Proteomics Improves the New Understanding of Honeybee Biology

Zewdu Ararso Hora, Solomon Zewdu Altaye, Abebe Jemberie Wubie, and Jianke Li*

Institute of Apicultural Research/Key Laboratory of Pollinating Insect Biology, Ministry of Agriculture, Chinese Academy of Agricultural Sciences, Beijing 100081, China

ABSTRACT: The honeybee is one of the most valuable insect pollinators, playing a key role in pollinating wild vegetation and agricultural crops, with significant contribution to the world’s food production. Although honeybees have long been studied as model for social evolution, honeybee biology at the molecular level remained poorly understood until the year 2006. With the availability of the honeybee genome sequence and technological advancements in protein separation, mass spectrometry, and bioinformatics, aspects of honeybee biology such as developmental biology, physiology, behavior, neurobiology, and immunology have been explored to new depths at molecular and biochemical levels. This Review comprehensively summarizes the recent progress in honeybee biology using proteomics to study developmental physiology, task transition, and physiological changes in some of the organs, tissues, and cells based on achievements from the authors’ laboratory in this field. The research advances of honeybee proteomics provide new insights for understanding of honeybee biology and future research directions.

KEYWORDS: honeybee, proteomics, protein expression, development, physiology, behavior

INTRODUCTION

Bees belong to order Hymenoptera, superfamily Apoidea, and family Apidae. Apidae is one of the most diverse families of bees, containing more than 200 genera worldwide. Apis is an emblematic genus, and it is the most studied relative to other bee genera in the family of Apidae. Members of Apis, in which the honeybee is represented, play a crucial role in pollinating plant vegetation (Figure 1) and providing precious ecosystem services. Bees are also substantially important in the world’s agricultural economy, in that 35% of the world’s food production relies on pollinators, of which the honeybee accounts for the largest portion. This is attributed to the body structures and social and instinctive behavioral characteristics of the honeybee species. Importantly, some honeybee species such as Apis mellifera (A. mellifera) are managed in large numbers for their primary products such as honey, royal jelly, and beeswax. Motivated by honey production, this species became the most commonly managed pollinator, employed in 90% of active agricultural pollination and kept in large-scale commercial apiaries.

The honeybee is placed at the highest level of social organization among the eusocial insects. In eusociality, an evolutionarily advanced level of colonial existence, adult colony members have two or more overlapping generations, take care cooperatively of offspring that are not their own, display division of labor among sterile females, and are divided into reproductive and non-reproductive castes.

A honeybee colony typically consists of three kinds of adult castes: a queen, drones, and workers. Among these colony members, there is a division of labor and specialization in the performance of biological functions. Under normal conditions, a colony contains only one fertile female, the queen. At a very early stage of adult life, the queen mates with up to 20 or more males, the drones, which die immediately afterward. She stores the sperm and uses it to fertilize millions of eggs during her lifetime. Once mated, she can lay up to 2000 eggs per day during active seasons of the next 3 years, until she dies or her sperm stores are depleted. In a sex determination mechanism called haplodiploidy, in which female bees are developed from fertilized diploid eggs and males from unfertilized haploid eggs, the queen can control the sex of her progeny.

Each honeybee passes through four major developmental stages over the course of its life: egg, larva, pupa, and adult. The embryo develops inside the egg (embryogenesis) for 3 days, consuming the protein-rich egg yolk which results in a reduction of egg weight. The adult honeybee body plan is formed during embryogenesis. After 3 days, the larva, a whitish grub, hatches from the egg. The larval phase is mainly a period of feeding and growth during which the larva shows an astonishing increase in size. The end of the larval phase is marked by the beginning of metamorphosis, which consists of a complex reorganization of larval structures, which then leads to the non-feeding pupal stage. The pupa undergoes a series of changes in the pigmentation of its compound eyes and the body until the emergence of the imago. When worker bees emerge from their cells in the comb, all their anatomical features are fixed, but the full development of their glandular system takes place afterward, in a complex pattern which reflects the changes in the bees’ behavior over the course of their lives. During adult life, the worker bees perform all the necessary tasks involved in maintaining and protecting the colony, as well as providing food for all colony members. However, there is a division of labor among worker honeybees which progresses through a series of tasks from nursing to nest maintenance and then to foraging as the bees age. Although workers do have the potential to lay eggs that develop to male offspring, they can never become queens or replace a lost or old queen. A diploid egg, laid by a fertile queen, is either laid into a queen cell shaped for queen rearing or a cell of a pre-existing...
young female larva redesigned to accommodate queen larva. This larva is fed with a protein-rich brood food—royal jelly, secreted from the hypopharyngeal and mandibular glands of nurse bees when it is needed—and its differential feeding compared to other young larva triggers the larva to develop into a queen.

Although honeybees play an important role in human economy and ecological services, researchers did not start to investigate honeybee biology until the beginning of the 20th century. Before then, most of the studies in honeybee biology did not go beyond describing them, either at the colony level or at the single animal level. Hence, bees have been poorly studied at the molecular, biochemical, and cellular levels compared with other organisms such as Drosophila, because of a wide range of knowledge and technical limitations. Despite the importance of the honeybee as a model organism to understand social behavior for a long period of time, it has been merely explored at the molecular level in developmental biology, neurobiology, immunology, and aging.

However, the circumstances changed immensely with the advent of complete honeybee genome sequencing and technological advances in protein separation, identification, mass spectrometry (MS), and computational platforms. The A. mellifera genome was sequenced in 2006, which opened a new epoch of functional genomic study in the honeybee. Although having the complete genome is fundamental for understanding honeybee biology, the accumulation of a vast amount of genome sequences in the database is not sufficient to elucidate all of its complexity. This is because the genome sequence and protein functions cannot be correlated directly. Thus, studying dynamic gene expression through proteomics is needed. Although proteomics is a well-suited tool for studying honeybee biology, early two-dimensional gel electrophoresis (2-DE)-based proteomics were labor intensive and cumbersome, and high-quality results required semi-manual analysis of spectra for identification and quantification. Moreover, the downstream biological analysis of highly multivariate quantitative protein abundance data generated using MS-based analysis was a main bottleneck in the early proteomics studies of honeybee. Presently, MS-based shotgun proteomics, taking advantage of the technological advances in instrumentation and computational proteomics platforms, is reaching a level of maturity that makes it a powerful and largely employed technology. These technological advances help us to get new ideas about different aspects of honeybee biology, including physiology, behavior, and pathology. In this Review, we first summarize the advances made in honeybee proteomics, and then discuss the progress in light of honeybee developmental physiology and behavioral studies.

### ADVANCES IN HONEYBEE PROTEOME RESEARCH

Honeybee proteomics has undergone a major revolution over the past decade. The pioneer work published in 2005 identified only nine proteins in the venom of worker bees using 2-DE-based proteomics. As soon as the first honeybee genome was released a year later, some of the earliest work was done, identifying 324 proteins using 1-DE coupled with MS-based proteomics in hemolymph of queens, drones, workers, and worker larvae, revealing profound proteome differences among the castes. The increased proteome coverage applying 1-DE revealed the proteome differences of hemolymph of queens, drones, workers, and worker larvae. This young science has rapidly expanded with technological advances associated with protein separation, identification, and mass spectrometry, as well as protein chemistry and bioinformatics. 2-DE-based proteomics had been widely used in honeybee proteome analysis at a genome-wide scale since the beginning of 21st century. However, its laborious nature and technical limitations such as low sensitivity, low reproducibility, limited dynamic range of staining methods (Coomassie or Silver), and under-representation of membrane proteins hamper the throughput.

As a result, many earlier 2-DE-based proteomic works merely identified a small fraction of proteins in single honeybee samples. Through time, the field has moved forward, away from early methods of proteomics toward the more powerful methods of liquid chromatography coupled with tandem MS (LC-MS/MS) over the past decade. Thus, with MS-based proteomics, the large-scale analysis of proteins has greatly improved in speed and quality, such as resolution, sensitivity, and mass accuracy, which made identification of thousands of proteins from a single honeybee sample possible. For instance, recently >8600 proteins were identified across three sample types (hemolymph, mushroom body, and antenna), which significantly extended the depth of protein coverage in the honeybee proteome. Furthermore, a wide spectrum of the molecular underpinning of honeybee biology has been deciphered, which greatly improves the understanding of complex biological processes of honeybees, such as their physiology and behavior.

The milestone achievement of genome sequencing and MS-based analytical instrumentation were fundamental in improving our knowledge of honeybee biology by profiling all the proteins that exist in a specific cell, tissue, organ, or whole organism at a given time point. For instance, a complex proteome atlas of A. mellifera from 29 different organ/tissue

---

Figure 1. Honeybees forage on flowers, and as a result flowers get pollinated. (Photographs by Prof. Dr. Jianke Li.)
types has been analyzed for the adult stage of all three castes, identifying 2288 proteins. Therefore, we can study the overall performance and complex physiological and behavioral changes of a honeybee at a particular time by effectively employing these new technologies that can deal with such a vast amount of data. This innovation has changed our thinking about protein research from studying one individual protein at a time to understanding a complex system-level change. In addition, this new thinking helps us solve the difficulties caused by lack of well-developed gene editing tools in honeybees as for other model animals, which has astonished and continues to surprise us with numerous findings and implications on honeybee physiology, behavior, and many other biological characteristics.

### PROTEOMICS DISSECTS HONEYBEE DEVELOPMENTAL PHYSIOLOGY AND BEHAVIOR

#### Proteomics Uncover the Honeybee Pre-adult Developments

A honeybee egg is a small, rod-like shape, with considerably varying sizes. It remains an egg for 3 days before hatching to the first instar larva. During this period, it undergoes 10 successive embryonic developmental phases. As a consequence, noticeable and relatively rapid transformation happens in the embryo structure through cell growth and differentiation. A 2-DE-based proteomics study has uncovered the presence of 38 abundant proteins over the embryonic development of worker bees (A. mellifera), involved in different processes of cell growth and differentiation. The accumulation of most of the proteins during embryogenesis is the driving force for cell division, tissue metamorphosis, and self-protection, indicating their higher priority to the embryogenesis of honeybees. Specifically, it is suggested that the escalated levels of lethal (2) 37Cc and imaginal disc growth factor 4 (IDGF) proteins are because 3-day-old embryos need more growth factors to regulate the embryonic development. Moreover, the proteome of a drone embryo reveals that organogenesis and cell differentiation of the embryo largely occur at the middle to late stages of embryogenesis. Also, on the last day of the embryo stage, the stronger expression of a large number of developmental and energy-related proteins may enhance cellular activities of organogenesis, tissue elongation, and body segmentation.

With the advancement of proteomics technologies, the number of proteins identified increased, and several other aspects of embryogenesis have been addressed. For instance, when the proteome of worker embryos was again analyzed covering the whole course of development (24, 48, and 72 h), 1460 proteins were identified across the three ages. Interestingly, the proteome of each age is intensely changed to orchestrate the development events: the 24 h embryo produces proteins for nutrition storage and nucleic acid metabolism, and the 48 h embryo demands increased levels of proteins for organogenesis that are involved in pathways of aminocyt-tRNA biosynthesis, β-alanine metabolism, and protein exportation. At the last stage of the embryo (72 h), biological pathways of fatty acid metabolism and RNA transport are highly activated, which matches the physiological transition from egg to larva. Worker embryos start their morphogenesis later compared with drones, and the abundance of proteins associated with morphogenesis is lower in worker embryos across embryogenesis. In addition, drone embryos employ more cytoskeletal proteins to support a large body size and antioxidants for their temporal organogenesis.

During the 6 days of larval development, a 1500-fold increase in body weight occurs. In larva, ~22 proteins of the whole body proteome were quantified with respect to age, and the upregulation of proteins for carbohydrate metabolism at early stage of larval development is needed. The accumulation of carbohydrate-related proteins is suggestive of the fact that young larvae require more energy than at the later stages to promote development, whereas the higher level of storage proteins, specifically of larval serum protein 2 on day 6, indicates that larvae store amino acids for the subsequent metamorphosis. However, proteomic analysis of larval hemolymph across ages unveils that the levels of immunity-related proteins such as prophenoloxidase and apismin are positively correlated with development, while other immunity-related proteins are not differentially expressed with the aging of larvae. These findings dissect the molecular details of the reason why bee larvae are susceptible to major age-related

---

Figure 2. Comparison of functional classes enriched by the highly abundant proteins between the honeybee worker and drone (A. m. ligustica) embryos across three stages of embryonic development. Left, middle, and right graphs represent 24, 48, and 72 h aged embryos, respectively. Reprinted with permission from ref27. Copyright 2015 American Chemical Society.
infectious bee diseases such as American Foulbrood or chalkbrood.

Determination of morphologically and behaviorally distinct workers and reproductive castes occurs during larval development in honeybees depending upon differential nurturing by workers. To this end, specific genes are activated for regulating the caste determination. Several proteomic profiles as early as the third larval instar support the importance of the time-point of the nutritional switch for the caste fate. For instance, the significant differences in protein expression between queen-intended and worker-intended larvae at 72 and 120 h are indicative of the notion that the fate of the two castes has already been decided before 72 h. Moreover, subcellular proteomics (mitochondrial and nuclear) of the larvae indicates the existences of large differences in protein expressions between the two caste-intended larvae at day 3, day

Figure 3. Comparison of brain neuropeptides identified in newly emerged bees (day 0), nurse bees (day 7), nurse bees (day 14), and forager bees (day 21) of both Italian bees (ITBs) and high royal jelly producing bees (RJBs), showing number of neuropeptides identified (A) at each time point and (B) in each neuropeptide precursor protein. The x-axis in (B) is the number of neuropeptides under each precursor protein, represented by the bars. Reprinted with permission from ref 46. Copyright 2015 American Chemical Society.
4, and day 5 larval ages, manifesting the strong directional selection pressures due to the quantity and quality of larval food provided across the developmental stages.34,35 Interestingly, this subcellular proteome evidence is in agreement with what has been concluded from proteomic analysis on the whole larvae.33 Furthermore, royalactin can induce the differentiation of honeybee larvae into queens, as well as derive queen development through an epidermal growth factor receptor-mediated signal pathway.36

The non-feeding pupal stage of honeybees is the longest post-embryonic development, which lasts for \( \sim 13 \) days. During this period, the pupa undergoes a series of changes in body structure and pigmentation of the compound eyes.9 The earliest work done on pupa head development using 2-DE found 58 differentially expressed proteins across five time points (13, 15, 17, 19, and 20 days), of which 36 proteins are involved in the organogenesis of the head in early development. However, 22 of the proteins are involved in regulating the pupal head neuron and gland development that occur at a later stage of development of the pupae heads.37 Furthermore, a 2-DE proteomic map of worker red-eye pupae hemolymph identified \( \sim 129 \) proteins.38 Most of the proteins identified during the non-feeding period are known to be involved in the mechanism of metamorphosis,36,39 which results in the dramatic decrease of the overall protein quality as the red-eye pupa develops into a newly emerged bee.

**Proteome Changes Drive Social Ontogeny of Adult Workers.** With age development, the worker honeybees normally progress from performing in-nest activities such as cleaning, nursing, comb building, and guarding to foraging for nectar, pollen, and resin in the field.1 A whole-body proteome profiling of worker bees reveals the divergence of nest bees from foragers.40 Likewise, a significant difference between foragers before and after reversal from foraging to nursing activities suggests that the plasticity and robustness of protein expressions are associated with the physiological and behavioral role changes in the honeybee.41 The metabolic specialization that occurs during the social ontogeny of worker bees is to meet their metabolic energy demand and utilization as worker bees initiate intense flight.42,43 This social ontogeny of worker bees is further supported by the overexpression of proteins involved in energy production, iron binding, metabolic signaling, and neurotransmitter metabolism in experienced foragers compared with nurse bees’ brains.44 Similar proteome-wide analysis of the brains of forager and nurse bees has also justified that proteins involved in energy production and conversion, with elevated levels of expression in experienced foragers, indicate an increased brain activity demands higher energy during learning and memorization processes that are triggered upon foraging.44 Moreover, the higher levels of proteins implicated in translation, ribosomal structures, and biogenesis in nurses than in foragers reflect more active protein synthesis to develop the protein machinery necessary for the changes in brain structure, which precede ontogenesis to forager.44 Moreover, in addition to the main role in modulating caste differentiation,45 differential expression patterns of major royal jelly proteins (MRJPs, i.e., MRJP1, MRJP2, and MRJP7) in the nurse bee brain possibly suggest that they are working as endogenous participants of various brain activities.

Recently, a time-resolved study of the brain neuropeptidome of *A. mellifera ligustica* (A. m. ligustica, ITBs) and high royal jelly producing bees (RJBs) selected from ITBs to enhance royal jelly outputs has found that both bee lines employ a similar neuropeptidome to adjust their respective physiology to age-dependent task transitions (Figure 3).46 Comparing neuropeptidomes across ages, almost all of the neuropeptides in the brain of newly emerged bees are found to be down-regulated, indicating neuron signal networks are still immature and neuromodulators have not been fully employed, whereas the highly abundant neuropeptides such as FMRFamide, diuretic hormone, pigment-dispersing hormone, and allatotropin are supposed to tune biological functions during age-dependent task shifts via regulating excretory system, circadian clock system, and juvenile hormone synthesis.48 More recently, a comparison of brain phosphoproteome between nurse and forager bees revealed the age-dependent phosphorylation in tuning of protein activity to regulate brain function according to the biological duties as nursing and foraging bees.47 Moreover, an age-resolved dynamics of brain membrane proteome and phosphoproteome is found to be associated with the neuro-biological requirements during the development of adult workers.48 In newly emerged bees, differentially expressed proteins involved in metabolism of carbohydrates, nucleosides, and lipids are to enhance brain cell maturity; a higher number of membrane proteins and phosphoproteins in nurse and forager bees compared with in young bees is an indication of their significance in sustaining in-depth information processing during nursing and foraging activities.48

Similar to the brain, a time-resolved proteome comparison of the mandibular gland and hemolymph of both ITBs and RJBs has shown great differences over age. The different proteome programs have been defined by the mandibular glands of newly emerged, nurse, and forager bees to underline their specific roles in the colony.49 In newly emerged bees, the proteome drives the initiation of young mandibular gland development; in nurse bees, it plays key roles in priming high secretory activity in lipid synthesis, whereas in forager bees, the mandibular gland proteome is found to enhance colony defense or scent markers to increase foraging efficiency. Age-specific hemolymph proteome settings have also been adapted by the larva and adult ages of ITBs and RJBs to prompt tissue development and immune defense in newly emerged bees, gland maturity in nurse bees, and carbohydrate energy production in forager bees.40 All these findings clearly highlight the role of proteomics in deciphering honeybee age-dependent task transitions and behavioral changes. However, as ontology of worker bees relies on differential expression of proteins in organs and tissues in concert with their distinct age-dependent physiology, most previous works have focused on individual organs and tissues. Thus, further work focusing on many organs and tissues at the same time is needed to fine-tune how and when age-dependent transitions appear to happen exactly.

### ORGAN AND TISSUE PROTEOME UNDERLIE MOLECULAR DIFFERENCES IN HONEYBEES

**Antennal Proteome Changes Modulate Olfactory and Neural Activities in Honeybees.** As social insects living in a colony, honeybees have a complex odor cue (pheromone) communication system and rely on their odor signals to attract their potential mates, locate food sources, and detect possible enemies.51 In honeybees, the reception of odor signals takes place in the antennae via placoide sensilla and bind to odorant binding proteins (OBPs) and chemosensory proteins (CSPs) to activate sequential neural responses and last attain their social activities. Due to its importance to olfaction, the honeybee antenna has been an active domain of proteomics
study since 2010. The first antennal proteome study focusing on the individual expression of OBPs and CSPs has revealed the presence of four OBPs (OBP1, OBP2, OBP4, and OBP5) and two CSPs (CSP1 and CSP3) in the antennae of A. mellifera forager bees. On the other hand, a comprehensive antennal proteome comparison of forager and drone bees reveals a sex-biased protein expression in both bees, suggesting that odorant response mechanisms are sex-specific because of natural selection for different olfactory functions in the two castes. A more comprehensive proteome comparison among drone, worker, and queen bees again confirms that the differential expressions of antennal proteins are associated well with the different requirements of cast-dependent olfactory activities.

To investigate olfactory mechanisms employed by honeybees for ecological and pest resistance behavioral adaptations, the proteomes of drones and workers of A. m. ligustica and A. cerana cerana (A. c. cerana) have illustrated that the two bee species have developed their respective olfactory mechanism to adapt to distinct ecologies during their evolution. Moreover, a proteome-wide analysis of disease tolerance behaviors, colony-level hygienic behavior (HB), and Varroa-sensitive hygienic (VSH) behavior (Figure 4) identifies that several proteins that are highly predictive of behaviors contributing to reduced hive infestation via antennal proteome changes of hygienic bees. These alterations in antennal proteome of hygienic bees stimulate the speed of HB through facilitating chemosensory and neurological processes by providing specificity for detection of Varroa destructor in the antennae. Recently, correlation of individual proteins’ expression patterns with HB scores (Figure 5) have found seven proteins may be putative biomarkers of HB. All the seven proteins are also found to be involved in semiochemical sensing, nerve signal transmission, and signal decay, which are required to respond to an olfactory signal from dead brood inside the cell. Similarly, the highly expressed proteins associated with sensitivity of olfactory senses and signal transmissions in the antennae of VSH bees further signify that antennal proteins do have vital roles in carrying important signals to the mushroom bodies (MBs) in order to activate VSH behavior in VSH bees. Hence, these findings provide molecular clues of the most likely mechanism behind a complex social immunity behavior that allows bees to coexist with pathogens and may help the future protein-based selective breeding of better performing bee lines resistant to Varroa.

**Molecular Neurobiological Basis Underlies Honeybee Behavior.** Processing sensory clues and altering functional dynamics are higher order mental functions that are carried out by the brain. To this effect, several proteomic studies on honeybee brain encourage our comprehensive understanding of the molecular basis of neurology that triggers different social behaviors in honeybees. The first 2-DE-based proteomics has found that the juvenile hormone diol kinase (JHDK) is preferentially expressed in worker bees’ MBs, while glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is selectively expressed in optical lobes (OLs), suggesting the importance of JHDK in the formation of JH diol phosphate to facilitate Ca2+ signaling in the MB and the involvement of GAPDH in sugar metabolism in the OL. Further, the preferential expression of reticulocalbin, an endoplasmic reticulum (ER) Ca2+ transport protein, in the large-type Kenyon cells of MBs supports the notation that the function of ER Ca2+ signaling is specifically enhanced in the large-type Kenyon cells in the honeybee brain. In addition, differential gene expressions of reticulocalbin and ryanodine receptor, involved in ER Ca2+ channel, and non-significant expression of genes related to Ca2+ signaling pathways in the MBs highlight the importance of the Ca2+ signal pathway in honeybee brains for learning and memory. Furthermore, the levels of kinases, synaptic, and neural growth-related proteins decline in the central brain with increased foraging duration, whereas the calyx region of the MB remains intact both structurally and biochemically. Thus, distinct brain areas are differently affected by biological aging of the honeybee, and the calyx region is suggested not to be in charge of the foraging-dependent performance decline. Although the forager bees’ brain function declines with age, learning ability can recover in aged foragers that revert to nursing tasks, and the recovery is directly related to the levels of stress response/cellular maintenance proteins in the central brain.
Recently, to understand the molecular neurobiology bases underlying behavioral divergences between and within honeybee species, a comparative proteome of three brain regions (MBs, OLs, and antennal lobes (ALs)) of *A. m. ligustica* and *A. c. cerana* (Figure 6) revealed that both species have evolved similar proteome signatures in MBs and OLs to drive the domain-specific neural activities. However, both species have developed distinct AL proteome architectures to prime their respective olfactory learning and memory. In ALs of *A. c. cerana*, olfactory learning and memory are supported by the enriched cytoskeleton organization that modulates glomeruli flexibility and intracellular transport, while in *A. m. ligustica*, functional groups related to hydrogen transport (Figure 7) are to promote olfactory processes by regulation of synaptic transmission. On the other hand, neural peptides regulating behaviors of RJ secretion in workers brain of RJBs are enhanced via regulation of water homeostasis, brood pheromone recognition, foraging capacity, and pollen collection to underline the elevated physiology of RJ secretion compared with ITBs. Again, phosphatidylinositol signaling and arachidonic acid metabolism are vital in the stronger olfaction sensation of RJB nurses in response to larval pheromone stimulation, and enriched pathways related to signal processing are to enhance nerve sensitivity in RJB foragers to promote stronger tendency in pollen collection. Furthermore, the higher expression levels of MB proteins implicated in neural sensitivity of VSH bees over non-VSH bees may support the performance of VSH behavior by activating synaptic vesicles and calcium channel activities.

**Proteome of Mandibular and Hypopharyngeal Glands Drives Caste- and Age-Dependent Differences**

**in Honeybees.** Mandibular glands (MGs), a pair of sack-like glands, are located inside the head. Secretions from the MGs have important caste-specific functions that are associated with the social evolution of honeybees. The molecular bases of caste-specific MG functions have been elucidated by several proteomic reports. Three proteins (aldehyde dehydrogenase, medium-chain acyl-CoA dehydrogenase, and electron-transfer flavoprotein a) are selectively expressed in queen MGs, whereas fatty acid synthase is selectively expressed in worker MGs. Moreover, the molecular expression of OBPs and CSPs are caste- and age-dependent in queen, worker, and drone MGs. Recently, the MGs of newly emerged, nurse, and forager bees of RJBs and ITBs were found to employ different proteome repertoires to fit with their distinct age-dependent physiology. The reshaped proteome in nurse bees of RJB consolidates the desired amount of lipids for increased RJ production by enhancing the rate of lipid synthesis and minimizing its degradation to increase 10-hydroxy-2-decenoic acid synthesis, which is a major component of RJ. Furthermore, the hypopharyngeal glands (HGs) are an important organ to synthesize and secrete RJ (the major larval and queen food). Time-resolved proteomic profiling has illustrated that protein synthesized in the HG of the worker bees matches with age-dependent role occurrence. For instance, age-dependent expression changes of MRJPs in the HGs are concurrent with task-switching, and their expression reached peak levels during days 6–12 in the nurse bees. Moreover, proteomic comparison in the expression of HG proteins between ITB and RJB workers indicate that HGs are associated with their age-dependent roles, and RJB workers have adapted a different strategy to increase RJ production by enhancing wide ranges of proteins as compared to ITB workers. Also, the HGs have experienced important changes in protein expression during their ontogenic development, which supports the secretion of proteins involved in diverse functions in adult workers beyond their traditional role in RJ production. Phosphoproteome analysis of HGs of worker bees across ages has found that most proteins are regulated by phosphorylation independent of their expression levels. In addition, proteins in key biological processes and pathways are dynamically phosphorylated with age development, and complementary protein and phosphoprotein expression is required to support the unique physiology of secretory activity in the HGs.

**Different Honeybee Species/Stocks Evolve Distinct Hemolymph Proteomes To Support Their Respective Physiology.** Hemolymph is a blood equivalent fluid in arthropods that bathes tissues. Its major function is to transport nutrients and immune components throughout the body. As in other insects, the hemolymph of honeybees is composed of a broad spectrum of proteins such as enzymes, nutrient and pheromone transporters, structural proteins, immune response proteins, MRJPs, and so on. Hence, hemolymph holds a key for a breakthrough in investigating phenotypical and physiological differences among honeybees. The earlier hemolymph proteomics works were focused on investigating differences in hemolymph composition between honeybee castes. Profound differences among the castes, especially between larvae and adult stages and between male and female castes, as well as between adult workers and queens were found. In addition, higher level of vitellogenin in adult workers hemolymph than in larvae manifest disease resistance of adult workers, and adult workers also have more immunity-associated proteins compared with larvae.
With aging of the honeybee, hemolymph protein components change accordingly. During the life transition from larva to pupa, larval hemolymph expresses proteins involved in the metabolism of carbohydrate and MRJPs, whereas young pupae express more energy storage related proteins for the non-feeding pupation process. Although many of the proteins identified are previously reported in the hemolymph of adult summer workers, adult winter workers, and larvae, the majority of these proteins are involved in the metamorphosis. However, some major proteins in the hemolymph of red-eye worker pupae are not detected in a newly emerged worker hemolymph, indicating that energy depletion occurs in non-feeding pupae during body reconstruction. On the other hand, relatively higher level proteins like ferritin, glutamine S-transferase, and toll-like receptor detected in the newly emerged bees over red-eye pupae indicate their vital roles in innate immune adaptation to the new environment upon emerging.

Comparative hemolymph proteome between honeybee species, and even within lines of subspecies, is also a promising strategy to better understand resistance to some pathogens and enhanced RJ production in RJBs. For instance, the stronger expression of proteins related to energy production, protein folding, cytoskeleton, and development in A. m. ligustica over A. c. cerana indicate that both bees evolved their distinctive strategies in using hemolymph for nutrient transport and immune defense during larval to pupal development stages. Moreover, the VSH-line, a line selected for its VSH behavior from A. m. carnica, has tailored a unique hemolymph proteome cascade compared to non-VSH bees to boost social immunity and drive pupal organogenesis through enhancing energy metabolism and protein synthesis. A viral protein load difference between sterile and egg-laying workers suggests the reproductive workers have a stronger immune system over the sterile ones. Furthermore, a large number of highly abundant proteins enriched in protein synthesis and energy metabolism in day 4 larvae and nurse bee stages of RJBs relative to ITBs imply that RJB larvae and nurse bees have reprogrammed their proteome to initiate a different developmental trajectory and higher RJ secretion, in response to selection-enhanced RJ production.

Figure 7. Comparative analysis of GO terms enriched in mushroom bodies (MBs), antennal lobes (ALs) and optical (OLs) in A. m. ligustica brain. Bars A, B, and C, show enriched functional groups, and bars D, E, and F show enriched kegg pathway, in MBs, ALs, and OLs, respectively. Different color bars represent different term in functional groups. The single asterisk and double asterisks indicate significant enrichment at the p < 0.05 and p < 0.01 statistical levels, respectively. Reprinted with permission from ref 62. Copyright 2018 The American Society for Biochemistry and Molecular Biology, Inc.
indeed enhance sperm survival, whereas spermathecal gland secretion proteins have a comparable positive effect on sperm viability.\textsuperscript{77} Seminal fluid contains a similar set of proteins in intra-species of \textit{A. mellifera}, but protein abundance or state of protein modification has varied significantly.\textsuperscript{78} Proteins with altered abundances have diverse biological functions that are linked to male reproductive success, energy metabolism and cellular structural proteins, and immune-competence.\textsuperscript{79} Several studies have also confirmed that honeybee seminal fluid contains enzymes, regulators, and structural proteins used for energy production, antioxidation, interacting with female physiology, and maintaining the stability and viability of the sperm.\textsuperscript{77,78,81,83} In addition, in the spermathecal fluid proteome, the majority of the proteins are enzymes of energy metabolism and antioxidant defense, indicating that this fluid might facilitate long-term storage of sperm.\textsuperscript{79} Prolonged survival of sperm could be underpinned by substantial changes in only a specific subset of sperm proteins that allow physiological adaptation to storage.\textsuperscript{80} However, due to plasticity of sperm cellular machinery between sperm within an ejaculate and within the female’s storage organ,\textsuperscript{83} we are not confident enough whether the changes in protein abundances are because of active adaptation or sperm senescence. Moreover, it is still not clear whether these proteins could adjust sperm performance or not in different chemical environments. At this point, it is also important to display that there is no available information on individual protein effects and how they modulate sperm physiological adaptations for storage and male reproductive success.

As a conclusion, the advents of honeybee genome sequencing, and proteomics advancements in instrumental and computational analysis, created a fertile ground for researchers to study honeybee biology at molecular and biochemical levels over the past decade. Using this fertile ground, the uncovering of molecular mechanisms in honeybee biology has shown substantial progress, revealing diverse expression-based proteome changes associated with development and behavioral physiology. Both the diversity of molecular mechanisms and social functions widen our understanding of cellular- and molecular-level underpinnings in honeybee developmental and behavioral physiologies. However, examining honeybee proteome profile to test which proteins and protein expression changes specifically underlie their complex phenotypes and social behavior will reveal only part of the multistep processes. Thus, looking to the future, further studies are needed to explore the process of molecular mechanisms and social functions. A more detailed understanding of molecular mechanisms in honeybee development and behavioral physiology will help to identify possible key proteins/genes and signaling pathways that participate in particular developmental and behavioral physiological changes. Moreover, the development of protein biomarkers and implementation of protein-based selective breeding programs potentially illuminates lines of research and will help intentions and progressive desires to make current proteomic studies more complete. If possible, research on transgenic or mutant honeybee with traits of interest through overexpression or knockdown of target protein/gene might be needed. However, transgenesis in honeybee is currently a big challenge.

**ORCID**

Jianke Li: 0000-0003-4183-7336

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work is supported by the Agricultural Science and Technology Innovation Program (CAAS-ASTIP-2015-IAR) and the earmarked fund for Modern Agro-Industry Technology Research System (CARS-44) in China.

**REFERENCES**


(18) Li, J.; Li, H.; Zhang, Z.; Pan, Y. Identification of the Proteome Complement of High Royal Jelly Producing Bees (\textit{Apis mellifera}) during Worker Larval Development. \textit{Apidologie} \textbf{2007}, \textit{38}, 545–557.

**AUTHOR INFORMATION**

**Corresponding Author**

* E-mail: apislijk@126.com. Tel./Fax: +86 10 8210 6448.


(70) Qf, Y.; Fan, P.; Hao, Y.; Han, B.; Fang, Y.; Feng, M.; Cui, Z.; Li, J. Phosphoproteomic Analysis of Protein Phosphorylation Networks in the Hypopharyngeal Gland of Honeybee Workers (Apis mellifera ligustica). *J. Proteome Res.* **2015**, 14, 4667–4671.
