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# Spatial Distributions of Inorganic Elements in Honeybees (*Apis mellifera L.*) and Possible Relationships to Dietary Habits and Surrounding Environmental Pollutants

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**ABSTRACT:** In this study, the laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) was adopted to determine the distribution of inorganic elements, including Ca, Cu, Fe, Mg, Mn, S, P, Pb, and Zn, in honeybees (*Apis melifera L.*). Two features are particularly noteworthy. First, it was found there is a significant amount of Fe located at the fringe of the abdomen in worker bees; ultrasonic imaging, scanning electron microscopy, and magnetic resonance imaging revealed that it arose from magnetic Fe-bearing nanoparticles (NPs) having an average diameter of approximately 40 nm. Interestingly, only worker bees contained these magnetic Fe-bearing NPs; no similar features appeared in larvae, pupae, wasps, or drones. Second, a detectable amount of Pb accumulated particularly in the alimentary canals of worker bees. Again, no detectable amounts of Pb in larvae, pupae, drones, or wasps, yet a level of 0.24 ± 0.05 mg/kg of Pb in pollen; therefore, the diet appears to be the primary pathway for environmental pollutants entering the honeybees' food chain.

**KEYWORDS:** *laser ablation inductively coupled plasma mass spectrometry, honeybee, bioindicator, multielement distribution, environmental contaminants*

## INTRODUCTION

Honeybees (*Apis melifera L.*) perform several irreplaceable environmental roles. Directly, the honey and other apiary products produced by honeybees are important nutrient sources, not only providing energy but also having certain medical applications. Indirectly, honeybees are the most efficient pollenators, enabling crops to fertilize, fruit, and undergo sexual reproduction.

When bees collect nectars and pollens, attached foreign objects, including anthropogenic pollutants, are brought back to their hives and, hence, enter into the apiary products. Given that they usually forage nectars and pollens covering an area of several square kilometers, bees are ideal bioindicators for monitoring the local levels of soil, plant, water, and air pollution.[1] For example, Cd, Cr, and Pb accumulate significantly in the pollen, propolis, and wax and in the honeybees themselves, more so than in their honey.[2] Honeybees residing next to an airport have been found to accumulate high levels of Pb.[3] In addition, the mineral contents in bee pollen produced in the northeastern states of Brazil have been measured as higher than those from southern regions.[4] Moreover, metal contents in bee pollen samples from an urban site in Brazil have been reported to be higher than those in a rural site, with these metals accumulating particularly during the dry season (July−October).[5] Temporal variation also affects the concentrations of various metals, including Cd, Cr, Al, Co, Cu, Mn, Sr, Ti, and V, in honeybees.[6]

In addition to the pollens and nectars that honeybees forage, fine particles, including aerosol dusts, will adhere to either their hairy bodies or to the pollens, ultimately being delivered to their hives. It has been reported that fine anthropogenic particles can result in various elements concentrating on the surfaces of pollens, particularly in urban areas.[7] Together, these studies convey the notion that honeybees and their associated apiary products are sensitive to the level of local pollution.

Although many relevant studies have been performed regarding the total concentrations of elements of interest in honeybees, to the best of our knowledge none have dealt with the elemental distributions in honeybees and, particularly, their spatial distributions, which should provide further precious information. Accordingly, in this study the laser ablation (LA) system was used to locally evaporate thinly sliced honeybee samples. These evaporated samples were then introduced into an inductively coupled plasma-mass spectrometry (ICP-MS) system for qualitative and semiquantitative analyses, allowing the construction of images displaying the spatial distributions of the elements of interest. In addition, samples of larvae, pupae, drones, and worker bees as well as wasps were examined to reveal the elemental distributions during different growth stages. Further, other sophisticated techniques, namely, high-resolution *in vivo* X-ray computed microtomography (micro-CT) and magnetic resonance imaging (MRI), were selected to probe the forms of the studied elements. Finally, a series of apiary products, including honey, pollen, and royal jelly, were digested and their mineral contents analyzed using ICP-MS.

## EXPERIMENTAL DETAILS

**Sample Preparation.** Samples of honey, pollen, and royal jelly were obtained from a local apiary vendor, located in northern Hsinchu in a suburban area surrounded by paddy fields with no heavy industry within a radius of 10 km. All examined honeybees, including worker bees, drones, pupae, and larvae, were provided by the same apiary

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vendor; they were kept in 10% formalin prior to analyses. Wasps (genus *Vespa basalis*) were provided by the fire department of Hsinchu city and stored in the same way as were the honeybees.

To prepare thin specimens for imaging analysis, all examined bees were cooled to −50 °C and, when completely frozen, cut into slices (Leica CM 1850) having a thickness of approximately 30 μm. Given the pronounced difference in hardness between the bee's external skeleton and internal organs, these specimens were quite fragile and the overall yields of prepared samples were rather low; nevertheless, all image collections were conducted in triplicate analyses. As-obtained specimens were carefully fixed onto glass slides for further analyses using LA-ICP-MS and scanning electron microscopy (SEM).

**LA-ICP-MS.** Laser ablation was performed using a UP 213 laser ablation system (New Wave Research) with a Nd:YAG laser (wavelength, 213 nm) operated in Q-switched (pulsed) mode; the pulse length was 4 ns with a repetition rate of 10 Hz, a dwell time of 8 s, and an intersite pause of 1 s. During ablation, the laser beam (diameter, 0.11 mm; defocused distance, 1.5 mm) with a fluence of 15−20 J $cm^{-2}$ was used to vaporize the elements within a selected area. At a scan rate of 0.1 mm $s^{-1}$ and a frequency of 10 Hz, an area of approximately 1.0 mm × 1.0 mm was ablated and introduced into the ICP-MS system using Ar as the carrier gas (1.0 L $min^{-1}$). Multielement analysis was conducted using a quadrupole ICP mass spectrometer (Agilent 7500a); the operating rf power was 1.5 kW, with the detector operated in time-resolved analysis acquisition mode; the plasma gas flow rate and auxiliary gas flow rate were 15 and 2.0 L $min^{-1}$, respectively. Individual calibration curves were set up by dipping a series of known concentration solutions onto the substrate.

**MRI Specimen Preparation.** Because any residual air within a specimen would lead to severe interference in the MRI data, worker bees were immersed overnight at 4 °C in a solution containing 4% formalin/0.1% Triton X. They were then rinsed with iso-osmotic sucrose solution (450 mOsm $L^{-1}$) and placed in a test tube filled with sucrose solution for MRI analysis. Operating conditions (Bruker ClinScan 70/30 USR): repetition time (TR), 50 ms; echo time (TE), 7, 8, or 9 ms; matrix size, 512 × 256; slice thickness, 0.5 mm; flip angle, 15°; field of view (FOV), 22 mm × 11 mm; resolution, 43 μm $pixel^{-1}$.

**Micro-CT and SEM.** Operating conditions for micro-CT (Skyscan 1176): rotation degree, 0.3°; average, two pieces; resolution, 9 μm. The scanning electron microscope (Zeiss Supra 60) was operated under relatively ambient pressure.

**Image Construction.** To compose the pixel-by-pixel information acquired from the LA-ICP-MS system, a home-built image processing code, based on the Matlab interface, was used. The intensity of these elements was directly converted into an 8-bit color image with variable darkness. A map with the spatial distribution of each element was thus obtained.

## ■ RESULTS AND DISCUSSION

First, it has to be mentioned that, although only duplicate or triplicate samples of larvae, pupae, drones, wasps, and worker bees were employed in this study (depending upon the goodness of the prepared specimens), all observed patterns among these samples were quite consistent. We are, therefore, confident that the images reported herein are highly representative. Given the difficulty in matrix matching, we prefer to interpret our results in a relatively qualitative manner and focus on the connections between the studied elements and the physiological functionalities involved. The unit shown in the figures is defined as $ng/mm^2$. Our strategy in image construction will be a great benefit to demonstrate the distribution of some environmentally hazardous elements since their concentrations are quite low (vide supra).

**Appearance of Sliced Larva, Pupa, and Worker Bee Samples.** Before getting into a detailed discussion, it is good to present a general overview of the anatomies of honeybees at their different growth stages. Figure 1 displays images of a larva (top), pupa (middle), and worker bee (bottom), recorded using the built-in charge-coupled device (CCD) of the LA-ICP-MS system. In the larval stage, two major components are evident: the alimentary canal (dark) and the fat body (light yellow). The alimentary canal is a very important organ of the larva; it digests the fed nectar, diluted honey, and pollen. The fat body is actually a mixture of many undifferentiated cells, the individual functionalities of which are rather difficult to discern; these cells will eventually develop into specific organs during the pupal stage. Comparing the anatomical images of the pupa and larva, it is clear that the percentage covered by the dark areas has decreased greatly. Thus, during the pupal stage, the alimentary canal develops into individual cavities. Along with the developing alimentary canal, the fat body undergoes self-aggregation; accordingly, several clusters of fat bodies are evident. These clusters undergo further transformation into the various organs of adult worker bees, wasps, and drones. The body of an adult honeybee comprises three parts: head, chest, and abdomen. Several individual organs (including the stomach), the alimentary canal, and muscles are discernible in the CCD image. The sliced images of drones and wasps were very similar to that of the worker bee. The most obvious difference between a worker bee and a drone or wasp is that a honey stomach is found only in the worker bee.

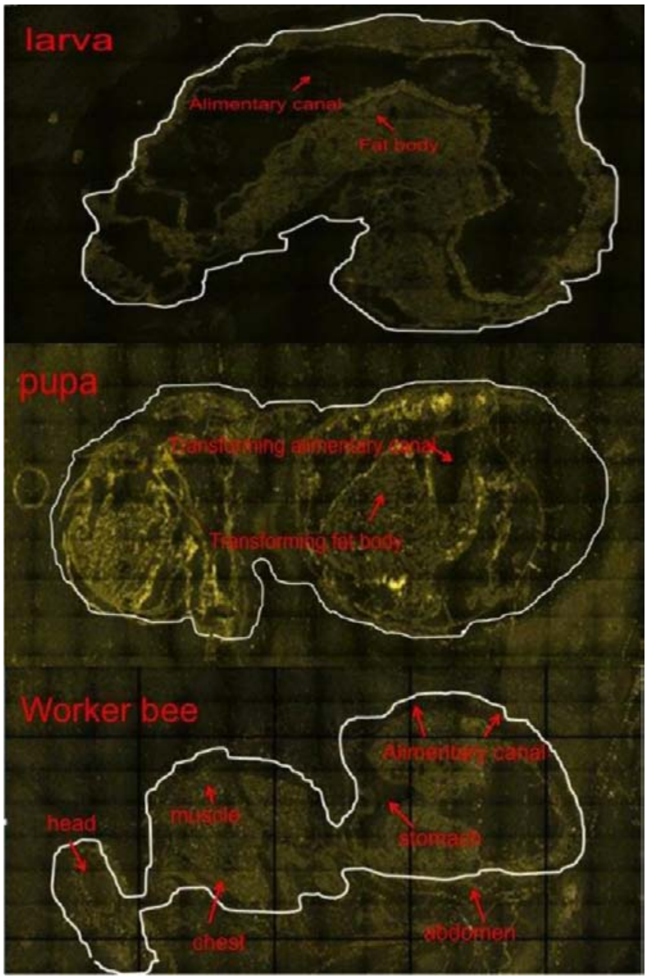


**Figure 1.** CCD images of larva, pupa, and worker bee samples.

**Spatial Distribution of Elements in a Worker Bee.** Figure 2 reveals the spatial distributions of nine elements (Ca,

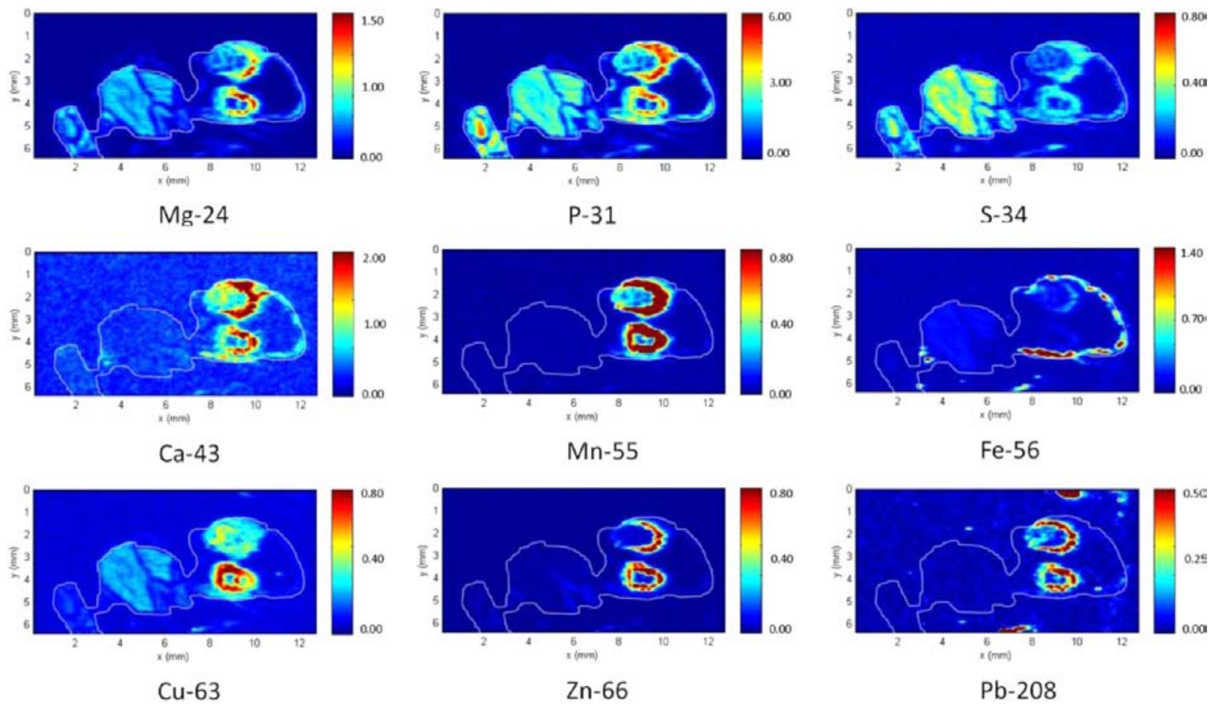


**Figure 2.** Spatial distributions of inorganic elements in a honeybee, measured using LA-ICP-MS. The unit shown in the figure is ng/mm$^2$.

S, Mg, P, Mn, Fe, Cu, Zn, Pb) in a worker bee. Phosphorus is an essential nutrient and a basic element in many biomaterials, including nucleic acids, phospholipids, phosphoproteins, membrane structures, and a variety of enzymes. Sulfur is another essential element in all living species; it appears in the structures of the amino acids cysteine, methionine, homocysteine, and taurine, with disulfide bonds playing important roles in determining the conformations of various proteins and enzymes. This may explain the high concentrations of S were observed in the muscle, fat body, as head; for P, several hot zones in the head and alimentary canal as well as in the wing muscles were noted. Calcium was located particularly around the alimentary canal and along the fringe of the abdomen. No significant amounts of Ca appeared in the worker bee's head or chest. Magnesium appeared in several hot zones in the head, chest, and alimentary canal, very similar to the distribution patterns of the P and S elements.

A balance between P, Ca, K, Na, S, and Mg ions is crucial in regulating the activity level of ATPase.[8] This biochemistry is likely responsible for the quite comparable distribution patterns of the elements P, S, and Mg. A detailed study of the inorganic elements found in the fat bodies of insects has been reported previously.[9] The hot zones of the P element appear to be associated with the places requiring a higher energy demand for ATP activity; that is, higher energy is required for metabolism in the alimentary canal, the chest muscles that power the wings, and the heavy activities of the brain. The reasons behind the distribution pattern of Ca element being different from that of the P, Mg, and S elements remains unclear, but one possible explanation might be the fact that the Ca element also participate in the programming of apoptosis; that is, pronounced Ca element accumulation occurred along with the preparation of a larva transforming into a pupa.[10,11]

For transition metals, it was observed that Cu, Zn, and Mn all shared a similar distribution pattern, with a clear hot zone in the alimentary canal, very similar to the distribution of Ca. Notably, Ca, Mg, and Zn elements participate in the immune response and directly affect the reactivity of tissues involved in the processes of encapsulation and phagocytosis by interfering with the spread and network formation of plasmatocytes.[12,13] In addition, it has been reported that the availability of the Cu element is strongly related to regulation of the synthesis and melanization of new cuticles.[14] Furthermore, Zn also play important roles in the homeostasis of organisms and in the processes of cell growth and differentiation.[15,16] Manganese has been reported to participate in the ATP activity of insects.[17] Accordingly, the hot zones of the transition metals Mn, Cu, and Zn appear to reflect the sites of heavy enzyme activity. These enzymatic reactions require huge amounts of ATP as the energy supply; therefore, it is quite understandable that hot zones of P element appear in the alimentary canal, superimposing those of the Mn, Cu, and Zn elements.

In addition to these biochemical reactions, the hot zones of the elements appearing in the alimentary canal seem to reflect the dietary habits of the worker bee. For example, several unexpected hot zones of Pb element appear in the alimentary canal. The spatial distribution of Pb, a hazardous heavy metal, is closely related to local anthropogenic activity. Accordingly, the observed Pb element was likely contributed from pollens that the worker bees had consumed.

It is noted that most of the essential elements were distributed in the head, chest, and abdomen of the worker bee. Transition metals, which are indispensable in the structures of many enzymes, appear particularly in the chest (flying muscle) and alimentary canal. In addition, a detectable amount of Pb also appeared in the alimentary canal, revealing that environmental pollutants can be introduced into worker bees and, consequently, into their apiary products as a result of their dietary habits.

**Magnetic Nanoparticles (NPs) in a Worker Bee.** The nature and distribution of Fe element within honeybees is a topic that attracts much attention.[21] In addition to the crucial

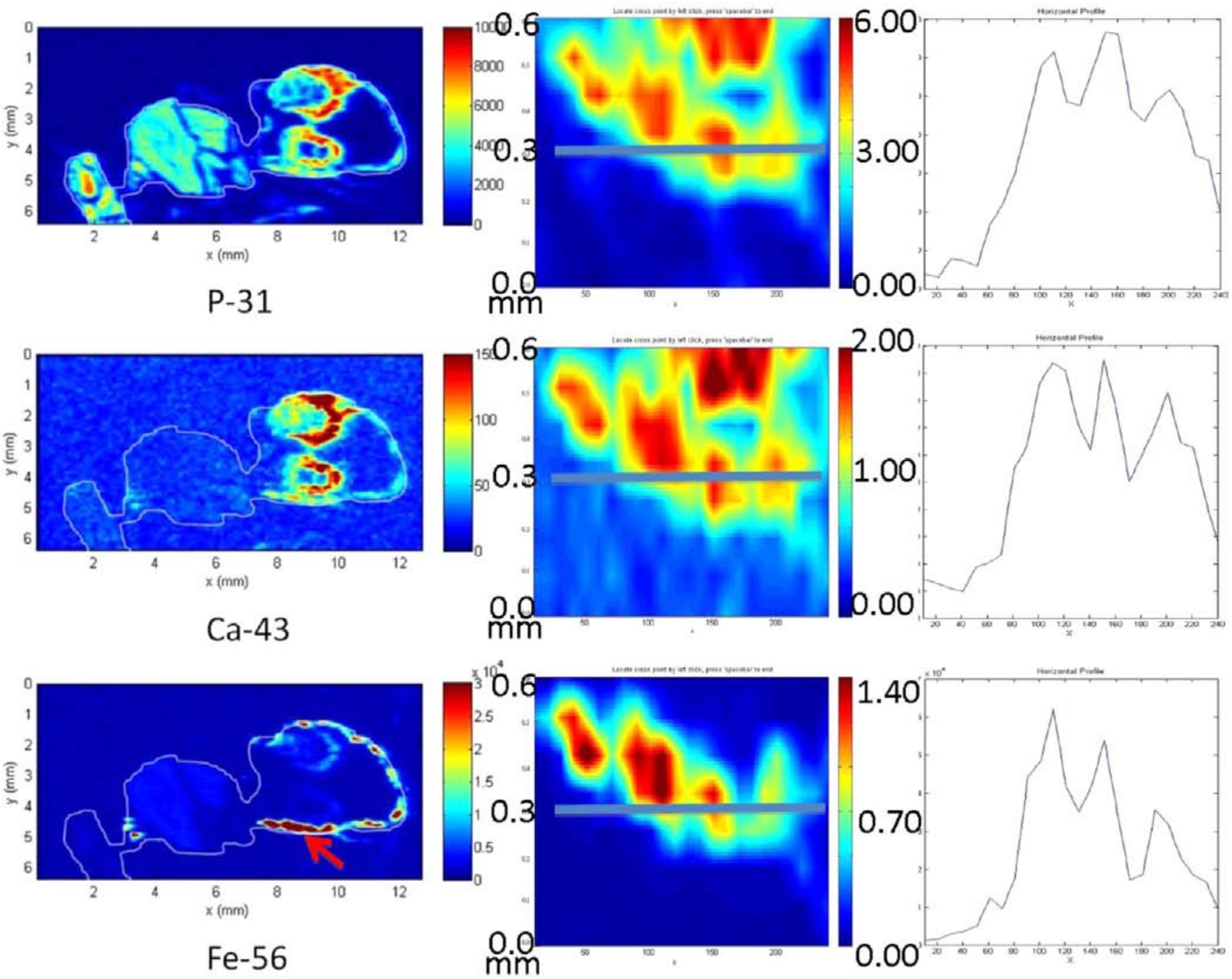

**Figure 3.** Correlation between the P, Ca, and Fe element distributions in the lower abdomen of a worker bee. (Left) Spatial distributions determined using LA-ICP-MS; (middle) finer scans along the point denoted by the red arrow in the right panel, the unit in *y*-axis is millimeters and the *x*-axis is in micrometers, in this region (250 × 600 $\mu$m$^2$) and the unit of corresponding concentration is ng/mm$^2$; and (right) corresponding intensity profiles along the finer scan.

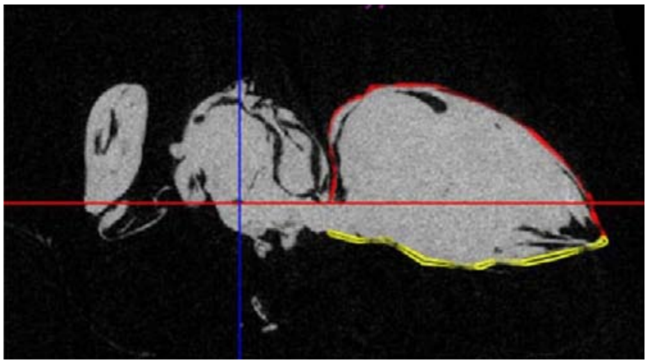

**Figure 4.** Micro-CT analysis of a worker bee. The zones featuring high-density materials are emphasized in yellow and red.

roles of Fe element participating in the bioactivity of some enzymes, Fe-bearing materials, particularly magnetic granules, are believed to be responsible for worker bees' awareness of the Earth's magnetic field. Such interactions assist the spatial orientation of worker bees, a particularly important aspect during foraging. Figure 2 reveals an intense hot zone for Fe element concentrated along the fringe of the abdomen.

Interestingly, P and Ca elements also appeared in hot zone rings along the fringe of the abdomen, similar to the distribution of the Fe element. To reveal correlations between these three elements, a horizontal scan along the lower abdomen (as indicated by the red arrow in Figure 3) was conducted. The intensity fluctuations of these three elements superimposed one another, suggesting that the P, Ca, and Fe elements coexisted along the lower abdomen and did not merely happen to appear at the same time. Assuming that the matrix effects can be negligible, the concentrations of P, Ca, and Fe were estimated as approximately 500, 110, and 110 ng/cm$^2$, respectively, corresponding to an atomic ratio of 8.19:1.39:1. The structure $CaFe_2P_2$ is Pauli paramagnetic; its paramagnetism arises from the magnetic moments of the nearly free electrons in their conduction bands.[18] Given that $CaFe_2P_2$ is usually formed only at high temperatures (>900 °C) and that its stoichiometry differs from the ratio determined from Figure 3, it is suspected that $CaFe_2P_2$ is unlikely to be the material responsible for the magnetic response of worker bees.

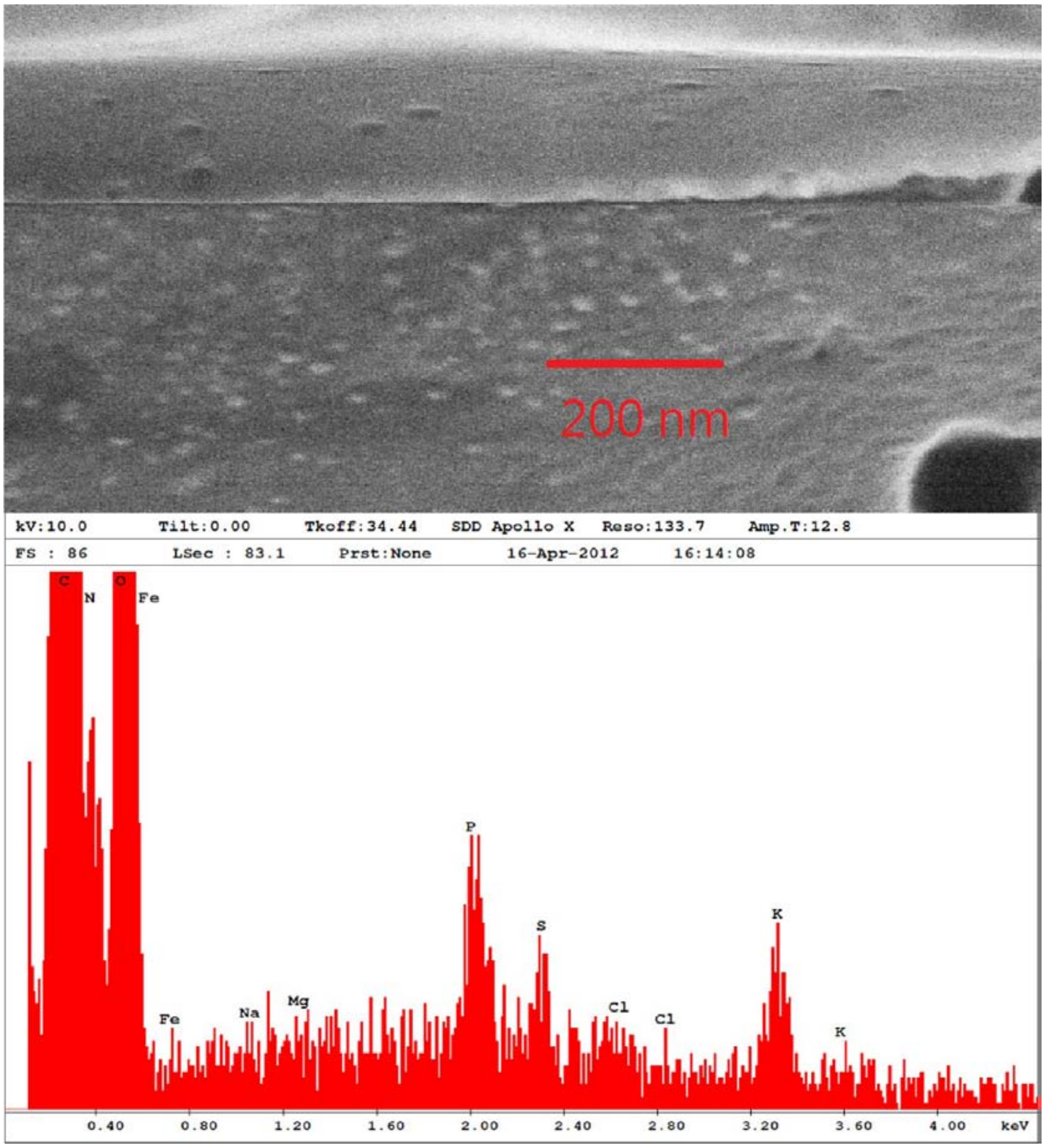


**Figure 5.** SEM image (top) and EDS data (bottom) of the Fe hot zone highlighted by the red arrow in Figure 3. The scale bar inside the SEM figure is 200 nm. The *y*-axis in the EDS figure is the read intensity.

Unfortunately, LA-ICP-MS cannot be used to determine the O element contents of oxides; therefore, it is possible that other undetermined Fe-bearing oxide components might be responsible for worker bees' spatial awareness. It has been reported that the Fe-bearing granules in worker bees arise through the degradation of ferritin.[19,21,25] During this process, the compromised protein shell causes the Fe element at the core of ferritin to become exposed to the medium of the endoplasmic reticulum. This slightly acidic medium facilitates the precipitation of Fe element in the form of granules. Simultaneously, P and Ca elements in the medium are released from the protein shell of ferritin and undergo deposition onto the precipitated Fe.[19] Accordingly, the granule possesses a structure very similar to that of ferritin.[20]

Because it is difficult to use LA-ICP-MS to determine the structures of these Fe-bearing granules directly and because isolating them is not a simple task, other alternative sophisticated techniques, namely, micro-CT, SEM, and MRI, were adopted to obtain further structural information.

Micro-CT revealed a pattern of dense components spread along the fringe of the abdomen, as indicated by the red and yellow lines in Figure 4. Consistent with Fe element distribution pattern in Figure 3, the micro-CT data provided evidence for the existence of Fe hot zones in a dense manner, rather than representing cationic Fe ions in solution. The white spots in the SEM image in Figure 5a represent Fe-bearing granules having a diameter of approximately 20−40 nm. The corresponding energy-dispersive X-ray spectroscopy (EDS) data in Figure 5b reveal that these white granules are comprised of Fe, P, Ca, and most importantly, O elements. Although the resolution of the EDS system was insufficient to precisely quantify the atomic ratio of these elements, it did provide evidence suggesting that, in addition to $CaFe_2P_2$, the Fe element in these hot zones might exist in the form of magnetite ($Fe_3O_4$).[21] Because the isolation of these potential structures from the matrix of the abdomen is rather challenging, it is difficult to identify the structure accurately using XRD. Instead,

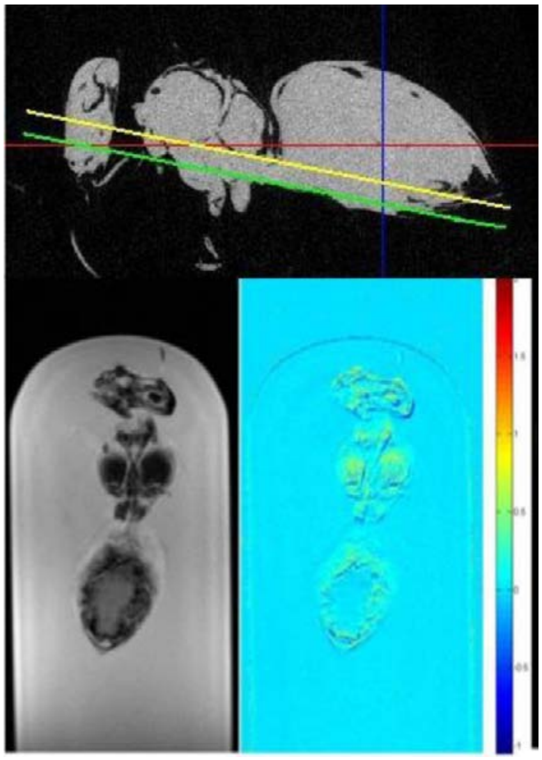

**Figure 6.** MRI image recorded along the (left) green and (right) yellow lines.

**Table 1. Contents (ppm) of Inorganic Elements in Samples of Local Honey, Pollen, and Royal Jelly ($n$ = 3)**

| element | honey | pollen | royal jelly |
|---|---|---|---|
| Li | 0.06 ± 0.01 | 0.10 ± 0.01 | 0.14 ± 0.01 |
| Na | 30.4 ± 0.19 | 89.3 ± 2.71 | 125 ± 1.25 |
| Mg | 5.5 ± 0.66 | 377 ± 75.3 | 320 ± 12.3 |
| Al | ND | 173 ± 51.1 | ND |
| K | 297 ± 32.8 | 1970 ± 300 | 1960 ± 80 |
| Ca | 47.5 ± 5.22 | 946 ± 216 | 189 ± 7 |
| Mn | 0.51 ± 0.02 | 60.9 ± 14.6 | 0.56 ± 0.01 |
| Fe | 0.38 ± 0.01 | 17.4 ± 4.41 | 8.53 ± 0.07 |
| Ni | 0.10 ± 0.01 | 0.91 ± 0.17 | ND |
| Cu | 0.37 ± 0.03 | 2.21 ± 0.34 | 6.08 ± 0.16 |
| Zn | 0.55 ± 0.06 | 6.87 ± 1.76 | 18.0 ± 0.50 |
| As | ND | 0.03 ± 0.01 | ND |
| Pb | ND | 0.24 ± 0.05 | 0.09 ± 0.02 |

an MRI technique that has an extremely high sensitivity toward magnetic magnetite was applied.[22]

To identify the magnetic properties of these Fe-bearing granules, several MRI scans along the cross sections of the yellow and green lines in Figure 6 were conducted. The strong magnetic response along the fringe of the abdomen confirmed that these Fe-bearing granules were magnetic. It is suspected that the shadows appearing in the head and chest were artifacts stemming from air bubbles within the cavities. Furthermore, when analyzing the Fe distributions in a drone wasp, pupa, and lava, none of them revealed an Fe distribution pattern similar to that of the worker bee. This finding is interesting because worker bees are responsible for the foraging of pollen; they must develop an efficient navigation system, such as that provided by magnetic magnetite granules, to guide the way. On the other hand, drones and wasps do not have to collect and return home any pollen or nectar; correspondingly, no such magnetic navigation system in drones or wasps were found.

Results from MRI imaging experiments suggested that the Fe-bearing granules in the worker bees were magnetite particles. It is possible that the P and Ca elements appearing in these hot spots once have resided within the protein shell of ferritin; during the degradation of ferritin, its Fe element were exposed to an acid environment, where biomineralization occurred to transform the Fe element into magnetite particles, with the released P and Ca elements depositing onto these granules. Because no similar features in wasps or drones or in pupae or larvae were found, it is believed that these magnetic magnetite granules are the most important component of the navigation system of worker bees.

**Content of Inorganic Elements in Apiary Products.** The spatial distribution of Pb in the honeybee is an interesting topic deserving deeper study. Lead is a legislated environmental pollutant that can cause serious damage to the nervous system. Its content in aquifer, soil, and air samples is monitored routinely.[23] In a carefully controlled experiment in which Pb cations were intentionally added into sugar syrup, a significant amount of Pb was found to accumulate in the digestion systems of worker bees.[24] This result suggested that environmental pollutants can be transported into apiary products through the medium of the worker bee. However, no reports regarding the spatial distribution patterns of environmental pollutants in honeybees were presented. To the best of our knowledge, this paper is the first to describe the spatial distribution of Pb in the honeybee. Although obvious Pb hot zones along the alimentary canal of the worker bee were found, no other clear patterns of the Pb element in the pupa, larva, wasp, or drone were obtained. To explain this behavior, the roles of these insects in the hive as well as their dietary habits should be addressed in advance.

Worker bees are responsible for most of the heavy-duty activities in the colony, including the collection of food, the production of honey and royal jelly, and the raising of baby honeybees (larvae). When a worker bee finds nectar, it crawls into the petals of the flower, sucks up the nectar, and stores it in the honey sac, in which the collected nectar is concentrated and treated with some enzymes. When the worker bee returns to its hive, it places the processed nectar into the honeycomb, which is further sealed with a layer of beeswax. These processed nectar are called "honeys" when they are harvested from the hive. Pollens that adhere onto the furry legs of worker bees are also brought into the hive and stored in honeycomb. The collected honey and pollen are the main sources of food that the worker bees use to feed the larvae, the drones, the queen, and themselves. Nonetheless, no obvious Pb hot zones appearing in the larva, pupa, and drone were found. Thus, the transfer of Pb from the worker bees into honey, and consequently, into the larvae, pupae, and drones, is very limited. This argument is supported by elemental analysis: Table 1 lists the inorganic element contents in local apiary products, including honey, pollen, and royal jelly. Notably, the amounts of Pb present in pollen and royal jelly were 0.24 ± 0.05 and 0.09 ± 0.02 mg/kg, respectively, whereas no Pb was detectable in the local honey, consistent with the absence of any detectable Pb in the insects that had mainly been fed with honey. Because wasps, in contrast, mainly consume proteins (meats) rather than honey and pollen, it is not surprising that no significant amounts of Pb were found within them.

Here, 13 elements that have analyzed are roughly divided into three groups: main group elements (Li, Na, Mg, Al, K, Ca); transition metals (Mn, Fe, Ni, Cu, Zn); and environmental pollutants (As, Pb). In principle, pollen possesses the highest concentrations of all of the studied elements. Among the main group elements, Na, K, and Ca had the highest concentrations, from dozens to hundreds of parts per million, in pollen, honey, and royal jelly. A high concentration of Al appeared in pollen, but not in honey or royal jelly, presumably because of airborne dust that had deposited or adhered onto the pollen. All of the transition metals (Mn, Fe, Ni, Cu, Zn) appeared at the parts-per-million level in pollen, honey, and royal jelly, direct evidence for a dietary pathway being responsible for the transition metals found within worker bees and other samples. Finally, small amounts of As and Pb in the pollen and royal jelly were pointed out but not in honey. Given that a detectable amount of Pb was found within the worker bees, it is a clue these elements originated from the pollen; that is, this environmental pollutant is passed on to humans through the food chain.

In conclusion, in this study, the LA-ICP-MS was adopted to probe the distributions of inorganic elements in the worker bee, particularly their spatial distributions. Among all of the distribution patterns, a series of hot zones of Fe element spread along the fringe of the abdomen was noted. Micro-CT data suggested that these zones featured dense Fe-bearing materials, with SEM/EDS revealing that they were small granules (~20−40 nm) comprising Ca, P, Fe, and O elements. MRI confirmed that they had the structure of magnetite. In contrast, no similar magnetic granules were found in larvae, pupae, drones, or wasps. In addition, a significant amount of Pb that had accumulated in the alimentary canal of the worker bees was observed, conveying the notion that the food chain (via honey) is a main pathway that brings this environmental pollutant into our bodies.

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### Notes

The authors declare no competing financial interest.

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## REFERENCES

(1) García, J. C. R.; Rodríguez, R. I.; Crecente, R. M. P.; García, J. B.; Martín, S. G.; Latorre, C. H. *J. Agric. Food Chem.* **2006**, *54*, 7206−7212.

(2) Conti, M. E.; Botre, F. *Environ. Monit. Assess.* **2001**, *69*, 267−282.

(3) Perugini, M.; Manera, M.; Grotta, L.; Abete, M. C.; Tarasco, R.; Amorena, M. *Biol. Trace Elem. Res.* **2011**, *140*, 170−176.

(4) Morgano, M. A.; Martins, M. C. T.; Rabonato, L. C.; Milani, R. F.; Yotsuyanagia, K.; Rodriguez-Amayac, D. B. *J. Braz. Chem. Soc.* **2012**, *23*, 727−736.

(5) Morgano, M. A.; Martins, M. C. T.; Rabonato, L. C.; Milani, R. A.; Yotsuyanagi, K.; Rodriguez-Amayac, D. B. *J. Agric. Food Chem.* **2010**, *58*, 6876−6883.

(6) van der Steen, J. J. M.; de Kraker, J.; Grotenhuis, T. *Environ. Monit. Assess.* **2012**, *184*, 4119−4126.

(7) Okuyamaa, Y.; Matsumotoa, K.; Okochib, H.; Igawa, M. *Atmos. Environ.* **2007**, *41*, 253−260.

(8) Williams, R. J. P. *Bioenergetics* **1970**, *1*, 215−225.

(9) Pinheiro, D. O.; Zucchi, T. D.; Zucchi, O. L. A. D.; Nascimento Filho, V. F.; Almeida, E.; Cônsoli, F. L. *Comp. Biochem. Physiol., Part B* **2010**, *156*, 273−278.

(10) Jones, R. G.; Davis, W. L.; Vinson, S. B. *J. Histochem. Cytochem.* **1982**, *30*, 293−304.

(11) Chamberlain, M. E. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2004**, *287*, R314−R321.

(12) Willott, E.; Hallberg, C. A.; Tran, H. Q. *Arch. Insect Biochem. Physiol.* **2002**, *49*, 187−202.

(13) Willott, E.; Tran, H. Q. Zinc and Manduca sexta hemocyte functions. *J. Insect Sci.* **2002**, *2*, 6−12.

(14) Vinson, S. B.; Pennachio, F.; Consoli, F. L. The parasitoid−host endocrine interaction from a nutritional perspective. In *Endocrine Interactions of Insect Parasites and Pathogens*; Edwards, J. P., Weaver, R. J., Eds.; The Cromwell Press: Trowbridge, Wiltshire, U.K., 2001; pp 187−205.

(15) Wellinghausen, N.; Kirchner, H.; Rink, L. *Immunol. Today* **1997**, *18*, 519−521.

(16) Marklová, E. *Acta Med.* **2002**, *45*, 129−133.

(17) Maruyama, K.; Allen, S. R. *Comparative Biochem. Phys.* **1967**, *21*, 713−718.

(18) Jia, S. A.; Chi, S. X.; Lynn, J. W.; Cava, R. J. *Phys. Rev. B* **2010**, *81*, 214446.

(19) Keima, C. N.; Cruz-Landimb, C.; Carneiroa, F. G.; Farina, M. *Micron* **2002**, *33*, 53−59.

(20) Kuterbach, D. A.; Walcott, B.; Reeder, R. J.; Frankel, R. B. *Science* **1982**, *218*, 695−697.

(21) Hsu, C-Y; Ko, F-Y; Li, C-W; Fann, K.; Lue, J.-T. *PLoS ONE* **2007**, *2*, e935.

(22) Huang, S.; Yan, W.; Hu, G. F.; Wang, L. Y. *J. Phys. Chem. C* **2012**, *116*, 20558−20563.

(23) Izquierdo, M.; Tye, A. M.; Chenery, S. R. *Sci. Total Environ.* **2012**, *433*, 110−122.

(24) Raes, H.; Cornelis, R.; Rzeznik, U. *Sci. Total Environ.* **1992**, *113*, 269−279.

(25) Hsu, C. Y.; Chan, Y. P. *PLoS ONE* **2011**, *6*, e19088.