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Original Article

FIRST IDENTIFICATION OF NANOPARTICLES ON THORAX, ABDOMEN AND WINGS OF THE WORKER BEE *APIS DORSATA* FABRICIUS

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Abstract

The presence of nanoparticles on the body of the honeybee *Apis dorsata* Fabricius, was investigated for the first time to better understand the bee's behaviour. These have been observed by using Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and confirmed by Atomic Force Microscopy (AFM). Our study clearly denotes that the Indian rock honey bee *Apis dorsata* possess calcium silicate and calcium phosphate nanoparticles on its body surface of 5-50 nm in diameter. In particular, the nanoparticles on the abdomen and thorax of *A. dorsata* have an average diameter of about 10 nanometers and they are smaller than those found on wings of the same bees which are about 20 nanometers. The nanoparticles found are different of the ones previously observed on honey bees or other insects. The origin and role of these natural nanoparticles on the body of the Indian rock bee need to be to be further investigated; more research in the subject might raise important aspects in relation to the conservation of these unique pollinators.

Keywords: nanoparticles, calcium silicate, calcium phosphate, *Apis dorsata* Fabricius, behaviour, pollinator

INTRODUCTION

General

It is a well known fact that the honey bee is a major pollinator of many of our food crops such as almonds, apples, avocados, blueberries, cantaloupes, cherries, cranberries, cucumbers, sunflowers, watermelon, and there are many other crops that rely on honey bees for pollination. The honey bee's economical role in worldwide pollination has been valued to be around 153 billion Euros in the year 2005 (Gallai et al., 2009) although the bee's primary interest is the 'search for food'. The dance language of the honey bees (*Apis* spp.) is one of the best known communicative adaptive feature among the honey bees (von Frisch, 1967; Gould, 1976). This language of honey bee is used to convey landscape information to other nest mates. The geomagnetic field has been shown to have an effect on the performance of the waggle dance of *A. mellifera* L. (Lindauer et al., 1986). More specifically, it has been shown that honey bees use geomagnetic field information for orientation, homing, and foraging (Binhi, 2004). Furthermore, it has been reported that honey bees posses large numbers (~105) of super crystals. The crystals are called nanoparticles because their diameter is >100 nm. The word "nano" developed from the Greek word 'nanos' meaning "dwarf". In more technical terms, the word "nano" means 10-9 or one billionth of something. For example, the size of a nanoparticle is usually between 10–100 nm while viruses are between 20–450 nm, proteins between 5–50 nm, and genes between 2 nm wide by 10–100 nm long.

Up till now, those nanoparticles found in the bee abdomens ranged between 30 and 35 nm in diameter. They were assumed to be involved in magnetic field detection (Gould et al., 1980), probably due to iron protein ferritin and the ferrimagnetic responses of nanoscale magnetite (Fe_3O_4) particles (Desoil et al., 2005). Nanoparticles were also found in body parts of other social insects, such as ants (Wajnberg et al., 2004; Abracado et al., 2005), termites (Alves et al., 2004), and meliponinae bees (Lucano et al., 2006).

Nanoparticles, in general, demonstrate unique characteristics with elevated strength, high electrical conductivity, and extra chemical reactivity (Nykypanchuk et al., 2008), as well as distinctive chemical, physical, and biological properties (Leiderer & Dekorsy, 2008; Bhattacharyyal et al., 2008; Sabbour, 2013). That is why nanotechnology has been increasingly used in many fields as well as in agriculture (Gul et al., 2014).

Most studies concerning nanoparticles on bees have been performed on *A. mellifera* bees. Our study, however, demonstrates for the first time that the rock bee, *A. dorsata* F., possess nanoparticles on its body surface. These particles have been identified by Scanning Electron Microscopy (SEM), and Transmission Electron Microscopy (TEM), and confirmed by Atomic Force Microscopy (AFM). The role that these particles could play on the behaviour of *A. melifera dorsata* is discussed.

Background information on *Apis dorsata* Fabricius

Apis dorsata Fabricius honey bees are the second largest species of honey bees. *Apis dorsata* F. is commonly called the rock bee or giant bee. It is distributed throughout the Indo-Malayan region. It occurs in India, Pakistan, Sri Lanka, Nepal, and southern China, northern Burma, Indonesia, and all throughout the Philippine islands (Koeniger & Koeniger, 1980; Ruttner, 1988).

Apis dorsata Fabricius construct single and huge nests under open conditions on tall trees, cliffs of rocks, tanks, buildings etc. An aggregation of colonies on a particular nesting site is a very common feature seen in this species. The shape of the comb is more or less semicircular or cuneiform. The surface of the comb varies from 0.1 to more than 1 square meter. The comb contains a thick honey comb with a thickness of 15-20 cm in the high section of the comb; a brood comb of 3-4 cm thick. Pollen is stored between the two zones. A functional division of the colony, like nest service, a protective curtain, and a mouth or active zone, is also present (Neupane et al., 2013).

The differences among the different castes (i.e. the workers, drones, and the queen) are very small. Worker and drone cells are also of the same size except that the drone cell capping is elevated and flat. The queen is slightly larger than the worker. The developmental periods of the worker, queen, and drone are 16-20, 13-13.5, and 20-23.5 days, respectively. The migratory distance can be up to 200 km and on the way, they may stop 2 to 3 times. These honey bees are known to forage throughout the day. This is the only species of honey bees foraging even during bright night hours. The extrapolated sun is used for compass orientation and not the position of the moon. These honey bees are also attracted to street lights (Dyer, 1985).

Apis dorsata F. are the most dominant pollinators visiting all agricultural, horticultural, and farm crops. They are quite predominant on crops like sunflower, niger, sesame, mustard, cotton, coconut, cardamom, coffee, fruit, and vegetables. The foraging range of these bees is more than 5 km.

MATERIAL AND METHODS

Sample preparation for Field Emission Scanning Electron Microscopy (FE-SEM)

The bees were collected from the southern part of Dharawar, in the southern Indian state of Karnataka. Nanoparticles were determined on the abdomen, thorax, and wings of the bees. All samples were initially washed with double distilled water (DDW). For fixation, they were kept in glutaraldehyde 2.5% in 0.1M cacodylate buffer (pH 7.4) for 24 h and then washed with cacodylate buffer©. Then they were kept again in glutaraldehyde 2.5% for other 24 h and washed with glutaraldehyde 2.5% in cacodylate buffer (GLC) according to Chambarelli et al. (2008).

Transmission electron microscopy

Transmission electron microscopy (TEM) imaging and energy-dispersive X-ray spectroscopy (EDS) were conducted using a JEOL 2200 FS TEM with attached EDS, at the Advanced Microscopy Center, Michigan State University, USA. Copper grids were coated with ultra thin carbon films. By using the thin film copper grids, the sample could be deposited on the film instead of falling through the mesh of the grid. Grids were then coated with Sundew adhesive in the same manner, using the technique described earlier. Briefly, the copper grids were grasped using sharp electron microscopic forceps and gently brushed against the tentacles of the Sundew. After coating with the adhesive, the samples were dried overnight for subsequent analysis.

Atomic force microscopy

Atomic force microscopy (AFM) is a very high resolution type of Scanning probe microscopy with demonstrated resolution on the order of fractions of a nanometer, more than 1000 times better than the optical diffraction limit. It is a modern tool for imaging nanostructures. The machine can measure single molecule interaction forces. These forces play an important role in fundamental processes occurring in physical and biological systems.

Atomic force microscopy imaging was conducted using both an Agilent 5500 AFM and an Agilent 6000 ILM/AFM. The purpose of using both systems was to control for potential artifacts, and to allow for microscopic imaging of the samples to determine the targeted scanning areas. In addition, all samples were examined by two independent investigators who prepared their samples separately to further eliminate the possibility of creating artifacts in the data sets. All imaging for both systems was conducted in air in AC mode. Both systems were equipped with intermittent contact mode tips, Budget Sensors® Tap150AL-G, with aluminum reflex coating. The tips had a resonant frequency of 150 kHz and a force constant of 5 N/m. Due to tip variation, manual sweeps were conducted on all tips prior to scanning to determine the actual frequency of the tip. Prior to scanning, a calibration grid was used to assure that the distance measurements of the Picoview® software were accurate. Publication quality scans were conducted at a scan speed of less than 1 In/s and a resolution of 1024 × 1024 pixels.

RESULTS

Field Emission Scanning Electron Microscopy (FE-SEM) images showed the presence of nanoparticles on the body parts of the bees. Figure 1 depicts the nanoparticles detected on the different parts of the worker honey bees (wings, abdomen, and thorax, respectively). The size distribution of the nanoparticles collected from the different parts is between 5-50 nm. The abdomen (Fig. 1b) and the thorax (Fig. 1c) contain smaller sized particles compared with the ones on the wing (Fig. 1a). A little aggregation of the nanoparticles has also been observed which indicates their interactions on the surface. The size distribution of the particles was also confirmed by the TEM images (Fig. 2, A, B; as abdomen and thorax of the bees contain similar sized particles, we show them selectively, choosing the best image).

Further clarifications have been seen by Atomic Force Microscopy (AFM). Figure 3 represents

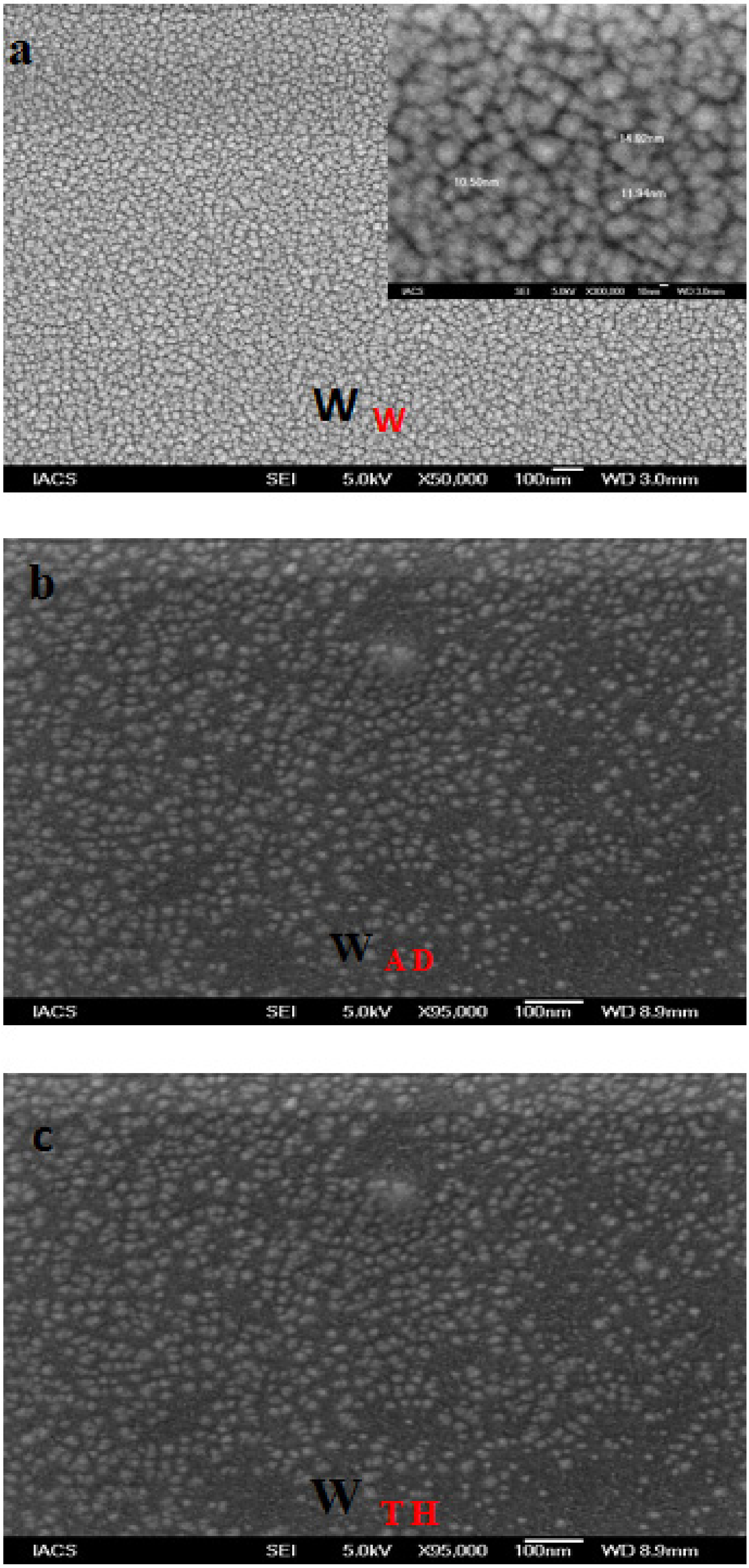

Fig. 1. FE-SEM images of nanoparticles extracted from different parts of *Apis dorsata*; a. Ww -Worker wing; b. WAD - Worker Abdomen; c. WTH - Worker thorax

the AFM images for WW (Worker Wing) sample. A topographical image represents the two-dimensional distributions of nanoparticles throughout the body matrix (depicted in Fig. 3a). The histrogram in Figure 3b suggests that the particle size distribution of the nanoparticles is around 20 nm. The three dimensional AFM-image shows the particle height on a measured surface (Fig. 3c). Similar observations have been made for the nanoparticles collected from a WAD (Worker Abdomen) sample (Fig. 4). The histogram data (Fig. 4b) show that particles are somehow smaller in size with respect to the WW sample (as also seen in SEM data in Fig. 1). We also need to keep in mind that, in general, the size obtained from an AFM image should be higher than that seen in FE-SEM images because in tapping mode, AFM image particles are somewhat exaggerated. The image in Figure 4c gives almost a similar height for consecutive particles. The diameter of the particles measured by using two or three consecutive particles (see Fig. 5A, B, for abdomen and thorax, respectively) matches with the particle diameter seen in SEM and TEM data (Fig. 1 and 2).

Furthermore, it was interesting to investigate the nature of the nanoparticles detected. For this reason electron dispersive X-ray spectroscopy analysis was performed. The results are presented in Fig. 6. The presence of calcium in higher amounts, including Si and P, may suggest the presence of calcium silicate and calcium phosphate as nanoparticles. Cu and C elements have also been found but probably represents crystals of cacodylate buffer used in the procedure. It is believed that the Cu and C elements come from the TEM grid or from contamination as they are being associated with different eco-condition. Cu and C elements have been found in samples from different nests.

DISCUSSION

Field Emission Scanning Electron Microscopy (FE-SEM) images have shown the presence of nanoparticles on the body parts of *A. dorsata* bees (wings, abdomen, and thorax). Till now, nanoparticles have never been investigated in *A. dorsata* F. The above work clearly demonstrates the presence of nanoparticles on all three body parts of the Indian rock bee, although different from the nanoparticles previously reported by other researchers in honey bees, meliponinae bees or other insects (Alves et al., 2004; Wajnberg et al., 2004; Abracado et al., 2005; Desoil et al., 2005; Lucano et al., 2006; Hsu et al., 2007). According to Sharma et al. (2011), the *A. mellifera* bee's thorax, abdomen and wings contain small

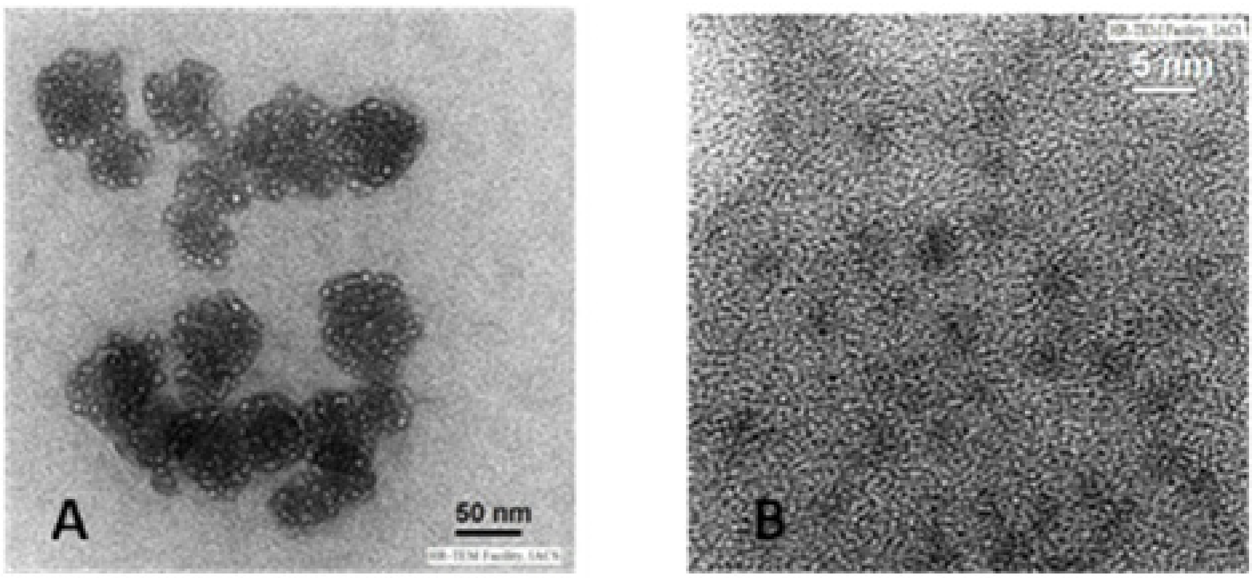

Fig. 2. TEM image showing the size distribution of the nanoparticles Worker wing and Worker thorax; A. the biggest and B. the smallest nanonparticles

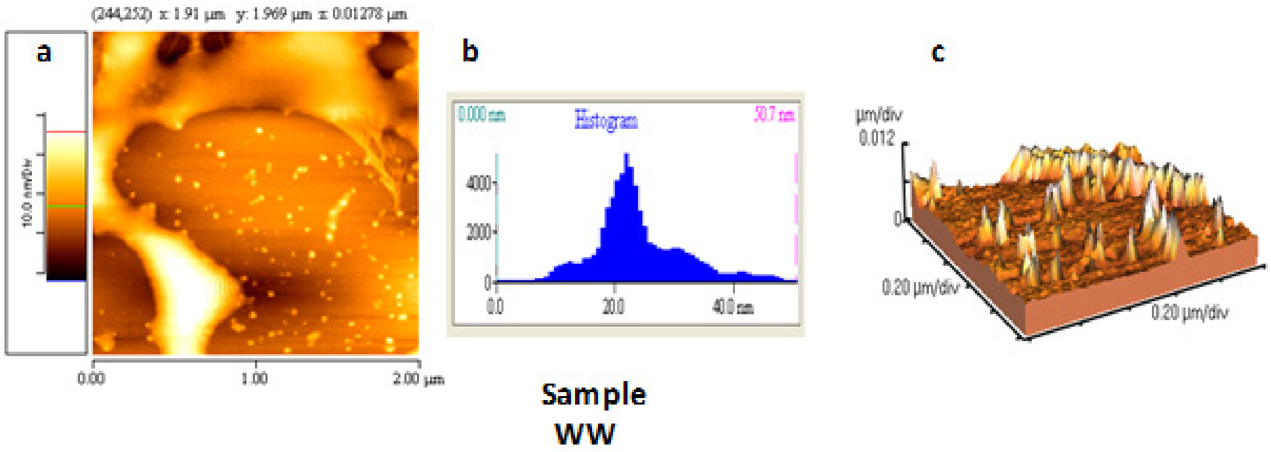

Fig. 3. AFM images of WW -Worker wing; a. in 2D; b. histogram of particle distribution; c. height distributions in 3D

to large particles, with a size from 5 to 50 nm. Gould et al. (1980) observed that nanoparticles found in *A. mellifera* abdomens ranged between 30 and 35 nm in diameter. Particles of that size (around 30 nm) are classified as 'large' and were absent in the heads and thoraxes (Desoil et al., 2005). In this study, we found that the nanoparticles on the abdomens of *A. dorsata* have a diameter of about 10 nanometers and they are smaller than those found on the wings of the same bees. The size distribution of the nanoparticles collected from the different parts is between 5-50 nm.

All previous works describe iron nanoparticles on the *A. mellifera* body (Could et al., 1980; Schiff, 1991), as well as on trophocytes (Hsu & Li, 1994; Hsu et al., 2007; El-Jaick et al., 2001). In our study, we clearly defined that the particles on the body of the *A. dorsata* bee were not iron but most probably calcium silicate and calcium phosphate in nature. Such findings have not been noted before. Still there is no indication, at least from this study, on what the function is of the nanoparticles detected on the body of the Indian rock bee. It is also impossible at this stage, to know where these particles come from, since calcium silicate is, in fact, a white free-flowing powder derived from limestone or diatomaceous earth (fossilised remains of algae) and that calcium phosphate is a mineral found in food.

It is possible that nanoparticles in the insect wings help to increase the aerodynamic effectiveness of the insect. Therefore, the nanopar-

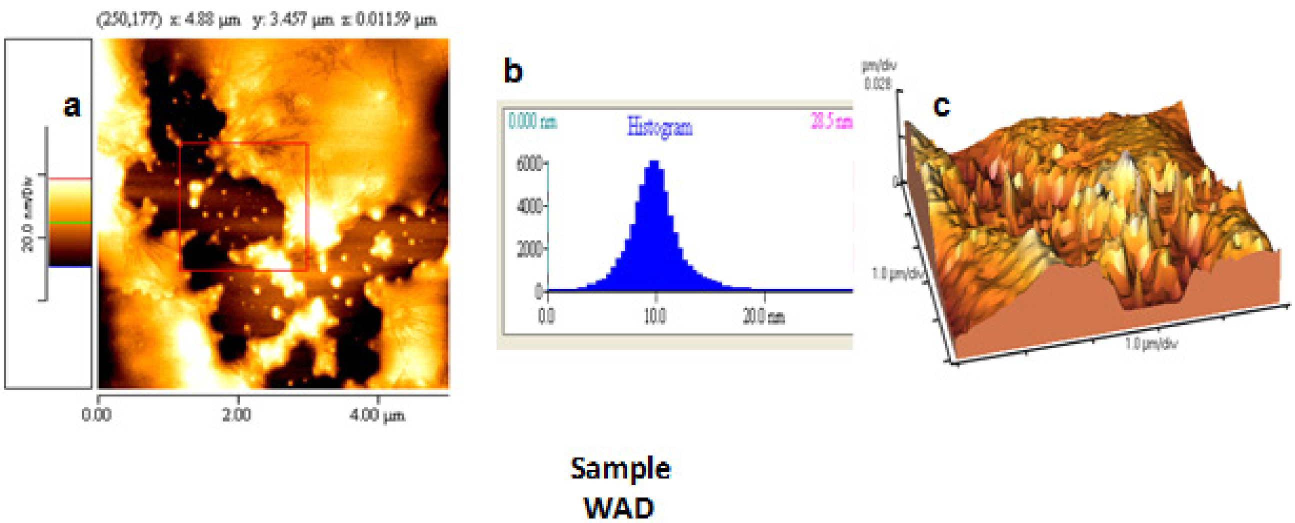

Fig. 4. AFM images of WAD -Worker Abdomen a. in 2D; b. histogram ofparticle distribution; c. height distributions in 3D

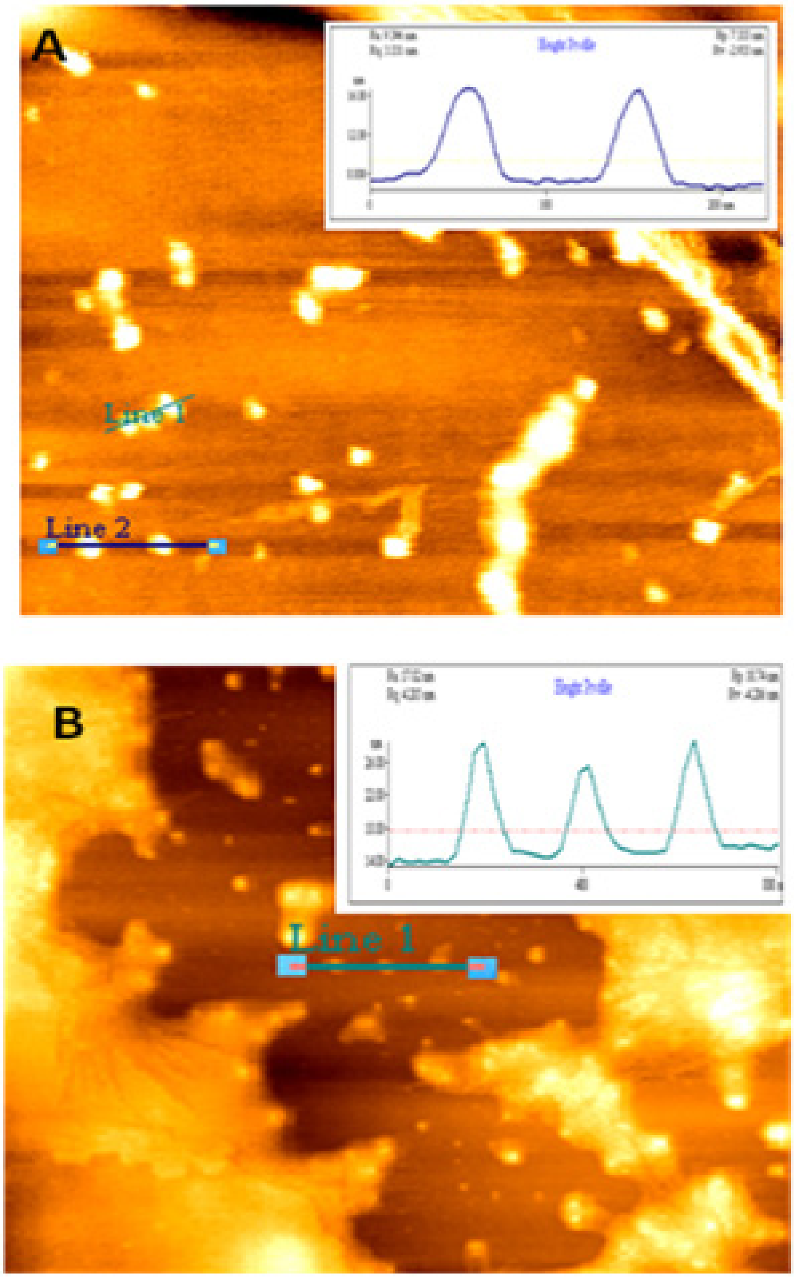

Fig. 5. Obtained diameter of the nanoparticles by using Pro-Scan software for AFM analysis. The measurements were done for two or three consecutive nanoparticles. A. WAD - Worker Abdomen; B. WTH - Worker thorax

ticles might play an important role in pollination efficiency of the giant bee. It is essential to investigate if these nanoparticles play any role in the behavior of *A. dorsata* bees in relation to their orientation (diurnal or nocturnal) (Dyer, 1985), colony migration (Dyer & Seeley 1994), and absconding or swarming behavior, considering the special environmental conditions in which this particular bee survives.

This is the first study to suggest that the *Apis dorsata* honey bee posses calcium silicate nanoparticles on their body surface. A future study should make comparisons between *Apis dorsata* and *Apis* mellifera as well as to define the nature of these nanoparticles in order to allow us to understand their use by *Apis dorsata*. Future work in conservation studies of the *Apis dorsata* species might also help. These nanoparticles are sufficiently small enough to move inside the body without disrupting normal functions and can access spaces unapproachable by other pathological means. This means that nanoparticles could be used in an application for insect pest management, instead of

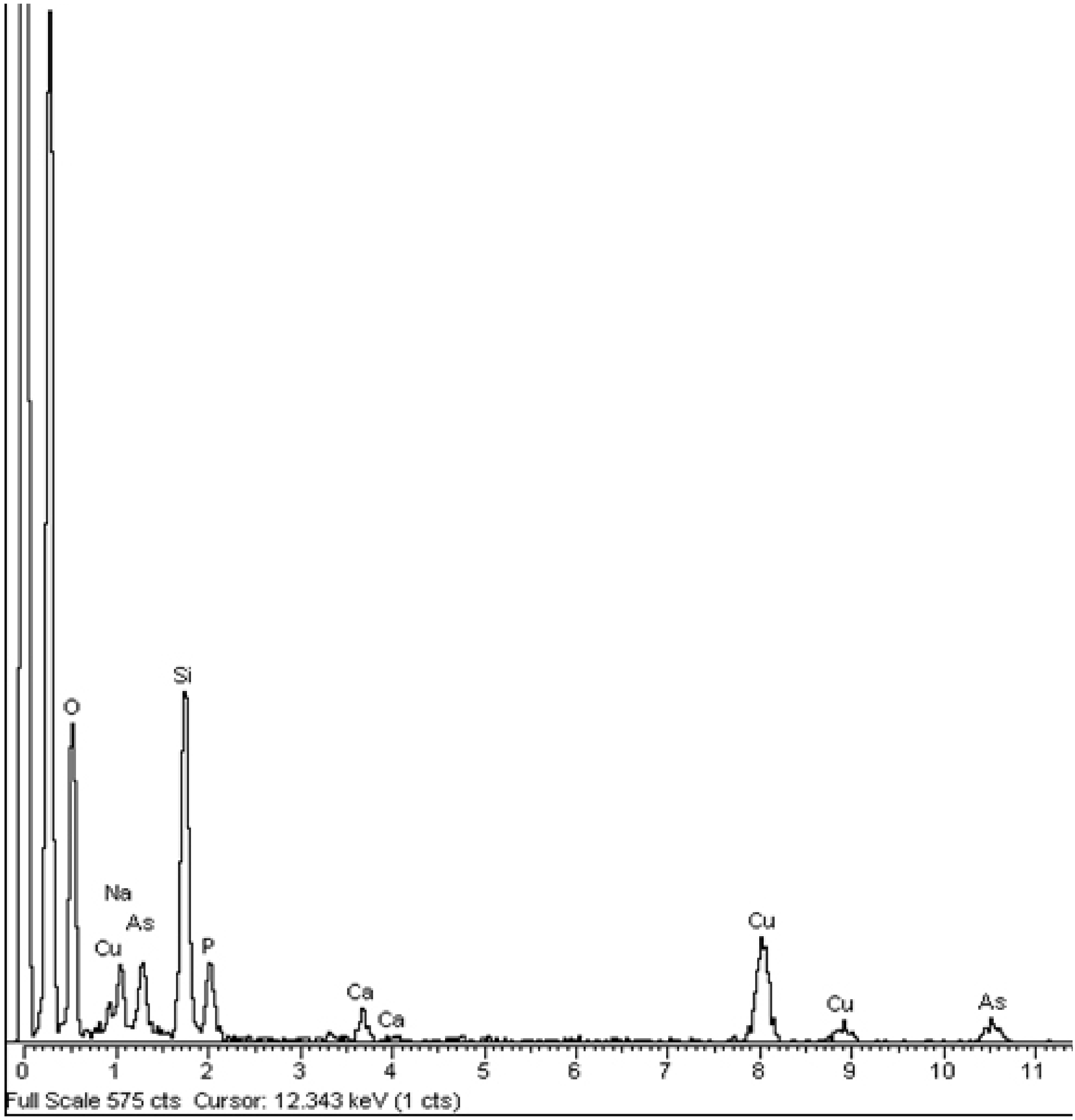

Fig. 6. Presence of calcium in higher amounts, including Si and P, may suggest the presence of calcium silicate and calcium phosphate as nanoparticles. Cu and C elements have come from TEM grid

synthetic chemicals (see also Bhattacharyya et al., 2010; Gul et al., 2014).

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