activated, the aim of these being to increase survival by preventing pathogen invasion and haemolymph loss (Theopold *et al.* 2002; Dushay, 2009). For unknown reasons, this does not occur at the bite site of *V. destructor,* which remains open. In the case of larvae of the ectoparasitic wasp, *Eulophus pennicornis*, which develop on the outside of their caterpillar host, it has been demonstrated that feeding is facilitated by larval secretions which damage host haemocytes, resulting in suppression of key immune responses (Richards and Edwards, 2002). Ectoparasitic arthropods feeding on vertebrates (e.g. mosquitoes, tsetse flies, reduuvid bugs, ticks, fleas etc) overcome the host defences by the numerous components in their saliva (Ribeiro, 1995; Ribeiro and Francischetti,

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2003). The ability of *V. destructor* to repeatedly feed off its bee host, suggests that they also secrete anti-immune/anti-wound healing factors, most likely derived from their salivary glands. In view of this, the main aims of the current work were 3-fold. First, to develop a procedure for collecting *V. destructor* salivary gland secretions, second, to make a preliminary biochemical characterization of the secretions and third, to determine whether the salivary gland secretions can affect the function of insect haemocytes.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma-Aldrich (Poole, Dorset, UK) unless stated otherwise.

Mites and insects

Varroa destructor mites were obtained from the National Bee Unit at Fera, York, UK. The mites were collected from hives untreated for control of *V. destructor*, and were obtained from adult bees. *Lacanobia oleracea* larvae were reared in a constant environment room under standard conditions of 22 °C, 70% relative humidity, and a light:dark cycle of 16 h:8 h, and fed on artificial diet (Bioserve, USA).

Preparation of varroa salivary gland secretions

Varroa destructor salivary gland secretions were collected using a method developed for collecting secretions from ticks (e.g. Campbell *et al*. 2010*b*) with modifications for the much smaller size of the mites. All procedures were performed under sterile conditions, using sterile equipment and reagents, and all solutions were prepared using sterile, pyrogen-free water (Baxters, UK). Briefly, immediately after collecting mites from bees, the mites were washed twice in distilled water containing 0·01% methylparaben (p-hydroxybenzoic acid methyl ester; antifungal agent). A ball of cotton wool (about 10 mg) was placed into a Petri dish and 120 μl of TC-100 (an insect tissue culture medium) added. Then 33 V. *destructor* were placed onto the cotton wool, and 0·5–1 μl of 50 mg/ml pilocarpine solution (prepared in ethanol) was placed onto the dorsum of each mite. The Petri dish was closed and incubated in a moist chamber at room temperature. After 4 h, the mites were removed from the ball of cotton wool and the latter soaked in a further 120 μl of TC-100 containing 50 μM phenylthiocarbamide, 0·008% methylparaben and 200 μg/ml ampicillin. This solution was then centrifuged at 13 000 ***g*** for 5 min at 4 °C, and the supernatant containing putative mite secretions removed (= V + pilo). Prior to use, the solution was centrifuged again, as described above.

To control for the effects of pilocarpine on mites, mites on a cotton wool ball without pilocarpine treatment (= V no pilo) were processed as described above. To control for the effects of pilocarpine on *L. oleracea* haemocytes (see below), 33 μl of 50 mg/ml pilocarpine solution were added directly to a ball of cotton wool (this represents the maximum amount of this solution which could get onto the cotton wool [= no V + pilo]). In addition, balls of cotton wool with 120 μl of TC-100 added but without mites and without pilocarpine (= TC-100), were also processed.

Preparation of V. destructor *whole body homogenate*

Twenty *V. destructor* were homogenized in 120 μl of ice-cold TC-100 in a sterile glass homogenizer, and the homogenate transferred to an Eppendorf tube on ice. A further 120 μl of TC-100 were then added to the homogenizer to recover any material left behind and transferred to the Eppendorf tube. After centrifugation (13 000 ***g***, 5 min at 4 °C), the supernatant was transferred to a clean Eppendorf tube and stored at −20 °C.

SDS discontinuous polyacrylamide gel electrophoresis

To estimate the profile of proteins present in the *V. destructor* salivary gland secretions, SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Routinely, samples generated (see above) were diluted in 5X SDS sample buffer and 20X DTT (Fermentas Life Sciences, York, UK) to give 1X final concentration of these reagents. The samples were boiled for 4 min and subsequently resolved at a constant voltage of 180 V with a 4% stacking gel and a 12·5% resolving gel along with prestained protein molecular weight markers (Fermentas Life Sciences). Following electrophoresis, protein bands were visualized by silver staining (Bio-Rad, Hemel Hempstead, UK).

Electroblotting

To increase the sensitivity of the method used to detect proteins following SDS-PAGE, proteins were electroblotted to a PVDF membrane and then stained. Samples were resolved using SDS-PAGE as described above, and the resolving gel equilibrated in blotting buffer (10 mM CAPS [3-(cyclohexylamino)-1-propane sulphonic acid] pH 11, and 10% methanol (v/v)). Proteins were electroblotted (50 mA for 90 min) to low porosity (0·2 μm), PVDF membrane (Sequi-Blot; Bio-Rad) that had been prewetted in methanol and equilibrated in blotting buffer. The SDS gel was stained with ProtoBlue Safe (National Diagnostics) so that the transfer efficiency of proteins could be established. The membrane was washed in distilled water, air-dried

and the proteins attached to it visualized by staining with 0·005% sulforhodamine B in 30% methanol, 0·2% acetic acid and double-distilled water.

Preparation of insect haemocytes and haemocyte anti-aggregation assays

Haemocytes were prepared and a semi-quantitative haemocyte anti-aggregation assay performed as described previously (Richards and Dani, 2008). Basically, haemolymph was collected in at least 6 volumes of an iso-osmotic, ice-cold anticoagulant solution (10 mM Na EDTA, 30 mM trisodium citrate, 26 mM citric acid, 100 mM glucose, pH 4·6). The haemocyte number per ml was estimated using an improved Neubauer haemocytometer. After centrifugation at 254 ***g*** for 8 min at 4 °C, the haemocyte pellet was resuspended in TC-100 (containing phenylthiocarbamide, methylparaben and ampicillin at final concentrations of 25 μM, 0·004% and 100 μg/ml, respectively), at a concentration of 3×10^6 cells/ml. For the haemocyte anti-aggregation assay, 50 μl of sample (i.e. eluates from balls of cotton wool, or mite whole body homogenate, see above) were serially titrated in 50 μl of TC-100 in the flat-bottomed wells of a 96-well plate (Costar, Fisher Scientific, UK). In addition, 50 μl of TC-100 (not eluted from cotton wool) were titrated instead of sample. Then 50 μl of the haemocyte suspension were added to each well and the plates incubated in a moist chamber at RT for 18 h. After assessment of haemocyte monolayers using a Leitz Labovert FS inverted microscope, haemocytes were fixed and stained in 0·125% Coomassie blue G250 (Bio-Rad) in 10% acetic acid, 40% methanol, destained in 10% acetic acid, 40% methanol, and then covered in PBS. Each monolayer was examined and the lowest concentration of sample that inhibited haemocyte aggregate formation was determined. Note that a haemocyte aggregate is defined as a closely associated group of approximately 10 or more haemocytes making contact with each other. Furthermore, the end point for each sample is defined as the dilution of sample in the well before the one that most resembles the control (Richards and Dani, 2008).

RESULTS

Detection of V. destructor *salivary gland proteins by SDS-PAGE and electroblotting*

The protein profile of the whole body homogenate of *V. destructor* is complex, containing several proteins and polypeptides with molecular weight estimates ranging from >130 kDa to <17 kDa (Fig. 1). When TC-100 (data not shown) or TC-100 plus 30 μl of pilocarpine (Fig. 1; No V + pilo) were added to a cotton wool ball, and the cotton wool then incubated in TC-100, no proteins eluted from it. When mites were incubated on cotton wool and but not treated with pilocarpine, a small amount of protein was subsequently eluted into TC-100 and could be visualized as 3 very faint bands (Fig. 1; V no pilo, bands a, b and c). When the mites were treated with pilocarpine, a 'spitting' response was stimulated and a number of putative salivary gland proteins could be subsequently eluted from the cotton wool ball (Fig. 1; V + pilo). As indicated in Fig. 1, under the experimental conditions utilized, 5 main protein bands were detected (Fig. 1; V + pilo, bands d, e, f, g and h), with molecular weight estimates of 72 kDa, 23 kDa, 21 kDa, 19 kDa, and 17 kDa, respectively. Three of these bands (Fig. 1, bands d, f and h) stained more strongly with silver compared to the other 2 bands (e and g).

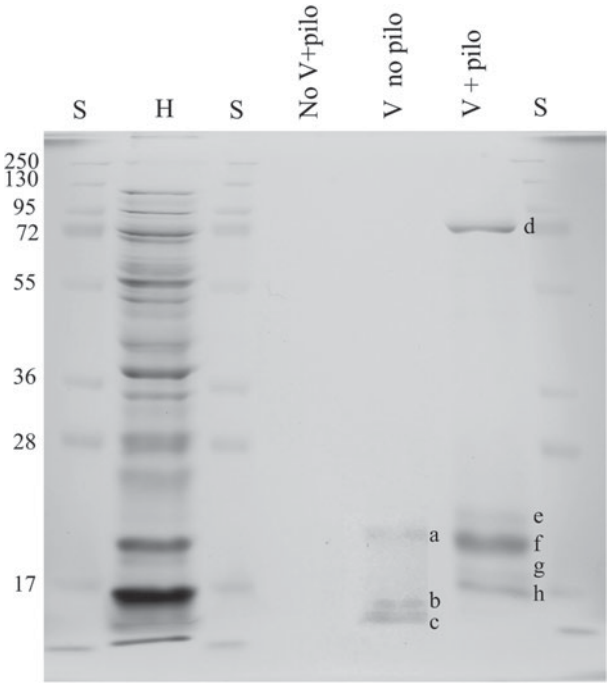

Fig. 1. SDS-PAGE analysis under reducing conditions and silver staining of *Varroa destructor* whole body homogenate and salivary gland secretions. Gel lanes are: (H) Mite whole body homogenate; (V + pilo) proteins released by *V. destructor* treated with pilocarpine; (V no pilo) mites without pilocarpine treatment; (No V + pilo) the eluate from a cotton wool ball treated with pilocarpine but without mites; (S) molecular weight standards (in kDa).

Following electroblotting of samples and staining of the membrane in sulforhodamine B, the protein profile for the V no pilo sample (mites incubated on cotton wool and neither treated with pilocarpine), was similar to that obtained with in-gel silver staining in that only very faint-staining, low molecular weight bands were present (Fig. 2. V no pilo; arrow). Conversely, electroblotting and sulforhodamine-B staining of the V + pilo sample (mites on cotton wool treated with pilocarpine), enabled visualization of a larger number of secreted salivary gland proteins

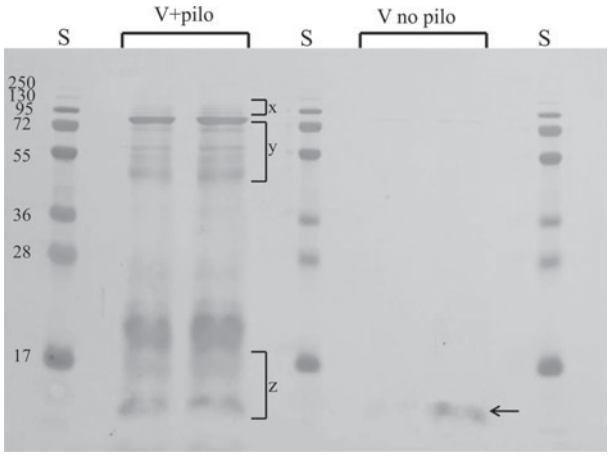

Fig. 2. Sulforhodamine B-stained electroblot of *Varroa destructor* salivary gland secretions. Mites on cotton wool balls were either treated (V + pilo) or not treated (V no pilo) with pilocarpine. X, y and z indicate the presence of proteins released by mites treated with pilocarpine and not detected on a silver-stained SDS gel (see Fig. 1). The arrow indicates the presence of a low molecular weight protein(s) for pilocarpine-untreated mites, previously detected on a silver-stained SDS polyacrylamide gel (see Fig. 1). Molecular weight standards (S; in kDa) are indicated on the right- and left-hand side.

(compare the V + pilo sample for Fig. 2 with that in Fig. 1). In particular, several proteins with molecular weight estimates >72 kDa (Fig. 2, x), ranging from 45 to 72 kDa (Fig. 2, y), and with a molecular weight estimate <17 kDa (Fig. 2, z), were detected following electroblotting.

Effect of V. destructor *whole body homogenate and salivary gland secretions on insect haemocytes*

When incubated in TC-100, the majority of *L. oleracea* haemocytes present adhered to the tissue culture plastic surface and extended pseudopods (Fig. 3a). Then, over an 18-h period, some haemocytes migrated towards their neighbours to form haemocyte aggregates (Fig. 3a). Note that the number of haemocytes making up each aggregate is indicated not only be the size of the aggregate, but also by the density of the staining. When *V. destructor* whole body homogenate was present at a 1:2 dilution aggregation of haemocytes still occurred (Fig. 3b). However, in the presence of the homogenate, the majority of haemocyte aggregates formed tended to be smaller and less densely stained compared to those formed under control conditions (compare Fig. 3b with 3a). At a 1:4 dilution of the homogenate, the haemocyte aggregates formed resembled those in the TC-100 control (data not shown).

Addition of 120 μl of TC-100, or 120 μl of TC-100 plus 33 μl of pilocarpine solution to a cotton wool ball followed by elution in TC-100, had no effect on the ability of *L. oleracea* haemocytes to extend pseudopods and form aggregates *in vitro* (Fig. 4). Similarly, when *V. destructor* mites without pilocarpine treatment were incubated on a ball of cotton wool, the subsequent eluate did not damage *L. oleracea* haemocytes, nor prevent them from forming aggregates (Fig. 4c). However, when *V. destructor* mites on a ball of cotton wool were treated with pilocarpine, the eluate damaged the haemocytes, causing many to disintegrate (Fig. 4d). In addition, the ability of the haemocytes to extend pseudopods and migrate towards each other to form aggregates was severely impaired (Fig. 4d). The effect of the putative salivary secretions on haemocyte aggregation was observed at 1:2 and 1:4 dilutions of the eluate, but not at 1:8 dilution. In particular, as shown in Fig. 4, at a 1:2 dilution of the secretions, the majority of the haemocytes present on the monolayer had broken down, and only clumps of haemocyte debris were present.

DISCUSSION

The ability of the honey bee mite, *V. destructor*, to feed repeatedly off the haemolymph of its bee host is well documented (Tewarson *et al.* 1992; De D'Aubeterre *et al.* 1999; Garedew *et al.* 2004). The puncture wound at the feeding site produced by the varroa bite in the cuticle of the bee remains open and is revisited as a communal feeding site over several days by all the mites infecting that bee (Kanbar and Engels, 2003, 2005). Artificial punctures in the pupal bee heal and no bacterial infection was observed in such puncture sites (Herrmann *et al.* 2005). In contrast, varroa bite punctures do not heal and bacterial infections are routinely observed (Kanbar and Engels, 2003). Since insects prevent haemolymph loss by mobilizing wound healing and wound plugging responses (Brey *et al.* 1993; Gillespie *et al.* 1997; Theopold *et al.* 2002; Dushay, 2009), we have hypothesized that *V. destructor* introduces into the bee some bioactive factors that overcome the host defences. Additionally, we postulate that these factors affect the bee's immune system permitting bacteria to colonize the feeding site and perhaps inadvertently facilitate pathogen transmission from varroa to the bee. We believed that these bioactive factors are synthesized in the mite's salivary gland and are introduced into the puncture wound of the bee via the saliva.

As a first step in testing the above hypotheses, an *in vitro* procedure was developed for collecting *V. destructor* salivary gland secretions. Based on the assumption that salivation in *V. destructor* was regulated in a manner similar to the well-studied salivary glands in the fellow Acari ticks we employed topical application of the cholinomimetic agent pilocarpine which is thought to cross the cuticle, interact with the acetylcholine receptor in the syngagnlion ('brain') and cause dopamine to be released from neurones innervating the salivary

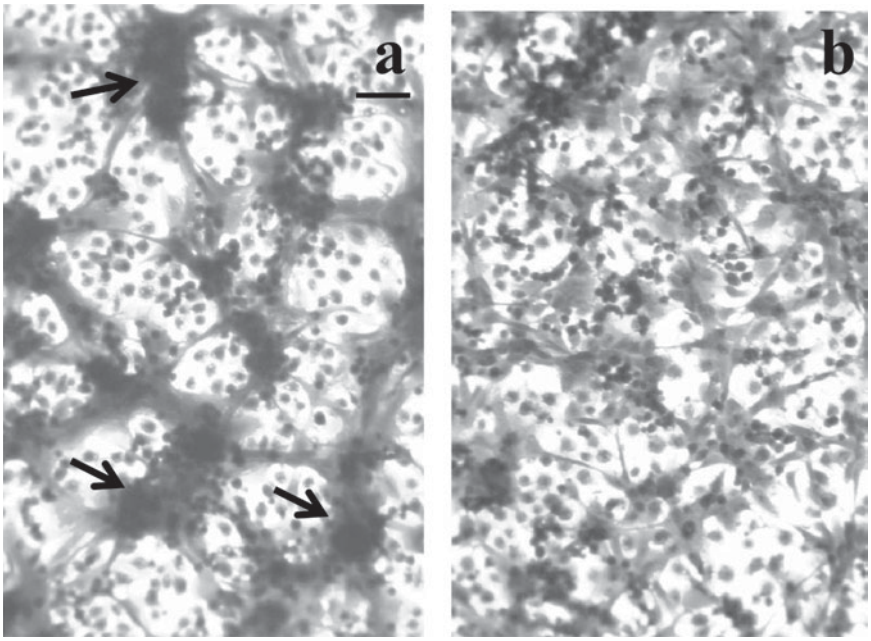

Fig. 3. Bright-field micrograph of Coomassie blue-stained haemocytes demonstrating effects of (a) TC-100 or (b) *Varroa destructor* whole body homogenate on haemocyte aggregation activity. Note that in (a), the haemocytes are healthy and have extended pseudopods and migrated towards each other to form aggregates (arrows). By contrast, in (b), although some haemocyte aggregates are present, they tend to be smaller and less dense than those in (a). The Scale bar = 200 μm.

gland resulting in salivation (reviewed by Bowman and Sauer, 2004 and Bowman *et al*. 2008). Because *V. destructor* are so much smaller than ticks and microcapillary tubes could not be placed over their mouthparts to collect saliva, we utilized the 'cotton wool ball' approach for collection of saliva (Richards and Edwards, 2001, 2002). In a previous study, varroa mites were allowed to feed through an artificial membrane into culture medium for 24 h (Shen *et al*. 2005*a*). Subsequent analysis of this culture medium established the presence of varroa-associated viruses demonstrating that the mites had salivated into the medium. Our pharmacologically induced salivation method allows collection of mite saliva in a smaller volume and, hence, more concentrated form and a more rapid method that would favour capture of bioactive factors that may be more labile.

In the current work, it was determined that when *V. destructor* mites on a cotton wool ball are induced to 'spit' with pilocarpine (= V + pilo sample), a number of proteins can be eluted from the cotton wool and subsequently detected on an SDS polyacrylmide gel stained with silver. That these proteins are derived from the mites is indicated by the fact that no proteins are eluted from cotton wool balls without mites (TC-100 and No V + pilo samples). Furthermore, the suggestion that the proteins are secreted from the mite salivary gland is supported by the finding that hardly any protein is recovered when the mites are not treated with pilocarpine (V no pilo sample). We cannot totally rule out the possibility that some of the proteins derived from mites treated with pilocarpine (V + pilo sample) are constituents of their bloodmeal and have been vomitted from the gut, but this is considered to be unlikely, since no such action occurs in ticks.

For *V. destructor* salivary secretions, 5 protein bands were detected by SDS-PAGE and silver staining. As expected, this is significantly less than the number of proteins detected in the mite whole body homogenate (about 30 to 40). However, when the eluate from a cotton wool ball incubated with mites treated with pilocarpine was electroblotted to a PVDF membrane and the latter stained with sulforhodamine-B, several additional protein bands were detected. This suggests that these mite proteins are either present at too low a level to be detected when the polyacrylamide gel is stained with silver, and/or have an amino acid composition which is not favourable for use with this stain. Either way, a total of approximately 15 separate protein bands were detected using this more sensitive approach, with molecular weight estimates ranging from 130 kDa to <17 kDa. However, since electrophoresis was performed under reducing conditions, it is not known if some of the bands present represent subunits of higher molecular weight proteins, normally held together by disulphide bonds. Furthermore, the fact that this more sensitive procedure for detecting proteins did not show up any additional proteins in any of the other mite samples, supports our conclusion that the proteins in the V + pilo sample represent proteins secreted from the salivary glands of the mites.

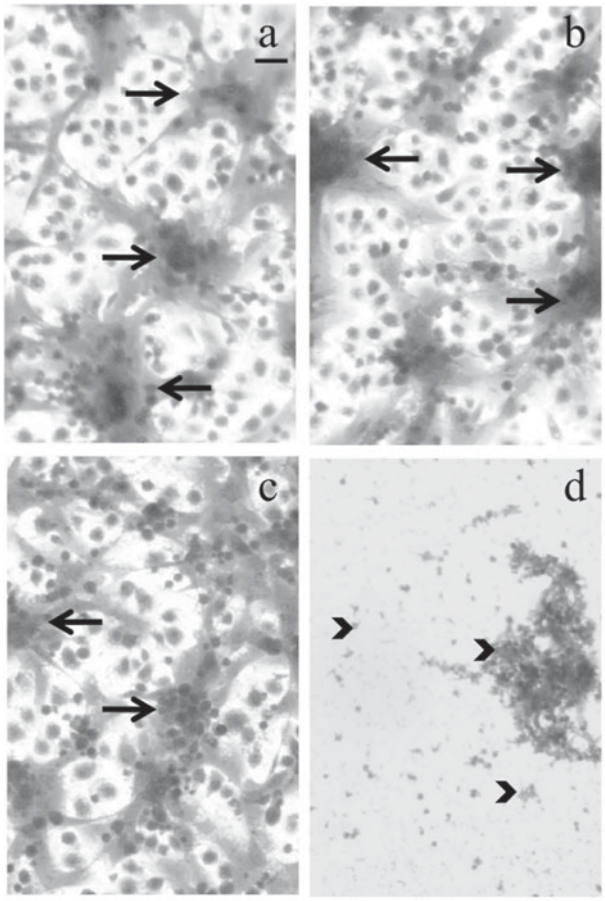

Fig. 4. Bright-field micrograph of Coomassie blue-stained haemocytes, demonstrating haemocyte anti-aggregation activity of *Varroa destructor* salivary gland secretions. *Lacanobia oleracea* haemocytes were incubated for 18 h with either (a) Eluate from a cotton wool ball incubated with TC-100; (b) TC-100 plus pilocarpine but without mites; (c) TC-100 and mites but not treated with pilocarpine; or (d) TC-100 and mites treated with pilocarpine. Note that in (a), (b) and (c), the haemocytes are healthy, have extended pseudopods and have migrated towards each other to form aggregates (arrows). By contrast, in (d), the majority of haemocytes have broken down (arrow heads), and only clumps of haemocyte debris are present. The Scale bar = 50 μm.

With regard to the function(s) of *V. destructor* salivary gland secretions, incubation of the proteins (V + pilo sample) with haemocytes from *L. oleracea* larvae *in vitro,* indicates that the secretions greatly impair the normal behaviour of these cells. In particular, the secretions damage the haemocytes (causing many to disintegrate) and inhibit their ability to extend pseudopods and form aggregates. That this effect is due solely to *V. destructor* salivary gland secretions is supported by the finding that other samples (including the eluate from cotton wool balls without mites but treated with pilocarpine [no V + pilo sample], and the eluate from cotton wool balls incubated with mites not treated with pilocarpine [V no pilo sample]), did not exhibit haemocyte anti-aggregation activity. In contrast to *V. destructor* salivary gland secretions, the mite whole body homogenate exhibited only weak haemocyte anti-aggregation activity. It is not known, however, whether the effects of the homogenate are due to the activity of low levels of salivary gland proteins, or enzymes (including digestive enzymes and non-secretory lysosomal enzymes) released from cells during homogenization.

Salivary gland secretions from many haematophagus ectoparasites have also been collected *in vitro* and assessed including insects (mosquitoes, reduuvid bugs); leeches and acarines (ticks, scabie mites, chiggers etc). The most studied salivary secretions are those of ticks and have been shown to contain a great array of anti-coagulants, anti-platelet aggregatory factors, vasodilators, immunosuppressants and anti-inflammatory factors (reviewed by Brossard and Wikel, 2004; Nuttall and Labuda, 2004; Valenzuela, 2004 and Titus *et al*. 2006). To our knowledge, the current work represents the first account of proteins secreted from the salivary glands of *V. destructor* but we hypothesize that saliva of these fellow acarines is likely to contain an equally impressive array of factors as seen in ticks to counter the honey bee's defences. An important, well-reported phenomenon is that tick saliva greatly enhances the transmission and establishment of pathogens, including viruses (Jones *et al*. 1987; Labuda *et al*. 1993), which is thought to be brought about by immunosuppression (Nuttall and Labuda, 2004). In honey bees, deformed-winged virus (DWV) remains largely benign but becomes pathogenic when *V. destructor* feeds on the bee (de Miranda and Genersch, 2010) as is the case for Kashmir bee virus (Shen *et al*. 2005*b*). It has already been demonstrated that varroa-infested bees exhibit suppressed immune gene expression (Gregory *et al*. 2005; Yang and Cox-Foster, 2005). We postulate that the trigger for DWV and some other bee viruses becoming pathogenic is the suppression of the bee's immune system by some factors in the *V. destructor* saliva.

We report here, for the first time, studies into the bioactivity of *V. destructor* saliva. A simple procedure is presented for the collection of *V. destructor* saliva demonstrating that the small size of these mites is not a major obstacle to proteomic or functional studies. Further, we clearly demonstrate the bioactivity on insect haemocyte function of *V. destructor* saliva collected by this procedure. Overall, our presented approach for saliva collection and bioactivity testing combined with the recently published *V. destructor* genome data (Cornman *et al*. 2010) and a non-invasive approach for gene knockdown (Campbell *et al*. 2010*a*) allows researchers to study the role of saliva in the *V. destructor* – *A. mellifera* interaction and pathogen transmission.

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