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Western Honeybee Drones and Workers (*Apis mellifera ligustica*) Have Different Olfactory Mechanisms than Eastern Honeybees (*Apis cerana cerana*)

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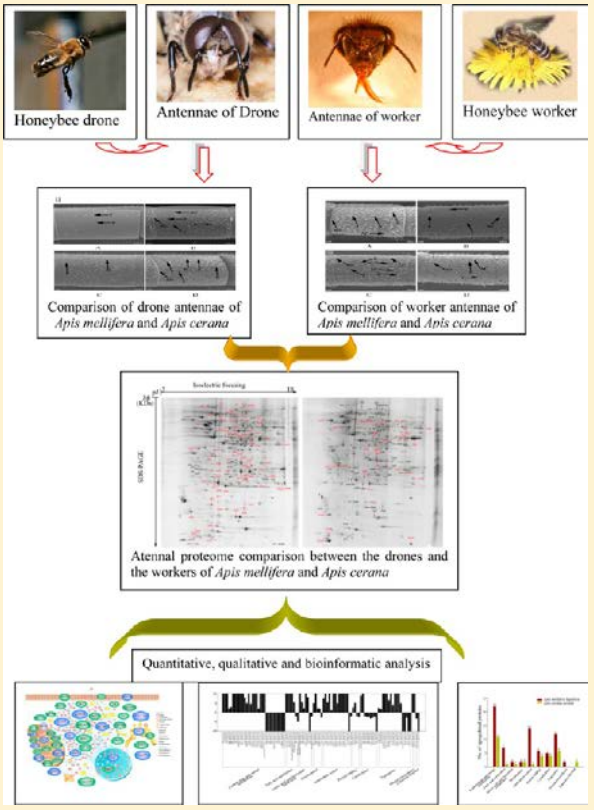
Supporting Information

ABSTRACT: The honeybees *Apis mellifera ligustica* (*Aml*) and *Apis cerana cerana* (*Acc*) are two different western and eastern bee species that evolved in distinct ecologies and developed specific antennal olfactory systems for their survival. Knowledge of how their antennal olfactory systems function in regards to the success of each respective bee species is scarce. We compared the antennal morphology and proteome between respective sexually mature drones and foraging workers of both species using a scanning electron microscope, two-dimensional electrophoresis, mass spectrometry, bioinformatics, and quantitative real-time polymerase chain reaction. Despite the general similarities in antennal morphology of the drone and worker bees between the two species, a total of 106 and 100 proteins altered their expression in the drones' and the workers' antennae, respectively. This suggests that the differences in the olfactory function of each respective bee are supported by the change of their proteome. Of the 106 proteins that altered their expression in the drones, 72 (68%) and 34 (32%) were overexpressed in the drones of *Aml* and *Acc*, respectively. The antennae of the *Aml* drones were built up by the highly expressed proteins that were involved in carbohydrate metabolism and energy production, molecular transporters, antioxidation, and fatty acid metabolism in contrast to the *Acc* drones. This is believed to enhance the antennal olfactory functions of the *Aml* drones as compared to the *Acc* drones during their mating flight. Likewise, of the 100 proteins with expression changes between the worker bees of the two species, 67% were expressed in higher levels in the antennae of *Aml* worker contrasting to 33% in the *Acc* worker. The overall higher expressions of proteins related to carbohydrate metabolism and energy production, molecular transporters, and antioxidation in the *Aml* workers compared with the *Acc* workers indicate the *Aml* workers require more antennal proteins for their olfactory mechanisms to perform efficient foraging activities than do the *Acc* worker bees. These data decipher the mechanisms of the western and eastern drone and worker bees acting in response to their different olfactory system in their distinct ecosystem.



KEYWORDS: *honeybee, drone, worker, species, antennae, proteome, olfaction*

1. INTRODUCTION

The honeybee society is a highly complex social organization consisting of a single reproductive queen, hundreds of males, and thousands of sterile female workers.[1] A key feature of this eusocial species is an intricate division of labor that has developed through highly specialized sensory organs used as signals in many aspects of their lives.[2−4]

In the honeybee society, chemical communication plays a fundamental role in mediating their main behaviors.[5,6] Thus, honeybees rely on their olfactory system to locate food sources, recognize potential mates, and detect possible dangers (such as fire or predators) as well as allowing for social interactions

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through olfactory sensilla located on the antennae.[7] Honeybee antennae perceive chemical signals with sensilla,[8,9] and there is a variation in sensillar patterns between males and females. There are about 6,500, 3,000 and 18,600 of sensilla per antenna of the worker, queen, and drone, respectively.[10−15] Furthermore, the chemical signals of the antennae of each caste are evolutionarily shaped to ensure success in the social behavior of each species.[6,7] They employ a rich repertoire of pheromones to ensure intraspecific communication in many behavioral contexts, and their social organization is determined by chemical signals produced by both the queen and the workers.[16,17]

The Italian bee (*Apis mellifera ligustica*, *Aml*) and the Chinese indigenous honeybee (*Apis cerana cerana*, *Acc*) have evolved in distinct ecologies, and their social organization as well as mating behavior have been successfully shaped by their respective ecosystems. However, because of the high production rate of honey and royal jelly, the *Aml* is widely distributed in the world and most extensively used in the beekeeping industry of China since the 1920s.[18] On the other hand, the indigenous *Acc*, with a smaller body size than *Aml*, has a long apicultural history and survives with strong merits in resisting bee mites, wasps, diseases, extreme climates (cold/hot weather), and adverse conditions even with low productivities.[19,20] The two bee species have evolved significant differences in behavior and biology in regards to their distinct species' successes that resulted in variability in productivity and product quality. For instance, royal jelly and the abundance of major royal jelly proteins are produced in significantly higher quantity by the *Aml* than by the *Acc*.[21] These two bee species also differ in several mandibular gland characteristics, despite being close phylogenetically.[22−24] In *Apis mellifera*, the queen's mandibular pheromone consists of 9-hydroxy- and 9-keto-2(*E*)-decenoic acids (9-HDA and ODA), methyl *p*-hydroxybenzoate (HOB), and 4-hydroxy-3-methoxyphenyl-ethanol (HVA) and is responsible for retinue attraction, among other functions. In retinue bioassays with workers of *Apis cerana* (whose queens lack HVA), ODA, 9-HDA, and HOB are sufficient to elicit maximal retinue behavior.[25]

Overall, the diversity and complexity of social communication in honeybees are supported by various specific proteins involved in odorant detections.[26] It is known that 12 of the 21 odorant binding proteins (OBPs) predicted in the genome of the *Aml* and two of the six chemosensory proteins (CSPs) are present in the foragers' antennae,[27] but only one CSP and the *Apis cerana*-antennae-specific proteins (Ac-ASP3) are analyzed in the *Acc* antennae.[28,29] Also, honeybee antennal specific proteins,[30,31] pheromone binding proteins (PBPs), and general odorant binding proteins in both drone and worker antennae of the *Aml*[30−32] have been characterized. Recently, our group has reported a caste-biased protein expression in the antennae of the *Aml* drone, worker, and queen bees that is used for their corresponding biological mission attainment.[33,34] On the other hand, despite significant differences in behavior and biology between the *Aml* and the *Acc* bees, no contrasting information is available on how their olfactory mechanisms are shaped in regards to the success of their own specific species. Therefore, the main objective of this work was to compare the antennal morphology and proteome between respective sexually mature drones and foraging workers of the two bee species on the basis of their differences and move toward better understanding of how the olfactory functions between the western and the eastern honeybee.

2. MATERIALS AND METHODS

2.1. Chemical Reagents

All chemicals used for 2-DE were purchased from Sigma (St. Louis, MO, USA) except for Biolyte and immobilized pH gradient (IPG) strips that were from Bio-Rad (Hercules, CA, USA), α-cyano-4-hydroxycinnamic acid (CHCA) from Bruker Daltonics (Billerica, MA. USA), and trypsin from Roche (Modified, Sequencing grade, Roche, Mannheim, Germany). The entire chemical array used for RNA isolation and qRT-PCR was from Bio-Rad (Hercules, CA, USA). Chemicals used but not specified here are noted with their sources in the text. The reagents used for the analysis were analytical grade or better.

2.2. Biological Samples

To ensure the exact age of the sexually mature honeybee drones and forager workers to be sampled from the western (*Apis mellifera ligustica*) and the eastern (*Apis cerana cerana*) species, the queen bees with five replicates of bee colonies from each species were confined to a single wax comb frame containing the drone or the worker cells for 18 h with a cage made of a queen excluder, through which the workers but not the queen could pass. Then, the queens were removed and the worker or the drone eggs contained in the frames were maintained in the honeybee colony for further development. Newly emerged (<12 h, $N > 2000$) drone bees from five colonies of each species were marked on their thoraxes and placed back into colonies. Then, 1000 marked drone bees of each species were collected on day 15 (the age at which the drone is sexually mature),[35] and their antennae were dissected. Similarly, 1000 foraging workers of the correct age (with pollen load) were collected on day 20[35] at the entrance of the hive at 8:00−10:00 a.m. from five colonies of each species. The antennae collected from the drones and the workers of the two bee species were randomly assigned to each of the three replicates with equal numbers of antennae. Then, the antennae were washed with phosphate buffered saline (PBS) to remove the contaminants on the surface and stored at −80 °C until later use.

2.3. Morphometric Measurements of Antennae

The antennae from both species of the drones and workers were randomly selected, and ultrastructure photographs were carried out using a scanning electron microscope (SEM) as was recorded in our previous report.[33,34] The counting of sensilla on the image was done manually segment by segment.

2.4. Protein Extraction and Two-Dimensional Gel Electrophoresis (2-DE)

The antennae were homogenized with liquid nitrogen and then mixed with PBS (1 mg/10 μL). The following protein extraction was done according to our previously published protocol,[33,34] and the protein concentration was determined according to Bradford.

A 550 μg sample of protein was suspended in 84 μL of LB and mixed with 336 μL of rehydration buffer (8 M urea, 2% CHAPS, 0.001% bromophenol blue, 45 mM DTT, 0.2% Biolyte, pH 3−10). The mixture was loaded onto a 17 cm IPG strip (immobilized pH gradient, pH 3−10, linear, Bio-Rad). Isoelectric focusing (IEF) was performed (Protean IEF Cell, Bio-Rad) at 18 °C according to the following program: 14 h at 50 V; 250 V for 30 min × 4 times; 1000 V for 60 min; 9000 V for 5 h; 9000 V, for 60,000 Vh. The IPG strips were first equilibrated for 15 min in equilibration buffer 1 (6 M urea,

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0.375 M Tris-HCl, pH 8.8, 20% glycerol, 2% SDS, 2% DTT) and later in equilibration buffer 2 (6 M urea, 0.375 M Tris-HCl, pH 8.8, 20% glycerol, 2% SDS, 2.5% iodoacetoamide) for 15 min. The second dimension electrophoresis, SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis, 12% T separating gel, 1.00 mm) was performed in a Protean II Xi Cell (Bio-Rad) at 25 mA/gel for about 6 h.

2.5. Image Acquisition and Statistics Analysis

Gels were fixed overnight in 50% (v/v) ethanol with 10% (v/v) acetic acid, washed in water, and stained with Flamingo fluorescent dye (Bio-Rad) for image analysis and then further stained with CBB G-250 to visualize spots for mass spectrometry (MS) analysis. Three independent 2-DE gel images from triplicate samples of respective honeybee drone and worker bees from both species were digitized with Image Scanner III (GE Healthcare) at 16 bit and 300 dpi resolution. Image filtration, background subtraction, spot detection, spot matching, and quantitative intensity (all of the pixels making up the spot) analysis were performed using PDQuest software (version 8.0.1, Bio-Rad). All gels were matched with one of the selected reference gels. The match analysis was performed in an automatic mode, and further manual editing was performed to correct the mismatched and unmatched spots. The expression level of a given protein spot was expressed in terms of the volume of the spot. To compare spot quantities between gels accurately, the spot volumes were normalized as a percentage of the total volume of all of the spots in the gel. The means and standard deviations from the triplicate experiments were calculated, and the statistical significance of the expression levels of the proteins between the antennae of respective drones and workers of both species was performed by one-way ANOVA (SPSS version 16.0, SPSS Inc.) using a Duncan's multiple-range test. An error probability of $p < 0.05$ (corrected for multiple testing) was considered to be statistically significant of at least 1.5-fold changes.

2.6. Trypsin Digestion and Protein Identification by MS

The CBB stained spots were manually excised from each gel of drone and worker samples and destained for 30 min using 100 mL of acetonitrile (50%) and 25 mM NH_4HCO_3 pH 8 (50%) three to four times until gels were transparent. The protein digestion and peptide extraction were carried out according to our established methods in the previous work.[33,34]

The matrix was prepared by dissolving CHCA in 50% acetonitrile/0.1% trifluoroacetic acid. The solution (10 μL) was added on to the dried digests and vortexed for 30 min. A total of 1.5 μL of the reconstituted in-gel digest sample was spotted initially on an Anchorchip target plate (600/384F, Bruker Daltonics), followed by adding 1 μL of matrix solution. The dried sample on the target was washed twice with 1 μL of 0.1 TFA and left for 30s before solvent removal. Mass spectra were acquired on an Ultra flex MALDI TOF-TOF/MS (matrix assisted laser desorption ionization time-of-flight/MS) (Bruker Daltonics) in reflectron, positive mode in the mass range of 700−4000 Da. About 300 laser shots were averaged for each mass spectrum manually. Spectra were calibrated by a protonated mass signal from a standard peptide calibration mixture consisting of eight peptides covering mass range of 700−3100 Da (Bruker Daltonics) for MALDI TOF-TOF/MS.

Before MS/MS data searching, peak lists of peptide mass fingerprinting (PMF) were generated with MASCOT distiller (version 2.3.2.0, Matrix Science) using default parameter settings. All known contaminants (trypsin autoproteolysis and known keratin peaks) were excluded during the process. The data were stored in a combined mgf file and transferred through MASCOT daemon (Version 2.3.0, Matrix Science) as inputs to search against the non-redundant database of the national center for biotechnology information (NCBInr, release date, January 22, 2010) using MASCOT 2.3 (Matrix Science). Search parameters were as follows: taxonomy, all entities; trypsin cleavage, allow up to one missed cleavage; peptide mass tolerance, 0.2 Da; fixed modification, carbamidomethyl (C); variable modification, oxidation (M). A total of 10,348,164 sequences and 3,529,470,745 residues in the database were actually searched.

When the identified peptides matched with multiple members of a protein family or when a protein appeared under the same name and accession number, the match was considered in terms of a higher Mascot score, the putative function and differential patterns of protein spots on 2-DE gels. Protein identification was accepted when it contained at least two identified peptides having both minimal cutoff Mascot score of 83 and probability of 95%.

2.7. Bioinformatics Analysis

The identified proteins were searched against the Uniprot database and combined Flybase search results and grouped on the basis of their biological process of Gene Ontology terms. To understand protein interaction networks in the honeybee olfactory context, the biological interaction networks (BIN) of the identified proteins were predicted using Pathway Studio (Ariadne Genomics). Briefly, the protein lists were run against the *Drosophila* database that was equipped with functional relationships from other scientific literature. The filters that we applied included "all shortest paths between selected entities". The information received was narrowed to our proteins of interest. Each link was built on the basis of evidence from at least three publications. Protein entities which belong to different functional groups were represented by different shapes according to the default settings of the software as shown in Figure 6.

2.8. Test of Protein Expression by Quantitative Real-Time PCR

Total RNA was extracted from respective drones ($n = 50$) and worker ($n = 50$) antennae of the *Aml* and *Acc* bees using TRIzol reagent (Bio-Rad). Reverse transcription was performed using an RNA PCR Kit (Bio-Rad), according to the manufacturer's instructions. The analysis of qRT-PCR was conducted on 12 differentially expressed proteins selected from seven major functional groups (carbohydrate metabolism and energy production, transporter, cytoskeleton, fatty acid metabolism, antioxidation, development, and protein biosynthesis) on the basis of their connectivity in the network. Actin was used as the reference (Table S1 of the Supporting Information). Real-time PCR was performed using an iQ5Multicolor Real-Time PCR Detection System (Bio-Rad) in a 25 μL reaction system containing 1 μL of cDNA, 5 pmol of forward and reverse primers, 12.5 μL of SYBR Green Supermix (Bio-Rad), and water. Fold-change was calculated using the $2^{-\Delta\Delta Ct}$ method. Each sample was analyzed independently and processed in triplicate. The values from the three independently obtained results were compared by one-way ANOVA (SPSS version 16.0, SPSS Inc.) using a Duncan's multiple-range test. An error probability $p < 0.05$ (corrected for multiple testing) was considered statistically significant.

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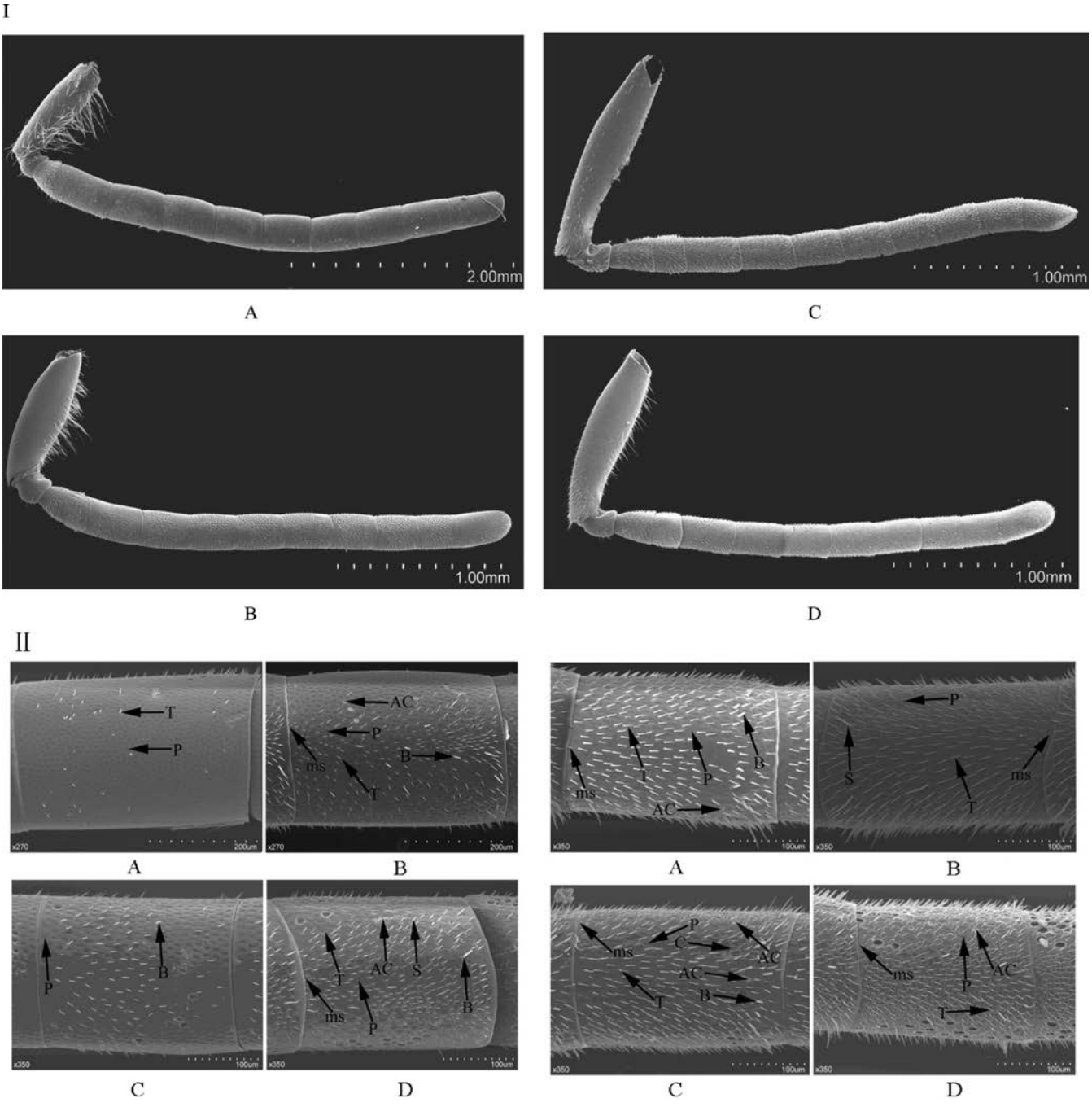

Figure 1. Comparison of antennal images of drones and workers from western (*Apis mellifera ligustica*) and eastern (*Apis cerana cerana*) honeybees. Panel I is morphological contrast of the drones antennal image between the two bee species, where A and B (western) and C and D (eastern) stand for ventral and dorsal sides, respectively. Panel II is an enlarged segment of drone (left) and worker (right) where A and B independently represent the dorsal and ventral sides of an Italian honeybee drone flagellum, while C and D individually represent the dorsal and the ventral sides of a Chinese honeybee worker flagellum. The letters in the image indicate different kinds of sensilla where AC denotes ampullaceal or coeloconic sensilla, B is basiconic sensilla, C is coelocapitular sensilla, ms is margin sensilla, P is poreplate sensilla, S is seta sensilla, and T is trichoid.

3. RESULTS

3.1. Morphology Comparison

The antennal structure showed no differences either between the drones (11 flagella) or between the workers (10 flagella) corresponding to the two bee species (Figure 1I). Even though the antennal structures of both species of bees have no differences, we observed significant variations between the drone antennae of the two bee species in terms of hair-like and poreplate sensilla number on both the ventral and dorsal sides on the basis of the SEM photograph aided assay count. The numbers of hair-like sensilla on each segment were significantly higher on the *Acc* drone ($p < 0.05$) than on the *Aml*, whereas the numbers of poreplate sensilla on each segment were significantly higher ($p < 0.05$) on the antennae of the *Aml* drone than on the *Acc* (Figure 1II, Figure 2A,B, and Supplementary Table S2). Nevertheless, both numbers of hair-like and poreplate sensilla had no significant differences ($p > 0.05$) between the workers of the two bee species (Figure 1II, Figure 2C,D, and Supplementary Table S2).

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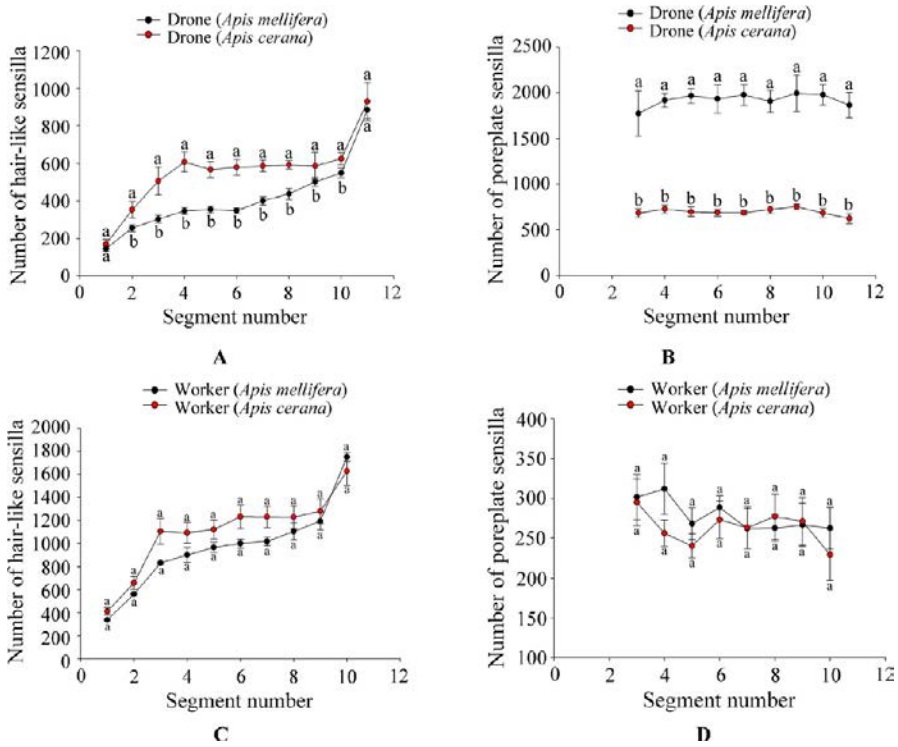

Figure 2. Comparison of the sensillum number between western (*Apis mellifera ligustica*) and eastern (*Apis cerana cerana*) honeybees. A and B and and C and D represent the number of hair-like sensilla and poreplate sensilla on the drone and the worker antennae of western and eastern honeybees, respectively, where a is significantly higher than b ($p < 0.05$) in the drones (*Aml* vs *Acc*), but there is no significant difference between the workers from both species ($p > 0.05$).

3.2. Comparison of Antennal Proteome Profiles

The 2-DE images detected about 400 protein spots on the antennae of respective drones and workers of the two species. Quantitative analysis showed that there were ~150 and ~130 protein spots altered in their expression (>1.5-fold change, $p < 0.05$) in the antennae of both species of the drones and the workers, respectively. Subsequent MS analysis identified 106 and 100 protein spots in the drones and the workers of both bee species, respectively (Supplementary Tables S3 and S4, Figure 3). The remaining unidentified differential protein spots on 2-DE images could be attributed to either their low abundance to produce enough spectra or that the search scores in the databases did not produce unambiguous results. Since the genome of *Apis cerana* has not been sequenced yet, this is likely to cause an imbalance in the number of proteins identified from *Acc* versus *Aml* samples.

3.3. Qualitative and Quantitative Comparisons of Differentially Expressed Proteins

To estimate biological significances, the quantitative expression levels of each protein from the antennae of the drones and the workers of the two bee species were analyzed using the ANOVA log ratio ($|\log_{1.5}$ ratio$| \geq 1$). The *p*-values for the differentially expressed proteins were calculated as the ratio of the protein abundance (drone/drone and worker/worker) and protein spots with $p \leq 0.05$ were considered (Figure 4). Among the total 106 identified proteins in the drone antennae of both bee species, 92 proteins (86.8%) were in the antennae of the *Aml* drone, of which 72 (78.3%) of them were with high abundance including 24 uniquely expressed proteins. In the antennae of the *Acc* drone, 82 (77.4%) proteins were identified comprised of only 34 (41.5%) with higher abundance, of which 14 proteins were exclusively expressed in this bee species. Subsequent analysis showed that, of the 72 proteins abundantly expressed in the antennae of the *Aml* drone, 27 proteins were with >10-fold changes, whereas there were 15 proteins with a level of expression >10-fold in the antennae of *Acc* drone (Figure 5A). The 106 differentially expressed proteins in the drone antennae between the two bee species were grouped into nine functional categories and one unknown group (Supplementary Table S3, Figure 4A). It was observed that the largest proportion of those proteins with higher abundance in the antennae of *Aml* drone were associated with carbohydrate metabolism and energy production, molecular transporters, antioxidation, and fatty acid metabolism compared to those abundantly expressed proteins in the *Acc* drone bees (Figures 5A and 6A). Likewise, of the 100 proteins that altered their expression in the worker antennae of the two bee species, 85 proteins (85%) were identified in the antennae of *Aml* workers, of which 67 (78.8%) showed high abundance and 24 proteins were unique to this bee species. In the *Acc* worker antennae, 74 proteins (74%) were identified, 33 (44.6%) of them were with higher abundance, and there were 15 unique proteins to this species of bees. These 100 proteins with differential abundances in the worker antennae between the two bee species were grouped into nine functional categories and one unknown group (Supplementary Table S4, Figure 4B). Among the proteins with higher abundance in the antennae of both species of worker bees, those involved in the carbohydrate metabolism, antioxidation activities, and molecular transporter functions represented the largest proportions in the *Aml* worker compared to the *Acc* worker bees (Figures 4B and 5B).

3.4. Biological Network Analysis

Most proteins exist as integral parts of protein complexes rather than a single entity in a living cell. The biological relationship of

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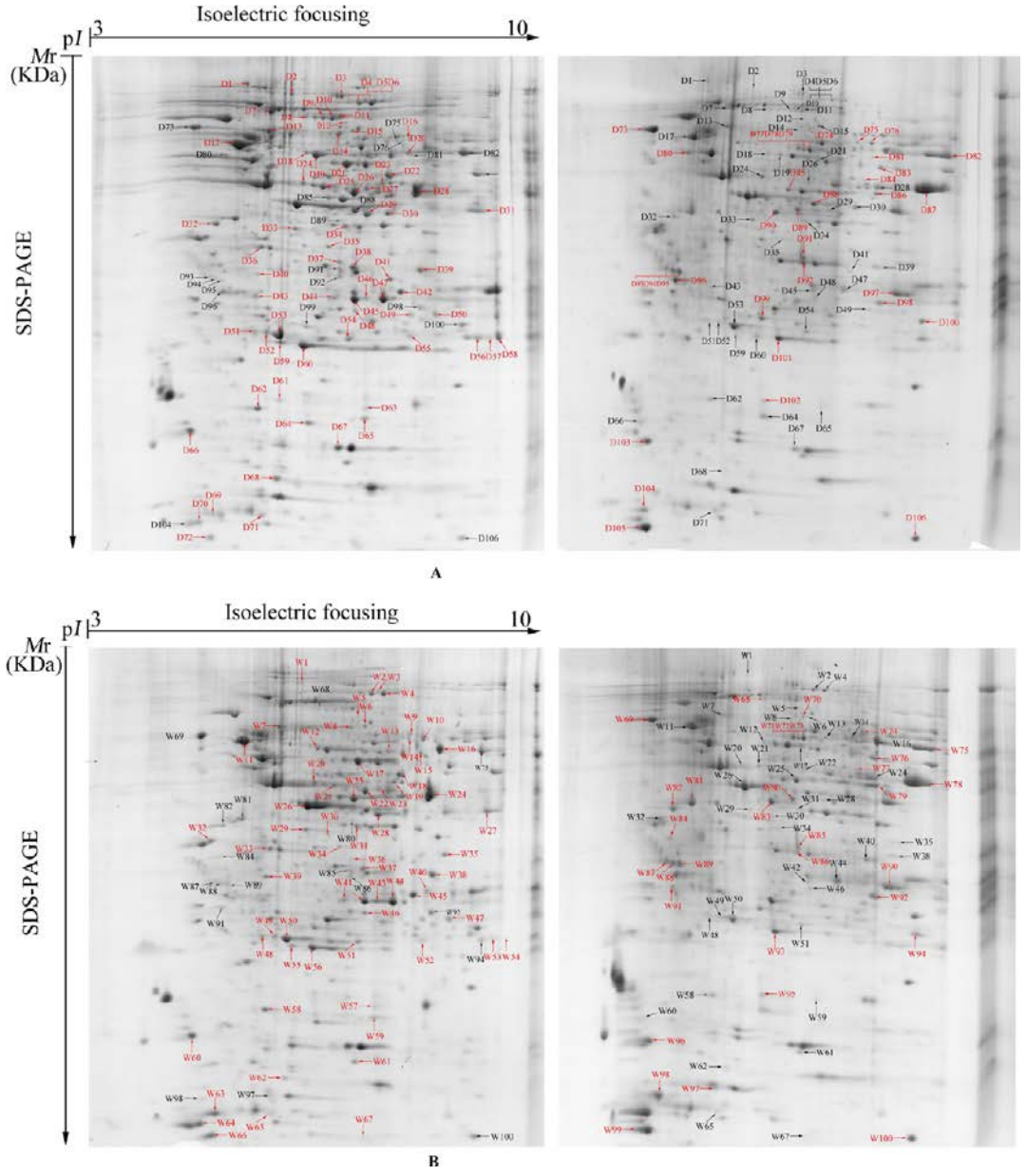

Figure 3. 2-DE antennal profiles of honeybee drones (A) and workers (B) of western (*Apis mellifera ligustica*) and eastern (*Apis cerana cerana*) honeybees. A 550 μg sample of each was subjected to 2-DE and stained by CBB G-250. The red and the blue labels indicate up and down regulated proteins.

the proteins involved in the olfactory functions of the drones and the workers of the *Aml* and the *Acc* was predicted by the constructed BINs (Figure 6). Of the 106 proteins that altered their expression in the drone antennae of both bee species, 44 proteins were linked to the BIN through the shortest paths. Among these, 27 proteins were with high abundance in the drone antennae of the *Aml* bee, while the remaining 17 proteins had high abundance in the antennae of the *Acc* drone. Nine of 11 proteins (the major represented in the BIN) related to carbohydrate metabolism and energy production pathways were found in the *Aml* drone (Vha26, idh, Aldh, pglym78, Scs, men, Argk, l(1) G0230, and CG9360) and the remaining two proteins (l(1) G0156 and ARY) were in the *Acc* drone (Figure 6A). Likewise, four of nine proteins in molecular transporters (Vib, obp56a (obp5513), obp57c (obp16) and CG31974) showed high abundances in the *Aml* drone, while five others (Porin, Cg1540, CG10513, Chea87a, and Ran) were highly expressed in the antennae of the *Acc* drone. Four of six proteins involved in protein folding (Cg11267, HIP-R, crc, and l(2)efl) had higher expression in the antennae of the *Acc* drone, while two proteins (Gp93 and HSP83) were abundantly expressed in the *Aml* drone. Three of four proteins related to the cytoskeleton (Tsr, TM1, and beta tub56d) were highly expressed in the antennae of the *Aml* drone, whereas only one protein (Mlc2) showed high level of expression in the *Acc* drone. All of the four proteins in the antioxidation group (GstD1, GstS1, Sod, and Phgpx) in this BIN were abundantly expressed in the antennae of the *Aml* drone. Also, of the proteins related to metabolism of amino acids and nucleotides, one (HtrA2) and two (Gdh and Gs1) were abundantly expressed in the *Aml* and *Acc* drones, respectively. Of the three proteins related to development, one was enhanced in its

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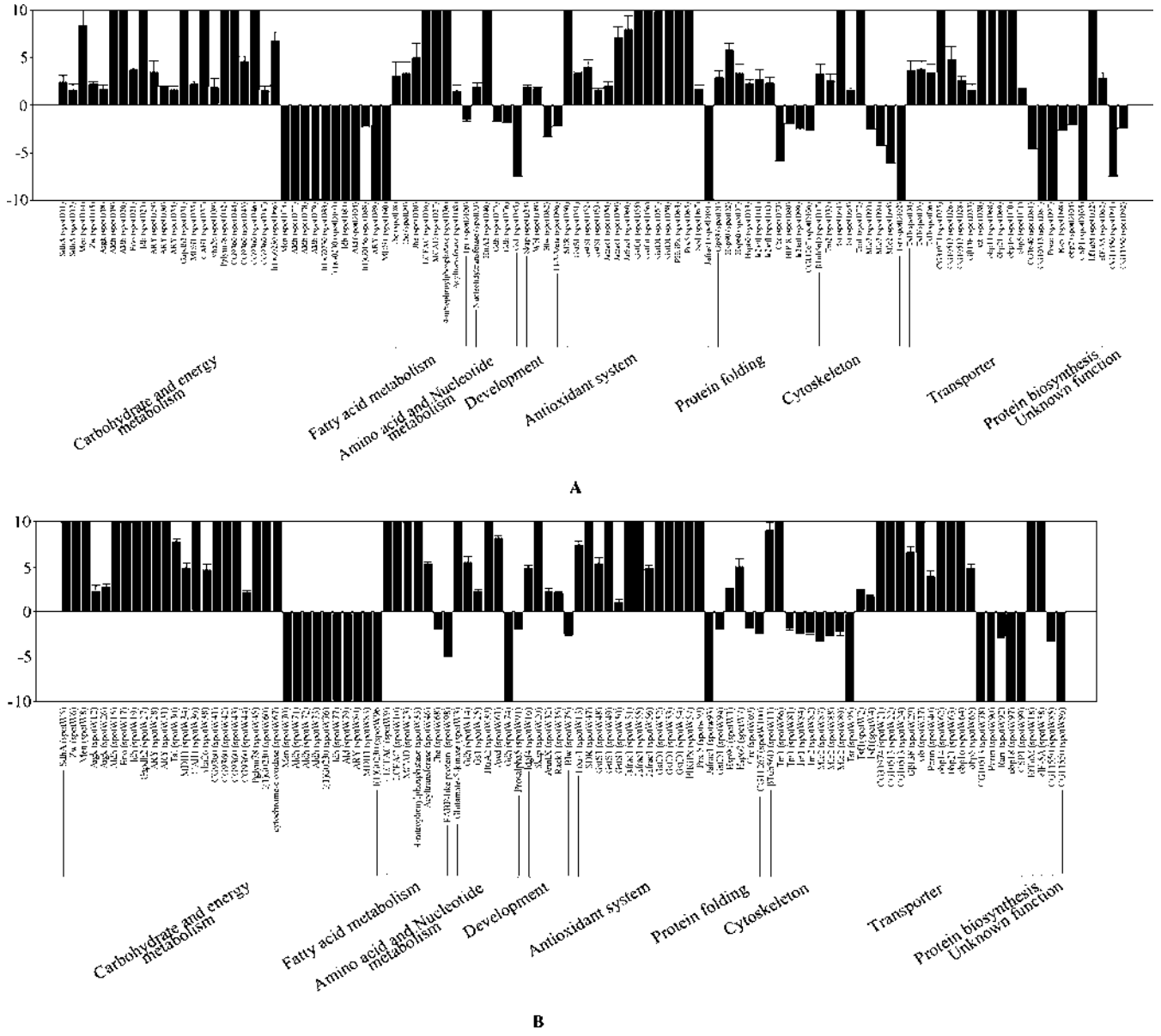

Figure 4. Quantitative comparison of differentially expressed proteins in the antennae of drones (A) and workers (B) between western (*Apis mellifera ligustica*) and eastern (*Apis cerana cerana*). Positive values indicate western honeybee expression over eastern honeybees, and negative values are eastern honeybee expression over western honeybees. The ratios of protein abundance between the two species of bees were transformed, and the protein spots with $|\log_{1.5}$ ratio$| \geq 1$ and p values ≤ 0.05 were selected as the differentially expressed proteins. Protein names (in abbreviations) and protein numbers are listed. The $|\log_{1.5}$ ratio$|$ of the uniquely expressed proteins is limited to 10.

expression in the *Aml* drone; two were in the antennae of the *Acc* drone. Among the two proteins related to fatty acid metabolism, one (MCAD) was highly expressed in the *Aml* and another one (tpi) in the *Acc* drone. However, two proteins in protein biosynthesis (Eif-5a and Eftum) were both abundantly expressed in the *Aml* drone (Figure 6A).

Similarly, from 100 proteins differentially regulated in the antennae of both species of the worker bees, 44 proteins were networked to the BIN. The major proportions of these proteins (25 proteins) were in the antennae of the *Aml* worker and the remaining 19 proteins in the antennae of the *Acc* worker. Among these 44 proteins, 14 proteins (31.8%) were related to carbohydrate metabolism and energy production pathway, of which 10 proteins (idh, ARY, Gapdh2, Vh26, HtrA2, CG9360, Pglym78, Scs-fm, zw, and CoxuB) were abundantly expressed in the antennae of the *Aml* worker and four others (Aldh, MDH1, l(1) g0230 and Ald) in the antennae of the *Acc* worker. Likewise, molecular transporter proteins were the second most abundant (20.5% or 9 proteins) in the BIN, as three of them, Cg 31974, Gbeta13f, and obp99a (obp14) were expressed with high abundance in the *Aml*, and the other six (CG 10513, Porin, obp57c (obp16), obp56a(obp5), Ran and chea87a-(Cp1)) were highly expressed in the antennae of the *Acc* worker. Also, among the five proteins involved in the metabolism of amino acids and nucleotides linked to the BIN, three (CG7470, GS1, and Awd) and two (Gdh and prosma5) were highly expressed in the antennae of the *Aml* and *Acc* worker, respectively. From the four cytoskeleton proteins in the BIN, two (betatub56d and Tm1) were highly expressed in the *Aml* and the other two (Tm2 and Tsr) in the *Acc* worker. Three of four proteins in the antioxidant group (Trxr1, GstS1 and GstD1) were highly expressed in the antennae of the *Aml*

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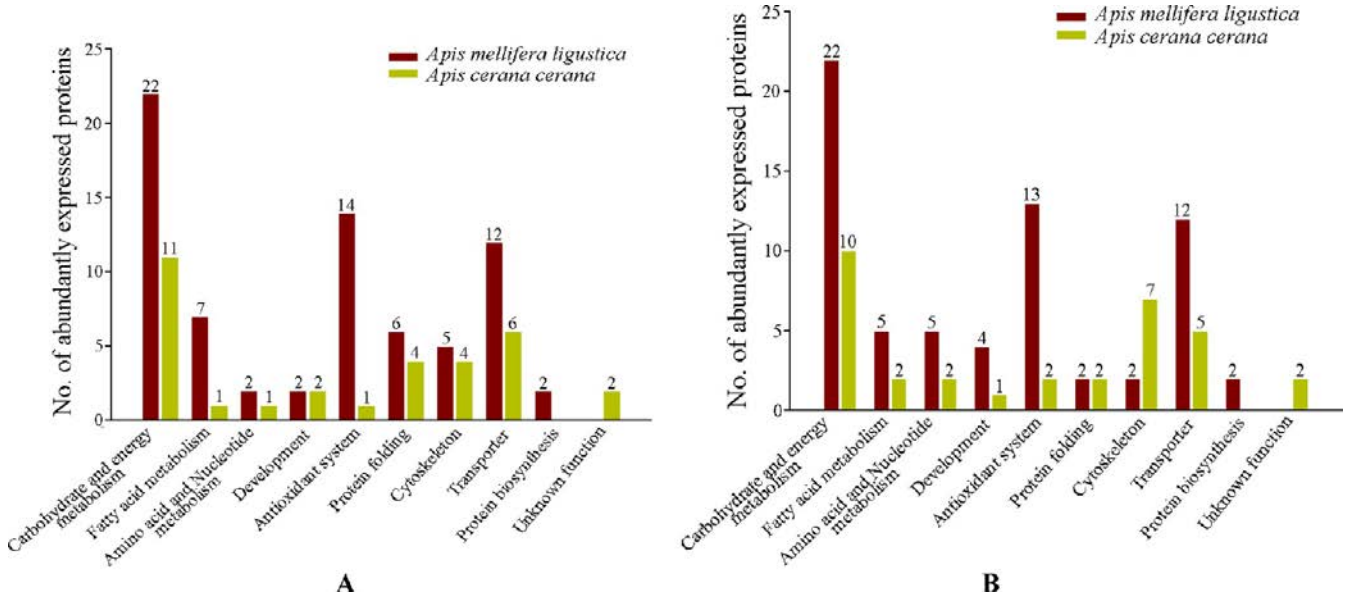

Figure 5. Comparisons of the upregulated number of proteins in the antennae of drones (A) and workers (B) of western (*Apis mellifera ligustica*) and eastern *Apis cerana cerana* honeybees.

worker and only one (Phgpx) in the *Acc* worker's antennae. From the three proteins related to development, two (idgf4 and Rak1) and one (blw) were highly expressed in the *Aml* and the *Acc* workers' antennae, respectively. Among the two proteins related to fatty acid metabolism, one (MCAD) was abundantly expressed in *Aml* and one (Rfabp) in the *Acc* worker. However, two proteins in the protein folding group (crc and CG11267) and one protein involved in protein biosynthesis (Emftu) were all found with high abundance in the *Acc* worker (Figure 6B).

3.5. Test of Differentially Expressed Proteins by qRT-PCR

Twelve proteins from seven major functional groups (carbohydrate metabolism and energy production, antioxidation, molecular transporter, cytoskeleton, fatty acid metabolism, protein biosynthesis, and development) were selected and tested using qRT-PCR. The result of mRNA expression showed that 11 genes, Argk (D18), Pglym78 (D42), vha26 (D39) scs-fp (D11), MACD (D 27), Blw (D82), GstS1 (D51−53), Obp14 (D68), Obp21 (D69), Obp16 (D70), and EftuM (D22), from the drone antennae were consistent with the protein expression analyzed by 2-DE gel images. However, one gene, βTub56D (D17), showed mRNA-protein expression inconsistency. Likewise, 10 genes, Argk (W12), Pglym78 (W45), vha26 (W38) scs-fp (W5), MACD (W 23), GstS1 (W48−50), Obp14 (W62), Obp21 (W63), Obp16 (W64), and EftuM (W18), were consistent with protein expression, but two genes, Blw (W75) and βTub56D (W11), were not consistent with protein expression in the antennae of worker bees (Figure 7).

4. DISCUSSION

In this study, the antennal morphology and the proteome of sexually mature drones and foraging workers between the *Aml* and the *Acc* bees were compared to understand their mechanisms of olfactory function. The 3-fold higher number of poreplate sensilla on the drone antennae of the *Aml* (western bees) than its eastern counterparts (*Acc*) might have attributed to an increase of flagellar surface and poreplate density. This is supported by the higher sensilla placodea (olfactory discs, 7.36 times) in the *Aml* drone that covers nearly all of the male antennomeres, thereby suggested to enhance the detection of molecules from the environment and boost olfactory functions.[23,36] The higher number of poreplate sensilla in the antennae of *Aml* also matches with an increase in sensory neurons sensitive to (*E*)-9-oxodec-2-enoic acid (9-ODA) of the drone.[37] Although detailed physiological investigations are still lacking, the morphology of the antennae and the organization of antennal lobes both suggest that the *Aml* drones have a more highly alert sex pheromone processing olfactory subsystem with a large number of pheromone-sensitive receptor neurons converging onto its four macroglomeruli.[13] The observed fewer number of olfactory poreplate sensilla in the *Acc* drones may be a reason for a lower number of sensory neurons[38] since morphological features of the antennae are thought to have a strong impact on olfactory sensitivity.[39] On the contrary, the absence of significant differences in the number of hair-like and poreplates sensilla between the antennae of the *Aml* and *Acc* foraging workers may not imply that they have an equal olfactory sensitivity. Their difference could be because of variations in body size modifications, specific social organization, and evolutionary adaptations to different ecosystems.

In addition to the antennal morphology, the proteome of the two honeybee castes (drone vs drone and worker vs worker) of the *Aml* and the *Acc* were compared to gain deep insight into their olfactory functions at the protein level. Obviously, significant differences in olfactory mechanism between each respective drone and worker of the *Aml* and the *Acc* are most likely derived from their respective protein expression levels. The abundant expression of more proteins in the antennae of the *Aml* drone compared to the *Acc* drone was anticipated due to the large number of respective poreplate sensilla. These are the main parts of the olfactory system in their antennae that perceive and process a large number of sex pheromones (detecting and discriminating various specific olfactory messages).[37] This gives us sound clues that the *Aml* drone bee presumably requires more antennal proteins to enhance its performance compared to the *Acc* drone to increase success in the tough competition for mating with the queen[40−42] in the

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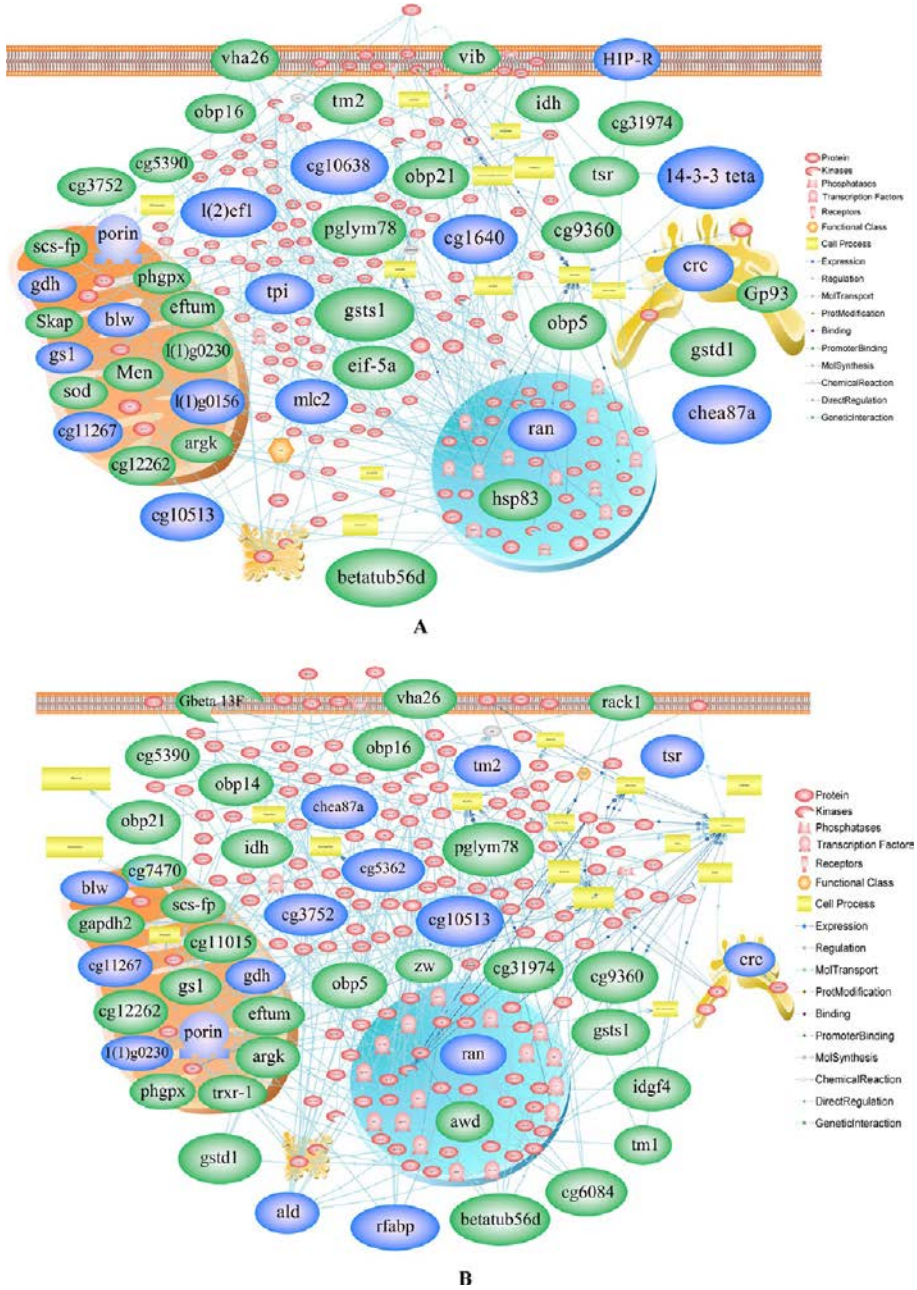

Figure 6. Network analysis of the differentially expressed proteins in the antennae of drones (A) and workers (B) of western (*Apis mellifera ligustica*) and eastern (*Apis cerana cerana*) using Pathway Studio software. Those highlighted in green and blue in A represent the key node proteins upregulated in the drone antennae of western and eastern bees, whereas those highlighted in green and blue in B represent the key node proteins upregulated in the worker antennae of western and eastern bees, respectively. Protein entities that belong to distinct functional groups were represented in different shapes according to the default settings of the software as described in the legend.

open field at the height of 5−40 m.[43] Different species and even subspecies have different drone congregation areas; the *Acc* drones make their congregation for the mating flight usually at limited areas near the top of big trees,[44,45] and thus may not involve strong competition or need much energy as in the case of the *Aml* drones. Likewise, the higher number of the proteins with high level of abundance in the antennae of the *Aml* worker as compared to that of the *Acc* worker is probably related to their higher performance in honey production that requires more efficient foraging activities supported by well equipped olfactory systems.[46,47]

The proteins related to energy metabolism are required as a key metabolic fuel by the worker bees for foraging flights[41,42,48] and as nutrients for neurons involved in learning and memorization processes.[49,50] The *Aml* worker bee abundantly expressed several key proteins of carbohydrate and energy metabolism such as enolase (spot W17), an energy-yielding metalloenzyme in glycolysis,[51] present in the muscles of the forager bees and which acts in carbohydrate metabolism,[52−55] and glyceraldehyde-3-phosphate dehydrogenase 2 (GAPDH II,spot W27), which was uniquely expressed here, catalyzes the important step of glycolysis and thus serves to break down glucose for energy and carbon molecules.[56] Arginine kinase (argk, spots W12 and W26), which was highly expressed in the *Aml* worker, plays a great role in maintaining high levels of ATP at the sites of flight muscle contraction.[57] Also, its high

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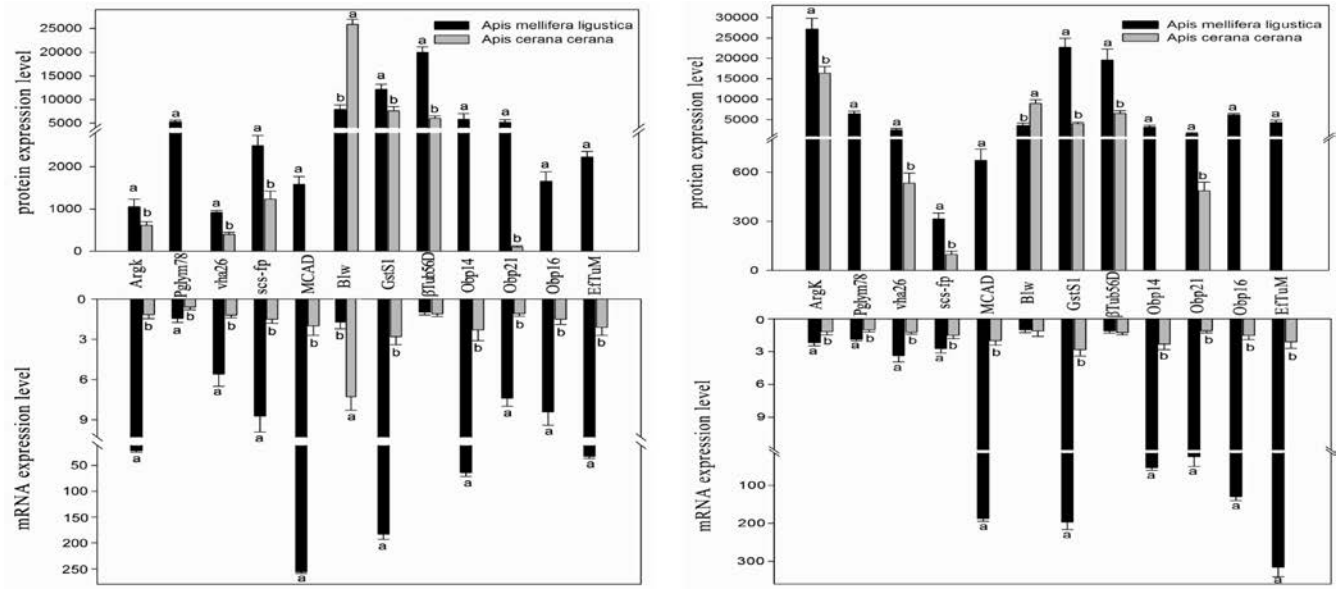

Figure 7. Validation of 12 differentially expressed proteins at mRNA level between the antennae of western (*Apis mellifera ligustica*) (dark colored) and eastern (*Apis cerana cerana*) (light colored) drones and workers honeybees. The values of protein and mRNA abundance are the average of three replicats. The lower case letters (a and b) indicate significant differences between the antennae of *Aml* and *Acc* drones (left panel) and between the antennae of *Aml* and *Acc* worker honeybees (right panel) ($p < 0.05$), where "a" is significantly higher than "b".

abundance can be associated with greater locomotory performance, which is in demand due to the need for rapidly released energy and for maintenance of adequate ATP/ADP ratios during foraging flights.[58,59] The exclusive expression of carbonic anhydrase (CAH1, spot W36) in the antennae of the *Aml* worker bee is probably associated with the more cognitively demanding tasks performed by foragers[60,61] because it plays important roles in synaptic plasticity and cognition as in spatial learning and memory.[61,62] Yet, the unique expression of aldehyde dehydrogenase (aldh, spots W71−73) in the *Acc* worker antennae indicates promotion and formation of reduced coenzymes from their oxidated forms (NAD+ or NADP+), which is similar to its function in the hypopharyngeal gland in the worker larvae.[63]

The proteins with metabolism enhancing activities generally play significant roles in the process of bees' chemical communications with each other and with the queen for their normal social organization and competition during mating flights.[17,41,64,65] In view of that, abundant expression of major key node proteins in the antennae of the *Aml* drone, particularly aldh (spots D19 and D20), with unique expression and enzymes catalyzing the oxidation of aldehydes to carboxylic acids, suggests its involvement in the synthesis or degradation of pheromone compounds.[17,66,67] The metabolism of aldehyde dehydrogenase is selective in the queen mandible,[68,69] and thus its higher expression in the *Aml* drone antennae is most likely to equip its antennae with particular olfactory systems required for an enhanced response to queen pheromone signals compared with its eastern counterparts. Additionally, aldh serves as a biotransformation enzyme for pheromones and possibly xenobiotics in a manner similar to that of the antenna-specific GST of *Manduca sexta*.[70−72] One of the major proteins, malic enzyme (men, spot D74), which was uniquely expressed in the *Acc* drone antennae, is thought to be very important for the accumulation of storage lipids in the drone antennae for the generation of nicotinamide adenine dinucleotide phosphate (NADP) for lipid biosynthesis.[73] The higher abundance of specific proteins such as argk (spot D18) and exclusive expression of phosphateglyceromutase (pglym78, spot D42) in the *Aml* drones indicate their explicit function toward enhancing the metabolic energy requirements that assist in attaining its distinctive physiological and behavioral characters.[74] The argk is suggested to play a key role for quick genearation of energy during the mating flight[57] because it maintains high levels of ATP at the site of muscle contraction in insect flight muscles.[58,59]

Most proteins related to the molecular transporters are the major contributors to the activation of odorant responsive chemo-sensory neurons and perform as selective filters in odor recognition.[75] In this study, large numbers of molecular transporters were abundantly expressed in the *Aml* worker antennae compared to the *Acc* worker. Above all, the unique expression (>10-fold) of odorant responsive chemo-sensory neuron activator proteins,[75] obp14 (spot W62), obp21 (spot W63), and obp16 (spot W64), in the antennae of the *Aml* worker bees and porin (spot W90), obp18 (spot W97) and CSP1 (spot W99) in the *Acc* workers, indicate differences in their degree of importance in the olfactory system of each species that evolved in different ecosystems.[77] Such diversity between the two species of worker bees is supported by variations in specific proteins involved in odorant detection[78] and shows that they are key role players in enhancing the foraging activities of the *Aml* workers than their eastern counterparts, predominantly detection of various flowering plant odors.[79,80]

On the other hand, the unique expression (>10-fold) of OBPs, particularly, >10-fold of obp14 (spot D68), obp21 (spot D69), and obp16 (spot D70) in the antennae of the *Aml* drone compared to the *Acc* drone could be attributed to boosting its response to the queen pheromone in mating flights[16,81] with species-specific/sex-specific roles.[26] This agrees with different OBPs found within a single or between insect species that display distinct odorant-binding specificities.[82] On the other hand, the unique expression (>10-fold) of porin (spot D97)

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and CSP1 (spot D105) in the antennae of the *Acc* drone most likely indicates their particular importance in the olfactory functions of this species of drone bee. Most of these proteins identified in the antennae of both species of drones are thought to be carriers for odorants and pheromones across the sensillar lymph in the sensory organs.[30,83] They are also involved in the detection of semiochemicals,[26] increasing odorant solubility and protection of odorants from enzymes in the sensillum lymph prior to activating olfactory neurons.[81]

Antioxidant metabolites and enzymes work together to prevent cellular components from oxidative damages.[67,84,85] This protein family has been well documented in the honeybee embryogenesis,[86−88] hypopharyngeal gland development,[89] sperm storage facilitation,[90] and caste development,[42,64] in order to protect cells from damages. In the present study, the antioxidant proteins were expressed with higher abundance in the antennae of the *Aml* drones and the *Aml* workers than the *Acc* drones and worker bees, respectively. This implies the performances of the *Aml* drones and workers require a more enhanced antioxidant system to lessen the overall damages to their cells during higher oxidation activities for their olfactory activities. Consequently, this is supposed to help the rapid degradation of odorants for the detection and response to changes in the concentration of odorants in their antennae.[91] In particular, the unique expression (>10-fold) of proteins of metabolizing peroxide glutathione *S*-transferase 1-1 (gstD1, spots D55−58) and cellular detoxificant glutathione S transferase S1 (gstS1, spots D51−53) in the *Aml* drone antennae signify their centralities in preventing oxidation reactions because of extreme metabolic activities in the *Aml* drone antennae during the processing of sex pheromones.[17,64,67] The gstD1 also plays a key role in the degradation of odorants and alternatively, protects the olfactory system from harmful xenobiotics.[71] On the other hand, the exclusive expression (>10-fold) of jafrac1 (spot D101) in the *Acc* drone antennae coincides with its function in protecting various oxidative stresses and intracellular signal transductions.[92] It also illustrates its crucial role in changing xenobiotics to harmless products such as one of the super family of detoxication proteins.[73] Likewise, the abundant expression (>10-fold) of gstS1 (spot W48−50) and unique expression (>10-fold) of gstD1 (spots W52−54) in the antennae of the *Aml* worker suggests that they avoid oxidation reactions owing to higher metabolic activities during their intensive foraging activities.

The cytoskeletal proteins function in both intracellular transport and cellular division.[93] Actin filaments serve as stabilizing structures in the scolopale of insect sensilla[94−96] and are functionally important during sensillum development.[97,98] The higher expression of proteins associated with cytoskeletal function, particularly, twinstar (tsr, spot D61, D62), and tropomyosin 1, (tm1,spot D72) exclusively in the antennae of the *Aml* drone indicate their sensilla have evolved prominent cytoskeletal elements as part of their buildup of olfactory functional specialization as compared to the *Acc* drones. Tsr is the *Drosophila* homologue of cofilin (Cfl1)/ADF[87,99] that accomplishes various processes such as cell motility[100,101] and cell polarity during migration.[102] The tm1 has co-localization with actin filaments to stabilize them, promote their assembly by preventing disassembly, and increase their rigidity.[103,104] On the other hand, the higher abundance of cytoskeletal proteins in the antennae of the *Acc* worker probably helps in the stabilization of its olfactory systems to assist in foraging activities. The higher expression of mlc2 (spots W87−89) in this *Acc* species is possibly related to the molecular motor myosin, responsible for defining both structural and mechanical properties of the insect flight muscle.[105] Yet, the exclusive expression of tm1(spot W66) in the antennae of the *Aml* worker indicates its involvement in the stretch-activated indirect flight muscle because the antennae can be used as a tool to measure flight speed, perceive odors, and detect even the direction of the odor .[17,106,107]

HSPs support the transportation, folding, and rearrangement of other proteins as a molecular chaperone under normal physiological conditions.[108,109] Hence, the higher expressions of hsp90 (spot D2), hsp60 (spot D7) and glycoprotein (gp93, spot D1) in the antennae of the *Aml* drone compared to the *Acc* drone may indicate their great contribution to assist proper protein conformation and prevention of unwanted protein aggregation. This most likely helps the antennae of the *Aml* drone perform higher oxidation of fatty acids with less stress and enhances the processing of complex pheromones during the mating flight[109,110] than its eastern counterparts. Also, the overexpression of hsp90 (spot W1) and hsp60 (spot W7) in the antennae of the *Aml* workers suggest they are to maintain the higher oxidation and metabolism processes required for their chemical communication during foraging activities as compared to the *Acc* workers. Hsp60 assists as molecular chaperones and promote proper protein folding by preventing aggregation[111] and catalyzes the folding proteins,[112] while hsp90 plays a key role in antennal olfactory communications through its interaction with steroid hormone receptors which are essential for ligand binding and activation of the receptors.[113,114]

Proteins involved in fatty acid metabolism carry out multiple functions in insects as precursors in the biosynthesis of pheromones[115,116] and essential components in the function of the cuticle.[9] The overexpression of most of these proteins in the *Aml* drone reveals their high contribution to support its response to queen pheromones (a combination of various fatty acids)[117] because the drone antennae require rapid degradation of the female sex pheromone during their mating flight.[118] Among the proteins with high abundance, LC-FAC (spot D16) with unique expression in the *Aml* drone is suggested to help the physiological regulation of various cellular functions via the production of long chain fatty acyl-CoA esters to promote protein transport, enzyme activation, cell signaling, and transcriptional regulation.[119,120] Medium-chain acyl-CoA dehydrogenase (MCAD, spot D27), exclusively expressed in the *Aml* drone antennae, is a mitochondria cell fatty acid β-oxidation catalyzing protein and also participates in the process of carbon chain shortening.[121] Apart from this, the higher and unique expression of proteins related to fatty acid metabolism, particularly LCFAC (spot W9 and W10) and MCAD (spot W 83) in the antennae of the *Aml* worker indicates their high demand for a further energy source to enhance their olfactory functions as compared to the *Acc* worker bee.[121−123] However, the higher expression of FABP in the *Acc* worker antennae may fine-tune the balance between the availability of metabolic resources.[124−127,76]

The mapped BIN revealed that the greater part of the total 44 key node proteins were highly expressed in the antennae of the *Aml* drone compared to the *Acc* drone. Of these, proteins related to carbohydrate metabolism and energy production and molecular transporters were represented in higher proportion in the antennae of the *Aml* than in the *Acc* drone. Similarly, these two groups of proteins played a major part in the

antennae of the *Aml* worker compared to the *Acc* worker. These variations in proteins between the antennae of the two bee species indicate that each of the drones and the workers of *Aml* and *Acc* have their own definite quantitative and qualitative biological requirements to perform specific duties. In general, the highest key node proteins in these two groups are likely to work together to regulate the process of olfactory functions in the antennae of both bee species. The tested results between gene and protein expression further provide important information to manipulate the honeybee antenna olfactory system at the gene level. This represents a step toward performance improvement for some important organs in the olfactory system of honeybees.

5. CONCLUSION

The differences in olfactory mechanisms between the western and eastern honeybee drones and foraging workers were explored at both the molecular and morphological levels. Despite the existence of similarities in the antennal morphology between the respective drones and the workers of the two bee species, there were significant differences in their protein expression abundances. In general, the overexpression of the four major functional groups of the antennal proteins (carbohydrate metabolism, molecular transporter, antioxidant activities, and fatty acid metabolism) in the *Aml* drones are believed to be the major role players in enhancing the olfactory functions of the western bee compared to its eastern counterparts. Likewise, three functional groups of proteins (carbohydrate metabolism, molecular transporter, and anti-oxidation activities) are thought to play vital roles in boosting the olfactory functions of the western honeybee worker compared to its eastern counterparts. These data reveal species-biased protein expression that shapes differences in olfactory mechanisms between respective drones and worker bees of the two bee species. The tested relationships between proteins and their encoding genes in the BIN help us to detect important key node proteins for future targeting and functional analysis. To our knowledge, this report extends our understanding of how ecology has shaped honeybees, pursuing their olfactory function in terms of its roles in social, biological, and biochemical signals.

■ ASSOCIATED CONTENT

Supporting Information

Primer sequences used for qRT-PCR analysis of the differentially expressed proteins, antennae morphology data set, and identification of differentially expressed proteins in the antennae of sexually mature drone and worker of *Apis mellifera ligusitca* and *Apis cerana cerana*. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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