

Phosphoproteomic Analysis of Protein Phosphorylation Networks in the Hypopharyngeal Gland of Honeybee Workers (*Apis mellifera ligustica*)

Yuping Qi,^{†,||} Pei Fan,^{†,‡,||} Yue Hao,^{†,||} Bin Han,[†] Yu Fang,[†] Mao Feng,[†] Ziyou Cui,^{†,§} and Jianke Li^{*,†}

[†]Institute of Apicultural Research, Chinese Academy of Agricultural Science, No. 1 Beigou Xiangshan, Beijing 100093, China

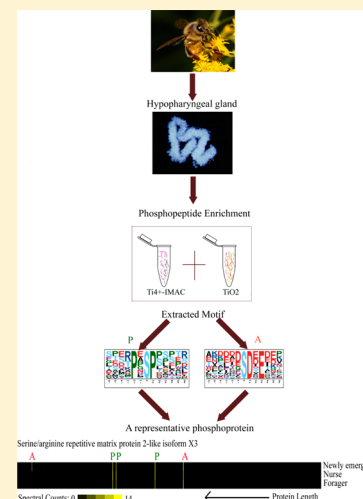
[‡]College of Bioengineering, Henan University of Technology, No. 100 of Science Road, Zhengzhou 450001, China

[§]Department of Pediatrics, Medical School, and Lillehei Heart Institute, University of Minnesota, Twin Cities 4-240 CCRB, 2231 Sixth Street SE, Minneapolis, Minnesota 55455, United States

Supporting Information

ABSTRACT: The hypopharyngeal gland (HG) in honeybee workers changes functions according to physiological age in the bee colony from producing royal jelly (RJ) in nurse bees to digestive enzymes in foragers. The same set of secretory cells expresses different genes or proteins to create these age-dependent changes; however, it is unknown precisely how the phosphorylation network regulates physiological differences across the development of the adult worker HG. We employed high-accuracy mass-spectrometry-based proteomics to survey phosphoproteome changes in the newly emerged, nurse, and forager bees. Overall, 941, 1322, and 1196 phosphorylation sites matching 1007, 1353, and 1199 phosphopeptides from 549, 720, and 698 phosphoproteins were identified in the three ages of the HG, respectively. Specialized, interconnected phosphorylation networks within each age were found by comparing protein abundance and phosphorylation levels. This illustrates that many proteins are regulated by phosphorylation independent of their expression levels. Furthermore, proteins in key biological processes and pathways were dynamically phosphorylated with age development, including the centrosome cycle, mitotic spindle elongation, macromolecular complex disassembly, and ribosome, indicating that phosphorylation tunes protein activity to optimize cellular behavior of the HG over time. Moreover, complementary protein and phosphoprotein expression is required to support the unique physiology of secretory activity in the HG. This reported data set of the honeybee phosphoproteome significantly improves our understanding of a range of regulatory mechanisms controlling a variety of cellular processes and will serve as a valuable resource for those studying the honeybee and other insects.

KEYWORDS: hypopharyngeal gland, phosphoproteome, honeybee



1. INTRODUCTION

The HG is quite a unique organ that changes its biological function from secreting RJ in nurse bees to producing digestive enzymes in foragers, in accordance with age or role of the adult honeybee in a colony.^{1,2} The grape-like HG is tightly coiled and connected to many acini; each of these contains a dozen secretory cells on both sides of the head of a honeybee worker. The acini size in the HG attains its highest volume around days 6–12. After day 15, the acini begin to shrink.^{3,4} The nurse bees are the major producers of RJ, a proteinaceous secretion that is both functionally important for honeybee biology and human health promotion as a nutrient and defense agent. Conversely, the HG in forager bees begins to secrete enzymes that are responsible for processing nectar into honey by dissociating disaccharides into monosaccharides, the main constituents of honey.^{5,6} These physiological role changes of the HG in nurse and forager bees are also reflected in gene expression, from major RJ proteins (MRJPs) in nurses to α -glucosidase, amylase, and glucose oxidase in foragers.⁶

Advances in genomic and proteomic technologies permit an increasing breadth and depth of new insight into honeybee biology at the molecular level. For instance, genome profiles of the HG have been compared among different honeybee species to better understand the molecular details that underpin HG development at the gene level.⁷ Recently, proteomics has grown as an emerging tool for revealing the molecular mechanism that supports honeybee biology. This advancement has made significant headway in the study of reproductive biology,⁸ embryogenesis,^{9,10} brain,¹¹ brood,^{12,13} and HG development at the proteome level.^{3,14} Applying the proteome approach, we found that a honeybee line, selected for increasing RJ production from Italian bees (*A. m. ligustica*) in China, has an altered proteome that permits the HG to produce more RJ than the control line.³ Overall, with age, the HG undergoes morphological and physiological transitions to adjust its

Received: June 8, 2015

Published: September 18, 2015

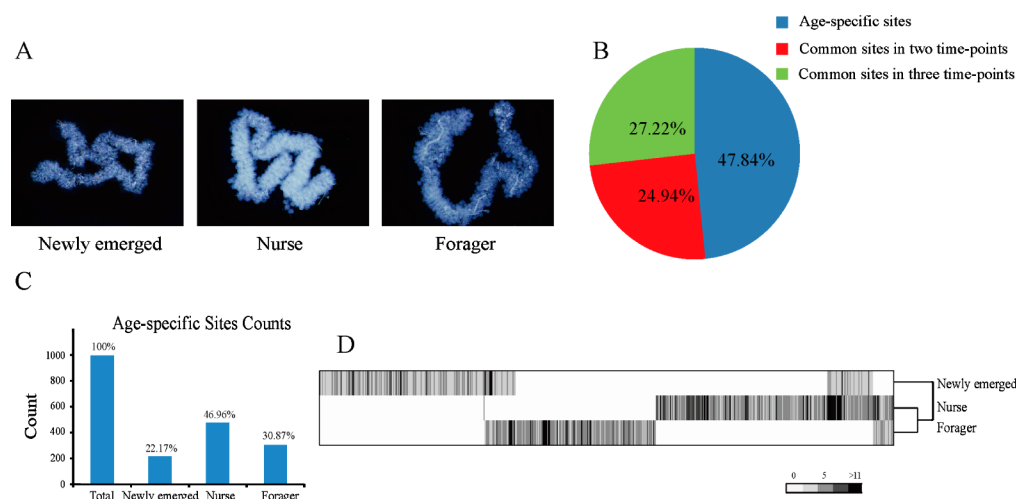


Figure 1. Overview of age-dependent phosphorylation in the hypopharyngeal gland of honeybee workers (*A. m. ligustica*). (A) Morphological images of the HG newly emerged, nurse, and forager bees. (B) Numbers of sites detected at distinct ages of the hypopharyngeal gland. (C) Distribution of age-specific sites across three time points of the hypopharyngeal gland. (D) Hierarchical clustering of sites and different ages of the hypopharyngeal gland based on the number of spectral counts.

biological imperative in the shift from nurse to forager bee. These age-related morphological and functional changes during the HG development are reflected in the changes in protein/gene expression or pathways.^{3,6,7}

Reversible protein phosphorylation is one of the most important post-translational modifications (PTMs) in eukaryotes and plays a critical role in regulating diverse cellular processes, such as cell cycle control, differentiation, proliferation, and metabolism. In PTM, protein kinase modifies the target proteins by adding a phosphate group to amino acid residues of serine (Ser), threonine (Thr), and tyrosine (Tyr), and a protein phosphatase removes the phosphate group that has been attached by protein kinase.^{15–18} It is reported that dynamic phosphorylation not only plays a key role in regulating the embryogenesis in *Drosophila*¹⁹ but also changes the abundance of phosphoproteins as a driving force for the embryo-larva transition of worker bees.²⁰ Moreover, phosphoproteomic technology has been widely used for interrogation of protein level dynamics and the definitive role of phosphorylation in a protein's function, activity, localization, and ligand/protein interactions in humans, mice, and other organisms.^{21,22} These observations indicate that modification of the phosphorylation pattern is important for differentiation and proliferation in living cells. Although proteome surveys have revealed the mechanism that matches the physiological role shift of HG over the age growth,^{3,6,7} the mechanism of the phosphorylation dynamic that underlines the physiological role transition of HG during the whole life circle of adult honeybee workers has not yet been discovered.

To understand the phosphorylation events that drive the change in biological functions in honey bee glands, we performed the first time-dependent study on the comprehensive phosphoproteome of the HG throughout the entire adult life of worker bees. Many proteins are regulated by phosphorylation independent of their expression level. The phosphorylated biological processes and pathways are uncovered in an age-dependent way; the phosphoproteome plays vital complementary roles in the proteome to fine-tune their biological functions in preparation for the normal physiological shift of the HG. This data constitutes an important first step

into the study of the signaling mechanism in the honeybee and other social insects.

2. MATERIALS AND METHODS

2.1. Reagents

Unless otherwise specified, all chemicals were bought from Sigma-Aldrich (St. Louis, MO). Titanium oxide (Titansphere, 5 μ m particles) was purchased from GL Science (GL-Sciences, Japan). Ti⁴⁺-IMAC material was obtained from Dalian Institute of Chemical Physics, Chinese Academy of Sciences.

2.2. HG Dissection and Protein Sample Preparation

Five honeybee (*A. m. ligustica*) colonies were maintained at the apiary of the Institute of Apicultural Research, Chinese Academy of Agricultural Science, in Beijing. Specific ages of bees were sampled in accordance with the methods described by our group.³ In short, the newly emerged bees were taken once they emerged from cells (>12 h), and nurse and forager bees were collected on days 10 and 20, respectively. More than 100 bees were collected from each of the five colonies; then, their HGs were dissected, and the samples were pooled and stored at -80°C for later analysis. Three independent biological replicates were produced. To ensure the consistency of morphological modifications with age, all colonies were managed with almost the same population, food, and brood during the nectar flow of chasteberry (*Vitex negundo* L.) in June. These factors controlled the variability of HG, which can be affected by the environment. The HG image of each age was taken by dissecting 30 bees, from the five colonies, under anatomical microscopy (Figure 1A). The average acini diameter of 30 HG at three ages was taken by a scanning electron microscope for statistical analysis, as our method describes (Figure S1).³

The HG tissue was mixed in ice by pestle homogenization prior to protein extraction. The sample was then mixed with a lysis buffer containing 8 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 20 mM Trisbase, 30 mM dithiothreitol (DTT), and 2% Biolyte (pH 3–10). To remove insoluble fractions, we centrifuged the sample at 15 000g at 4°C for 15 min. Proteins were precipitated by adding ice-cold acetone to the recovered

supernatant at -20°C for 30 min, then centrifuging twice at 15 000g at 4°C for 10 min. The pellets were dissolved in 40 mM $(\text{NH}_4)\text{HCO}_3$, then reduced with DTT (final concentration 10 mM) for 1 h to prevent reformation of disulfide bonds, and finally alkalinized with iodoacetamide (final concentration 50 mM) for 1 h in the dark. The protein sample was digested using sequencing-grade-modified trypsin (Promega, Madison, WI) at 37°C overnight.

2.3. Phosphopeptide Enrichment and LC–MS/MS Analysis

To produce an in-depth profile of the HG phosphoproteome, a protocol with complementary efficiency of phosphopeptide enrichment was applied using Ti^{4+} -IMAC and TiO_2 chromatography, as previously described elsewhere in another study.²¹ Phosphopeptides from each of the HG samples were analyzed on a Q-Exactive mass spectrometer coupled to a two-column EASY-nLC 1000 nanoflow system (Thermo Fisher Scientific). Samples were loaded onto a 2 cm long, 100 μm inner diameter fused silica trap column containing 5.0 μm Aqua C18 beads (Thermo Fisher Scientific) for 2 min in buffer A (0.1% acetic acid) at a flow rate of 5 $\mu\text{L}/\text{min}$ prior to analytical separation. Peptides were eluted from the analytical column (15 cm long, 75 μm inner diameter fused silica trap column filling with 3.0 μm Aqua C18 beads, Thermo Fisher Scientific) using 90 min gradient with following conditions: 100% buffer A (0.1% formic acid) to 8% buffer B (0.1% formic acid, 80% acetonitrile) for 5 min, 8–20% buffer B for 55 min, then 20–30% buffer B for 10 min, followed by increase to 100% buffer B for 5 min and 100% buffer B for an additional 15 min. The eluting peptides were directly infused into a Q-Exactive mass spectrometer (Thermo Fisher Scientific) via ESI. MS and MS/MS information were collected in a data-dependent mode using the following settings: one full scan (resolution 70 000 at m/z 400; m/z 300–1800), followed by top 20 MS/MS scans using high-energy dissociation in the linear ion trap mass spectrometer (resolution: 17 500, isolation window: 2 m/z , normalized collision energy: 27) using dynamic exclusion (charge exclusion: unassigned 1, >8; peptide match: preferred; exclude isotopes: on; dynamic exclusion: 10 s).

2.4. Database Search and Site Localization

Phosphoproteome raw data were retrieved using Xcalibur (version 2.2, Thermo Fisher Scientific) and were analyzed and processed using in-house PEAKS software (version 7.0, Bioinformatics Solutions). A database containing protein sequences of *Apis mellifera* (downloaded October 2013) and common contaminants was downloaded with a total of 21 778 entries. Search criteria were trypsin specificity, fixed modification of carbamidomethyl (C)/+57.02 Da, variable modifications of oxidation (M)/+15.99 Da and phosphorylation (S, T, Y)/+79.96 Da, and two allowed missed cleavages per peptide; one nonspecific cleavage at either end of the peptide; and three maximum allowed variable PTM per peptide. Precursor mass tolerance was set at 15.0 ppm, and fragment ion tolerance was set at 0.05 Da. The false discovery rate (FDR) was controlled at both the protein and peptide levels using a fusion-decoy database search strategy with a threshold $\leq 1.0\%$, an enhanced target-decoy approach that makes more conservative FDR estimations.²³ The phosphorylation sites were localized in HG proteins using Scaffold PTM (version 1.1.3; Proteome Software, Portland, OR) on the basis of Ascore algorithm.²⁴ Only when the site confidence was >95% was the site itself considered a mapped phosphorylation site. Abundances of phosphorylation sites were quantified via

spectral counting for each localized site for accurate quantitation of phosphorylation sites.²⁵

2.5. Validation of Phosphorylation Sites

To confirm the localized phosphorylation sites on HG proteins, we commercially synthesized four phosphorylation peptides using a solid-phase, peptide synthesis process (China Peptides, Shanghai, China). The MS/MS spectra compared the digested phosphorylation peptides from HG proteins and the synthetic phosphorylation peptides. When all major ions from the experimental spectrum aligned very closely with the ions from the synthetic version of the same peptide sequence at similar HPLC retention times, it was considered to have validated the sequence (retention time shift tolerance <0.2 min and >90% b or y ions were completely consistent).

2.6. Motif Analysis

Protein phosphorylation is catalyzed by its kinases and these enzymes work by recognizing specific sequence motifs which are defined by surrounding serine, threonine, or tyrosine residues in their substrates.²⁶ To extract motif from all phosphopeptides in the HG, all single phosphorylation sites with a localization confidence >95% were used by the motif-X algorithm (<http://motif-x.med.harvard.edu/motif-x.html>).²⁷ The background was the uploaded *Apis mellifera* proteome (a <10 m database that was randomly generated from *A. mellifera* proteome), the motif width was 13, occurrences were 20, significance was 1×10^{-6} , and motifs were extracted separately by pS, pT, and pY sites at position 7. The extracted motifs were used to determine the kinase classes (acidic, basic, proline-directed, tyrosine and others) based on substrate sequence specificity because the kinase specificity is often defined by amino acid motif surrounding Ser, Thr, and Tyr residue on the substrate proteins.²²

2.7. Quantitation of Phosphoprotein Abundance Level

To quantify the level alteration of phosphoprotein abundance during the three time points of HG development, the label-free strategy was performed with Progenesis LC–MS software (version 4.1, Nonlinear Dynamics, U.K.). Triplicates of each sample were subjected to software for subsequent quantitation analysis. After the data were quality controlled, one run was selected as a reference, followed by automatic feature matching for all runs, with manual editing to correct mismatched and unmatched feature detection. Thereafter, normalization and quantitation were carried out according to our previous method.⁹ The differences in the levels of phosphoprotein expression throughout the three development stages were determined by one-way ANOVA using a q value for multiple tests in the software. The differences of phosphoprotein abundance levels were taken as statistically significant when they contained at least two-fold changes and $p < 0.05$.

2.8. Bioinformatics Analysis

To better understand the biological implications of the identified phosphoproteome in the HG of the honeybee, we assigned *Drosophila* orthologs of bee proteins a Uniprot identifier by searching the Uniprot database. The unique identifiers of *Drosophila* orthologs of bee proteins, identified in the HG, were used as an input for functional analyses using ClueGOv2.1.6, a Cytoscape plug-in (<http://www.ici.upmc.fr/cluego/>).²⁸ The significantly enriched, functional gene ontology (GO) categories in biological processes and pathways were reported using a right-sided hypergeometric test. This test compares the background set of GO annotations in the entire

Drosophila genome and then does an FDR by Bonferroni step-down test to correct the *p* value. The nodes in functionally grouped networks were linked based on their kappa score level (0.4) in ClueGO.

Phosphorylation is one of the important effects in regulating physical interactions among proteins. To better understand these interactions among the phosphoproteins that are regulated during the HG development, we constructed protein–protein interaction (PPI) networks from large-scale interaction and functional association databases with GeneMANIA.^{29,30} High-confidence *Drosophila* data sets (passing the cutoff threshold in 3/3 experiments) were entered into GeneMANIA with the following settings: all networks enabled, equal weighting by biological process, and 10 related genes displayed. The GO category enrichment in the input data set was performed using a right-sided hypergeometric test in GeneMANIA as above, and the FDR was done by *q* values. Significantly enriched GO categories and their associated *q* values were reported. For each GO category enriched in the interaction network, the number of genes in the interaction network annotated in that category as well as the number of total genes in the *Drosophila* genome annotated with the same GO category were also reported.

2.9. Quantitative Real-Time PCR (qPCR)

To survey the differentially expressed proteins at the gene level in the ribosome pathway, we also ground the HG tissue by pestle in ice. Total RNA was extracted from the HG of differently aged bees using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions and quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The quality and the integrity of total RNA were tested by 1.0% agarose gel electrophoresis by visualizing the bands of 28S RNA, 18S RNA, and 5S RNA. Then cDNAs were generated using reverse-transcriptase kit reagents (Transgen, China). Two highly abundant proteins in the ribosome pathway were selected for qPCR analysis (60S acidic ribosomal protein p1, RpLP1, and 60S acidic ribosomal protein p2, RpLP2). qRT-PCR was performed using specific primers (RpLP1, forward: 5'-TGTTGATGTGGAATCTTAT-3', reverse: 5'-CAGGTTCTTCTTCTTCT-3'; RpLP2, forward: 5'-AATTATTATCTATGCCAGTTG-3', reverse: 5'-CCATATCATCATCTTCTGA-3') in triplicate. PCR amplification and data collection were conducted by a iQ5Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The reference gene used to normalize the data was glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Both the gene expression and statistical analysis of gene expression were performed according to our previous protocols.^{9,21}

3. RESULTS

3.1. Phosphoproteomic Profiling of Three Ages of HG

To obtain a large-scale data set of phosphorylation sites and phosphoproteins in the HG throughout the entire life cycle of the adult worker bees, we sampled the HG of newly emerged, nurse, and forager bees. A systematic investigation approach was performed with the complementary phosphopeptide enrichment method, using IMAC⁺⁺ and TiO₂ chromatography and MS-based high-throughput proteomics. Applying FDR cutoff criteria at the peptide and protein levels, localization of each site on each phosphopeptide was done using an Ascore algorithm at a 95% confidence level. (The annotated spectra of all phosphorylated peptides are shown in Figure S2; data

available at http://apisomics.com/publication/data/Supplementary_Figure_S2.pdf.) Given that the secreted proteins or RJ proteins may be present in the HG tissues, we predicted secreted proteins in our data set using SignalP³¹ (D-cut off for SignalP-noTM networks was 0.5 and the whole data set was analyzed) and carefully checked the currently 81 known RJ proteins.^{32–34} There were 17 proteins identified as secreted ones and removed from our proteins list (Table S1). Of the proteins expressed by the HG, 601, 755, 643 phosphopeptides carrying 555, 737, and 616 phosphorylation sites corresponding to 480, 586, and 569 proteins were identified by TiO₂ chromatography, while 786, 1100, and 976 phosphopeptides harboring 738, 1111, 1027 phosphorylation sites in 523, 681, 661 proteins were identified by IMAC⁺⁺ enrichment in newly emerged, nurse, and forager bees, respectively (Tables S2 and S3). By applying the complementary strategy of phosphopeptide enrichment using IMAC⁺⁺ and TiO₂ consecutively, a substantial increase in the number of phosphopeptides was identified and in turn produced a significant increase in phosphoproteome coverage in the HG (Figure S3, Table S2). The final data set contained 941, 1322, and 1196 phosphorylation sites, matching 1007, 1353, and 1199 phosphopeptides (Table S2) from 549, 720, and 698 phosphoproteins (Table S3) in the newly emerged, nurse, and forager bees, respectively. Overall, the identified phosphorylation sites and phosphoproteins across the three ages of the HG were assigned as 1936 nonredundant sites and 954 protein forms (Table S3). All of the observed phosphorylation sites and phosphoproteins were previously unreported. Four representative phosphorylation sites were validated from artificial synthetic phosphopeptides (Figure S4).

3.2. Age-Dependent Distribution of Phosphorylation Sites

After evaluating the number of matched phosphopeptide spectra, unique sites, and total phosphoproteins, an age-specific variation was observed. The nurse bees have the highest numbers of identified phosphopeptides, phosphorylation sites, and phosphoproteins, while newly emerged bees have the lowest numbers (Figure S5A), indicating the difference in complexity and intracellular signaling across age-related HGs.

When examining the frequency among all identified phosphosites, Ser phosphorylation was the most observed (88.0%), followed by Thr (11.4%) and Tyr (0.6%). Phosphorylation at Ser residues, rather than Thr and Tyr, comprised the vast majority of identified phosphosites, reflecting a strong priority for Ser phosphorylation in this study (Figure S5B), and this tendency is consistent over the three ages (Figure S5B). Through analyzing the numbers of sites within each phosphoprotein, 57.1% of proteins were phosphorylated on 1 residue, whereas 42.8% were phosphorylated on 2 or more residues and only 1.5% were phosphorylated on more than 10 sites (Figure S5C). In all, the most HG proteins were phosphorylated on single site and some were modified on multiple sites.

Of the identified phosphoproteins, only a small number of Ser, Thr, and Tyr residues within each protein were phosphorylated (Figure S5D). In general, 2.07% of these residues were phosphorylated, whereas the extent of modification for each residue varied: Ser, 3.82%; Thr, 0.83%; Tyr, 0.06%. Although similar extents of phosphorylation were observed between nurse and forager bees, a relatively low extent occurred in the HG of newly emerged bees (Figure S5D).

Table 1. Abundant Age-Specific Phosphorylation Sites in the Hypopharyngeal Gland of Honeybee Workers at Three Ages, As Determined by Spectral Counts^a

age	annotation	accession	novel? ^b	site	class ^c	newly emerged ^d	nurse ^d	forager ^d	motif sequence
newly emerged	intracellular protein transport protein USO1	gil328787343	Y	S1169	A	5	0	0	NNKDYGSDAELGS
	periodic tryptophan protein 1 homologue	gil328787457	Y	T155	P	3	0	0	EEIIQCTPPTSTS
			Y	S111	A	15	0	0	TKEDDDSEKEDDI
	nucleolar protein 12	gil328785951	Y	S45	A	31	0	0	ELQDVESDLDEEH
			Y	S166	A	11	0	0	SSLADESDEDD
	AP2-associated protein kinase 1 isoform X1	gil328784406	Y	S142	P	5	0	0	AIPSSPSVPSPGG
nurse	zygotical DNA replication licensing factor mcm3	gil328788939	Y	S702	A	11	0	0	DPYNFESDDDSYI
	putative glycogen [starch] synthase	gil66549280	Y	S671	A	12	0	0	AASVHGSDEEDEV
	calcium homeostasis endoplasmic reticulum protein isoform X2	gil328787605	Y	S612	P	11	0	0	QKSKSPILRPR
	splicing factor 3B subunit 2-like	gil110756801	Y	T596	P	0	5	0	EMEGGDTPALYTV
	heat shock protein beta-1-like isoform X1	gil110757651	Y	S127	P	0	34	0	WLDGLNSPLIQDE
	histone-lysine N-methyltransferase SETD1B-like isoform 1	gil328778989	Y	T906	P	0	7	0	TPTGRETPIPNIK
	putative leucine-rich repeat-containing protein DDB_G0290503-like isoform X3	gil328784596	Y	T684	P	0	27	0	EQSGYNTPPKLDLP
	trichohyalin	gil328789935	Y	S541	A	0	28	0	PSKPSESEEEPEA
			Y	S539	A	0	20	0	PKPSKPSESEEEP
	caskin-1-like	gil335892850	Y	S332	P	0	8	0	GGPDLPSPDEDCS
forager	eukaryotic translation initiation factor 3 subunit B isoform 1	gil66548942	Y	S33	A	0	9	0	DEEPNFSDPGEFI
	autophagy 1	gil66564768	Y	S199	A	0	57	0	DKWESFSEGEIHH
	elongation factor 1-beta'	gil66565249	Y	T73	P	0	32	0	LPGEKKTVPVLGN
	microfibrillar-associated protein 1	gil110766351	Y	T86	P	0	0	4	NEISADTPTHSQI
	heat shock protein 90	gil229892248	Y	S207	A	0	0	21	SGDEMCSLKDYVG
	zinc finger protein DPF3-like isoform X2	gil328778645	Y	S388	P	0	0	26	HLPAVPSPGTAVP
	nitrilase homologue 1-like	gil328782156	Y	S107	P	0	0	1	LSNGNASPQIITK
	forkhead box protein O isoform X1	gil328784251	Y	S50	P	0	0	15	HTWPLPSPEEYIE
	histone deacetylase Rpd3 isoform 1	gil328788017	Y	S383	A	0	0	4	PGVQVQSIPEDGA
	another transcription unit protein	gil328792720	Y	S545	A	0	0	20	TYHDDGSDDEGAI
	eukaryotic translation initiation factor 3 subunit E-like isoform X2	gil328792782	Y	S397	P	0	0	4	MGGQPASPYQQLV
	raf homologue serine/threonine-protein kinase phl	gil66554913	Y	S363	P	0	0	15	SQSTQASPTSTLR

^aTable 1 chooses abundant phosphorylation sites that were found in only one of newly emerged, nurse, and forage hypopharyngeal gland, listed by protein and bees of different age. Age-specific sites are shown for all three aged hypopharyngeal glands of honeybee workers. ^bNovel site is decided according to whether the site has been reported with protein post-translational modifications in *Apis mellifera* or not. ^cSite class is assigned by a decision tree algorithm: A = acidic; B = basic; O = other; P = proline-directed; T = tyrosine. ^dNumber of spectral count in a protein of newly emerged, nurse, and forager bees.

To evaluate site distribution, we counted the number of phosphorylation sites observed in each age of HG samples (Figure 1B). Nearly 47.84% of the sites were uniquely detected in a single-aged sample, while about 27.22% were shared among the three-aged samples and about 24.94% were found in two of the samples. Of the unique sites observed in three of the HG samples, most were found in nurse bees (46.96%), foragers had 30.87%, and the newly emerged bees contained 22.17%. To better assess age-dependent distribution, we show in Table 1

the representative age-specific phosphorylation sites. These sites are derived from proteins with variable levels of abundance, including nucleolar protein 12 and periodic tryptophan protein 1 homologue, which were highly expressed in newly emerged bees. On the contrary, heat shock protein beta-1-like isoform X1 and the trichohyalin were found in low quantities in newly emerged bees and foragers, respectively. For comparison, Table 2 shows the representative proteins bearing global phosphorylation sites. Examples were 60S acidic

Table 2. Abundant “Common” Phosphorylation Sites in the Hypopharyngeal Gland of Honeybee Workers at Three Ages^a

annotation	accession	novel? ^b	site	class ^c	newly emerged ^d	nurse ^d	forager ^d	motif sequence
60S acidic ribosomal protein P2	gil 110765226	Y	S101	A	364	764	545	KPAKEESESEDDDD
stomatin-like protein 2 mitochondrial-like isoform 1	gil 328783826	Y	S374	A	20	39	41	ETFEYFSDKEEAE
AP2-associated protein kinase 1-like isoform X1	gil 328784406	Y	S145	P	7	13	6	AIPSSPSVSPGG
RNA-binding protein Nova-1-like isoform X1	gil 328784994	Y	S12	P	12	9	7	GMETCPSPEIADS
ralBP1-associated Eps domain-containing protein 2-like isoform X1	gil 328786568	Y	T344	P	10	12	11	TTTTTQKTPPNTSG
DNA topoisomerase 1 isoform 2	gil 328786781	Y	S376	A	32	21	24	IKTEEESDEDVPL
septin-1-like	gil 328787592	Y	S271	A	38	30	43	PLPDCDSDEDEDY
putative oxidoreductase GLYR1 homologue	gil 328789575	Y	T196	P	67	22	23	NIARPVTPLDVE
tropomodulin isoform 1	gil 328780029	Y	S127	P	16	7	8	SYECDKSPTGPLN
transcription factor E2F4	gil 328787371	Y	S223	A	11	10	8	KPDPVISDEDEED
serine/arginine repetitive matrix protein 2 isoform X1	gil 328780193	Y	S172	P	49	37	16	ELVRTSPAPDTT
calcium homeostasis endoplasmic reticulum protein-like isoform X2	gil 328787605	Y	T170	P	43	33	16	VYELVRTSPAPD
		Y	S691	P	52	49	20	MDRQDRSPTPPSF
CD2 antigen cytoplasmic tail-binding protein 2 homologue	gil 328787392	Y	T693	P	52	49	20	RQDRSPTPPSFLG
		Y	S125	A	13	25	21	THGLADSDSEDEG
enhancer of mRNA-decapping protein 4	gil66500463	Y	S127	A	13	20	14	GLADSDSEDEGPD
		Y	S421	P	6	16	5	ESLEDLSPCEDDI
splicing factor 1-like isoform X2	gil66509531	Y	S416	A	5	13	5	VRPTGESLEDLSP
		Y	S144	P	41	29	49	LNPEERSPSPEPI
RNA-binding protein 26	gil 328780081	Y	S146	P	43	29	48	PEERSPSPEPIYS
		Y	T91	P	43	12	10	DPDGGGTTPPGINP
zinc finger CCCH domain-containing protein 18 isoform X4	gil 328782605	Y	T284	P	11	8	5	GSPGGGTPTQDSN
		Y	S619	P	51	55	50	GTPHSVSPPHTR
probable ATP-dependent RNA helicase DDX46	gil 328786029	Y	S813	P	48	38	44	LNPPAPSPLSSHQ
		Y	S241	A	15	15	12	LDGEQISDGDIED
5'-AMP-activated protein kinase subunit beta-1 isoform 1	gil48113496	Y	S182	A	13	7	7	WSLEDDSDDEETPV
		Y	S800	A	2	8	6	ALGLQSDDEDIE
u4/U6.U5 tri-snRNP-associated protein 1 isoform 1	gil66513420	Y	S177	A	20	25	11	LVSRESDFEVFQ
		Y	S32	P	34	27	46	KEYPPSPGKEGQ
		Y	S53	A	45	38	43	ERKDYDSVIEVN
		Y	S179	A	13	9	7	SLGECSDDDDTKN

^aTable 2 chooses abundant phosphorylation sites that were found in the hypopharyngeal glands of newly emerged, nurse, and forager bees (*Apis mellifera* L.), listed by protein. See also Tables S1 and S2. ^bNovel site is decided according to whether the site has been reported with protein posttranslational modifications in *Apis mellifera*. ^cSite class is assigned by a decision tree algorithm: A = acidic; B = basic; O = other; P = proline-directed; T = tyrosine. ^dNumber of spectral counts in a protein of newly emerged, nurse, and forager bees.

ribosomal protein P2, and 5'-AMP-activated protein kinase subunit beta-1 isoform 1. Although some sites were modified in all three ages of samples, extensive age-specific phosphorylation signifies the biological implication of time-resolved phosphoproteomics. First, even if commonly expressed proteins displayed significantly different phosphorylation profiles across ages, the heavily phosphorylated intracellular protein transport protein USO1 (10 sites) harbored age-specific sites (S1169 and T155). Second, many proteins were expressed only at a distinct age and could be phosphorylated only at the specific age of the HG in which they were expressed. The proteins nitrilase homologue 1-like (forager), putative leucine-rich repeat-

containing protein DDB_G0290503-like isoform X3 (nurse), and nucleolar protein 12 (newly emerged) were found only in single ages. These observations indicate that temporal analysis is effective for comprehensive profiling of phosphoproteomics in the honeybee HG.

To compare the phosphorylation profiles for the three time-points of HG development, we used total spectral counts for hierarchical clustering to estimate each site's abundance level within each age of the HG (Figure 1D). On the basis of their site clustering phosphorylation profiles, the nurse and forager bees showed similarly high levels of site abundance, likely

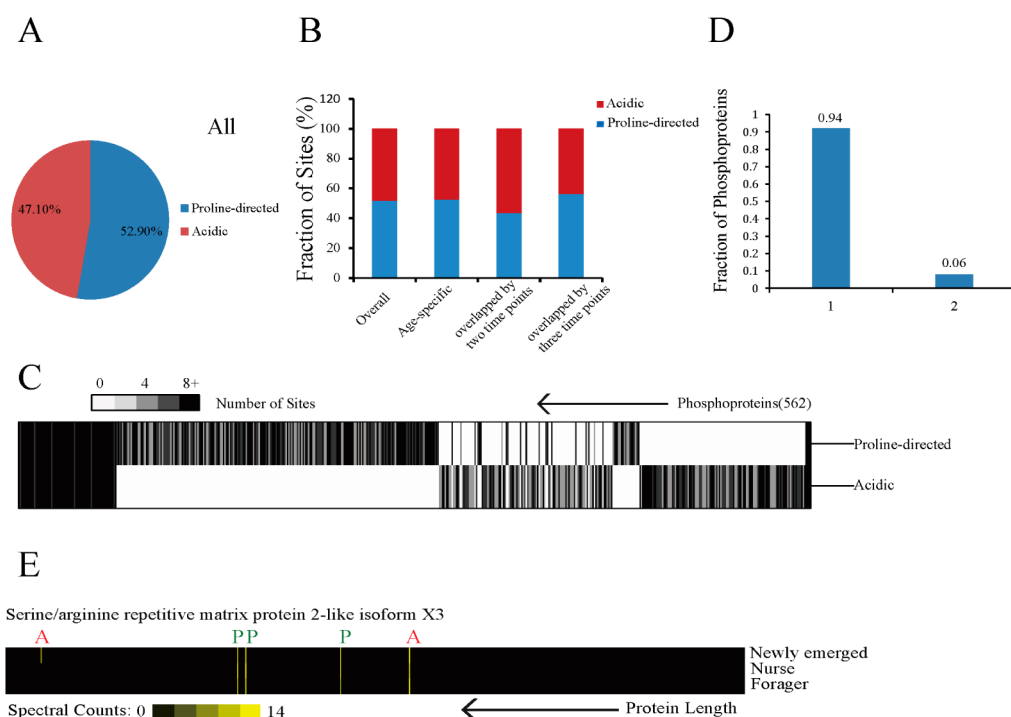


Figure 2. Overview of phosphosite classes across different ages of the hypopharyngeal gland and phosphoproteins. Phosphosites are grouped as acidic, basic, proline-directed, tyrosine, or other. (A) Frequencies with which each class is observed overall and for each age of hypopharyngeal gland are plotted. (B) Numbers of sites of each class observed for 301 phosphoproteins. Proteins and site classes are clustered to highlight similarities. (C) Proportions of phosphoproteins containing phosphorylation sites from different numbers of classes. (D) Relative proportions of age-specific and shared phosphorylation sites in each class. (E) Age-specific distributions and relative abundances of phosphosites along the lengths of selected proteins. Sites are labeled according to their classes: A = acidic; P = proline-directed. See also [Tables S1, S2, and S3](#).

indicating stronger and more distinct HG secretory activity of cell signaling compared with that of newly emerged bees.

3.3. Two Kinase Classes Phosphorylated Most HG Proteins

Kinases in different pathways often utilize the same general motif; therefore, using this simplified kinase categorization, all kinase can be divided into five classes (acidic, basic, proline-directed, tyrosine, and others) on the basis of a decision tree.²² This indicates that the known motif decides the specific kinase class. On the basis of the extracted motif from the phosphorylation data set, we found only proline-directed and acidic kinase classes with almost equal abundance (motif information seen [Table S4](#)). This suggests that phosphorylation events in the HG are mainly accomplished through these two distinct kinase classes to maintain its protein phosphorylation networks ([Figure 2A](#)). Again, when sites were grouped into age-specific, shared by two ages, and globally abundant phosphorylation, similar proportions of the two site classes were observed ([Figure 2B](#)).

When analyzing the distribution of phosphorylation classes within each phosphoprotein and hierarchical clustering, a heat map represented a quite distinct pattern ([Figure 2C](#)). 94% percent of phosphoproteins bore sites from single kinase classes, either proline-directed or acidic, and 6% had sites from two classes ([Figure 2D](#)). Among these, two variably phosphorylated proteins are serine/arginine repetitive matrix protein 2-like isoform X3, an enzyme involved in mRNA processing, and abhydrolase domain-containing protein DDB_G0269086-like isoform X1 ([Figure 2E](#)). Each was phosphorylated across its length and contained sites targeted to two kinase classes. Individual sites showed distinct age-related profiles. In some cases, pairs of sites within the same

class showed similar phosphorylation patterns; however, even within the same protein, different sites within the same class often showed variable patterns of modification.

3.4. Comparing Protein Abundance and Phosphorylation Measurements Reveals Real Differential Protein Phosphorylation

Protein phosphorylation is usually represented in a variation of protein abundance and site-specific phosphorylation. To differentiate between these two mechanisms, we compared our phosphoproteomic data with previously reported proteomic ones^{3,14,35} in the HG across adult life stages. Overall, 1834 proteins were identified in the HG, only 11.5% of which overlapped between two data sets ([Figure 3A](#), [Table S5](#)). This overlap was consistent over the age groups ([Figure 3B](#)). 880 proteins were exclusively identified in nonphosphorylated forms, whereas 744 proteins were detected in the phosphorylation data alone. The almost equal number of phosphoproteins identified here compared with the previously reported proteome of HG^{3,14,35} and other eukaryotic proteins (1/3 phosphorylated)³⁶ indicates that our protocol is robust and covers the phosphoproteome to an unprecedented degree in the honeybee.

To test whether changes in phosphorylation might be actually due to changes in protein expression, we also measured changes in protein abundance at each time period. The overall expression profiles of phosphoproteome and proteome^{3,14,35} were not always consistent over the age development ([Figure S6A](#)). To further focus on the overlap of phosphorylated and nonphosphorylated proteins (210 proteins), we clustered their abundance levels based on spectral counts within each age of the HG. Interestingly, the proteome profile was relatively stable

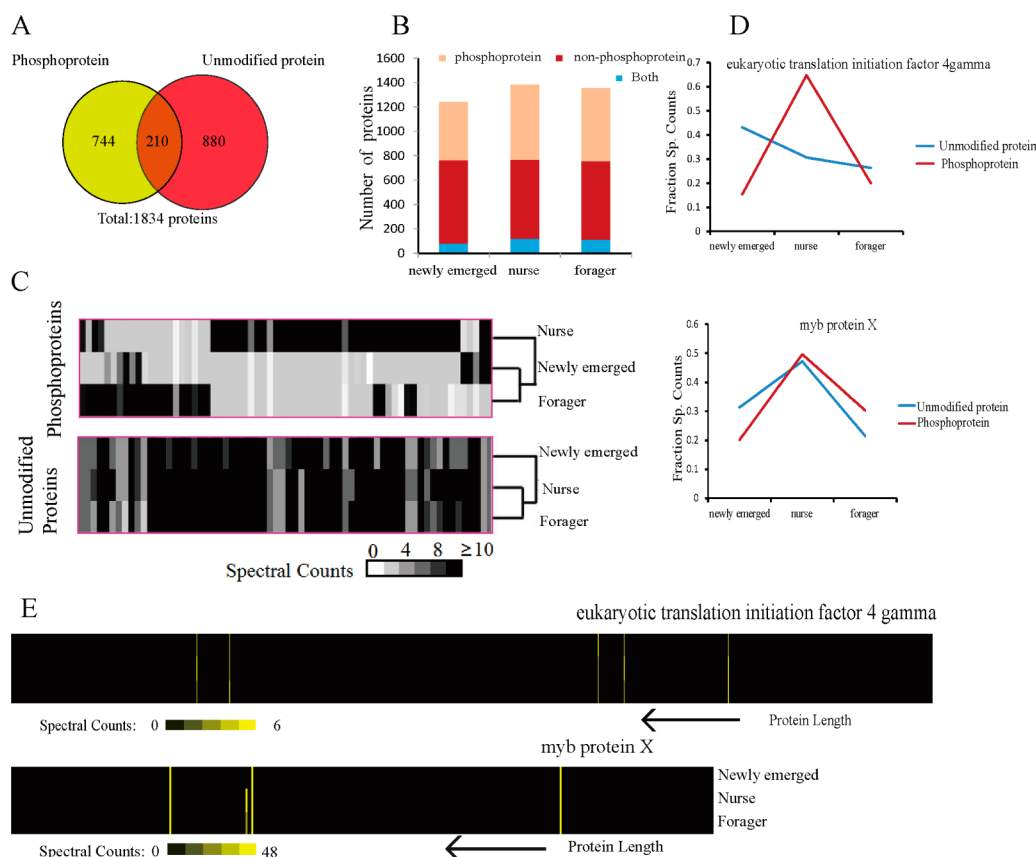


Figure 3. Cross-age comparison of phosphoprotein and nonphosphoprotein^{945,46} expression in the hypopharyngeal gland of honeybee workers. Overlap among phosphoproteins and unmodified proteins, overall (A) and in each age (B). (C) Clustering of proteins based on spectral counts of phosphoproteins and nonphosphoproteins. Columns indicate 211 overlapped proteins, and rows represent different ages of hypopharyngeal gland. Upper and lower panels represent expression profiles of 211 overlapped phosphoproteins and nonphosphoproteins. (D) Phosphorylated and nonphosphorylated abundance profiles for selected proteins. (E) Heat maps depicting spectral counts observed across different ages of the hypopharyngeal gland for sites along the length of each protein, reflecting variable phosphorylation within proteins. See also Tables S1, S2, S3, and S4.

over the course of HG development, whereas the phosphoproteins were more variably expressed than their unmodified forms (Figure 3C). For example, the expression profiles of phosphorylated and nonphosphorylated eukaryotic translation initiation factor 4 gamma (Figure 3D-1) and protein LSM14 homologue B-B were quite divergent (Figure S6B). On the contrary, phosphorylated and nonphosphorylated myb protein X (Figure 3D-2) and heterogeneous nuclear ribonucleo protein Upotein 1 were consistent (Figure S6B). Moreover, considerable variability was observed for four proteins' individual sites over the age development stages (Figure 3E, Figure S6C). Collectively, these results indicate that protein abundance and phosphorylation levels are not correlated and in turn reveal real an age-dependent differential phosphorylation event during HG development.

3.5. Functional Category and Pathway Enrichment of Phosphoproteins

To determine whether the identified phosphoproteins play a role in any biological/signaling pathways, the identified phosphoproteins in the three ages of the HG were analyzed and compared using ClueGO to detect significantly enriched KEGG pathways and GO terms. In the young HG, mitotic spindle elongation ($p = 1.53 \times 10^{-12}$), centrosome cycle ($p = 1.87 \times 10^{-17}$), negative regulation of protein complex disassembly ($p = 5.72 \times 10^{-6}$), and the ribosome ($2.39 \times$

10^{-15}) were significantly enriched (Figure 4A, Table S5). In nurse bees, centrosome cycle ($p = 1.62 \times 10^{-11}$), mitotic spindle elongation ($p = 1.68 \times 10^{-12}$), macromolecular complex disassembly ($p = 1.58 \times 10^{-7}$), and ribosome ($p = 5.25 \times 10^{-16}$) were also significantly enriched (Figure 4B, Table S5). Finally, mitotic spindle elongation (2.47×10^{-17}), centrosome organization (7.69×10^{-17}), and ribosome (1.75×10^{-25}) were also significantly enriched in the forager HG (Figure 4C, Table S5). The multiplicity of the enriched different functional categories suggests that the HG employs a distinct global phosphorylation modification signaling at different ages, with a role in modulating the function of different classes of proteins to support the physiological role of the HG. Strikingly, ribosome pathways were commonly enriched across differently aged developmental stages, showing that the translation process is highly regulated by phosphorylation. To gain a deeper insight into the differences of the biological roles, our phosphoproteome was compared with a nonphosphoproteome.³⁵ We found that the pyruvate metabolic process, muscle cell development, and pole plasm mRNA localization were complementarily enriched in both young and nurse bees, whereas only the first two groups were complementarily enriched in foragers in an unmodified data set (Figure 4D–F, Table S6).

To gain further insight into functional categories that are highly activated at different stages of the HG development, we

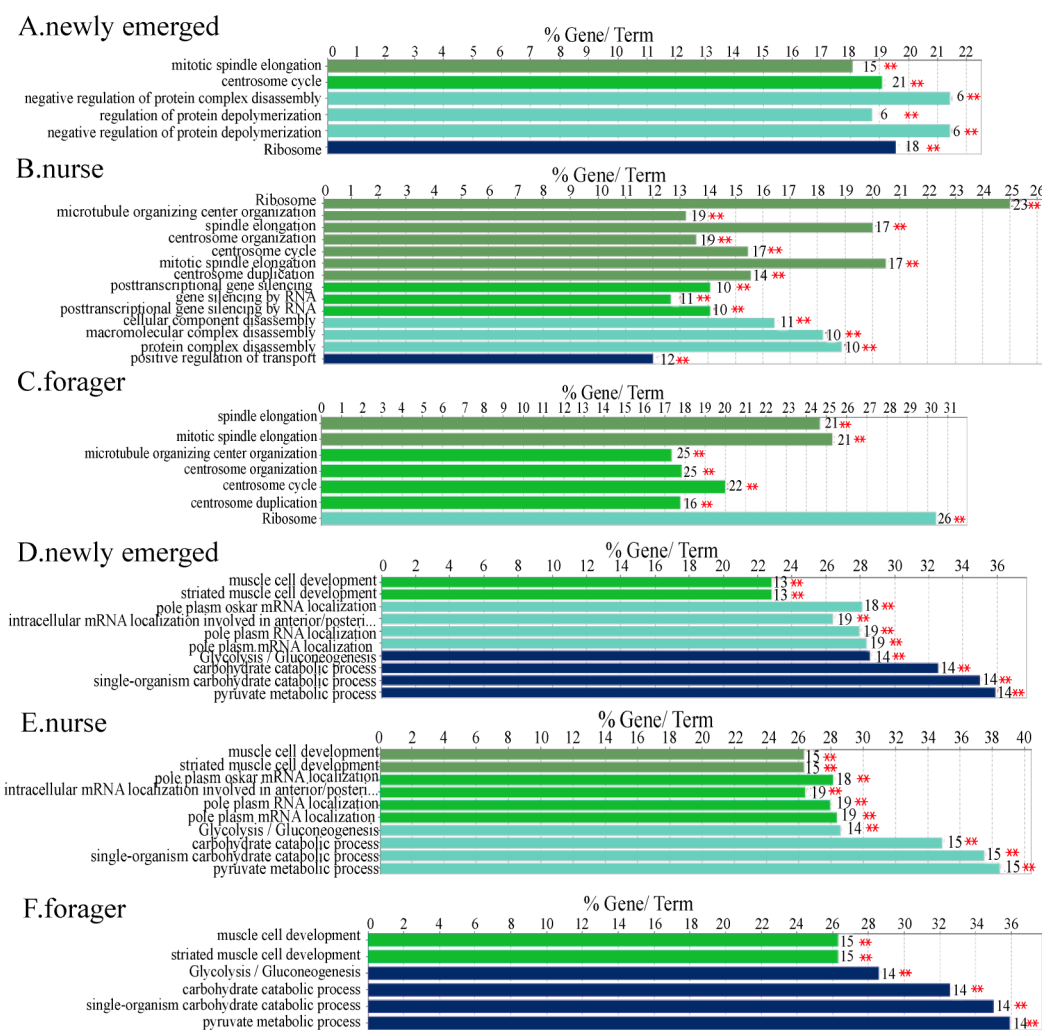


Figure 4. ClueGO analysis of phosphoproteins from the hypopharyngeal gland of honeybee workers. GO biological process and KEGG pathway terms (first letter capitalized) specific for phosphoproteins and nonphosphoproteins from the hypopharyngeal gland of honeybee workers (**, $p < 0.01$; *, $p < 0.05$). The number of corresponding genes associated with a specific term are indicated. The percentage of genes associated with a specific term is listed on the bars. (A–C) Enriched GO and KEGG pathway terms of phosphoproteins identified from the hypopharyngeal gland of honeybee workers at three ages. (D–F) Comparison of enriched GO and KEGG pathway terms between phosphoproteins nonphosphoproteins in the hypopharyngeal gland of honeybee workers at different ages.

also analyzed 238 phosphoproteins with a change of expression in levels throughout the HG growth (Figure 5, Table S7). Among the 238 differential phosphoproteins, expression levels of some proteins were in accordance with the site abundance levels such as 60S acidic ribosomal protein P2, while others were not, such as nipped-B protein precursor (Figure 5), further suggesting that phosphorylation is not related to the protein expression level. Of the 132 up-regulated phosphoproteins in the young HG, mitotic spindle elongation ($p = 7.63 \times 10^{-5}$), regulation of cell size ($p = 7.18 \times 10^{-5}$), and centrosome organization ($p = 1.84 \times 10^{-7}$) were enriched. Among the 45 up-regulated proteins in nurse bees, nucleus organization ($p = 6.25 \times 10^{-4}$), regulation of cell size ($p = 0.0013$), cellular component maintenance ($p = 5.03 \times 10^{-4}$), and maintenance of location in cells ($p = 0.0018$) were enriched. In the foragers, positive regulation of the cellular catabolic process ($p = 1.13 \times 10^{-5}$), lipid localization ($p = 3.64 \times 10^{-5}$), and extracellular structure organization ($p = 2.23 \times 10^{-5}$) were enriched for the 61 up-regulated proteins (Figure S7, Table S6). In general, phosphorylation is highly induced in these functional categories

to enhance the distinct biological demands of the HG during different physiological stages.

3.6. Age-Specific Kinome Profiles

Kinases are pivotal regulators of phosphorylation dynamics in cellular signaling and in turn are often regulated by phosphorylation. Of the 51 identified kinases, 29, 41, and 34 were found in newly emerged, nurse, and forager HGs with some being globally expressed and some being age-specific (Figure 6A). In general, similar kinome profiles were observed in the nurse and forager HG, whereas the young HG was distinct. For instance, 5'-AMP-activated protein kinase subunit beta-1 isoform 1, a positive regulator of AMP-activated protein kinase (AMPK) activity affecting the enzyme activity and cellular localization of AMPK via phosphorylation,³⁷ was down-regulated as age increased. Dual-specificity mitogen-activated protein kinase kinase hemipterous, associated with establishment of the dorsal epidermis,³⁸ was up-regulated, while cyclin-dependent kinase 10 was consistently expressed over the different ages (Table S8). Despite larger numbers of proteins having been identified in unmodified forms in the HG,³⁵ only

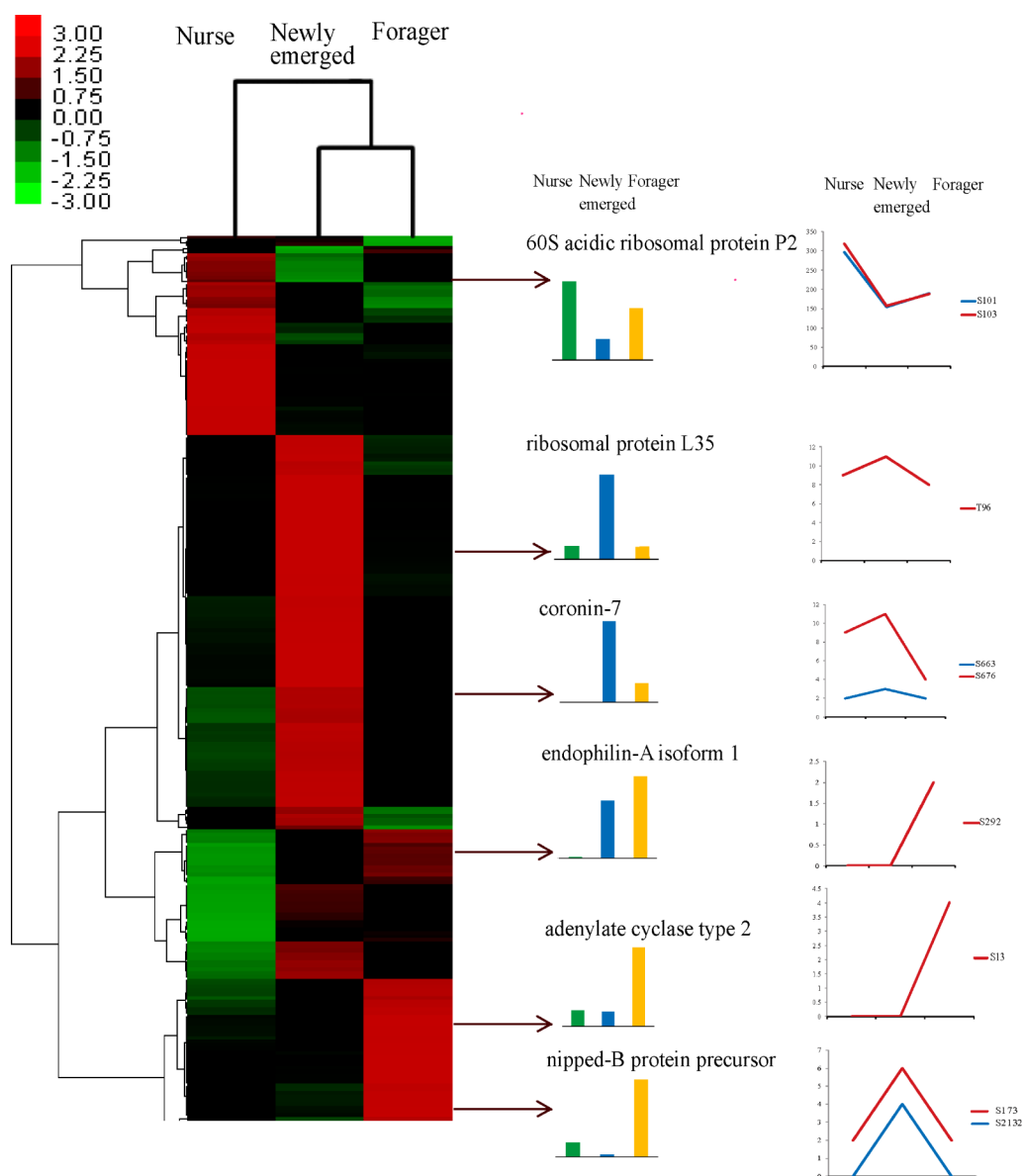


Figure 5. Hierarchical clustering of the differentially expressed phosphoproteins (fold change ≥ 2 and $p < 0.05$) in the hypopharyngeal gland of honeybee workers. The columns represent the age of the hypopharyngeal gland, and the rows represent the individual proteins. The up- or down-regulated phosphoproteins are indicated by a red and green color code, respectively. The color intensity changes within the phosphoprotein expression level are noted on the color bar on the top left. The histograms denote the expression trend of the representative proteins and their site abundance of a phosphoprotein expressed in the hypopharyngeal gland at three time points.

26 kinases were identified, and merely 5 overlapped in the current data set (Figure 6B, Table S8).

3.7. Age-Specific Phosphorylation within Protein Interaction Networks

When mapping phosphoproteins identified in each development stage of the HG onto a PPI network, all of the significantly enriched functional categories in the above GO functional analysis were also statistically overrepresented in the interaction network (Figure S8). This further supports the fact that phosphorylation is an important regulator of protein function in the PPI for a well-functioning HG. Protein phosphorylation was also significantly overrepresented in the network. Of the commonly represented functional categories and pathways, that is, mitotic spindle elongation and ribosomes, each interaction network shows distinct phosphorylation patterns (Figure S8A). One representative category is that of

mitotic spindle elongation, one of the most dramatic and highly regulated steps during the cell division process of the metaphase-to-anaphase transition. The high number of phosphorylated proteins implicated in mitotic spindle elongation in network phosphorylation is in line with the thinking that spindle elongation is highly controlled by phosphorylation, coordinating with cell cycle progression.³⁹ As for the protein phosphorylation, it also displayed age-specific network expression; some kinases commonly existed in the phosphorylation network such as CDK 12, while some were expressed in the network in an age-related manner such as wts (serine/threonine-protein kinase ULK2), which was only represented in the phosphorylation network of nurses and foragers.

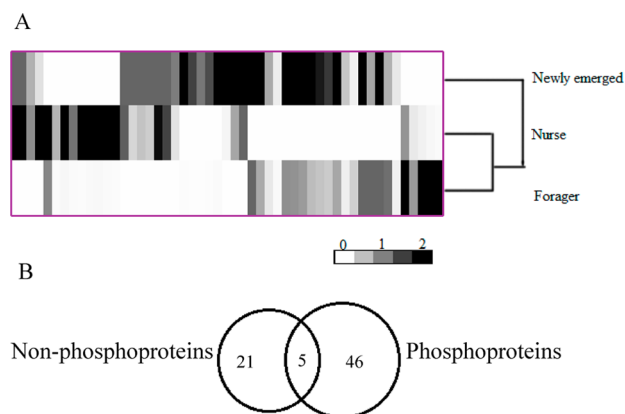


Figure 6. Overview of kinase in the hypopharyngeal gland of honeybee workers (*A. m. ligustica*). (A) Age distribution of protein kinase in the hypopharyngeal gland of honeybee workers at three ages. The kinase expression profile is visualized as a heat map according to Table S7. (B) Comparison of phosphorylated and nonphosphorylated kinase in hypopharyngeal gland of honeybee workers.

3.8. Combining Phosphorylation Data with Signaling Network Maps Reveals Age-Specific Differences

Reversible protein phosphorylation modifications are essential to cell-signaling networks in transmitting environmental cues. They must sense and respond to signals from the surroundings of a cell to relay and amplify the initial signal through tandem and ordered phosphorylation of constituent pathway proteins. As one of the most ubiquitous and important pathways in the HG, the ribosome is the workhorse of biological protein synthesis (translation). It mediates cellular responses that the small ribosomal subunit senses and reads the RNA message, and the large subunit forms amino acids into a polypeptide chain.⁴⁰ To survey differences in ribosome signaling across different ages of the HG, we overlaid each aged phosphoproteomic profile onto a ribosome pathway (Figure 7A). As expected for a central signaling pathway, much of the network was globally utilized; however, age-specific patterns were also apparent. Although signaling from 60S acidic ribosomal proteins P1, P2 (LP1, LP2) to 40S ribosomal proteins S3a (S3Ae) and 40S ribosomal proteins S27 (S27) was found in all three ages of HG, 40S ribosomal protein S16 (S16e) was phosphorylated in both nurses and foragers, and 60S ribosomal protein L7a (L7Ae) and 40S ribosomal protein S11 (S11e) were modified only in newly emerged and forager HGs, respectively (Figure 7A). We selected highly abundant LP1 and LP2 with altered levels of expression over the three ages to examine the tendencies of expression at both the gene and protein levels. The two proteins displayed a consistent trend in expression between the protein and its encoding gene (Figure 7B).

4. DISCUSSION

The HG of the honeybee is a functional center involved in both the production of brood food (RJ) in young nurse bees and the digestion of enzymes in older foraging workers.⁵ The same group of secretory cells of the HG expresses different genes/proteins in accordance with changes in behavior to accomplish this significant role change with over time.^{14,41} The HG could therefore be a unique and excellent model for a survey of signaling pathways in a single organ. A state-of-the-art technology combining complementary IMAC⁴⁴ and TiO₂

chromatography for phosphopeptide enrichment and high-accuracy mass spectrometric characterization was employed. We identified thousands of phosphorylation sites in HG proteins, demonstrating that this current strategy is effective for exploring the honeybee phosphoproteome in unprecedented depth. A holistic view of the HG phosphoproteome reveals that a large proportion of cellular proteins are phosphorylated during the course of HG development. Almost 60% of proteins were phosphorylated in a single phosphorylation site, suggesting that the majority of a protein's function in the HG is regulated by a single pathway or that a protein's cellular activity is achieved at single distinct sites to fulfill the function of the HG. For those multiply phosphorylated proteins, they may not necessarily occur on single protein molecules concurrently. Were they to do so, a single protein's cellular activities and interactions could arise from independent kinase regulation at distinct sites via multiple pathways. After analyzing phosphorylation events at various time points throughout HG development, a number of general interests have come to light.

4.1. Age-Dependent Phosphorylation Networks Are Specialized in Differently-Aged HG

When comparing phosphorylation profiles across the three age points of the HG, we have found several profound biological implications. (1) Only about 25% of phosphosites are shared; half of the sites are unique to a single age point in the HG. (2) Two kinase classes (acidic and proline-directed) span all signaling scenarios during the whole process of HG development. (3) The kinome shows an age-dependent profile. (4) Both individual proteins and the entire phosphoproteome display age-specific phosphorylation. All of these facts clearly suggest that phosphorylation networks start to rise upon physiological demand to optimize cell performance of the HG in different developmental stages.

The substantial evidence in support of age-specific phosphorylation in the HG is evident in the following cases. First, the distinct phosphorylation profiles identified at the three time-points of HG development suggest that phosphorylation signaling within the HG cells acts in accordance with the very distinct physiological roles of the worker bees; that is, the nurse HG mainly secretes RJ to feed the brood, while the forager HG mainly secretes enzymes for the conversion of nectars into honey.⁶ The highest numbers of phosphopeptides, phosphorylation sites, and phosphoproteins are identified in nurses, indicating that high RJ-secretion activity in the HG activates the complex phosphorylation networks to boost the ability of RJ secretion. Similarly, higher numbers in the forager HG are indicative of phosphorylation signaling networks still active in HG secretory cells, priming the forager for efficient conversion of nectar into honey; however, the low number in newly emerged bees suggests the fact that the young HG employs a relatively simple signaling cascade that initiates the main task of cell growth and proliferation at this age. Second, the observed similarity of the extent of phosphorylated proteins in the HG of nurses and foragers and a relatively low extent in newly emerged bees indicate that the different ages of HG employ distinct phosphorylation events to consolidate this age-dependent physiology. For example, some proteins with a high number of phosphorylation sites varied in the extent of residue phosphorylation at different time points: zinc finger CCCH domain-containing protein 18 isoform X4 (18, 16, and 17 sites in newly emerged bees, nurses, and foragers respectively) is implicated in transcriptional machinery; serine/arginine repet-

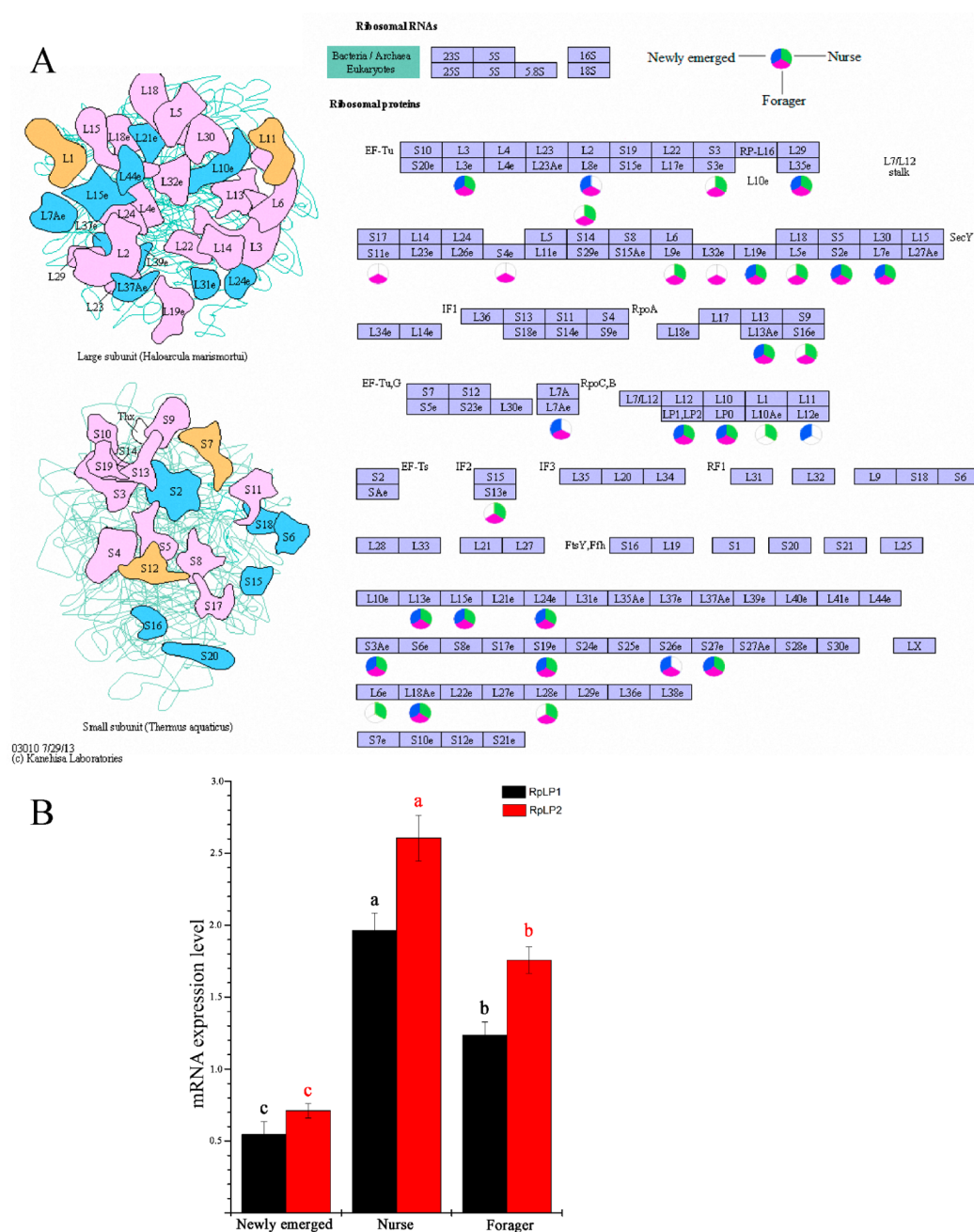


Figure 7. Mapping phosphorylation data with known signaling pathways and qPCR analysis of selected phosphoproteins in ribosome pathway. (A) Ribosome pathway that is significantly enriched among the phosphoproteome of the hypopharyngeal gland of honeybee workers at three ages of development. Red highlighted proteins indicate they were phosphorylated in each age of hypopharyngeal gland. Each protein is represented as a node containing up to three sections of a circle, each indicating the presence of phosphorylation in one age of hypopharyngeal gland. See also Tables S1, S2, and S5. (B) qPCR analysis of RplP1 and RplP2.

itive matrix protein 2-like isoform X3 (11, 11, and 9 sites in newly emerged, nurse, and forager respectively) is involved in mRNA splicing; and cyclin-dependent kinase 12-like isoform X2 (12, 8, and 13 sites in newly emerged, nurse, and forager respectively) is associated with mRNA processing, regulating transcription, and the differentiation of cells.⁴² Third, the presence of only two site classes and the distinctive age-specific profiles of kinome suggest that their activity depends upon many up and down stream intracellular and extracellular regulatory processes. Lastly, age-dependent phosphorylation events in the HG can be reflected in the age-specifically enriched and activated functional categories as well as distinct

network expression. As the workers emerge from their cells in the comb, their glands are already fully formed but not fully developed.¹⁴ The HG attains its maximum size in nurses immediately after their emergence and reduces its size in the forager.³ It is thus clear that cells in the young HG are still growing and differentiating to get ready to enter the secretory phase. To this effect, the highly activated category of the regulation of cell size, in both newly emerged bees and nurses, indicates that phosphorylation events are vital for the maintenance of cell shaping of the HG in response to gland growth and RJ secretion. Similarly, the highly induced category of the positive regulation of cellular catabolic processes in the

forager matches the fact that the gland at this age is converting polysaccharide nectar into monosaccharide honey. Another example is the ribosome pathway, a central signaling pathway for protein synthesis in the secretion of RJ proteins in the HG. The shared enrichment of ribosome pathways across the three time-points of the HG phosphoproteome reflects its fundamental role in solidifying the function of the HG as a protein biosynthesis center for RJ secretion; however, this pathway is tightly controlled by phosphorylation events reflected as age-specific expressions of different ribosome subunits. For example, larger subunits are more abundant in nurse and forager HGs. As an important mechanism for translational regulation, the phosphorylation of ribosomal proteins has been studied intensively in the regulation of protein synthesis or translational regulation.^{43–45} It is therefore tempting to speculate that the phosphorylation event regulates the ribosomal proteins at a specific time to control the translational machinery switch “on” or “off” by phosphorylation and dephosphorylation of different ribosome subunits and in turn modulate protein synthesis. The validated expression tendency between the protein and gene of LP1 and LP2 suggests that their protein phosphorylation and gene expression may have the same directions in regulating functionality in the translational machinery in the HG, thus providing potential clues for the investigation into the functionality of a phosphorylated specific protein in regulating the physiological changes of the HG. The wts pathway is functionally important in regulating autophagy,⁴⁶ maintaining energy homeostasis, promoting cell survival, and development in living cells.^{47–49} The fact that phosphorylated proteins are observed only in the wts pathway of the nurses and foragers suggests that phosphorylation is key to the wts pathway realizing the above roles for normal function of the HG at different ages.

4.2. Protein Phosphorylation is Independent of Its Expression but Complementary to the Proteome Profiles

When comparing our phosphoproteome with nonphosphoproteomes in the HG, several interesting points are found. (1) The protein abundance and phosphorylation levels are not correlated. (2) Only a very small proportion overlapped and many proteins are unique to each data set. (3) Very few kinases are shared between the phosphorylated and unmodified kinome, and most kinases are expressed in a phosphorylated form. (4) The complementary functional categories are enriched from the phosphoproteome and proteome.

These notions are supported by the subsequent facts. First, the observed, divergent expression profiles of phosphoproteome and proteome and drastically varied expression levels of phosphoproteins relative to the stable abundance level of their unmodified forms of the shared proteins, suggests that the protein abundance and phosphorylation levels are not correlated, or protein phosphorylation is regulated independently of its expression. For instance, among those proteins with consistent or inconsistent expression levels between phosphorylated and nonphosphorylated proteins, such as myb protein X, and eukaryotic translation initiation factor 4 gamma, the abundance level of the individual phosphorylation sites for each protein was significantly varied at different ages of HG. Second, the complementarily enriched functional classes are found in both young and nurse bees in phosphoproteome, such as pyruvate metabolic process, muscle cell development, and pole plasm mRNA localization, whereas only the first two classes enriched in foragers in a nonphosphoproteome. These

facts imply that the phosphoproteome and proteome are required to play complementary biological roles to underpin the unique age-dependent physiology of the HG. This notion is further supported by the complementary kinome profiles and kinase expression. These observations are consistent with the findings that the phosphoproteins and their unmodified forms function in a complementary manner to prime the normal physiological running of living organisms.²⁵ This is also similar to our recent findings that the phosphorylated MRJPs and their unmodified forms in RJ produced by Western and Eastern honeybees employ complementary phosphorylation strategies to achieve their roles as nutrient and immune agents.^{21,50} A representative protein functioning in a complementary way in both a phosphorylated and nonphosphorylated form is protein kinase C, an enzyme that transmits a myriad of signals to promote phospholipid hydrolysis in cells.⁵¹ Protein kinase C (PKC) must be processed by a series of ordered phosphorylations that are required for the enzyme to gain catalytic competence and correct intracellular localization to achieve this function in signal transduction.^{51,52} Often the activation of PKC is required by the upstream kinase phosphoinositide-dependent kinase (PDK-1). Although nonphosphorylated species of PKC are not capable of signaling, they are positioned at the cell membrane in a conformation as a target for phosphorylation by PDK-1.⁵³ Therefore, the presence of both phosphorylated and nonphosphorylated PKC may prime the enzyme for catalytic activity in the HG. The existence of phosphorylated kinases and their unmodified forms indicates that most kinases in the HG may be regulated by either interacting with proteins or through specific phosphorylation events, mediated by other protein kinases as signaling hubs during the HG development. Because the phosphorylation states of protein kinases can provide a readout for the signaling activities within a cellular system, these observations have the potential to become an important avenue for further study to reveal the signal transductions that drive age-dependent HG function. Together, these observations are a manifestation of the facts that the protein phosphorylation is independent of its expression, and the complementary protein and phosphoprotein expression profiles are required to drive the unique physiology of secretory activity in the HG.

4.3. Future Utilizations of Phosphorylation Data

To our knowledge, this is the first large-scale documentation of in vivo patterns of expression and phosphorylation for almost 1000 proteins in the HG of honeybee workers. The mapping of phosphorylation sites and quantifying site abundance changes are vitally important initial steps to better understand the biological functions of proteins. For instance, the function of tyrosine phosphatase, Ptp61F, has been implicated in the involvement of the regulation of actin reorganization and remodeling in *Drosophila*,⁵⁴ and the activation of protein kinase is required for phosphorylation at the site of Thr 198 in mammalian cells.⁵⁵ Hence, our data offer an extended overview of phosphorylation and protein expression regulating temporal signaling networks over age development in the HG. This data set acts as sound evidence of downstream functions of these phosphorylated proteins and sites in honeybee biology and other social insects.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.5b00530.

Figure S1. Ultrastructure image of hypopharyngeal glands of the worker honeybee (*A. m. ligustica*) is taken by SEM at three ages. Figure S2. Annotated spectra of all phosphorylated peptides in hypopharyngeal glands of worker honey bee at three ages of development. Figure S3. Efficiency comparison of phosphopeptide enrichment by TiO₂ chromatography and IMAC of hypopharyngeal glands of bees at the newly emerged, nurse, and forager stages. Figure S4. Tandem mass spectra comparison between the digested phosphorylation peptides from hypopharyngeal gland proteins and the synthetic phosphorylation peptides. Figure S5. Distribution of age-dependent phosphorylation in hypopharyngeal gland of honeybee workers at three ages. Figure S6. Overall expression trend of all the identified phosphoproteins and nonphosphoproteins in hypopharyngeal glands. Figure S7. Enriched GO functional category of up-regulated phosphoprotein in hypopharyngeal glands of young bees, nurses, and foragers. Figure S8. Interaction networks for phosphoproteins identified in the hypopharyngeal glands of honeybee workers. (PDF)

Table S1. Identification of secreted proteins presented in the hypopharyngeal gland tissue of honeybee workers. (PDF)

Table S2. Identification of phosphopeptides and phosphorylation sites in different ages of hypopharyngeal glands of honeybees. (PDF)

Table S3. Identification of phosphoproteins in hypopharyngeal glands of honeybee workers at different ages. (PDF)

Table S4. Motif extraction from the phosphoproteins identified in hypopharyngeal glands of differently aged honeybees. (PDF)

Table S5. Comparison of all identified phosphoproteins and nonphosphoproteins in hypopharyngeal glands of honeybees. (PDF)

Table S6. Functional GO term enrichment of phosphoproteins identified in hypopharyngeal glands of different ages of honeybees. (PDF)

Table S7. Differentially expressed phosphoproteins in hypopharyngeal glands of honeybees at three time-points. (PDF)

Table S8. Comparison of kinome between the phosphoproteome and nonphosphoproteome in hypopharyngeal glands of honeybees at different ages. (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Tel/Fax: +861062591449. E-mail: apislijk@163.com.

Present Address

[†]Y.Q., P.F., and Y.H. contributed equally to this work.

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-45) in China. We thank Katrina Klett from the USA for her help with the manuscript.

■ REFERENCES

- (1) Simpson, J.; Riedel, I. B.; Wilding, M. Invertase in the hypopharyngeal glands of the honeybee. *Tribology* **1968**, *7*, 29–36.
- (2) Silva de Moraes, R. L.; Bowen, I. D. Modes of cell death in the hypopharyngeal gland of the honey bee (*Apis mellifera* L.). *Cell Bio Int.* **2000**, *24*, 737–43.
- (3) Jianke, L.; Mao, F.; Begna, D.; Yu, F.; Aijuan, Z. Proteome Comparison of Hypopharyngeal Gland Development between Italian and Royal Jelly-Producing Worker Honeybees (*Apis mellifera* L.). *J. Proteome Res.* **2010**, *9*, 6578–6594.
- (4) Painter, T. S.; Bieseke, J. J. The fine structure of the hypopharyngeal gland cell of the honey bee during development and secretion. *Proc. Natl. Acad. Sci. U. S. A.* **1966**, *55*, 1414–1419.
- (5) Wegener, J.; Huang, Z. Y.; Lorenz, M. W.; Bienefeld, K. Regulation of hypopharyngeal gland activity and oogenesis in honey bee (*Apis mellifera*) workers. *J. Insect Physiol.* **2009**, *55*, 716–25.
- (6) Ohashi, K.; Sawata, M.; Takeuchi, H.; Natori, S.; Kubo, T. Molecular cloning of cDNA and analysis of expression of the gene for alpha-glucosidase from the hypopharyngeal gland of the honeybee *Apis mellifera* L. *Biochem. Biophys. Res. Commun.* **1996**, *221*, 380–5.
- (7) Liu, H.; Wang, Z.-L.; Tian, L.-Q.; Qin, Q.-H.; Wu, X.-B.; Yan, W.-Y.; Zeng, Z.-J. Transcriptome differences in the hypopharyngeal gland between Western Honeybees (*Apis mellifera*) and Eastern Honeybees (*Apis cerana*). *BMC Genomics* **2014**, *15*, 744.
- (8) Collins, A.; Caperna, T.; Williams, V.; Garrett, W.; Evans, J. Proteomic analyses of male contributions to honey bee sperm storage and mating. *Insect Mol. Biol.* **2006**, *15*, 541–549.
- (9) Fang, Y.; Feng, M.; Han, B.; Lu, X.; Ramadan, H.; Li, J. In-depth proteomics characterization of embryogenesis of the honey bee worker (*Apis mellifera ligustica*). *Mol. Cell. Proteomics* **2014**, *13*, 2306–20.
- (10) Li, J.; Zhang, L.; Feng, M.; Zhang, Z.; Pan, Y. Identification of the proteome composition occurring during the course of embryonic development of bees (*Apis mellifera*). *Insect Mol. Biol.* **2009**, *18*, 1–9.
- (11) Garcia, L.; Saraiva Garcia, C. H.; Calábria, L. K.; Costa Nunes da Cruz, G.; Sánchez Puentes, A.; Bão, S. N.; Fontes, W.; Ricart, C. A.; Salmen Espindola, F.; Valle de Sousa, M. Proteomic analysis of honey bee brain upon ontogenetic and behavioral development. *J. Proteome Res.* **2009**, *8*, 1464–1473.
- (12) Begna, D.; Fang, Y.; Feng, M.; Li, J. Mitochondrial proteins differential expression during honeybee (*Apis mellifera* L.) queen and worker larvae caste determination. *J. Proteome Res.* **2011**, *10*, 4263–80.
- (13) Begna, D.; Han, B.; Feng, M.; Fang, Y.; Li, J. Differential Expressions of Nuclear Proteomes between Honeybee (*Apis mellifera* L.) Queen and Worker Larvae: A Deep Insight into Caste Pathway Decisions. *J. Proteome Res.* **2012**, *11*, 1317–29.
- (14) Feng, M.; Fang, Y.; Li, J. Proteomic analysis of honeybee worker (*Apis mellifera*) hypopharyngeal gland development. *BMC Genomics* **2009**, *10*, 645.
- (15) Zolnierowicz, S.; Bollen, M. Protein phosphorylation and protein phosphatases De Panne, Belgium, September 19–24, 1999. *EMBO J.* **2000**, *19*, 483–488.
- (16) Alonso, A.; Sasin, J.; Bottini, N.; Friedberg, I.; Friedberg, I.; Osterman, A.; Godzik, A.; Hunter, T.; Dixon, J.; Mustelin, T. Protein tyrosine phosphatases in the human genome. *Cell* **2004**, *117*, 699–711.
- (17) Jimenez, C. R.; Verheul, H. M. Mass spectrometry-based proteomics: from cancer biology to protein biomarkers, drug targets, and clinical applications. *American Society of Clinical Oncology educational book/ASCO. American Society of Clinical Oncology. Meeting* **2014**, *34*, e504–10.

- (18) Knutson, T. P.; Daniel, A. R.; Fan, D.; Silverstein, K. A.; Covington, K. R.; Fuqua, S. A.; Lange, C. A. Phosphorylated and sumoylation-deficient progesterone receptors drive proliferative gene signatures during breast cancer progression. *Breast Cancer Res.* **2012**, *14*, R95.
- (19) Zhai, B.; Villen, J.; Beausoleil, S. A.; Mintseris, J.; Gygi, S. P. Phosphoproteome analysis of *Drosophila melanogaster* embryos. *J. Proteome Res.* **2008**, *7*, 1675–82.
- (20) Gala, A.; Fang, Y.; Woltdj, D.; Zhang, L.; Han, B.; Feng, M.; Li, J. Changes of proteome and phosphoproteome trigger embryo-larva transition of honeybee worker (*Apis mellifera ligustica*). *J. Proteomics* **2013**, *78*, 428–446.
- (21) Han, B.; Fang, Y.; Feng, M.; Lu, X.; Huo, X.; Meng, L.; Wu, B.; Li, J. In-depth phosphoproteomic analysis of royal jelly derived from Western and eastern honeybee species. *J. Proteome Res.* **2014**, *13*, 5928–43.
- (22) Villen, J.; Beausoleil, S. A.; Gerber, S. A.; Gygi, S. P. Large-scale phosphorylation analysis of mouse liver. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 1488–93.
- (23) Zhang, J.; Xin, L.; Shan, B.; Chen, W.; Xie, M.; Yuen, D.; Zhang, W.; Zhang, Z.; Lajoie, G. A.; Ma, B. PEAKS DB: De Novo Sequencing Assisted Database Search for Sensitive and Accurate Peptide Identification. *Mol. Cell. Proteomics* **2012**, *11* (4), M111.010587.
- (24) Beausoleil, S. A.; Villen, J.; Gerber, S. A.; Rush, J.; Gygi, S. P. A probability-based approach for high-throughput protein phosphorylation analysis and site localization. *Nat. Biotechnol.* **2006**, *24*, 1285–92.
- (25) Huttlin, E. L.; Jedrychowski, M. P.; Elias, J. E.; Goswami, T.; Rad, R.; Beausoleil, S. A.; Villen, J.; Haas, W.; Sowa, M. E.; Gygi, S. P. A tissue-specific atlas of mouse protein phosphorylation and expression. *Cell* **2010**, *143*, 1174–89.
- (26) Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. The protein kinase complement of the human genome. *Science* **2002**, *298*, 1912–34.
- (27) Schwartz, D.; Gygi, S. P. An iterative statistical approach to the identification of protein phosphorylation motifs from large-scale data sets. *Nat. Biotechnol.* **2005**, *23*, 1391–8.
- (28) Bindea, G.; Mlecnik, B.; Hackl, H.; Charoentong, P.; Tosolini, M.; Kirilovsky, A.; Fridman, W. H.; Pages, F.; Trajanoski, Z.; Galon, J. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics* **2009**, *25*, 1091–3.
- (29) Warde-Farley, D.; Donaldson, S. L.; Comes, O.; Zuberi, K.; Badrawi, R.; Chao, P.; Franz, M.; Grouios, C.; Kazi, F.; Lopes, C. T.; Maitland, A.; Mostafavi, S.; Montojo, J.; Shao, Q.; Wright, G.; Bader, G. D.; Morris, Q. The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function. *Nucleic Acids Res.* **2010**, *38*, W214–20.
- (30) Zuberi, K.; Franz, M.; Rodriguez, H.; Montojo, J.; Lopes, C. T.; Bader, G. D.; Morris, Q. GeneMANIA prediction server 2013 update. *Nucleic Acids Res.* **2013**, *41*, W115–22.
- (31) Petersen, T. N.; Brunak, S.; von Heijne, G.; Nielsen, H. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* **2011**, *8*, 785–6.
- (32) Zhang, L.; Han, B.; Li, R.; Lu, X.; Nie, A.; Guo, L.; Fang, Y.; Feng, M.; Li, J. Comprehensive identification of novel proteins and N-glycosylation sites in royal jelly. *BMC Genomics* **2014**, *15*, 135.
- (33) Fujita, T.; Kozuka-Hata, H.; Ao-Kondo, H.; Kunieda, T.; Oyama, M.; Kubo, T. Proteomic Analysis of the Royal Jelly and Characterization of the Functions of its Derivation Glands in the Honeybee. *J. Proteome Res.* **2013**, *12*, 404–411.
- (34) Han, B.; Li, C.; Zhang, L.; Fang, Y.; Feng, M.; Li, J. Novel royal jelly proteins identified by gel-based and gel-free proteomics. *J. Agric. Food Chem.* **2011**, *59*, 10346–55.
- (35) Ji, T.; Liu, Z.; Shen, J.; Shen, F.; Liang, Q.; Wu, L.; Chen, G.; Corona, M. Proteomics analysis reveals protein expression differences for hypopharyngeal gland activity in the honeybee, *Apis mellifera carnica* Pollmann. *BMC Genomics* **2014**, *15*, 665.
- (36) Cohen, P. The origins of protein phosphorylation. *Nat. Cell Biol.* **2002**, *4*, E127–E130.
- (37) Löffler, A. S.; Alers, S.; Dieterle, A. M.; Keppeler, H.; Franz-Wachtel, M.; Kundu, M.; Campbell, D. G.; Wesselborg, S.; Alessi, D. R.; Stork, B. Ulk1-mediated phosphorylation of AMPK constitutes a negative regulatory feedback loop. *Autophagy* **2011**, *7*, 696–706.
- (38) Glise, B.; Bourbon, H.; Noselli, S. hemipterous encodes a novel *Drosophila* MAP kinase kinase, required for epithelial cell sheet movement. *Cell* **1995**, *83*, 451–61.
- (39) Khmelinskii, A.; Roostalu, J.; Roque, H.; Antony, C.; Schiebel, E. Phosphorylation-dependent protein interactions at the spindle midzone mediate cell cycle regulation of spindle elongation. *Dev. Cell* **2009**, *17*, 244–56.
- (40) Benne, R.; Sloof, P. Evolution of the mitochondrial protein synthetic machinery. *BioSystems* **1987**, *21*, 51–68.
- (41) Ohashi, K.; Natori, S.; Kubo, T. Change in the Mode of Gene Expression of the Hypopharyngeal Gland Cells with an Age-dependent Role Change of the Worker Honeybee *Apis mellifera* L. *Eur. J. Biochem.* **1997**, *249*, 797–802.
- (42) Harper, J. W.; Adams, P. D. Cyclin-dependent kinases. *Chem. Rev.* **2001**, *101*, 2511–2526.
- (43) Mazumder, B.; Sampath, P.; Seshadri, V.; Maitra, R. K.; DiCorleto, P. E.; Fox, P. L. Regulated release of L13a from the 60S ribosomal subunit as a mechanism of transcript-specific translational control. *Cell* **2003**, *115*, 187–198.
- (44) Ruvinsky, I.; Sharon, N.; Lerer, T.; Cohen, H.; Stolovich-Rain, M.; Nir, T.; Dor, Y.; Zisman, P.; Meyuhas, O. Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. *Genes Dev.* **2005**, *19*, 2199–2211.
- (45) Ruvinsky, I.; Meyuhas, O. Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. *Trends Biochem. Sci.* **2006**, *31*, 342–348.
- (46) Lee, E. J.; Tournier, C. The requirement of uncoordinated 51-like kinase 1 (ULK1) and ULK2 in the regulation of autophagy. *Autophagy* **2011**, *7*, 689–95.
- (47) Levine, B.; Klionsky, D. J. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev. Cell* **2004**, *6*, 463–77.
- (48) Kuma, A.; Hatano, M.; Matsui, M.; Yamamoto, A.; Nakaya, H.; Yoshimori, T.; Ohsumi, Y.; Tokuhisa, T.; Mizushima, N. The role of autophagy during the early neonatal starvation period. *Nature* **2004**, *432*, 1032–6.
- (49) Komatsu, M.; Waguri, S.; Ueno, T.; Iwata, J.; Murata, S.; Tanida, I.; Ezaki, J.; Mizushima, N.; Ohsumi, Y.; Uchiyama, Y.; Kominami, E.; Tanaka, K.; Chiba, T. Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *J. Cell Biol.* **2005**, *169*, 425–34.
- (50) Yu, F.; Mao, F.; Jianke, L. Royal Jelly Proteome Comparison between *A. mellifera ligustica* and *A. cerana cerana*. *J. Proteome Res.* **2010**, *9*, 2207–2215.
- (51) Nishizuka, Y. Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J.* **1995**, *9*, 484–96.
- (52) Keranen, L. M.; Dutil, E. M.; Newton, A. C. Protein kinase C is regulated in vivo by three functionally distinct phosphorylations. *Curr. Biol.* **1995**, *5*, 1394–1403.
- (53) Newton, A. C. Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. *Chem. Rev.* **2001**, *101*, 2353–64.
- (54) Hilger, M.; Bonaldi, T.; Gnad, F.; Mann, M. Systems-wide analysis of a phosphatase knock-down by quantitative proteomics and phosphoproteomics. *Mol. Cell. Proteomics* **2009**, *8*, 1908–20.
- (55) Steinberg, R. A.; Cauthron, R. D.; Symcox, M. M.; Shuntoh, H. Autoactivation of catalytic (C alpha) subunit of cyclic AMP-dependent protein kinase by phosphorylation of threonine 197. *Mol. Cell Biol.* **1993**, *13*, 2332–41.