Journal of Zhejiang University-SCIENCE B 2026 27(3):295-309
www.jzus.zju.edu.cn; www.springer.com/journal/11585
E-mail: jzus_b@zju.edu.cn

Research Article
https://doi.org/10.1631/jzus.B2400624

Check for updates

Ectopic expression of structurally similar major royal jelly proteins reveals their distinct functions in *Drosophila*

Lingqi YU[1*], Danfeng WANG[3*], Xuanhao CHEN[1], Jiayu XIE[4], Dongjing WEN[1], Yi ZHANG[1], Lirong SHEN[5], Wenfeng CHEN[1,2✉], Zhenxing LIU[6✉], Yufeng YANG[1,2✉]

[1]*Institute of Life Sciences, College of Biological Science and Engineering, Fuzhou University, Fuzhou 350108, China*
[2]*Department of Biomedical Engineering, College of Biological Science and Engineering, Fuzhou University, Fuzhou 350108, China*
[3]*College of Bee Science and Biomedicine, Fujian Agriculture and Forestry University, Fuzhou 350002, China*
[4]*School of Medicine, Chongqing University, Chongqing 400044, China*
[5]*College of Biosystems Engineering and Food Science, Zhejiang University, Hangzhou 310058, China*
[6]*State Key Laboratory of Resource Insects, Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, Beijing 100093, China*

Abstract: Royal jelly (RJ), secreted by the hypopharyngeal and mandibular glands of young worker bees, is rich in proteins, 80%–90% of which are major royal jelly proteins (MRJPs). While MRJPs from RJ have been shown to exhibit specific biological functions and are also expressed in neuronal cells, their roles in vivo remain poorly understood. The aim of this study was to elucidate the functional roles of individual MRJPs (MRJP1–9) in vivo by ectopically expressing them in *Drosophila* neurons in a binary expression system using fluorescent proteins as controls. Transcriptome sequencing revealed that although MRJP1–9 share similar tertiary structures, their overexpression affects distinct gene sets. MRJP1, MRJP2, MRJP3, MRJP5, and MRJP7 induced more differentially expressed genes (DEGs), while MRJP4, MRJP6, MRJP8, and MRJP9 induced fewer such genes. Weighted gene co-expression network analysis (WGCNA) and gene set enrichment analysis (GSEA) revealed that MRJP1, MRJP2, MRJP3, MRJP5, and MRJP7 regulated overlapping gene sets, including an estradiol-responsive set, and activated cell proliferation pathways. MRJP6 lacked any significant gene set enrichment, while MRJP8 and MRJP9 modulated similar sets. Notably, the neuron-specific overexpression of MRJP1, MRJP2, MRJP3, and MRJP5 in *Drosophila* showed activated cell proliferation-related pathways and increased body sizes, highlighting their functional diversity and context-dependent effects. These findings expand our understanding of the functional roles of MRJPs and provide a foundation for further exploring their biological significance in honeybees and beyond.

Key words: *Drosophila melanogaster*; Major royal jelly protein (MRJP); Ectopic expression

1 Introduction

Royal jelly (RJ), a nutrient-rich mixture secreted by the hypopharyngeal and mandibular glands of nurse bees, plays a crucial role in the growth and development of honeybees. RJ has a relatively complex composition, with approximately 50% of its dry matter consisting of proteins, among which the major royal jelly proteins (MRJPs) account for over 80% of the total protein content (Collazo et al., 2021). To date, ten members have been identified in the MRJP family, namely MRJP1–10, with MRJP10 being a pseudogene, while the others (MRJP1–9) are all expressed in honeybees (Uversky et al., 2021). MRJP1 is the most abundant type (comprising 31%–66% of total RJ proteins), followed by MRJP3, MRJP2, and MRJP5 (Schmitzová et al., 1998; Bíliková and Šimúth, 2010). The function of MRJP1 has been shown to depend on its oligomeric state. In the monomeric form, MRJP1 is a 55 kDa protein, also known as royalactin, whereas its oligomeric form, apisin, is a complex of MRJP1 monomers bound to apisimin polypeptides and 24-methylenecholesterol.

✉ Wenfeng CHEN, chenwenfeng@fzu.edu.cn
Zhenxing LIU, liuzhenxing01@caas.cn
Yufeng YANG, yangyf@fzu.edu.cn
* The two authors contributed equally to this work
Wenfeng CHEN, https://orcid.org/0000-0001-5259-7214
Zhenxing LIU, https://orcid.org/0000-0001-8236-1931
Yufeng YANG, https://orcid.org/0000-0002-7738-1329
Lingqi YU, https://orcid.org/0009-0008-1862-0175

Received Dec. 9, 2024; Revision accepted Mar. 20, 2025;
Crosschecked Mar. 9, 2026; Published online Mar. 14, 2026

This oligomeric form of MRJP1 creates a fibrous network that provides RJ with the necessary viscosity (Fan et al., 2016; Tian et al., 2018). MRJP3 has been reported to have strong RNA-binding activity, which acts as an extracellular, non-sequence-specific RNA aggregation factor, protecting RNA from degradation and enhancing its bioavailability (Maori et al., 2019). Various bioactive MRJPs and their derivative peptides may benefit humans, as evidenced by studies in cell cultures, animal models, and computer simulations. These bioactivities include antioxidant, antibacterial, antitumor, antihypertensive, lipid-lowering, cell growth-promoting, wound-healing, anti-aging, neuroprotective, anti-inflammatory, and immunomodulatory effects (Matsui et al., 2002; Nagai and Inoue, 2004; Majtán et al., 2006; Guo et al., 2009; Xin et al., 2016; Vezeteu et al., 2017; Abu-Serie and Habashy, 2019; Kim and Jin, 2019; Lin et al., 2019; Zhang et al., 2019; Park et al., 2020; Wen et al., 2024). However, beyond these examples, the functions of MRJPs within honeybees remain largely unknown.

Although RJ is secreted primarily by the head glands of worker bees, various studies have revealed that various MRJPs are also expressed in the brain cells of honeybees. Research has identified three polypeptides in the bee brain, including MRJP1-related p57, MRJP3-related p70, and a potential MRJP1 dimer p128. In addition, proteins recognized by MRJP1 antibodies have been detected in the neural fibers of the mushroom body (MB), optic lobe (OL), and antennal lobe (AL) (Peixoto et al., 2009). MRJP1 has been observed to be associated with filamentous proteins in the brain, with elevated levels of MRJP2 and MRJP7 expression noted in the brains of nurse bees compared to other workers, although their specific functions remain to be clarified (Garcia et al., 2009). Further study has shown that MRJP1 expression in the MB varies with bee age and is reduced under conditions of social isolation, which impairs learning development (Hojo et al., 2010). It has also been confirmed that MRJP1–9 are all expressed in the bee brain, with expression levels positively correlated with those in the hypopharyngeal gland, albeit at lower levels (Dobritzsch et al., 2019). Dietary influences, such as increased 24-methylenecholesterol concentration, have been found to raise the relative abundance of MRJP1, MRJP4, MRJP5, and MRJP7 in the bee head (Chakrabarti and Sagili, 2020). Moreover, bees with gut microbial symbionts exhibited upregulated expression of odor-binding protein 14 (Obp14), olfactory receptor 115 (Or115), MRJP1, MRJP2, and MRJP7, suggesting that gut bacteria may regulate brain gene expression to enhance learning and memory in bees (Zhang et al., 2022). Experimental interventions, such as injecting MRJP1 and MRJP3 into the bee brain, have been shown to alter worker bee behavior, increasing task reversal from foraging to nursing and reducing the progression from nursing to foraging. Conversely, downregulating MRJP1 and MRJP3 via RNA interference (RNAi) injection into the brain increased the transition of worker bees from nursing to foraging, confirming the causal role of these proteins in regulating worker bee behavior and life history (Fang et al., 2023). The above findings indicate that MRJPs may play diverse functional roles in the bee nervous system; however, studying MRJPs directly in the brain cells of bees remains challenging, and the functional differences among MRJPs in vivo are still poorly understood. To investigate these differences, this study conducted an experiment to ectopically express MRJP1–9 in *Drosophila* neuronal cells and performed RNA-sequencing (RNA-seq) analysis to elucidate the potential functional variations of MRJPs in the brain.

2 Results

2.1 Highly similar tertiary structures of different MRJPs

MRJPs have evolved from an ancient protein family, YELLOW, with which they share a high degree of homology; thus, they are often grouped as the MRJP/YELLOW family. The honeybee genome contains ten *mrjp* genes (including one pseudogene) and ten *yellow* genes (Drapeau et al., 2006). The *mrjp* genes are found primarily in *Apis* species and other Hymenoptera and are organized in a single gene cluster within the honeybee genome (Fig. 1a). The *mrjp1–9* genes have highly similar structures (Fig. S1), with the gene family likely having emerged from multiple duplication events from a common ancestor (Drapeau et al., 2006). The coding sequences of *mrjp1–9* from *Apis mellifera* were amplified and sequenced using samples collected from a local apiary. Although some polymorphic sites were identified compared to the sequences published in genome databases, the similarity between

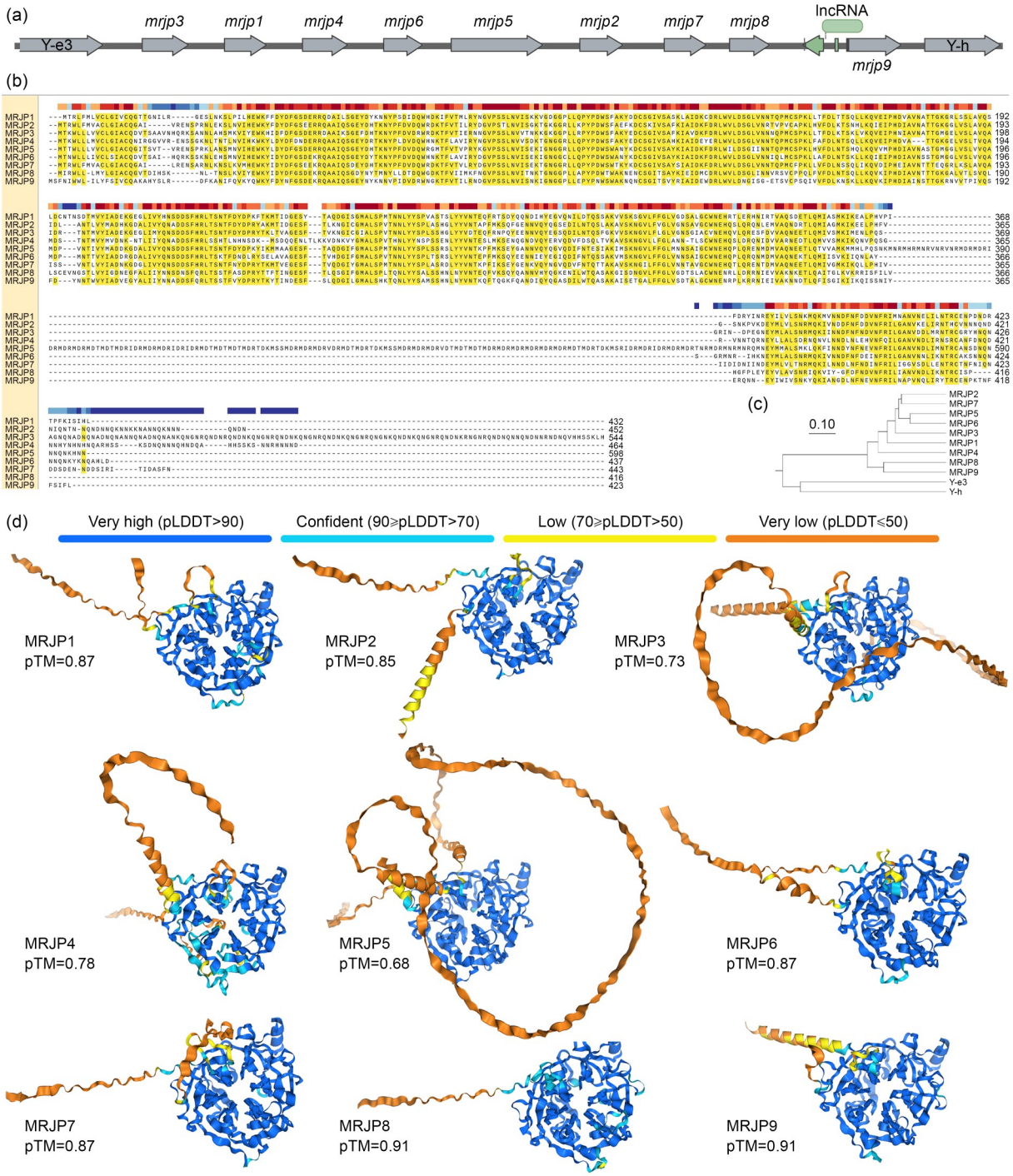

Fig. 1 Comparative analyses of protein sequences and tertiary structures of different major royal jelly proteins (MRJPs). (a) Schematic diagram of *mrjp* and partial *yellow* (Y) genes in the honeybee genome. (b) Alignment results of MRJP protein sequences obtained from our cloning and sequencing data. (c) Phylogenetic tree analysis of MRJP protein sequences based on our cloning and sequencing data. An unrooted phylogenetic tree based on the neighbor-joining method was constructed using molecular evolutionary genetics analysis across computing platforms (MEGA X) (Kumar et al., 2018). Y-e3: YELLOW-e3; Y-h: YELLOW-h. (d) Three-dimensional structures of different MRJPs predicted by AlphaFold 3. AlphaFold 3 produces a per-residue model confidence score (predicted local distance difference test (pLDDT)) between 0 and 100. Some regions below 50 pLDDT may be unstructured in isolation. The predicted template modeling (pTM) score is derived from the TM score, a metric that evaluates the accuracy of an entire structure. A pTM score above 0.5 indicates that the overall predicted folding of the complex may closely resemble the true structure.

different MRJPs remains high (Fig. 1b and Table S1). Using our amplified MRJP protein sequences and the YELLOW-e3 and YELLOW-h protein sequences from databases, an unrooted phylogenetic tree was constructed using the neighbor-joining method. Phylogenetic analysis revealed a clear clustering of MRJPs within the tree branches, specifically forming distinct groups of MRJP2+7, MRJP5+6, MRJP3, MRJP1, MRJP4, and MRJP8+9 (Fig. 1c), each encoded by tandemly arranged, adjacent *mrjp* genes (Fig. 1a), consistent with previous findings (Drapeau et al., 2006).

To further investigate the potential structural differences among MRJPs, their tertiary structures were examined. A prior study has identified a 16-molecule oligomeric structure of native MRJP1, comprising four MRJP1 proteins, four apisimin molecules, and eight 24-methylenecholesterol molecules (Tian et al., 2018). Nonetheless, the structures of other MRJPs remain unknown. The structures of the MRJP sequences that we amplified and sequenced were predicted using AlphaFold 3 (Abramson et al., 2024). The predicted structure of MRJP1 closely matched the experimentally determined structure of MRJP1 (Fig. S2), indicating the accuracy of the prediction. Furthermore, it was found that AlphaFold 3 predicted highly similar structures for MRJP1–9 (Fig. 1d), although the confidences (predicted local distance difference test (pLDDT) scores) at the N- and C-termini were lower. This raised an intriguing question: do proteins with such similar structures actually perform similar biological functions in vivo?

2.2 Efficient ectopic expression of different MRJPs in *Drosophila*

Due to the challenges associated with directly studying the functions of different MRJP proteins in honeybees, the model organism *Drosophila* was selected, as it offers a rich toolkit for genetic manipulation. *Drosophila* expresses *yellow* genes, and the YELLOW proteins in flies are ancestral members of the MRJP/YELLOW protein family, exhibiting various functions, such as controlling the sex determination pathway in *Drosophila* (Radovic et al., 2002; Drapeau et al., 2003), regulating the mating success rate in males (Massey et al., 2019), and influencing development and cellular functions (Hinaux et al., 2018). Since honeybee MRJPs and *Drosophila* YELLOW proteins belong to the same MRJP/YELLOW family, it seems feasible to study the functions of honeybee MRJPs through their ectopic expression in *Drosophila*. The galactose-activatable 4 (GAL4)/upstream activating sequence (UAS) binary expression system in *Drosophila* is a widely used tool for gene expression regulation (Fig. 2a). In this system, a tissue-specific promoter or enhancer drives the expression of the GAL4 transcription factor. By crossing flies carrying GAL4 with those carrying UAS, the resulting offspring can activate the target gene downstream of UAS in the tissues or cells expressing GAL4 (Fig. 2a). The pUAST-attB vector was selected for this purpose, and different *mrjp* genes along with the control fluorescent protein gene *mScarlet-i* were inserted between the *Xho*I and *Xba*I cloning sites (Fig. 2b). Importantly, different UAS-*mrjp* genes were chosen for site-specifical integration at the attP2 site on the third chromosome of *Drosophila* (Fig. 2c) to eliminate any unintended variations that may arise from insertion at different loci. By crossing *elav*-GAL4, which drives neuron-specific expression, with UAS-*mScarlet-i*, fluorescent protein expression in the *Drosophila* brain was clearly observed (Fig. 2d). In addition, via crossing *elav*-GAL4 with different UAS-*mrjp* genes, the expression of *mrjp* genes was confirmed (Fig. 2d).

2.3 Distinct signaling perturbations from ectopic MRJP expression in *Drosophila* neurons

Next, the *elav*-GAL4 driver was further utilized to induce ectopic expression of different MRJPs in neurons, followed by extracting total RNA from *Drosophila* heads for transcriptome sequencing analysis. The previous study has reported the expression of *Drosophila* YELLOW protein in certain subpopulations of neurons in both larval and adult brains (Hinaux et al., 2018). Following the pan-neuronal expression of MRJP1–9 in *Drosophila* heads, principal component analysis (PCA) revealed that each MRJP overexpression group was distinctly separated in overall gene expression from the control group (Fig. S3), indicating that the overexpression of different MRJPs indeed caused changes in endogenous genes in *Drosophila*. Although structural predictions suggested that MRJP1–9 share relatively similar tertiary structures, the genes disrupted by their overexpression varied. Notably, MRJP1, MRJP2, MRJP3, MRJP5, and MRJP7 overexpression resulted in a higher number of differentially expressed genes (DEGs), while MRJP4, MRJP6, MRJP8, and MRJP9 showed fewer DEGs (Fig. 3).

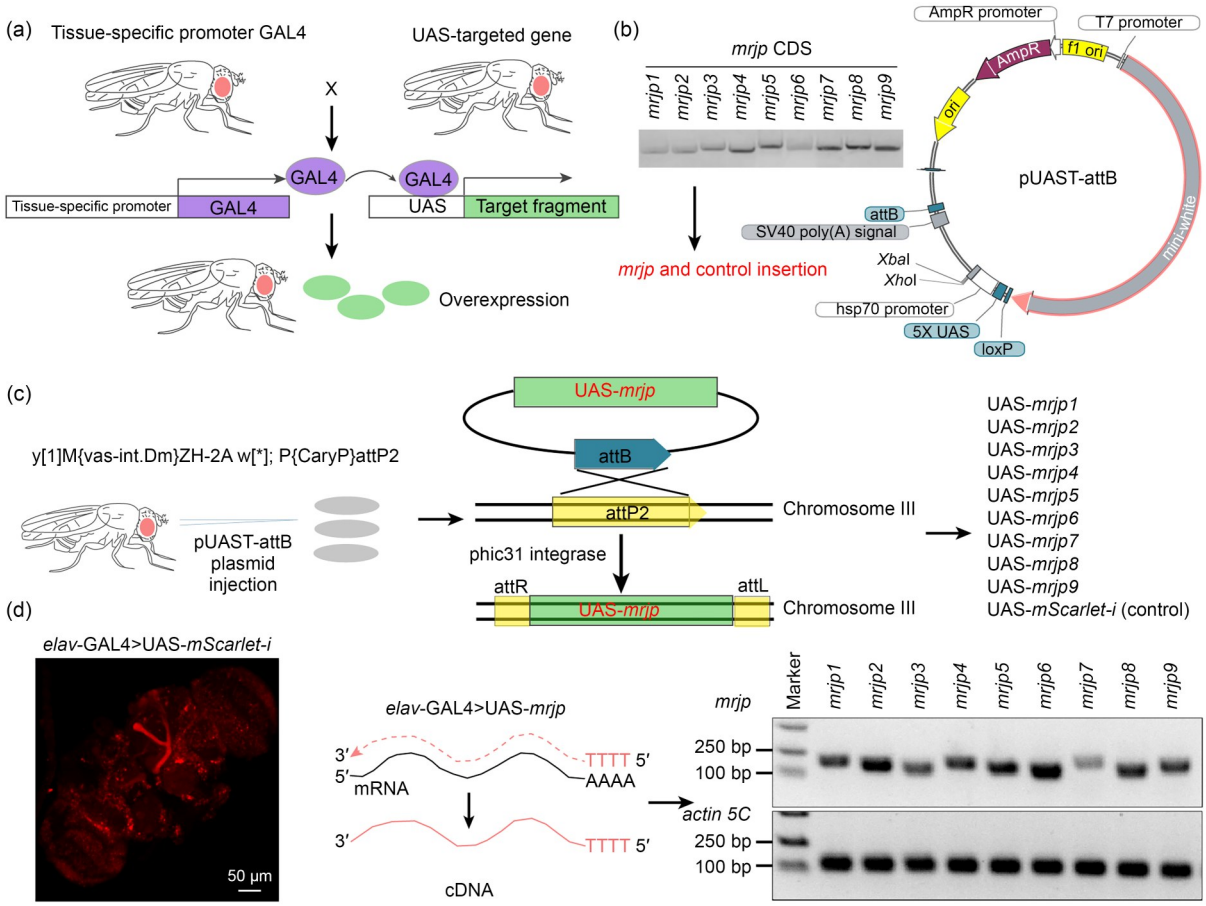

Fig. 2 Construction and expression analyses of transgenic *Drosophila* with different UAS-*mrjp* genes. (a) The galactose-activatable 4 (GAL4)/UAS binary expression system in *Drosophila*. (b) The pUAST-attB plasmid used for cloning various *mrjp* genes, enabling site-specific insertion into the *Drosophila* chromosome. (c) Site-specific insertion of pUAST-*mrjp*-attB into the attP2 locus on the third chromosome of *Drosophila*, mediated by integrase. (d) Validation of UAS-*mrjp* and UAS-*mScarlet-i* expression. UAS: upstream activating sequence; *mrjp*: major royal jelly protein; CDS: coding sequence; hsp70: heat shock protein 70; LoxP: locus of X(cross)-over in P1; attB: attachment site bacterial; attP2: attachment site phage, variant 2; attR: attachment site right; attL: attachment site left; mRNA: messenger RNA; cDNA: complementary DNA.

Subsequently, to identify gene association patterns between different MRJP overexpression groups and the control group, a comprehensive weighted gene co-expression network analysis (WGCNA) was conducted on the transcriptome datasets. WGCNA is a commonly used algorithm for constructing gene co-expression networks, grouping genes with shared expression characteristics across samples into the same network. Usually, genes within the same co-expression network exhibit similar expression patterns, while these patterns for genes in different networks are distinct. Genes with similar expression profiles are clustered into groups called modules. By identifying these co-expression gene modules, we can connect them to phenotypic information of interest to explore the relationships between gene networks and phenotypes (Langfelder and Horvath, 2008). Our transcriptome data revealed four primary modules (Fig. 4a). Cluster analysis of gene–phenotype associations showed that the MRJP1, MRJP2, MRJP3, and MRJP7 overexpression groups displayed distinct patterns compared to the other groups, with consistent module patterns within these groups (Fig. 4a).

Interestingly, comparative gene enrichment analysis using the screened DEGs revealed that MRJP1, MRJP2, MRJP3, MRJP5, and MRJP7 overexpression regulated similar gene sets, while MRJP6 showed no significant gene set enrichment. MRJP4 overexpression regulated a distinct gene set, whereas MRJP8 and MRJP9 regulated comparable gene sets (Fig. 4b). This finding aligns well with the phylogenetic tree clustering analysis (Fig. 1c). Notably, the gene sets regulated

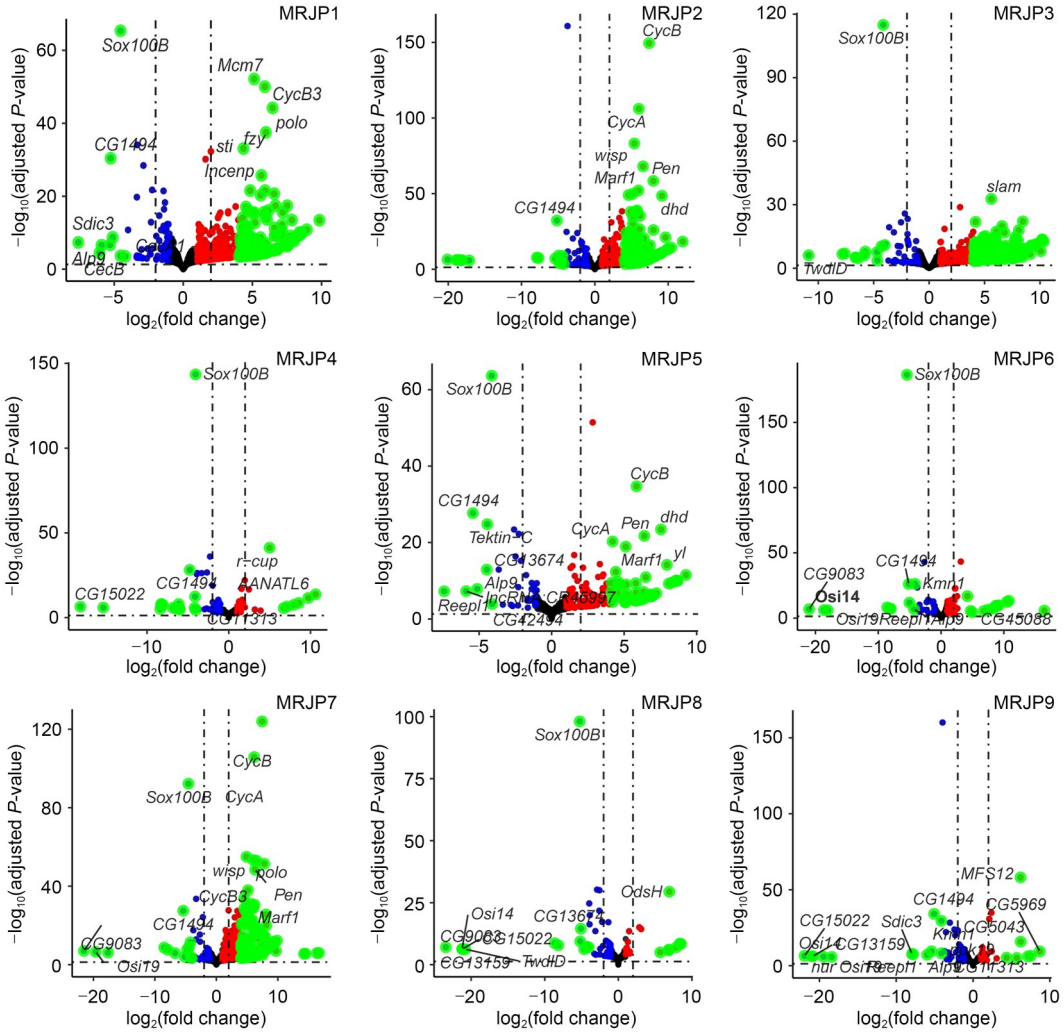

Fig. 3 Differentially expressed genes (DEGs) following the overexpression of different major royal jelly proteins (MRJPs) in neurons of *Drosophila* heads. The quantitative analysis of transcriptome data was performed using DESeq2, with the *x*-axis representing the $\log_2$(fold change) of DEGs and the *y*-axis representing the adjusted *P*-value. Genes with *P*-value<0.05 and |$\log_2$(fold change)|>0.5 were considered differentially expressed. The red dots indicate $\log_2$(fold change)>1, the blue dots indicate $\log_2$(fold change)<−1, and the green dots represent genes with |$\log_2$(fold change)|≥4.

by MRJP1, MRJP2, MRJP3, MRJP5, and MRJP7 overexpression were observed to include an estradiol-responsive gene set (Fig. 4b).

Furthermore, gene set enrichment analysis (GSEA) was performed to comprehensively detect gene sets with biological significance. By ranking genes based on their differential expression between two sample types, GSEA tests whether specific gene sets are enriched at the top or bottom of the ranking list, enabling the identification of biologically relevant genes without specifying a threshold of differential expression (Mootha et al., 2003; Subramanian et al., 2005). The hallmark gene sets were obtained by aggregating multiple Molecular Signatures Database (MSigDB) gene sets, designed to represent specific biological states or processes and display consistent expression (Liberzon et al., 2015). These gene sets are generated through computational methods that identify overlaps between gene sets in other MSigDB collections, retaining genes that exhibit coordinated expression. Hallmark gene sets help reduce noise and redundancy, providing a clearer biological space for GSEA. These gene sets were used to perform GSEA on the DEGs from various MRJP overexpression groups relative to the control group. The results indicated that only the MRJP1, MRJP2, MRJP3, MRJP5, and MRJP7 overexpression groups significantly activated certain signature gene sets, and the hallmark gene sets were consistently associated with

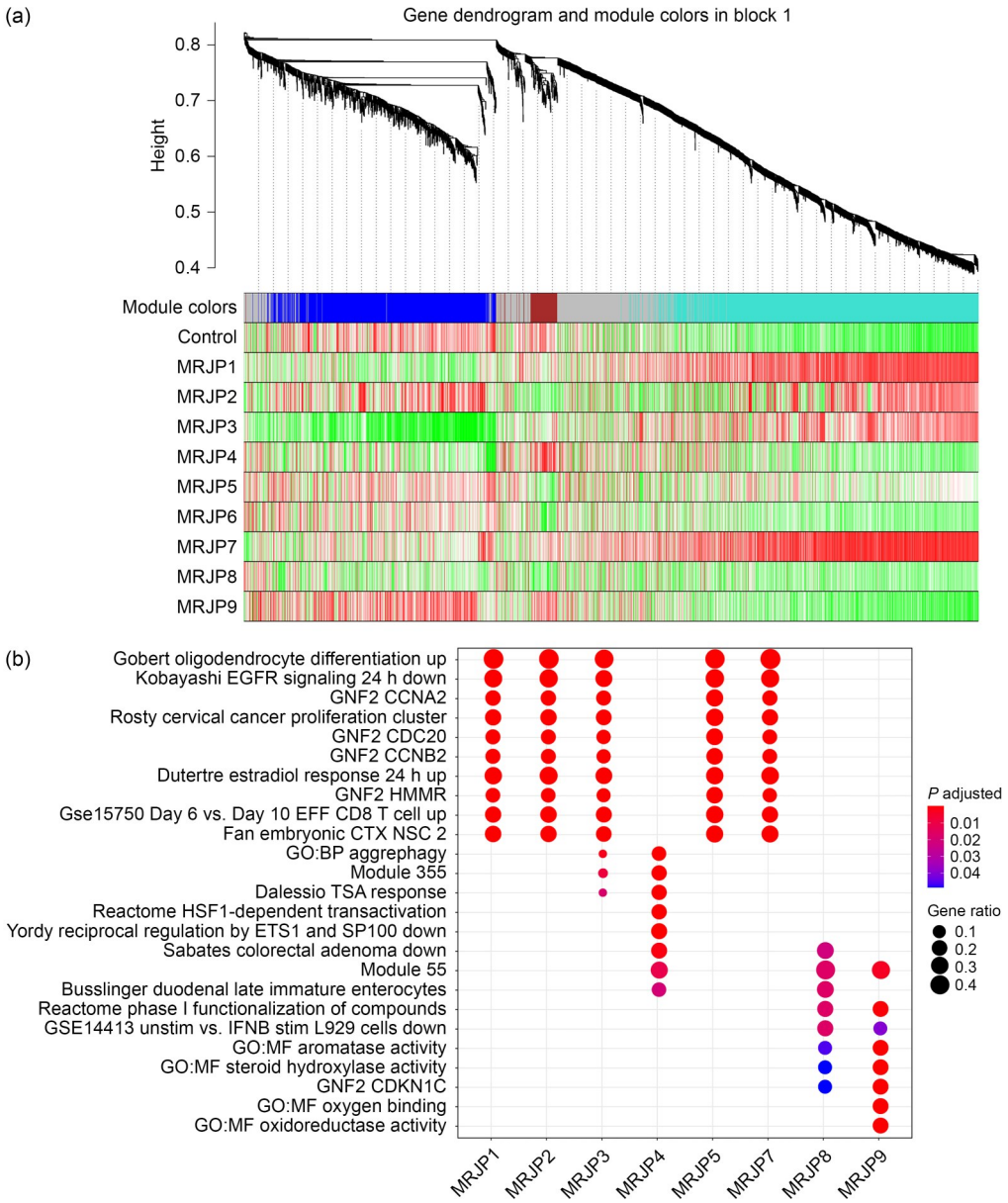

Fig. 4 Co-expression network analysis and gene enrichment comparison analysis of head transcriptomes in different major royal jelly protein (MRJP) overexpression and control groups. (a) Gene co-expression network analysis. The upper part of the image displays the hierarchical clustering tree of genes, the middle section shows the modules to which the genes belong, and the lower part illustrates the heatmap of gene–phenotype correlations within the modules, where each row represents a phenotype and each column corresponds to a gene within the module. The color indicates the strength of the correlation, with red denoting a positive correlation and green denoting a negative correlation. Module means a highly interconnected set of genes. In an undirected network, genes within a module are highly correlated. (b) Gene enrichment comparison analysis. We performed enrichment analysis of differentially expressed genes (DEGs) from various MRJP overexpression groups compared to the fluorescent protein *mScarlet-i* overexpression control group using the gene set resources annotated for *Drosophila* provided by the Molecular Signatures Database (MSigDB) for gene set enrichment analysis (GSEA) software. The analysis utilized the compareCluster function from the clusterProfiler package, including only genes with an adjusted *P*-value of <0.05 and $|\log_2(\text{fold change})|>1$. EGFR: epidermal growth factor receptor; GNF2: Genomics Institute of the Novartis Research Foundation (GNF) version 2 microarray platform; CCNA2: cyclin A2; CDC20: cell division cycle 20; CCNB2: cyclin B2; HMMR: hyaluronan-mediated motility receptor; EFF: effector (CD8 T cell); CTX: cortex; NSC: neural stem cell; GO:BP: gene ontology:biological process; TSA: trichostatin A; HSF1: heat shock factor 1; ETS1: ETS proto-oncogene 1, transcription factor; SP100: SP100 nuclear antigen; IFNB: interferon β; GO:MF: gene ontology:molecular function; CDKN1C: cyclin-dependent kinase inhibitor 1C.

cell proliferation signals, such as MYC proto-oncogene (MYC) targets, early region 2-binding factor (E2F) targets, Gap 2 to mitosis (G2M) checkpoint, and mitosis (Fig. 5).

2.4 Increased *Drosophila* body size induced by ectopic expression of certain MRJPs in neurons but not in fat bodies

MRJP overexpression activates cell proliferation-related signals. Accordingly, an earlier study has also indicated that the pan-expression or fat body-specific overexpression of MRJP1 in *Drosophila* leads to increased body size (Kamakura, 2011). This study aimed to verify this observation. Initially, the pan-cell expression driver *Act5C*-GAL4 was utilized to overexpress different MRJPs, and approximately one-week-old GAL4-MRJP female flies were selected for size observation. Unfortunately, no increase in body size was observed under the pan-expression of different MRJPs (Fig. S4). In addition, the fat body-specific

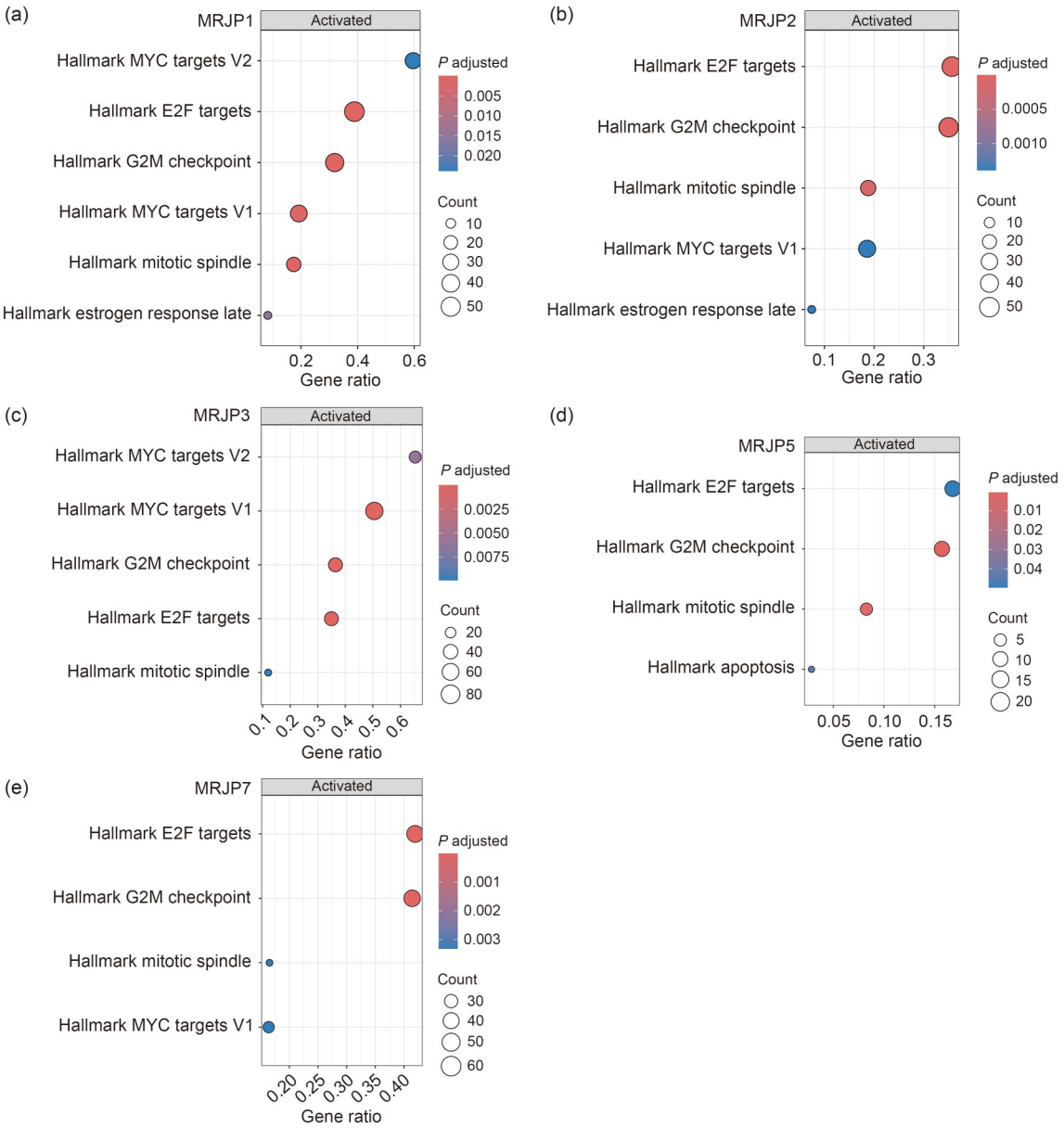

Fig. 5 Gene set enrichment analysis (GSEA) of hallmark gene sets in different major royal jelly protein (MRJP) overexpression groups. (a) MRJP1; (b) MRJP2; (c) MRJP3; (d) MRJP5; (e) MRJP7. E2F: early region 2-binding factor; G2M: Gap 2 to mitosis; MYC: MYC proto-oncogene.

driver *ppl*-GAL4 was employed to overexpress various MRJPs, but again, no phenotype with increased body size was observed (Fig. S5). However, when *elav*-GAL4 was used to drive the overexpression of different MRJPs in neurons, a significant increase in body size was observed in the MRJP1, MRJP2, MRJP3, MRJP5, and MRJP8 overexpression groups, as well as a small increase in the MRJP4 overexpression group (Fig. 6).

3 Discussion

Several unresolved questions remain regarding MRJPs, particularly their expression in tissues outside the hypopharyngeal gland, such as in brain cells. The molecular and biochemical characteristics of MRJPs in bumblebees, also members of the order Hymenoptera, suggest a non-nutritional role (Kupke et al., 2012). Consistent with findings in honeybees, MRJPs are also expressed in the bumblebee brain, particularly in the Kenyon cells of the mushroom bodies (involved in sensory integration) and in the optic lobes (Albert et al., 2014). In honeybees, *mrjp1–5* and *mrjp7* exhibit age-dependent expression patterns in both the worker hypopharyngeal glands and the brain, with lower abundance in the brain than in the glands. The expression levels increase from the hatchling to nurse stages but decrease in older worker bees. Unlike the other *mrjp* genes, *mrjp6* expression shows no significant variation in the brain. *mrjp8* and *mrjp9* exhibit low expression in both tissues, suggesting that they may function in

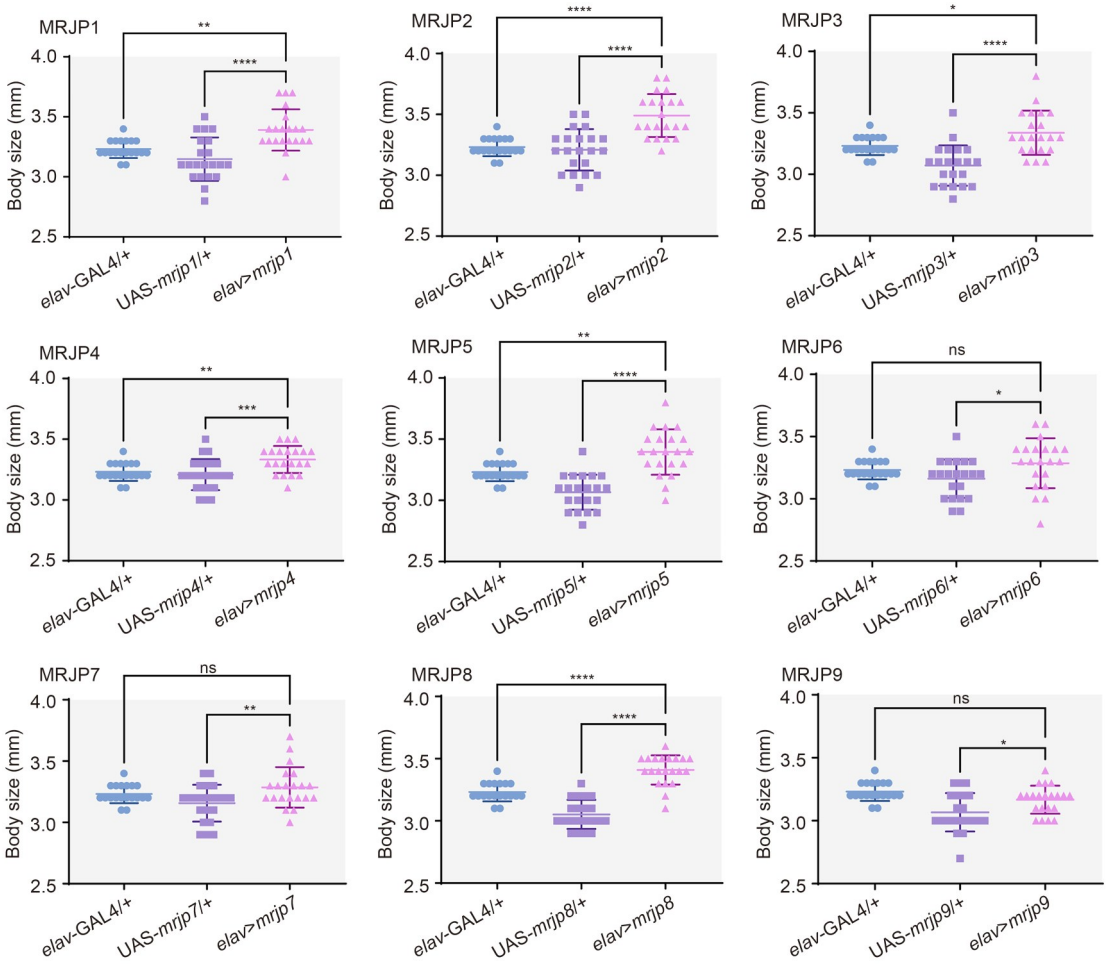

Fig. 6 Changes in the body size of *Drosophila* following neuronal overexpression of different major royal jelly proteins (MRJPs). Data are presented as mean±standard deviation (SD), with values displayed in scatter dot plots (n=19–24). *elav*-GAL4/+ represents the heterozygous control, and *elav>mrjp* indicates the experimental group in which UAS-*mrjp* expression is driven by the neuronal driver *elav*-GAL4. GAL4: galactose-activatable 4; UAS: upstream activating sequence. ns, no significance; * P<0.05, ** P<0.01, *** P<0.001, and **** P<0.0001.

other tissues, whereas *mrjp1–7* may modulate worker phenotype plasticity and age polymorphism (Dobritzsch et al., 2019). The expression of *mrjp1* through *mrjp7* is characteristic of worker bee heads, with *mrjp1–4* and *mrjp7* expressed at higher levels in nurse bees compared to foragers, while *mrjp5* and *mrjp6* are more highly expressed in foragers than in nurses, indicating additional functions beyond brood food proteins (Buttstedt et al., 2013). Our RNA-seq analysis of in vivo data further suggests that MRJP4, MRJP6, MRJP8, and MRJP9 play distinct functional roles compared to other MRJP proteins.

In *Drosophila*, the YELLOW protein is expressed in certain central neurons from the second larval stage to adulthood, including a group of neurons adjacent to clock neurons marked by the pigment-dispersing factor (PDF) neuropetide (Hinaux et al., 2018). The *yellow* gene, positioned downstream of *fru* in the third instar larval brain, is a crucial component of the somatic sex determination pathway in *Drosophila* and plays an important role in the development of male wing extension during courtship (Drapeau et al., 2003). One Study has also suggested that the influence of YELLOW on male mating success is mediated by pigmentation of a male-specific leg structure called the sex comb rather than through neural circuit regulation (Massey et al., 2019). Also, it has been reported that the *yellow* gene modulates behavioral plasticity by inhibiting courtship in butterfly species with dimorphic males (Connahs et al., 2022). While numerous studies have reported the expression of MRJPs in brain cells of honeybees, research into the biological functions of MRJPs in the brain remains limited. In one study, MRJP proteins were injected directly into the honeybee brain or, conversely, RNAi or corresponding antibodies were used to inhibit MRJP functions in the brain, demonstrating that certain MRJP proteins play a role in regulating worker bee behavior and life history (Fang et al., 2023). However, the precise neurobiological functions of MRJPs are yet to be understood.

The intriguing question is as follows: Why do MRJPs, which have such similar predicted structures, exhibit distinct functions in vivo? This functional divergence may be related to post-translational modifications of the proteins. Previous studies have shown that proteins in RJ undergo glycosylation, with asparagine-linked (N-linked) glycosylation being one of the most common types of post-translational modifications in eukaryotes. All MRJPs are predicted to have 1–8 *N*-glycosylation sites; the glycosylation of MRJP1–4, MRJP6, MRJP7, and MRJP9 has been experimentally confirmed (Okamoto et al., 2003; Kimura et al., 2010; Zhang et al., 2014). Research indicates that MRJP1 and MRJP2 exhibit different glycosylation patterns, and this differential glycosylation indeed affects the antibacterial activity of MRJP2 (Bíliková et al., 2009). Moreover, glycosylation may influence the immunoglobulin E (IgE)-binding capacity of MRJP1 and MRJP2 (Hayashi et al., 2011). These findings suggest that glycosylation plays a significant role in modulating the biological activity of MRJPs.

Several studies have reported the cell proliferation-promoting potential of MRJP proteins extracted from RJ (Mureşan et al., 2022). MRJP1, in particular, exhibits growth factor-like activity: it enhances DNA synthesis in rat hepatocytes, maintains proliferation, and inhibits apoptosis (Kamakura et al., 2001; Kamakura and Sakaki, 2006). Furthermore, MRJP1 stimulates the growth of human bone marrow cells (Watanabe et al., 1998) and human lymphocytes (Moriyama et al., 2015). The 290-kDa oligomer composed of MRJP1 and apisimin enhances lymphocyte proliferation in humans (Tamura et al., 2009). A protein component primarily containing MRJP2, MRJP3, and/or MRJP7 exhibits potential wound-healing bioactivity, promoting the proliferation and migration of human epidermal keratinocytes in an in vitro scratch model (Lin et al., 2019). In its monomeric form, MRJP1 can activate a pluripotency gene network to support the self-renewal of mouse embryonic stem cells (mESCs) (Wan et al., 2018). Even recombinant MRJP1 from *Apis cerana* stimulates the growth of lepidopteran cell lines, further validating these findings (Shen et al., 2010). MRJPs have also been shown to extend the lifespan of *Drosophila* by promoting signaling through the epidermal growth factor (EGF) receptor (EGFR) pathway (Xin et al., 2016), and monomeric MRJP1 similarly influences the lifespan of *Caenorhabditis elegans* by promoting the EGF-EGFR pathway (Detienne et al., 2014). Enhanced EGFR signaling is a key pathway for stimulating cell division and differentiation. In this study, we systematically compared the functions of different MRJPs for the first time and demonstrated at the molecular network regulation level that the overexpression of MRJP1, MRJP2, MRJP3, MRJP5, and MRJP7 significantly activated cell proliferation-related signaling pathways.

Previous research has proposed that royalactin (MRJP1) in RJ drives queen development in bees through an EGFR-mediated signaling pathway (Kamakura, 2011). In this study, the GAL4-UAS system was used to overexpress MRJP1 in *Drosophila*. Compared to the UAS-*mrjp1* control, *Drosophila* with pan-cellular MRJP1 expression exhibited increased body size, cell size, fertility, and lifespan, along with a shortened development time (Kamakura, 2011). Furthermore, similar phenotypic changes were observed when MRJP1 was specifically overexpressed in fat bodies, suggesting that this tissue-specific expression can also replicate the effects of pan-cellular overexpression (Kamakura, 2011). However, these findings have been challenged by subsequent research, arguing that royalactin does not play a definitive role in queen development (Buttstedt et al., 2016). An analysis of the *Drosophila* genetic experiments in the study of Kamakura (2011) revealed that only the UAS-*mrjp1* control was used, without a strictly designed additional GAL4 control group. In this study, we included control groups where GAL4 lines and UAS lines were crossed with wild-type lines to drive MRJP1 overexpression either pan-cellularly or in fat body cells. Contrary to the findings of Kamakura (2011), we did not reproduce the increased body size when MRJP1 was overexpressed with pan-cell or fat body-specific drivers in *Drosophila*. Interestingly, when MRJPs were overexpressed in neurons, some of them did show an increase in body size, a phenomenon warranting further investigation into its underlying mechanisms.

4 Conclusions

In summary, this study highlights the structural and functional diversity of MRJPs within the MRJP/YELLOW family (Fig. 7). It has been shown that despite their highly similar tertiary structures, MRJPs exhibit distinct biological impacts when ectopically expressed in *Drosophila* neurons. Phylogenetic and structural analyses have confirmed the evolutionary relationships among MRJPs, while transcriptome and gene network analyses revealed unique gene expression patterns and signaling perturbations associated with the specific MRJP-overexpression groups. Notably, MRJP1, MRJP2, MRJP3, MRJP5, and MRJP7 overexpression induced the significant activation of cell proliferation-related pathways, consistent with their phylogenetic clustering and gene enrichment profiles. Interestingly, among these five MRJPs, the neuron-specific expression of MRJP1, MRJP2, MRJP3, and MRJP5 led to increased body size in *Drosophila*, whereas their expression in the fat bodies or across all cells did not, highlighting the context-dependent effects of MRJPs on physiological phenotypes. These findings expand our understanding of the functional roles of MRJPs and provide a foundation for further exploring their biological significance in honeybees and beyond.

Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

Data availability statement

The original contributions presented in the study are included in the article and supplementary materials, and further inquiries can be directed to the corresponding author. The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) of the National Genomics Data Center (Nucleic Acids Res. 2022), China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA020151), which is publicly accessible at https://ngdc.cncb.ac.cn/gsa.

Acknowledgments

The work was supported by the National Key Research and Development Program (No. 2024YFF1106502), the Fujian Provincial Natural Science Foundation (No. 2024J01359), the National Natural Science Foundation of China (No. 31970461), and the Qi-Shan scholar grant of Fuzhou University (No. GXRC-20070), China.

We thank Dr. Wenchao YANG (College of Bee Science and Biomedicine, Fujian Agriculture and Forestry University, Fuzhou, China) for providing honeybee samples for RNA extraction. We thank Dr. Jiandong AN and Dr. Mao FENG (Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, Beijing, China) and Dr. Shaokang HUANG (College of Bee Science and Biomedicine, Fujian Agriculture and Forestry University) for critical comments and helpful advice. We thank Dr. Wei WU at the Core Facility of *Drosophila* Resource and Technology, Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences (CAS), Beijing, China, for providing fly stocks and reagents.

Author contributions

Wenfeng CHEN and Yufeng YANG conceived and designed the experiments; Lingqi YU, Danfeng WANG, Jiayu XIE, Dongjing WEN, Yi ZHANG, and Xuanhao CHEN performed the experiments; Wenfeng CHEN, Lingqi YU, Danfeng WANG,

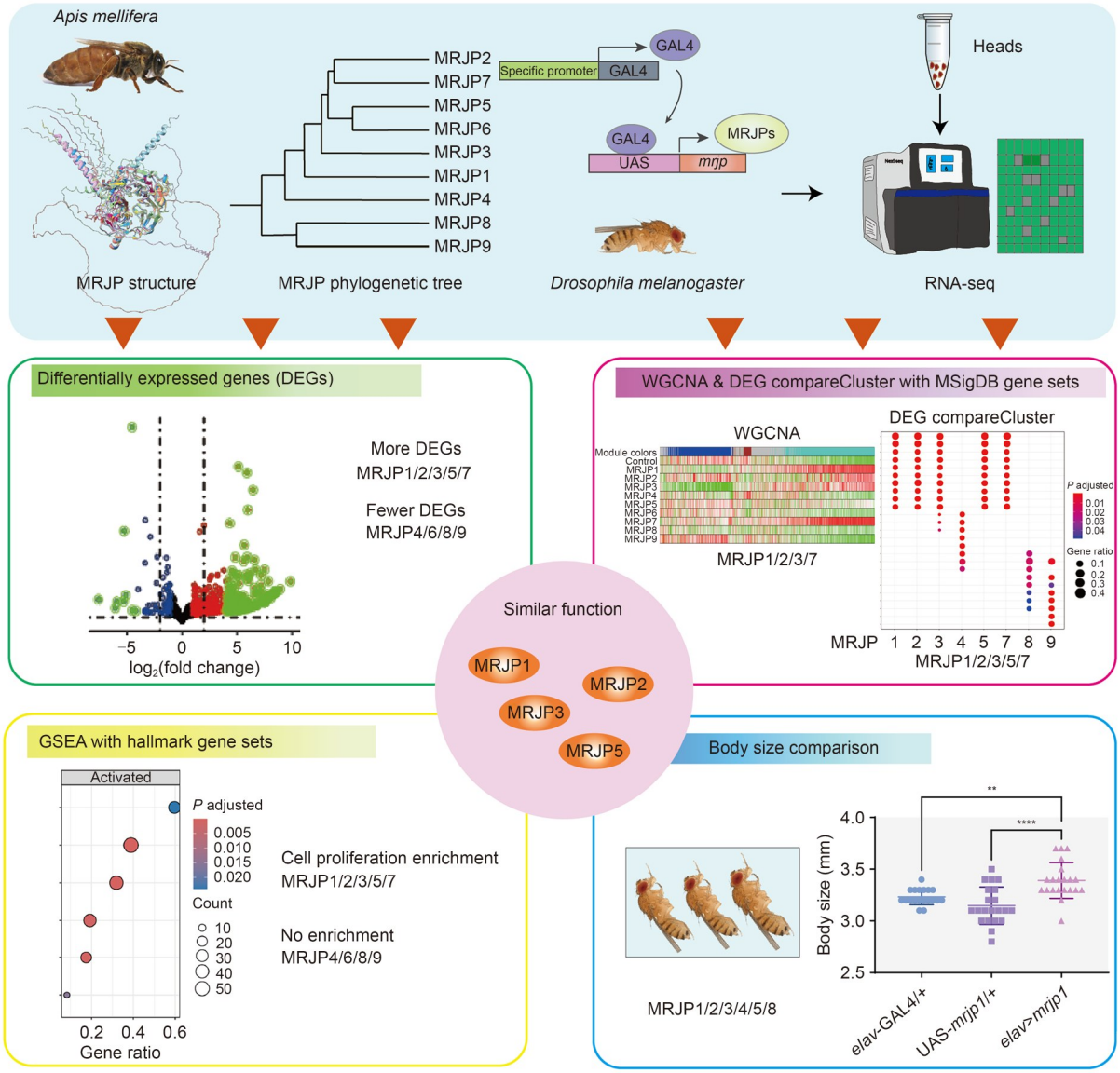

Fig. 7 Workflow summary. MRJP: major royal jelly protein; GAL4: galactose-activatable 4; UAS: upstream activating sequence; RNA-seq: RNA-sequencing; WGCNA: weighted gene co-expression network analysis; MSigDB: Molecular Signatures Database; GSEA: gene set enrichment analysis.

Zhenxing LIU, and Lirong SHEN drafted and revised the manuscript. All the authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Lingqi YU, Danfeng WANG, Xuanhao CHEN, Jiayu XIE, Dongjing WEN, Yi ZHANG, Lirong SHEN, Wenfeng CHEN, Zhenxing LIU, and Yufeng YANG declare that they have no conflicts of interest.

This study used the invertebrate model organism *Drosophila melanogaster*, which is exempt from ethical approval requirements.

Declaration on the use of generative AI tools

During the preparation of this work, the authors used ChatGPT in order to improve language and readability and check for grammatical errors. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

References

Abramson J, Adler J, Dunger J, et al., 2024. Accurate structure prediction of biomolecular interactions with AlphaFold 3. *Nature*, 630(8016):493-500. https://doi.org/10.1038/s41586-024-07487-w

Abu-Serie MM, Habashy NH, 2019. Two purified proteins from

royal jelly with in vitro dual anti-hepatic damage potency: major royal jelly protein 2 and its novel isoform X1. *Int J Biol Macromol*, 128:782-795. https://doi.org/10.1016/j.ijbiomac.2019.01.210

Albert Š, Spaethe J, Grübel K, et al., 2014. Royal jelly-like protein localization reveals differences in hypopharyngeal glands buildup and conserved expression pattern in brains of bumblebees and honeybees. *Biol Open*, 3(4):281-288. https://doi.org/10.1242/bio.20147211

Bíliková K, Šimúth J, 2010. New criterion for evaluation of honey: quantification of royal jelly protein apalbumin 1 in honey by ELISA. *J Agric Food Chem*, 58(15):8776-8781. https://doi.org/10.1021/jf101583s

Bíliková K, Mirgorodskayá E, Bukovska G, et al., 2009. Towards functional proteomics of minority component of honeybee royal jelly: the effect of post-translational modifications on the antimicrobial activity of apalbumin2. *Proteomics*, 9(8):2131-2138. https://doi.org/10.1002/pmic.200800705

Buttstedt A, Moritz RF, Erler S, 2013. More than royal food–*Major royal jelly* protein genes in sexuals and workers of the honeybee *Apis mellifera*. *Front Zool*, 10:72. https://doi.org/10.1186/1742-9994-10-72

Buttstedt A, Ihling CH, Pietzsch M, et al., 2016. Royalactin is not a royal making of a queen. *Nature*, 537(7621):E10-E12. https://doi.org/10.1038/nature19349

Chakrabarti P, Sagili RR, 2020. Changes in honey bee head proteome in response to dietary 24-methylenecholesterol. *Insects*, 11(11):743. https://doi.org/10.3390/insects11110743

Collazo N, Carpena M, Nuñez-Estevez B, et al., 2021. Health promoting properties of bee royal jelly: food of the queens. *Nutrients*, 13(2):543. https://doi.org/10.3390/nu13020543

Connahs H, Tan EJ, Ter YT, et al., 2022. The *yellow* gene regulates behavioural plasticity by repressing male courtship in *Bicyclus anynana* butterflies. *Proc Roy Soc B Biol Sci*, 289(1972):20212665. https://doi.org/10.1098/rspb.2021.2665

Detienne G, de Haes W, Ernst UR, et al., 2014. Royalactin extends lifespan of *Caenorhabditis elegans* through epidermal growth factor signaling. *Exp Gerontol*, 60:129-135. https://doi.org/10.1016/j.exger.2014.09.021

Dobritzsch D, Aumer D, Fuszard M, et al., 2019. The rise and fall of major royal jelly proteins during a honeybee (*Apis mellifera*) workers' life. *Ecol Evol*, 9(15):8771-8782. https://doi.org/10.1002/ece3.5429

Drapeau MD, Radovic A, Wittkopp PJ, et al., 2003. A gene necessary for normal male courtship, *yellow*, acts downstream of *fruitless* in the *Drosophila melanogaster* larval brain. *J Neurobiol*, 55(1):53-72. https://doi.org/10.1002/neu.10196

Drapeau MD, Albert S, Kucharski R, et al., 2006. Evolution of the Yellow/Major Royal Jelly Protein family and the emergence of social behavior in honey bees. *Genome Res*, 16(11):1385-1394. https://doi.org/10.1101/gr.5012006

Fan P, Han B, Feng M, et al., 2016. Functional and proteomic investigations reveal major royal jelly protein 1 associated with anti-hypertension activity in mouse vascular smooth muscle cells. *Sci Rep*, 6:30230. https://doi.org/10.1038/srep30230

Fang Y, Feng M, Ma C, et al., 2023. Major royal jelly proteins influence the neurobiological regulation of the division of labor among honey bee workers. *Int J Biol Macromol*, 225:848-860. https://doi.org/10.1016/j.ijbiomac.2022.11.150

Garcia L, Saraiva Garcia CH, Calábria LK, et al., 2009. Proteomic analysis of honey bee brain upon ontogenetic and behavioral development. *J Proteome Res*, 8(3):1464-1473. https://doi.org/10.1021/pr800823r

Guo H, Kouzuma Y, Yonekura M, 2009. Structures and properties of antioxidative peptides derived from royal jelly protein. *Food Chem*, 113(1):238-245. https://doi.org/10.1016/j.foodchem.2008.06.081

Hayashi T, Takamatsu N, Nakashima T, et al., 2011. Immunological characterization of honey proteins and identification of MRJP 1 as an IgE-binding protein. *Biosci Biotechnol Biochem*, 75(3):556-560. https://doi.org/10.1271/bbb.100778

Hinaux H, Bachem K, Battistara M, et al., 2018. Revisiting the developmental and cellular role of the pigmentation gene *yellow* in *Drosophila* using a tagged allele. *Dev Biol*, 438(2):111-123. https://doi.org/10.1016/j.ydbio.2018.04.003

Hojo M, Kagami T, Sasaki T, et al., 2010. Reduced expression of *major royal jelly protein 1* gene in the mushroom bodies of worker honeybees with reduced learning ability. *Apidologie*, 41(2):194-202. https://doi.org/10.1051/apido/2009075

Kamakura M, 2011. Royalactin induces queen differentiation in honeybees. *Nature*, 473(7348):478-483. https://doi.org/10.1038/nature10093

Kamakura M, Sakaki T, 2006. A hypopharyngeal gland protein of the worker honeybee *Apis mellifera* L. enhances proliferation of primary-cultured rat hepatocytes and suppresses apoptosis in the absence of serum. *Protein Expr Purif*, 45(2):307-314. https://doi.org/10.1016/j.pep.2005.08.004

Kamakura M, Suenobu N, Fukushima M, 2001. Fifty-seven-kDa protein in royal jelly enhances proliferation of primary cultured rat hepatocytes and increases albumin production in the absence of serum. *Biochem Biophys Res Commun*, 282(4):865-874. https://doi.org/10.1006/bbrc.2001.4656

Kim BY, Jin BR, 2019. Antimicrobial activity of the C-terminal of the major royal jelly protein 4 in a honeybee (*Apis cerana*). *J Asia-Pac Entomol*, 22(2):561-564. https://doi.org/10.1016/j.aspen.2019.04.004

Kimura Y, Nagai H, Miyamoto M, et al., 2010. Identification of a royal jelly glycoprotein that carries unique complex-type *N*-glycans harboring the T-antigen (Galβ1-3GalNAc) unit. *Biosci Biotechnol Biochem*, 74(10):2148-2150. https://doi.org/10.1271/bbb.100472

Kumar S, Stecher G, Li M, et al., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms.

Mol Biol Evol, 35(6):1547-1549. https://doi.org/10.1093/molbev/msy096

Kupke J, Spaethe J, Mueller MJ, et al., 2012. Molecular and biochemical characterization of the major royal jelly protein in bumblebees suggest a non-nutritive function. *Insect Biochem Mol Biol*, 42(9):647-654. https://doi.org/10.1016/j.ibmb.2012.05.003

Langfelder P, Horvath S, 2008. WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics*, 9:559. https://doi.org/10.1186/1471-2105-9-559

Liberzon A, Birger C, Thorvaldsdóttir H, et al., 2015. The Molecular Signatures Database hallmark gene set collection. *Cell Syst*, 1(6):417-425. https://doi.org/10.1016/j.cels.2015.12.004

Lin Y, Shao QQ, Zhang M, et al., 2019. Royal jelly-derived proteins enhance proliferation and migration of human epidermal keratinocytes in an in vitro scratch wound model. *BMC Complement Altern Med*, 19:175. https://doi.org/10.1186/s12906-019-2592-7

Majtán J, Kováčová E, Bíliková K, et al., 2006. The immunostimulatory effect of the recombinant apalbumin 1–major honeybee royal jelly protein–on TNFα release. *Int Immunopharmacol*, 6(2):269-278. https://doi.org/10.1016/j.intimp.2005.08.014

Maori E, Navarro IC, Boncristiani H, et al., 2019. A secreted RNA binding protein forms RNA-stabilizing granules in the honeybee royal jelly. *Mol Cell*, 74(3):598-608.e6. https://doi.org/10.1016/j.molcel.2019.03.010

Massey JH, Chung D, Siwanowicz I, et al., 2019. The *yellow* gene influences *Drosophila* male mating success through sex comb melanization. *eLife*, 8:e49388. https://doi.org/10.7554/eLife.49388

Matsui T, Yukiyoshi A, Doi S, et al., 2002. Gastrointestinal enzyme production of bioactive peptides from royal jelly protein and their antihypertensive ability in SHR. *J Nutr Biochem*, 13(2):80-86. https://doi.org/10.1016/s0955-2863(01)00198-x

Mootha VK, Lindgren CM, Eriksson KF, et al., 2003. PGC-1α-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet*, 34(3):267-273. https://doi.org/10.1038/ng1180

Moriyama T, Ito A, Omote S, et al., 2015. Heat resistant characteristics of Major Royal Jelly Protein 1 (MRJP1) oligomer. *PLOS One*, 10(5):e0119169. https://doi.org/10.1371/journal.pone.0119169

Mureşan CI, Dezmirean DS, Marc BD, et al., 2022. Biological properties and activities of major royal jelly proteins and their derived peptides. *J Funct Foods*, 98:105286. https://doi.org/10.1016/j.jff.2022.105286

Nagai T, Inoue R, 2004. Preparation and the functional properties of water extract and alkaline extract of royal jelly. *Food Chem*, 84(2):181-186. https://doi.org/10.1016/s0308-8146(03)00198-5

Okamoto I, Taniguchi Y, Kunikata T, et al., 2003. Major royal jelly protein 3 modulates immune responses in vitro and in vivo. *Life Sci*, 73(16):2029-2045. https://doi.org/10.1016/s0024-3205(03)00562-9

Park MJ, Kim BY, Deng YJ, et al., 2020. Antioxidant capacity of major royal jelly proteins of honeybee (*Apis mellifera*) royal jelly. *J Asia-Pac Entomol*, 23(2):445-448. https://doi.org/10.1016/j.aspen.2020.03.007

Peixoto LG, Calábria LK, Garcia L, et al., 2009. Identification of major royal jelly proteins in the brain of the honeybee *Apis mellifera*. *J Insect Physiol*, 55(8):671-677. https://doi.org/10.1016/j.jinsphys.2009.05.005

Radovic A, Wittkopp PJ, Long AD, et al., 2002. Immunohistochemical colocalization of Yellow and male-specific fruitless in *Drosophila melanogaster* neuroblasts. *Biochem Biophys Res Commun*, 293(4):1262-1264. https://doi.org/10.1016/S0006-291X(02)00366-2

Schmitzová J, Klaudiny J, Albert Š, et al., 1998. A family of major royal jelly proteins of the honeybee *Apis mellifera* L. *Cell Mol Life Sci*, 54(9):1020-1030. https://doi.org/10.1007/s000180050229

Shen LR, Zhang WG, Jin F, et al., 2010. Expression of recombinant AccMRJP1 protein from royal jelly of Chinese honeybee in *Pichia pastoris* and its proliferation activity in an insect cell line. *J Agric Food Chem*, 58(16):9190-9197. https://doi.org/10.1021/jf1007133

Subramanian A, Tamayo P, Mootha VK, et al., 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA*, 102(43):15545-15550. https://doi.org/10.1073/pnas.0506580102

Tamura S, Amano S, Kono T, et al., 2009. Molecular characteristics and physiological functions of major royal jelly protein 1 oligomer. *Proteomics*, 9(24):5534-5543. https://doi.org/10.1002/pmic.200900541

Tian WL, Li M, Guo HY, et al., 2018. Architecture of the native major royal jelly protein 1 oligomer. *Nat Commun*, 9:3373. https://doi.org/10.1038/s41467-018-05619-1

Uversky VN, Albar AH, Khan RH, et al., 2021. Multifunctionality and intrinsic disorder of royal jelly proteome. *Proteomics*, 21(6):2000237. https://doi.org/10.1002/pmic.202000237

Vezeteu TV, Bobiş O, Moritz RFA, et al., 2017. Food to some, poison to others - honeybee royal jelly and its growth inhibiting effect on European Foulbrood bacteria. *Microbiologyopen*, 6(1):e00397. https://doi.org/10.1002/mbo3.397

Wan DC, Morgan SL, Spencley AL, et al., 2018. Honey bee Royalactin unlocks conserved pluripotency pathway in mammals. *Nat Commun*, 9:5078. https://doi.org/10.1038/s41467-018-06256-4

Watanabe K, Shinmoto H, Kobori M, et al., 1998. Stimulation of cell growth in the U-937 human myeloid cell line by honey royal jelly protein. *Cytotechnology*, 26(1):23-27. https://doi.org/10.1023/A:1007928408128

Wen DJ, Xie JY, Yuan Y, et al., 2024. The endogenous antioxidant ability of royal jelly in *Drosophila* is independent of Keap1/Nrf2 by activating oxidoreductase activity. *Insect Sci*, 31(2):503-523. https://doi.org/10.1111/1744-7917.13252

Xin XX, Chen Y, Chen D, et al., 2016. Supplementation with

major royal-jelly proteins increases lifespan, feeding, and fecundity in *Drosophila*. *J Agric Food Chem*, 64(29): 5803-5812.
https://doi.org/10.1021/acs.jafc.6b00514

Zhang L, Han B, Li RL, et al., 2014. Comprehensive identification of novel proteins and N-glycosylation sites in royal jelly. *BMC Genomics*, 15:135.
https://doi.org/10.1186/1471-2164-15-135

Zhang XQ, Yu Y, Sun P, et al., 2019. Royal jelly peptides: potential inhibitors of β-secretase in N2a/APP695swe cells. *Sci Rep*, 9:168.
https://doi.org/10.1038/s41598-018-35801-w

Zhang ZJ, Mu XH, Cao QN, et al., 2022. Honeybee gut *Lactobacillus* modulates host learning and memory behaviors via regulating tryptophan metabolism. *Nat Commun*, 13:2037.
https://doi.org/10.1038/s41467-022-29760-0

Supplementary information

Materials and methods; Figs. S1–S5; Table S1