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Guanine visualization: a new method for diagnosing tracheal mite infestation of honey bees

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Summary — A new method for diagnosing tracheal mite infestation in honey bees is described. The method is based on the visualization of guanine, a purine which is the main end product of nitrogen metabolism in mites, but in bee excretions occurs at most in trace quantities. Thoraces of bees are homogenized in acidic or basic solutions, run on TLC plates and scanned by UV. The new assay was compared to the direct thoracic disk method and to the indirect ELISA method, and their relative advantages and disadvantages are discussed.

***Apis mellifera* / *Acarapis woodi* / diagnosis / guanine**

INTRODUCTION

The honey bee tracheal mite, *Acarapis woodi* (Rennie), a serious pest of honey bees (Eischen et al, 1989; Komeili and Ambrose, 1990), has recently been found in Israel (Gerson et al, 1994). Efforts towards its management, including various cultural and chemical methods, are usually hindered by difficulties in evaluating their effects. These difficulties stem from the fact that no single symptom fully characterizes the pests' effect (Shimanuki and Knox, 1991). Although affected bees may have disjointed wings and/or a distended abdomen, absence of these symptoms does not mean absence of mites, which are located mainly within the prothoracic tracheal system of their host bees. Several methods have been proposed and implemented for positive tracheal mite identification. These methods, reviewed by Shimanuki and Knox (1991), roughly fall into either direct or indirect categories. The former includes bee dissection and homogenization, and staining of tracheae, by which the mites are actually seen. The indirect method uses the ELISA technique (in which infestation is determined by a colorimetric

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reaction in microplates precoated with the tracheal mite antigen) (Ragsdale and Kjer, 1989; Grant et al, 1993; Grant and Nelson, in press).

Herein we describe a new, indirect method, in which a purine compound, guanine, was used to determine the presence of *A woodi*. Guanine (2-amino-6-oxypurine) is the main end product of nitrogen metabolism in many arachnids, including spiders and mites (Anderson, 1966; Bronswijk et al, 1989), but is almost absent from honey bee excretions (Atmowidjojo, 1992, cited by Erickson et al, 1994). Its occurrence in honey bees would therefore indicate that they are infested with mites. Guanine is also excreted by *Varroa jacobsoni* Oudemans (Erickson et al, 1994), another acarine attacking the honey bee, but this mite is an external parasite, not present within the bees' bodies.

MATERIALS AND METHODS

Bees

Older honey bees (recognized by carrying pollen) were collected from frames within commercial, non-treated colonies previously determined to be affected by tracheal mite. Older bees are known to be infested by larger numbers of tracheal mites (Pettis and Wilson, 1996). Several hundred worker bees were thus obtained during the spring of 1996 over a period of several weeks (group A). All bees were kept at –20 °C until assayed. A random sample of ca 300 bees from this group was then divided into four subsets. One included 35 and another 70 bees, and the two others were of 100 each, kept for the ACAREX® and ELISA tests (see below). The thoraces of the first 35-bee subset were dissected, the thoracic disks were cleared in NaOH (Shimanuki and Knox, 1991) and examined microscopically.

Thirteen additional samples (each of ca 200 older worker bees) (group B) were collected in the spring of 1996 from other commerial (treated and non-treated) apiaries located throughout Israel. These bees were likewise kept at –20 °C until they were assayed. These samples were used to compare essay methods (see below).

Guanine visualization

Thoraces of the second, 70-bee subset were separated into two equally-sized groups. Those of the first group were placed in 0.5 mL 0.1 HCl (acid solution), those of the second in 0.1 M NaOH (basic solution). All thoraces were individually homogenized by an Ultra-Turrax tissue homogenizer (2 min), followed by teflon-glass homogenization (2 min) and ultrasonic cell disruption (2 min) and finally centrifugation (12 000 *g* for 20 min, at 4 °C). The supernatant was collected for guanine determination via TLC. Two types of plates were compared for this purpose: Cellulose F_{254} (Merck) and Polygram® Cell 300 UV_{254}, (Macherey-Nagel, Doren, Germany). Supernatant samples of 10 µL were loaded 3 cm from the edge, with 2 cm between samples. Guanine (Sigma Ultra, 10 µL of 0.4 µg/µL) was loaded as a standard. An eluent of 95% ethanol/acetic acid/water (70:25:5) was most suitable for samples extracted in an acid solution, whereas a mixture of 5% w/v ammonium sulfate/13 M ammonia/1-propanol (60:30:10) was best for basic extracts. Guanine spots were visualized by a UV lamp and compared to spots obtained by the above standards. Ultraviolet spectral analyses (200–300 nm) of the homogenate were tested by means of an Uvicon-810 spectrophotometer and were compared to markers in basic and acidic solutions.

A preliminary evaluation of the method was conducted by dissecting the insects in one of the 100-bee samples from group A. Their tracheae were ranked in four categories of infestation (0: uninfested, meaning both tracheae were quite silvery; 1: incipient, one trachea with vague dark blemishes; 2: medium, one trachea clearly dark; 3: high, tracheae brown to black). The examined tracheae were then individually homogenized and visualized for their guanine content on TLC plates. The outcome was evaluated according to the degree of concordance or overlap (estimated by χ^2 tests) between the results of the dissection and guanine method.

Guanine presence has been used to evaluate the contribution of mites to house dust allergenicity; a commercial test kit is available for this purpose (Bronswijk et al, 1989; Twiggs et al, 1992). We tested this product (ACAREX®, Aller-

gopharma, Joachim Ganzer KG, Reinbeck, Germany), following the manufacturer's instructions, for its sensitivity to tracheal mite presence. About a dozen heavily infested bees from group A were individually homogenized in the fluid provided with the kit, and enclosed test stripes were dipped into various stock guanine preparations and into the bee homogenate. The resultant colors were compared to color standards provided by the manufacturer.

ELISA

A competitive monoclonal-antibody ELISA (enzyme-linked immunosorbent assay) kit which included microplates precoated with tracheal mite antigen (Grant and Nelson, in press) was used. A binding competition is set up on the plates between the fixed, precoated antigen and the free sample antigen, for a mite-specific monoclonal antibody. An enzyme-labeled anti-immunoglobin conjugate–substrate system is used to detect the specific antibody which binds the precoated antigen. Tracheal mites in the sample inhibit the binding of the monoclonal antibody to the tracheal mite-precoated antigen. Degree of inhibition is proportional to the logarithm of the amount of mite antigen in the sample. The resultant colors are measured by an ELISA colorimeter and the obtained values are then interpolated from a standard graph (% ELISA inhibition on tracheal mite infestation), sent with the 'ELISA kit for tracheal mite detection', supplied by Grant and Nelson (Beaverlodge, AB, Canada). Less than six infested bees in a 100-bee sample (6%), or more than 60 infested bees in such a sample (60%) could not be evaluated precisely by this method. Procedure and interpretation of results followed the protocol supplied by Grant and Nelson. Presence of the mite antigen was tested in the second 100-bee sample from group A. They were homogenized in 500 mL of 0.05% Tween 80 (Sigma), in phosphate-buffered saline (PBST), with a commercial blender. The homogenized mixture was allowed to settle for 2 min and a liquid sample of 5 mL was immediately assayed, or frozen at −20 °C, for replicate assays. Each ELISA test was replicated three times or more.

Comparison between methods

The 200-bee samples (group B) were individually subdivided into three batches, each assayed by a different method. Bees in one group (n = 35) were individually dissected, and mite presence in their thoraces established after clearing (Shimanuki and Knox, 1991). Mite infestation in bees of the second group (n = 35) was determined by the guanine method, whereas infestation of bees in the third group (n = 100) was assayed by ELISA. All data were converted to percentages.

RESULTS

Best guanine extraction was obtained with the basic solution. The Macherey-Nagel plates were more sensitive and required less running time than the Merck plates. Amounts of guanine as low as 2–4 µg were visualized by this procedure; they had an R_f value of 0.35 (fig 1).

The preliminary evaluation was promising in regard to the uninfested (category 0), medium infested (2) and highly infested (3) groups (fig 2). A good concordance, or fit, between the two methods was obtained in

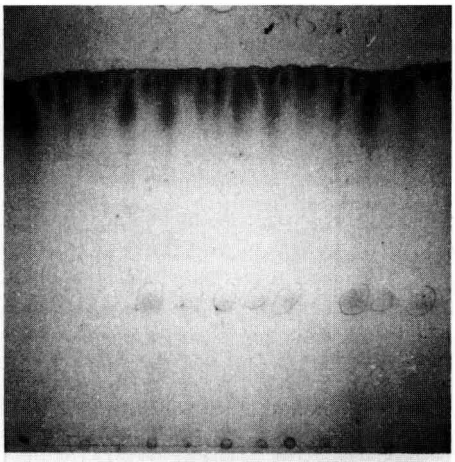

Fig 1. Guanine visualization with a UV lamp on TLC plates. G: guanine marker; numbers 1–10: visualization of individual bees, of which only 3–7 and 9–10 were infested.

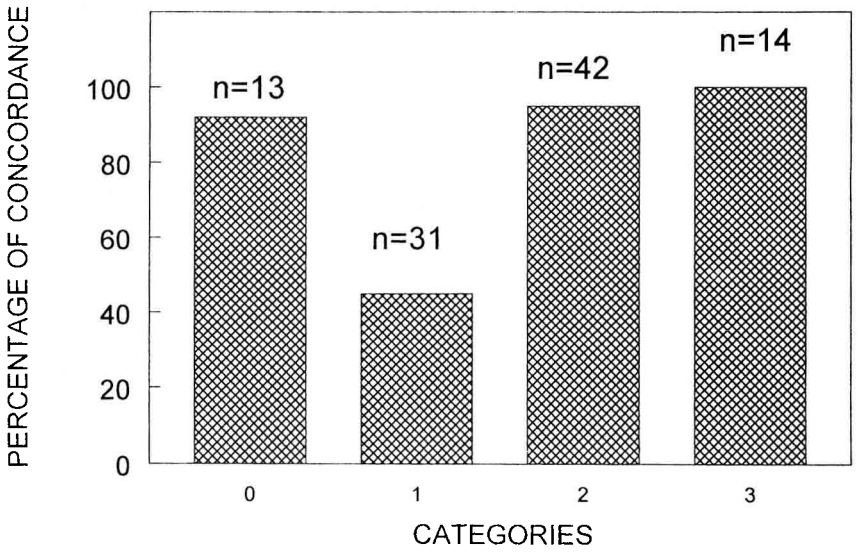

Fig 2. Percentage of bees (n = 100), in each of four tracheal mite infestation categories, which show concordance in the evaluation of mite presence by two diagnostic methods: visual inspection of the thorax and the guanine method (n = number of mites in each category). Categories: 0: tracheae silvery, without any blemishes; 1: minor, vague blemishes on tracheae; 2: one trachea with brown or darker markings; 3: both tracheae brown or black.

categories 0, 2 and 3 ($P < 0.05$; χ^2 tests), suggesting that the guanine method provided results comparable to the dissections. The data in category 1 (incipient infestation), on the other hand, were dissimilar ($P > 0.05$, χ^2 test), indicating lack of concordance between methods. In this category some slight blemishes may be due to causes other than mites, and very early mite attack may not result in blemishes. We conclude that the guanine method is as good as dissections for detecting *A woodi* in bees whose tracheae are clearly infested.

The ACAREX® test was neither able to detect the presence of guanine in a basic (NaOH extract) homogenate from infected thoraces nor from whole homogenated bees. With a prepared guanine solution the color scale indicated no result when 0.1 mg guanine or less was in the stock solutions, and a weak result with a 0.23 mg guanine solution (definitions according to the manufacturer). A strong reaction was seen with 1.5 mg guanine. These amounts are far greater (by a factor of 100) than the guanine concentrations detected on TLC plates by UV, as noted above, which negated the further use of ACAREX® for tracheal mite detection purposes.

A comparison between the three methods (table I) indicated that qualitatively the results of the three were consistent. Samples in which bees were infested (at or above 6%, the lowest level detectable by ELISA), tracheal mite presence was confirmed by all methods. Similarly, very low to no infestation was also uniformly detected. However, none of the methods were in consistent harmony with those of the other two. There was acceptable agreement between the dissections (representing the standard method) and the ELISA results in samples 1 and 8 (9 and 6%, and 14 and 15%, respectively). Samples 6, 7 and 11 had reasonable con-

cordance between dissections and guanine (80 and 71%, 62 and 59% and 71 and 69%, respectively); the latter method and ELISA provided similar results with sample 2 (28 and 30%); an acceptable fit was obtained with all three methods in regard to samples 9 and 10 (36, 37 and 33%, and 40, 34 and 30%, respectively) whereas sample 12 gave very different data (62, 48 and 37%). Samples 3, 4, 5 and 13 provided consistent similar results, of no or very low infestation.

DISCUSSION

As noted by Shimanuki and Knox (1991), each of nine methods listed for diagnosing tracheal mite presence had advantages and disadvantages. The common prothoracic disk method is labor intensive, monotonous and may not detect mites if they are very scarce or present only in other tracheae or in the head sacs. Other direct methods require additional (including microscopical) equipment and/or more elaborate preparations, such as staining.

The ELISA method, while accurate and sensitive, is probably most suitable for concomitantly assaying many bulk apiary samples. However, it requires special procedures, some technical expertise, may react to other species of *Acarapis* which could be present on the bees, and will deliver precise results only at infestation levels between 6–60%. Thus it is not suitable for work with individual bees. Initially this method could read only infestations of more than 90 mites per 100 bees, based on the assumption of an average of 15 mites per parasitized bee (Grant et al, 1993). Grant and Nelson (in

Table I. Numbers and percentages of tracheal mite-infested bees according to three diagnostic methods.

Sample no	*Infested bees according to guanine test (n = 35)*		*Infested bees according to dissections (n = 35)*		*Infested bees (%) according to ELISA (n = 100)*
	Number	*Percentage*	*Number*	*Percentage*	
1	6	17	3	9	6
2	10	28	16	46	30
3	0	0	0	0	0
4	0	0	0	0	6
5	0	0	0	0	0
6	25	71	28	80	36
7	22	62	20	59	NA
8	13	37	5	14	15
9	12	36	13	37	33
10	14	40	12	34	30
11	25	71	24	69	38
12	22	62	17	48	37
13	0	0	1	3	5

NA = not assayed.

press) recently reported a lower detectable level of 21 mites per 100 bees.

The guanine method, although simpler because it detects mite presence by specific excretions, and avoids the extensive preparations needed for immunological methods, is likewise unable to differentiate between *A woodi* and other bee-colonizing *Acarapis* spp. The results regarding incipient infestation (category 1, fig 2) indicate that the new method could not detect very low tracheal mite numbers. This drawback is shared by all other diagnostic methods. Another disadvantage of the guanine method is that it is very time-consuming when individual bees are to be examined, as each thorax must be homogenized separately. However, the guanine method has the advantage that it might be used as a quantitative tool in diagnosing infestations in individual bees (and hives), as well as a qualitative tool in bulk apiary samples, following whole bee homogenization. Such qualitative diagnoses would be quicker and less labor intensive than other available indirect methods.

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Résumé — Visualisation de la guanine : nouvelle méthode pour diagnostiquer l'acariose des trachées de l'abeille domestique. La présence de guanine (2-amino-6-oxypurine), purine qui est le principal produit final du métabolisme azoté chez les acariens mais n'est présent qu'à l'état de traces dans les excrétions des abeilles, a été utilisée comme test biologique pour diagnostiquer l'infestation des trachées de l'abeille par l'acarien *Acarapis woodi* (Rennie). Les abeilles infestées ont été homogénéisées dans des solutions acides ou basiques. Les cellules ont été déchirées aux ultrasons puis centrifugées. Le surnageant a été étalé sur des plaques pour chromatographie en couche mince avec les marqueurs de guanine appropriés et visualisé en lumière UV (fig 1). Dans un test préliminaire, la comparaison des résultats de la méthode classique (dissection de 100 abeilles) et de la méthode de visualisation de la guanine sur les mêmes abeilles prises individuellement, a montré que les deux méthodes donnaient des résultats identiques quel que soit le taux d'infestation (nul, moyen ou fort) (fig 2). La méthode de la guanine n'a pas pu mettre en évidence de façon nette les infestations débutantes. Les valeurs d'ACAREX®, produit commercial mis au point pour détecter la présence d'acariens de la poussière domestique par lecture de la guanine, étaient trop grossières pour diagnostiquer l'acarien des trachées. On a comparé trois types d'essais (la méthode du disque thoracique, la méthode ELISA et la méthode de la guanine) sur 13 échantillons de 170 acariens chacun (tableau I). La concordance entre les résultats, bonne sur le plan qualitatif mais seulement moyenne sur le plan quantitatif, indique que la méthode de la guanine pourrait être utilisée pour établir la présence d'*A woodi*. Les avantages et inconvénients de cette nouvelle méthode sont discutés.

***Apis mellifera* / *Acarapis woodi* / diagnostic / guanine**

Zusammenfassung — Guanin-Visualisierung: Eine neue Methode der Diagnose eines Tracheenmilbenbefalls bei Honigbienen. Der Nachweis von Guanin wurde als Biotest zum Erkennen eines Tracheen-

milbenbefalls von Honigbienen (*Acarapis woodi* (Rennie)) genutzt. Das Purin Guanin (2-Amino-6-Oxypurin) ist das Hauptendprodukt des Stickstoffwechsels bei Milben, kommt aber höchstens in Spuren in den Ausscheidungen der Bienen vor. Befallene Bienen wurden in sauren oder basischen Lösungen homogenisiert, die Zellen durch Ultraschall zerstört und zentrifugiert. Der Überstand wurde auf TLC Platten zusammen mit geeigneten Guanin-Markern aufgetrennt und mit einer UV-Lampe sichtbar gemacht (Abb 1). In einem vorläufigen Test wurde das Ergebnis einer klassischen Diagnose des Tracheenmilbenbefalls durch Präparation mit dem einer Guanin-Visualisation an 100 individuellen Bienen gegenübergestellt und ergab vergleichbare Ergebnisse für unbefallene, mittelstark und stark befallene Bienen (Abb 2). Ein beginnender Befall konnte mit der Guaninmethode allerdings nicht klar unterschieden werden. Die Bestimmung mit ACAREX®, einem kommerziellen Guanintest zum Nachweis von Hausstaubmilben, war für eine Diagnose der Tracheenmilben zu unempfindlich. Die Eignung von drei verschiedene Testmethoden zum Erkennen eines Tracheenmilbenbefalls (Thorax-Schnittpräparatmethode, ELISA und Guanin-Visualisierung) wurden anhand von 13 Proben mit jeweils 170 Bienen verglichen (Tabelle I). Die gute qualitative, allerdings nur mäßige quantitative Übereinstimmung belegt die Eignung der Guaninmethode zum Erkennen eines Tracheenmilbenbefalls. Die Vorteile und Nachteile der Methode werden diskutiert.

***Apis mellifera* / *Acarapis woodi* / Diagnose/ Guanin**

REFERENCES

Anderson JF (1966) The excreta of spiders. *Comp Biochem Physiol* 17, 973-982

Atmowidjojo AH (1992) Comparative behavior and physiology of feral and domestic honeybees, *Apis mellifera* L. Thesis, University of Arizona, AZ, USA

Bronswijk JEMH van, Bischoff E, Schirmacher W, Kniest FM (1989) Evaluating mite (Acari) allergenicity of house dust by guanine quantification. *J Med Entomol* 26, 55-59

Eischen FA, Cardoso-Tamez D, Wilson WT, Dietz A (1989) Honey production of honey bee colonies infested with *Acarapis woodi* (Rennie). *Apidologie* 20, 1-8

Erickson EH, Cohen AC, Cameron BE (1994) Mite excreta: a new diagnostic for varroasis. *BeeScience* 3, 76-78

Gerson U, Dag A, Efrat H, Slabezki Y, Stern Y (1994) Tracheal mite, *Acarapis woodi*, comes to Israel. *Am Bee J* 134, 486

Grant GA, Nelson DL The ELISA detection of honey bee tracheal mite, *Acarapis woodi*. In: *Acarology IX; Proc* (Mitchell R, Horn DJ, Needham GR, Welbourn WC, eds), Ohio Biological Survey, Columbus, OH, USA (in press)

Grant GA, Nelson DL, Olsen PE, Rice WA (1993) The ELISA detection of tracheal mites in whole honey bee samples. *Am Bee J* 133, 652-655

Komeili AB, Ambrose JT (1990) Biology, ecology and damage of tracheal mites on honey bees (*Apis mellifera*). *Am Bee J* 130, 253-257

Pettis JS, Wilson WT (1996) Life history of the honey bee tracheal mite *Acarapis woodi* (Acari: Tarsonemidae). *Ann Entomol Soc Am* 89, 368-374

Ragsdale DW, Kjer KM (1989) Diagnosis of tracheal mite (*Acarapis woodi*) parasitism of honey bees using a monoclonal based enzyme-linked immunosorbent assay. *Am Bee J* 129, 550-553

Shimanuki H, Knox DA (1991) *Diagnosis of Honey Bee Diseases*. USDA Agricultural Handbook No AH-690

Twiggs JT, Gray RL, Marx JJ Jr (1992) Correlation of the Acarex™ test for guanine contents of vacuum dust with the amount of mite allergen present as determined by RAST inhibition assay. *Immun Allerg Prac* 14, 334-339