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Review article

Molecular insights into the enhanced performance of royal jelly secretion by a stock of honeybee (*Apis mellifera ligustica*) selected for increasing royal jelly production

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Abstract – The genetic selection of a high royal jelly (RJ)-producing strain of bees (RJBs) from Italian bees (ITBs) has been conducted for nearly four decades in China since the 1980s. RJBs are the most important producers of RJ in the world and produce > 90% of the total output with an annual market value of > $40 million. With technological advancements in proteomics, the mechanism underpinning the high RJ production by RJBs has been explored to new depths in the last decades. Here, we give an overview that the mechanism driving the enhanced performance of RJ secretion by RJBs. First, the selection of RJBs, high-efficiency technology for RJ production, and advances in genetic characterization of RJBs are reviewed. Then, proteome and phosphoproteome characterization that decipher the augmented RJ production using honeybee organs and tissues are summarized. This may be potentially useful in gaining a complete mechanistic insight into the high performance of RJ yields in honeybees, and expands understanding of the honeybee biology.

Italian bees / royal jelly bees / royal jelly production / molecular basis - proteome

1. INTRODUCTION

Whole-genome sequencing revolutionized a wide cascade of biological research and brought an opportunity for a more thorough investigation of areas like insect development, physiology, and evolution (Suryamohan and Halfon 2015). It is with the completion of genome sequencing of *A. mellifera*, together with technological advances in protein separation and mass spectrometry (MS) resolution, sensitivity, and accuracy, that proteomics has become one of the most important tools in addressing the wide aspects of honeybee biology such as physiology, behavior, and pathology (Valcu and Kempenaers 2015; Hao and Li 2016).

Honeybees are commonly known for their production of valuable substances such as honey, RJ, propolis, and other products (Bogdanov et al. 2008). RJ is a special food for young larvae and the honeybee queen throughout her lifetime. Because of this, it is assumed that RJ extends the lifespan of queen bees relative to worker bees (Fratini et al. 2016). RJ is rich in nutrients such as proteins, sugars, vitamins, and a large number of bioactive substances, such as a 10-hydroxy-2-decenoic acid (10-HDA) (Viuda-Martos et al. 2008). The 10-HDA is a special component of a lipid fraction in RJ (Blum et al. 1959; Lercker et al. 1981), which has antibiotic and immunomodulatory activities (Townsend et al. 1959; Vucevic et al. 2007). Some of the RJ proteins such as major royal jelly proteins (MRJPs) (Mairesse et al. 1998), jelleines (Fontana et al. 2004), and royalisin (Fujiwara et al. 1990) are reported to have properties of antimicrobial, antifungal, anti-tumor, anti-diabetic, and anti-hypertensive

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activities (Bíliková et al. 2001; Oka et al. 2001; Tokunaga et al. 2004). In addition, RJ regulates inflammation, oxidative stress, and vasodilation activity (Okamoto et al. 2003; Liu et al. 2008; Nakajima et al. 2009; Kolayli et al. 2016; Yang et al. 2018). These RJ-induced activities are widely believed to help maintain homeostasis and recover from pathological conditions; therefore, RJ has been used as a cosmetic, health food, or dietary supplement (Ramadan and Al-Ghamdi 2012; Cornara et al. 2017; Yoneshiro et al. 2018). RJ affects the immune system, including malignant tumors, under a variety of physiological and pathological conditions, not only stimulating immune-active cells but also stimulating antibody production (Melliou and Chinou 2005; Izuta et al. 2009; Yuksel and Akyol 2016; Kocot et al. 2018). Based on some facts, there is a hypothesis that RJ may have some health benefits (Miyata and Sakai 2018), but without a conclusive evidence (e.g., clinical trials), no causal relationship can be established between the consumption of RJ and the claimed health benefits (EFSA Panel on Dietetic Products N.a.A.N 2011). Further information could be found from recent reviews on the composition of RJ and its potential utilization (Ramadan and Al-Ghamdi 2012), the origin and function of the honeybee MRJPs (Buttstedt et al. 2014), and its antimicrobial properties (Fratini et al. 2016) and medicinal value (Bogdanov 2017), and its potential applications for cancer treatment (Miyata and Sakai 2018).

To increase RJ outputs, huge efforts have been made for bee scientists and beekeepers in China. The genetic selection of a high RJ-producing strain of bees (RJBs, *A. m. ligustica*) from Italian bees (ITBs) has been conducted for nearly four decades in China since the 1980s. The RJBs can now produce 10 times greater amount of RJ than ITBs (Li et al. 2007a, b; Li et al. 2010; Feng et al. 2015; Han et al. 2015). Given the significantly elevated RJ outputs by RJBs, China is now the world's largest RJ producer and produces ~ 3500 tons of RJ per year, which accounts for more than 90% of the world RJ market (Krell 1996; Cao et al. 2016; Fratini et al. 2016). Many factors are likely to influence RJ production by honeybees (Cao et al. 2016). It was in the year 2003 that the performance of RJBs for enhanced RJ yield was identified as an inheritable trait (Li et al. 2003a, b). Since then, attempts have been made to elucidate the molecular basis of these augmented yields. The questions that have been given emphasis include the following: how do the RJBs achieve the high performance of RJ production? Is there any difference in protein components between the RJ of RJBs and the lower productions of other honeybee species and/or lines?

In order to attempt to answer these questions, various types of studies have been performed in recent decades (Cao et al. 2016). Some of the identified markers of RJBs are morphological. For instance, the correlation between length of the hypopharyngeal glands (HGs) and RJ production is positive, and length of the HGs has been recommended as one of the markers associated with RJ outputs (Su and Chen 2003). In addition, seven microsatellite DNA loci (159 bp at A29,100 bp and 104 bp at A24, 110 bp at A7, 126 bp at A43, 221 bp at A14, and 221 bp at A113) are found as possible molecular markers of the RJBs (Chen et al. 2005). Although these markers are important for marker-based selection, knowledge on the molecular mechanism that drives the stronger performance of RJ production by RJBs is still required. To be noted, recent proteome researches have revealed that RJBs have reshaped the proteome setting of the HGs (Li et al. 2010; Ji et al. 2014), mandibular glands (MGs) (Huo et al. 2016), hemolymph (Ararso et al. 2018), and nervous system (Han et al. 2015; Han et al. 2017), to support their biological performance for the elevated RJ production, as compared to ITBs and other honeybee species and/or lines. In addition, proteome, phosphoproteome, and glycoproteome comparisons of RJ from RJBs and/or other honeybee species/lines have been carried out (Li et al. 2007a, b; Chen and Li 2009; Fang et al. 2010). For instance, proteomic analysis of the RJB HGs reveals a group of up-regulated proteins involved in energy metabolism and protein biosynthesis etc. (Li et al. 2010), which matches with the fact that the HGs of the RJBs could secrete more RJ than those of ITBs. With regard to the protein complements in RJ produced by high and low RJ producing bee stocks/lines, there is a significant difference found between Carnica bees and RJBs or ITBs, whereas

there is no significant difference found between RJBs and ITBs (Li et al. 2007a, b). Moreover, there are significantly abundant protein species found in the RJ of RJBs relative to in that of *Apis cerana cerana* (*Acc*) (Fang et al. 2010). Here, we focus on the scientific community's attention to the cluster of current knowledge on the mechanistic understanding of enhanced RJ production. First, we give an overview of the selection process of RJBs, high-efficiency RJ production technology, advances in genetic characterization of RJBs, biological significance of RJ from different honeybee species or lines, and genetic and molecular basis of enhanced RJ secretion. Furthermore, the proteome and phosphoproteome of honeybee organs and tissues that underlie molecular differences for strengthened RJ production are also reviewed. This provides useful information on the mechanistic insight into the high performance of RJ yields by RJBs and it expands our understanding of the honeybee biology.

2. OVERVIEW OF THE SELECTION PROCESS OF RJBS

From as early as the 1930s, ITBs (*Apis mellifera ligustica*) from Japan were introduced in China by a Chinese ambassador. Since then, the ITBs have experienced tremendous expansion in colony number in China. In 1994, the number of Italian bees and other *Apis mellifera* sub-species reached over 6 million colonies, and now over 9 million colonies, accounting for 1/10th of the world total number (http://www.fao.org/faostat/en/#home).

RJ is one of the most important products for Chinese beekeepers. Regarding the technology of RJ production, China started producing RJ following the method reported by French literature in 1957 (Chen 1989). The history of the high RJ bee lineage is summarized by Cao et al. (2016). Initially, before the start of the high RJ production breeding program, China's RJ production was only 0.2–0.3 kg/colony/year (Chen 2005). In the 1950s, due to the increased demand for RJ, the beekeepers in Zhejiang Province invented the RJ production method using *A. m. ligustica* queenright colonies (CNCAGR 2011). The main concept of RJ collection from the colony is the application of handmade wax-based queen cells, which are fixed on wooden bars and put the wax queen cells with grafted larvae into the queenless chamber of the hive. Although this technological setup works well, the RJ yields are quite low. Normally, a colony can produce about 20 g in a 72-h cycle. During this process, beekeepers have recognized the importance of productive honeybee stocks and developed new tools. Since the 1960s, beekeepers in parts of Zhejiang Province have tended to select *A. m. ligustica* colonies for improved RJ production (CNCAGR 2011). After more than 20 years of semi-controlled breeding, the RJ yield of each colony in some apiary farms increased to 2.0–3.0 kg/year (Chen 2005). In 1979, the production of RJ started on a mass scale, and the output was about 150 tons that year (Qiu 1999). Since 1980, the Chinese government has noticed the importance of the product to the country and has begun investing in the selection program. In 1986, a stock of honeybees was reported to be selected from ITBs by beekeepers in Zhejiang Province, a stock that could produce an average of 87.43% higher amount of RJ than non-selected lines at that time (Wencheng et al. 1989). The selection process improved the production ranging from 6.0 to 8.0 kg/colony/year in the 2000s (Chen 2005). Through continuous selection, RJBs can now produce more than 10 times (normally 10 kg/colony/year) the amount of RJ of native ITBs (Li et al. 2003a, b; Huo et al. 2016; Ararso et al. 2018). Currently, China produces ~3500 tons of RJ each year, covering over 90% of the international RJ market of which Japan, the USA, and Europe are the major importers (Zheng et al. 2011).

3. HIGH-EFFICIENCY RJ PRODUCTION TECHNOLOGY

Over the past 40 years, the increase in the production of RJ in China has mainly been made possible due to developmental refinement of RJBs (Li 2000; Li et al. 2003a, b; Chen 2005; Li et al. 2007a, b; Feng et al. 2015; Hu et al. 2017), and the development and implementation of production techniques that allow the increase and optimization of RJ production (Li 2000; Li et al. 2003a, b; Li and Aiping 2005; Cao et al. 2016; Hu et al.

2017). A standard procedure for RJ production (Fig. 1a–f) in China has been summarized by Li and his colleagues (2003a, b). In brief, the requirements for high RJ production are found to include the following: good queen, large and strong colony throughout the bee season (the more populous the colony, the better for RJ production), sufficient food supply (including honey and pollen), agreeable temperature (20–30°C), efficient tools for RJ production, and experienced beekeepers. Of these, the good queen is the most important factor that influences the overall performance of RJ yields of a colony.

The development and implementation of powerful tools play an important role in the efficient production of RJ. The use of modern RJ production facilities such as plastic queen cells, larval grafting tools, and plastic spatulas made it possible to produce RJ on a large scale (Li 2000; Li et al. 2003a, b; Li and Aiping 2005). For instance,

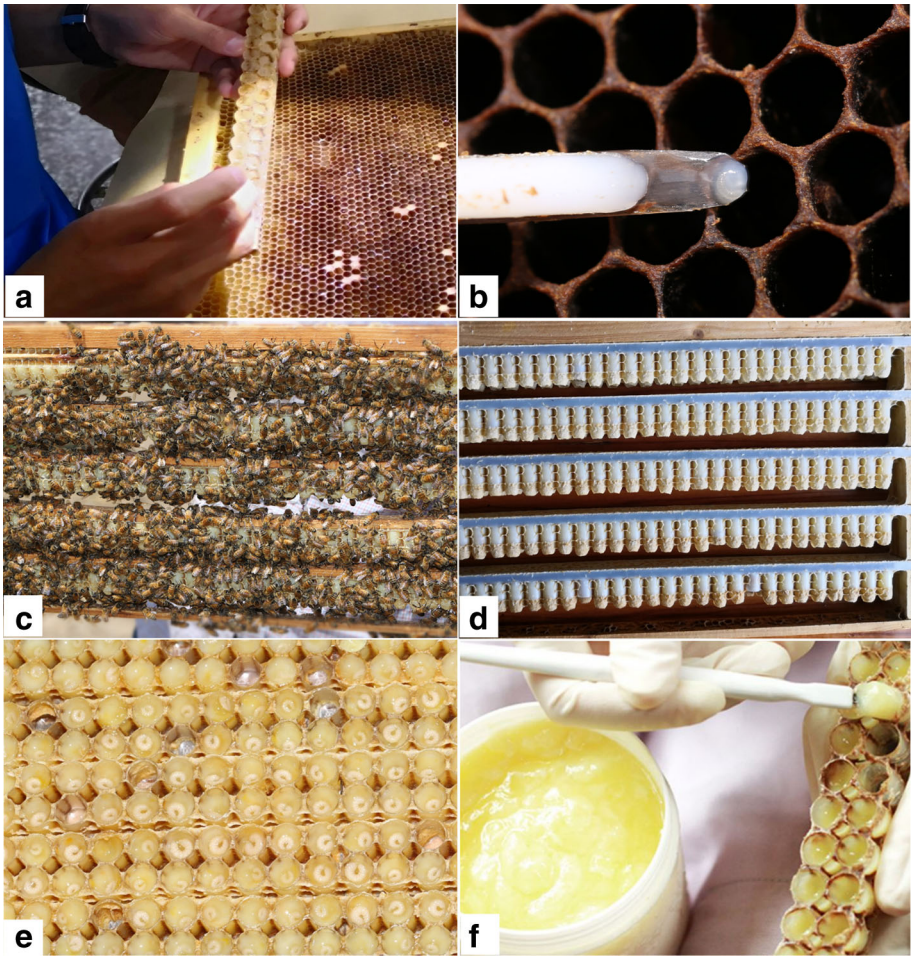


Figure 1. A standard procedure for RJ production. A standard procedure for RJ production in China (**a**, **b**, **c**, **d**, **e**, and, **f**) involves the following steps: 1-day-old larvae are grafted into plastic queen cups mounted on bars; the bars are put into the queenless portion of the colony, separated by a queen excluder (NBs then feed the larvae in the queen cups with royal jelly); the bars are removed from the colony after about 3 days; larvae are removed from the queen cups using forceps; and the RJ is collected with a plastic spatula and packed. In addition to this, the necessary tools for royal jelly production like plastic queen cells, transfer tools, forceps and plastic bottle, and experienced beekeepers are required. Moreover, a typical royal jelly producing colony should be two stories and must also be populous. Photographs are provided by Professor. Dr. Jianke Li.

the introduction of the plastic queen cell cups fixed on a wooden strip in the 1980s became an innovative step (Chen and Lin 1987; Liu et al. 2011). The replacement of the handmade wax-based queen cells with the plastic queen cells was a revolutionary step. The use of these plastic queen cells could improve the RJ production by 20–30% (Liu et al. 2011; Zheng et al. 2011). Moreover, recently, a bionic non-grafting larva ovipositor was designed, which excluded the larval grafting stage and significantly reduced the labor intensity required for RJ production (Zhang et al. 2013; Wu et al. 2015). Moreover, the development of RJ collecting machines has enabled large-scale RJ production by reducing labor and improving harvesting efficiency (Liu et al. 2011). For instance, the machine (Fig. 2a, b) could harvest at least 12 kg of RJ in 40 min once the larvae are taken out from the plastic queen cells. The integrated techniques and tools have improved the average RJ yield of RJBs per colony to about 200 g in a 72-h production cycle and 10 kg per year (Zheng et al. 2011). In general, owing to decades of research on the development of high RJ-producing strains, implementation of modern RJ production techniques and facilities, coupled with extensive experience of Chinese beekeepers in raising and managing colonies for efficient RJ production have greatly contributed to the increased RJ yields (Cao et al. 2016; Hu et al. 2017).

4. GENETIC CHARACTERIZATION OF RJBS

Since the successful selection of RJBs, the genetic characterization of this line has been conducted. The RJ producing ability of honeybees is a quantitative trait and is dominated by genotypic effects (Li et al. 2003a, b). Genetics of the RJB lineage is first described using allozymes as molecular markers (Yin et al. 2011). A microsatellite-based study shows a genetic differentiation between RJBs and ITBs, and a high degree of polymorphism within RJBs (Chen et al. 2005). In another study, genotype and allele frequencies, and degree of homozygosity reveals significant variation between workers of RJBs and ITBs (Liang-xian et al. 2004), which is due to the effects of genes

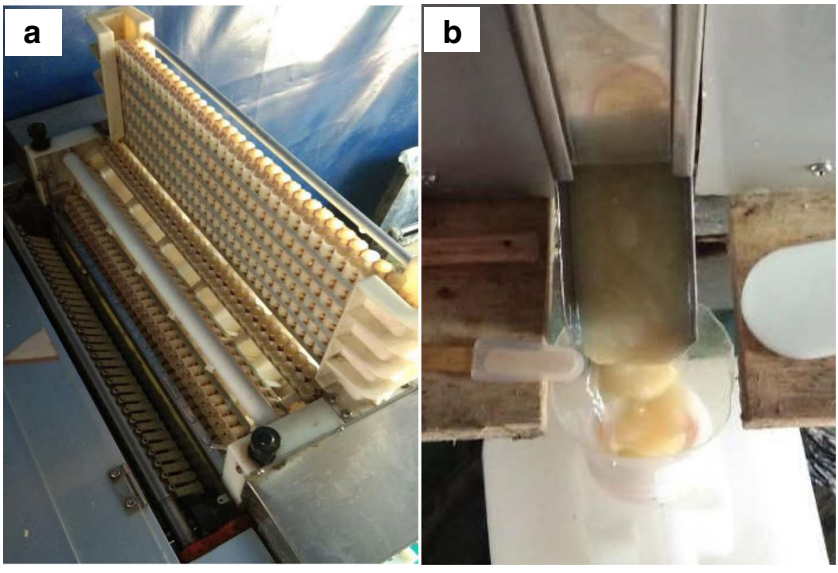


Figure 2. The development of automatic RJ collecting machines has enabled large-scale RJ production, both by reducing labor and improving harvesting efficiency. **a** shows the part of the machine where the plastic queen cells containing the RJ is inserted and extracted, **b** shows the outlet for the extracted RJ on the machine. Once the larva is taken out from the plastic queen cells and becomes ready for extraction, the machine could harvest at least 12 kg of RJ in 40 min.

that respond to artificial selection as a result of their favorable effect on a selected trait in the RJB lineage (Cao et al. 2016). Furthermore, the RJBs are found to contain a 632-bp of DNA fragment at a higher frequency than ITBs (Liang-xian et al. 2004), and compared to ITBs, the trait for high RJ production is found to associate with a 316-bp DNA fragment in RJBs (Zhang et al. 2001). Moreover, the frequency of certain alleles (A7, A14, A24, A29, A43, and A113) is found to correlate with RJ production, and these alleles are suggested as markers for high RJ production (Chen et al. 2005).

A cDNA microarray was used to asses gene expression levels and identify differentially expressed genes between the RJBs and ITBs (Nie et al. 2017). Specifically, three genes: MRJP4, 60 kDa heat shock protein, and heat shock 70 kDa protein cognate 3, and other genes, such as ribosomal protein, skeleton, and proteasome, are up-regulated in the head of RJBs relative to ITBs (Nie et al. 2017). This is consistent with the proteome data of the HG development of worker bees of RJBs and ITBs (Li et al. 2010). The up-regulated MRJP 4 in RJBs both at the transcriptional and proteomic levels suggest that the abundance of MRJPs may be increased, as MRJP 4 is a major protein for total RJ (Schmitzová et al. 1998; Albert et al. 1999). The ribosomal proteins, heat shock proteins, and proteasome in the head of RJBs (Nie et al. 2017) are consistent with other studies on the HGs (Feng et al. 2009; Li et al. 2010; Ji et al. 2014), suggesting that they may involve in nursing behavior by accelerating protein biosynthesis. In addition, a comparative genetic analysis of three stocks of Western honeybees: the RJBs, ITBs, and Chinese ITBs, reveal that RJ output and queen cell acceptance (Fig. 3) are genetically dominant traits (Li et al. 2003a, b). All above-mentioned information is suggestive of the facts that genetic selection for increasing RJ yields on RJBs may differentiate the genetic structures as compared with ITBs, thereby the molecular basis involved in high RJ production between both bee stocks may be further divergent.

5. MOLECULAR INSIGHTS INTO THE RJBS ACHIEVING HIGH PERFORMANCE OF RJ YIELDS

Proteomics is becoming an important tool for describing bee biology at the molecular level (Zewdu Ararso et al. 2018). The molecular basis of different organs and tissues that allow bees to perform their biological tasks has been explained on the proteome scale, for example in the brain (Hernandez et al. 2012; Chan et al. 2013; Han et al. 2015; Han et al. 2017; Meng et al. 2018), hemolymph (Ararso et al. 2018), embryo (Fang et al. 2014; Fang et al. 2015), antennae in Varroa resistance (Parker et al. 2012; Hu et al. 2016), and venom (Matysiak et al. 2014; Matysiak et al. 2016; Matysiak et al. 2017).

In the past few years, extensive research has been conducted to gain insight into the molecular basis for enhanced RJ production in RJB. To achieve a 10× higher RJ yield, RJB workers adjusted different proteomics strategies by enhancing

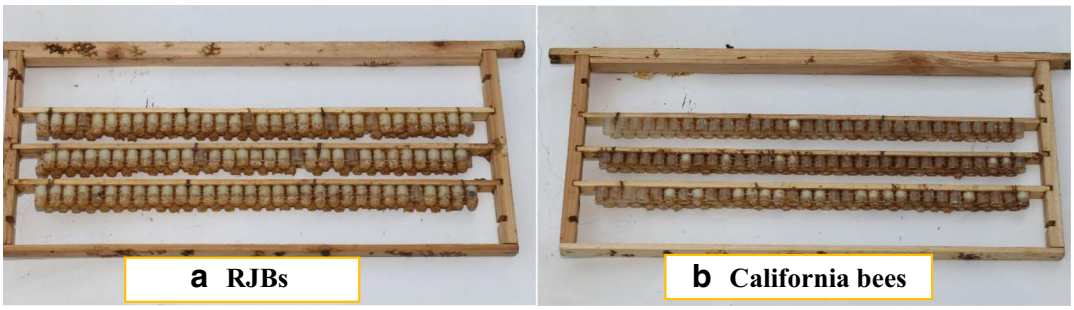


Figure 3. RJ output and queen cell acceptance are genetically dominated traits. Genome sequencing of RJBs and California bees have found significant genetic variation, and queen cell acceptance in RJBs (**a**) is higher than in California Bees (**b**). Photographs are provided by Professor. Dr. Jianke Li.

wider ranges of pathways as compared to the ITB workers (Li et al. 2010). For example, in HG of RJB, up-regulated proteins are involved in pathways such as protein biosynthesis, protein folding, and carbohydrate metabolism, suggesting that their biological effects are induced to enhance glandular activity, thereby enhancing RJ secretion (Li et al. 2010). In addition, phosphorylation has been shown to regulate the protein activity of HG at all ages of worker bees, suggesting that phosphorylation optimizes the biochemical activity of worker bees HG (Qi et al. 2015). In RJB NB, the pathways of protein synthesis and energy metabolism are functionally induced to consolidate enhanced RJ secretion compared to ITB (Hu et al. 2019). Also, in RJBs enhanced levels of neuropeptides are involved in regulating water homeostasis, brooder pheromone recognition, foraging ability, and pollen collection to regulate RJ secretion behavior (Han et al. 2015). Moreover, in nurses of RJB, the activity of phosphatidylinositol signaling and arachidonic acid metabolism is increased to enhance the olfactory response to larval pheromone stimulation (Han et al. 2017). The mandibular gland of RJB also selectively improved lipid synthesis-related pathways to maintain an appropriate ratio of 10-hydroxy-2-decenoic acid, an important fatty acid in RJ, for larval nutrition, also contributes to the increase RJ production (Huo et al. 2016).

The following subsections provide detail discussions of the molecular basis of different organs and tissues, enabling RJB to function in higher RJ production. At last, we also summarize the literature on the species-specific biological effects of RJ by comparing the proteome, phosphoproteome, and glycoproteome of RJ from RJBs and other honeybee species.

5.1. Proteome comparison of HGs of honeybee workers between RJBs and ITBs

The worker bees' ontogeny depends on the differential expression of proteins in organs and tissues in concert with their distinct age-dependent physiology. RJ is secreted by young workers, and RJ protein is a cocktail secretion of three glands: hypopharyngeal, postcerebral, and thoracic glands (Fujita et al. 2013), and most of the RJ proteins are secreted by HGs of the nurse bees (NBs) (Fujita et al. 2013; Ji et al. 2014). The HGs are located in the anterior part of the bee's head (Li et al. 2008) and each gland consists of hundreds of oval acini that are attached to an axial duct that opens into the sub-oral part of the hypopharynx (Ohashi et al. 1997; Deseyn and Billen 2005).

The HGs are the most important organ to secrete RJ. Morphologically, the size of the acini in HGs radically changes with age development (Albert et al. 2014, Ji et al. 2014), reaches their peak at about day 6, and decreases after day 15 during the active seasons (Feng et al. 2009). The activity of the HG is positively correlated with its size (Deseyn and Billen 2005). Specifically, the amount of RJ secretion is positively correlated with the acini size (Albert et al. 2014). Physiologically, the HGs and their acini size are well studied (Crailsheim and Stolberg 1989). The HGs develop mainly during the nursing stage of individual bees and degenerates in forager bees (FBs), although at an early stage of foraging, bees still have well-developed HGs (Sasagawa et al. 1989). With the age-dependent roles of worker bees, the proteins synthesized by the HGs vary (Kubo et al. 1996). In NBs, the HGs develop a high rate of protein synthesis, but the gland activity reverts in FBs (Ohashi et al. 2000). Moreover, depending on the role of the worker bee in the colony, the biological function of the HGs changes from secreting RJ in NBs to producing digestive enzymes (sucrose hydrolyzing enzymes) in FBs (Simpson et al. 1968; Kubo et al. 1996; Ohashi et al. 1997; Moraes and Bowen 2000). Biochemically, the gland expresses specific genes in the NBs and FBs to fit with their age-related roles. In the HGs, major proteins synthesized change from RJ in NBs to α-glucosidase in of FBs (Ohashi et al. 1996). In FBs, the produced proteins in HGs such as α-glucosidase, glucose oxidase, galactosidase, esterase, lipase, and leucine arilamidase are used in the process of converting nectar into honey (Kubo et al. 1996; Ohashi et al. 1999; Deseyn and Billen 2005; Santos et al. 2005). Furthermore, in the HGs, abundance level of MRJPs changes with age and with task (Li et al. 2010), and peak levels of proteins in the HGs occur during day 6–12 in NBs (Feng et al. 2009). Notably, the age at which the HGs of the RJBs and ITBs

potentially start RJ secretion varied. The HGs of RJB may have the potential of RJ secretion on day 3 after emergence, whereas this is day 6 for ITBs (Feng et al. 2009).

Proteomics has been widely applied to reveal the molecular basis of enhanced RJ production in RJBs. To attain a tenfold greater RJ yield, the RJB workers have adapted a different proteomic strategy by enhancing wider ranges of pathways as compared to the ITB workers (Fig. 4) (Li et al. 2010). For instance, in the HGs of RJBs, the up-regulated proteins are implicated in pathways such as in protein biosynthesis, protein folding, and carbohydrate metabolism, suggesting their biological roles are induced to boost the gland activity that in turn enhances RJ production (Li et al. 2010). Moreover, across ages of worker bees, phosphorylation is shown to regulate protein activity of HGs, showing that phosphorylation optimizes the biochemical activity of the HGs of worker bees (Qi et al. 2015). For instance, phosphorylation regulates proteins involved in key biological pathways, such as the centrosome cycle, mitotic spindle elongation, macromolecular complex disassembly, and ribosome. Here, in the RJBs, the trait of high RJ production is shown to associate with several important proteins and pathways. However, the specific activity of these proteins in related pathways still remains to be further investigated.

5.2. Proteome comparison of MGs during the adult life between ITBs and RJBs

The biological function of the MGs secretion has both a reproductive and a non-reproductive role in the colony (Plettner et al. 1996). Secretions from the MGs have important caste-specific functions that are associated with the social evolution of honeybees (Vallet et al. 1991; Wu et al. 2017). In worker bees, the MGs secrete lipids for larval nutrition and pheromones. Using proteomics, the regulatory mechanism that determines the development of MG and the metabolism involved in lipid and pheromone synthesis in both RJBs and ITBs is well investigated (Huo et al. 2016).

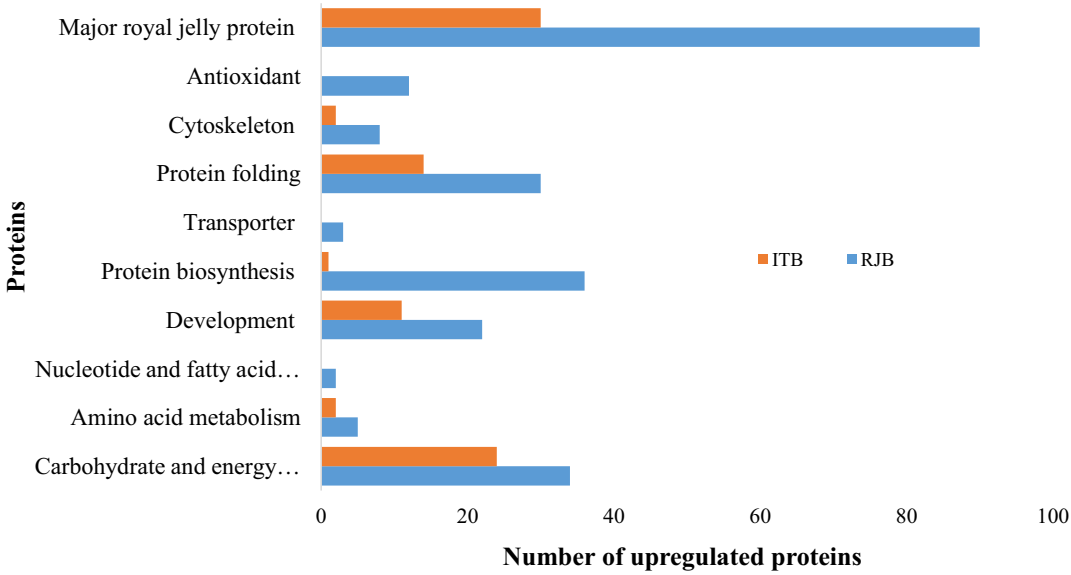


Figure 4. Proteome of HGs of RJB workers consolidates wide ranges of proteins as compared to ITB. In HGs of RJBs, the up-regulated proteins like protein synthesis and energy metabolism are implicated in a wide range of pathways to boost the HGs' functionality, such as protein biosynthesis, protein folding, and carbohydrate metabolism. Blue and red bars represent the number in the RJB and the ITB, respectively. Data were used from Li et al. (2010).

The proteome analysis of MGs of worker RJBs and ITBs across ages (NEBs, NBs, and FBs) shows a wide range of different biological process that defines different programs to underline the specific role of the sub-casts in the colony (Huo et al. 2016). For instance, in NEBs, the proteome shapes the initiation of young MG development; in NBs, it drives high secretory activity in lipid synthesis, whereas in FBs, it synthesizes scent markers to increase foraging efficiency by inducing activities such as lipid metabolism and small molecules. Notably, in NBs, specific and highly abundant proteins are mainly enriched in pathways related to substance transport and lipid synthesis, indicating their priority in priming high secretory activity in lipid synthesis as larval nutrition. It is through this process that the NBs of RJBs contribute to the elevated RJ production by maintaining the required proportion of lipids (a major component of RJ) through activated lipid synthesis and minimizing its degradation to increase 10-HDA synthesis (Huo et al. 2016).

5.3. Neurobiological basis of elevated RJ production: brain membrane proteome and phosphoproteome comparison of RJBs and other honeybee species and/or lines

Among the different roles of NBs, one is to feed the queen and the larvae aged less than 3 days with RJ. RJ is largely blended from the secretion of the HGs and MGs, these two glands reshaped their proteome to drive the gland development and functionality for augmented RJ production (see previous sections). The brain of RJB nurses has developed a unique neuropeptidome in response to enhanced RJ production, as RJ secretion is behavior performed by NBs. For instance, in RJBs, the enhanced level neuropeptide implicated in regulating water homeostasis, brood pheromone recognition, foraging capacity, and pollen collection, suggests their involvement in the regulation of RJ secretion (Han et al. 2015). Moreover, both membrane proteome and phosphoproteome have evolved unique settings to adapt to the elevated RJ secretion. Likewise, across all adult phases, more membrane proteins are expressed in the brain of RJBs than of ITBs (Fig. 5a, b). In each stage of both ITBs and RJBs, the identified membrane proteins are enriched in a similar pathway coverage (Fig. 5c) (Han et al. 2017). For instance, across the adult stages, significantly enriched and shared pathways include oxidative phosphorylation, protein processing in the endoplasmic reticulum, phagosome, endocytosis, and wnt signaling pathway. However, protein export and dorso-ventral axis formation pathways are only enriched in NEBs and on day 7, lysosome pathway is only found in NBs and FBs, and phototransduction is exclusively enriched in FBs.

The molecular basis of the enhanced performance of RJ secretion by RJBs is well explained by the up-regulated proteins and phosphoproteins in the brain (Han et al. 2015; Han et al. 2017). The up-regulated protein and phosphoprotein enhance the RJ production by facilitating larval pheromone and flower odor recognition. For instance, in the brain of RJB nurses and foragers, the up-regulated proteins are involved in pathways such as the wnt signaling pathway, endocytosis, and soluble N-ethylmaleimide-sensitive factor attachment protein receptor interactions in vesicular transport, which involve in facilitating larvae pheromone and flower odor recognition via the release and absorption of signal molecules that increase nerve sensitivity. Moreover, compared to ITBs, in RJBs the up-regulated phosphoproteins related to efficient neurotransmitter transmission and recycling may have played a role in increasing RJ yields via enhancing larval feeding of NBs and increasing the nutrient supply of the colony through facilitating efficient food collection or foraging.

Furthermore, the RJBs shaped their brain membrane proteome and phosphoproteome settings to match the nursing and foraging behaviors in response to enhanced RJ production (Han et al. 2015). For instance, in RJB nurses (Fig. 6a), the up-regulated proteins implicated in water and ion homeostasis and brood pheromone recognition (diuretic hormones and periviscerokinin, and tachykinins (TK), respectively), shows higher physiological activity involved in the RJ secretion process compared to ITBs (Han et al. 2015). Whereas in RJB forager (Fig. 6b), the highly abundant proteins are supposed to enhance

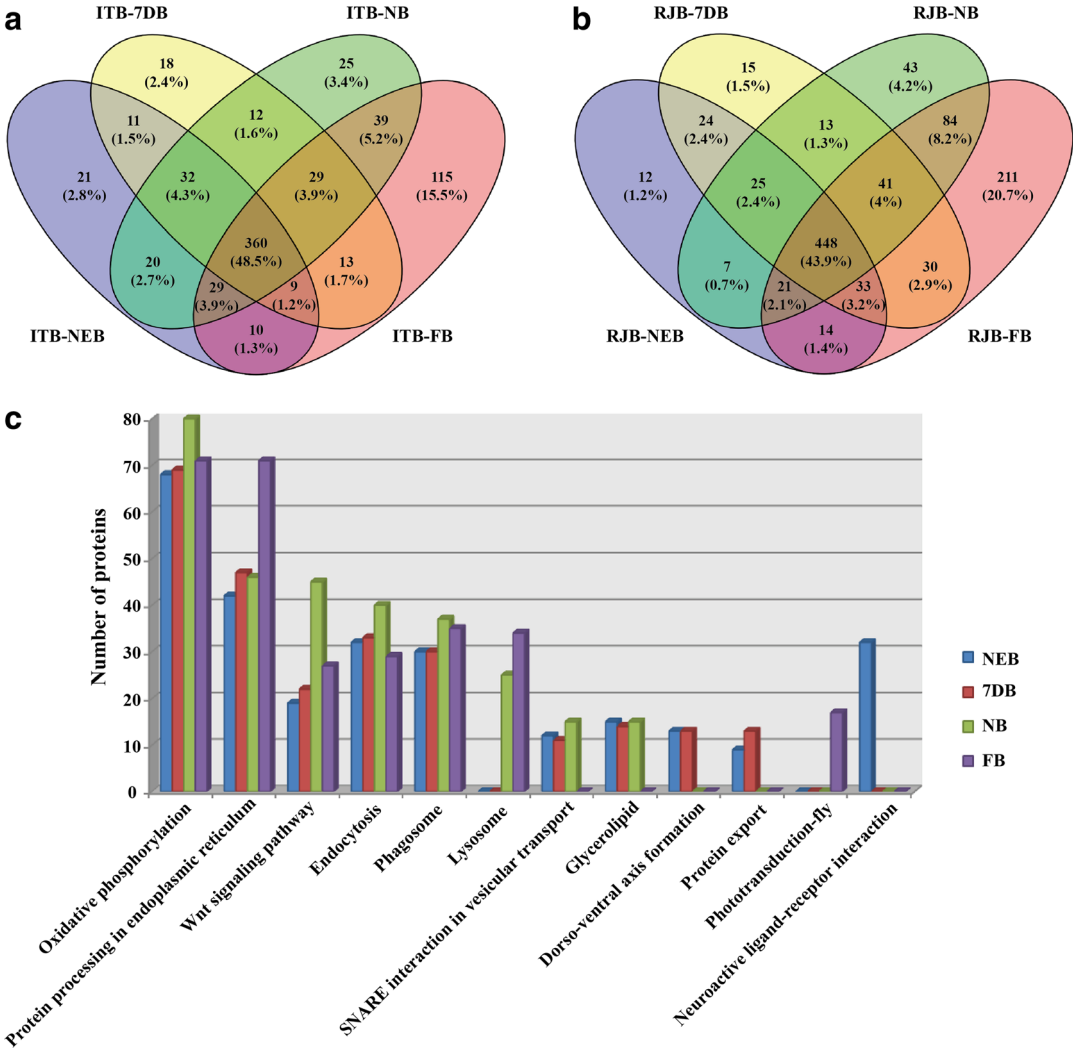


Figure 5. The brain membrane proteome comparison across the four stages during age-related polyethism in honeybee workers (*Apis mellifera* ligustica). Venn diagrams show the distribution of identified membrane proteins in **a** the Italian bee (ITB) and **b** the royal jelly bee (RJB) at four stages of adult worker bees: newly emerged bee (NEB), 7-day-old bee (7DB), nurse bee (NB), and forager bee (FB). **c** Significantly enriched KEGG pathways of the identified membrane proteins in each stage. Data were used from Han et al. (2017).

foraging capacity (PDH, sNPF, FMRFamide, Corazonin) and pollen collection (prohormone-4 and TK), which supports the required amount of food and protein supply to enhance the level of RJ production compared to ITBs. In sum, enhanced performance of RJ secretion by RJBs as compared to ITBs is associated with highly enhanced neural peptides that regulate the behavior and elevated RJ secretion via the regulation of water homeostasis, brood pheromone recognition, foraging capacity, and pollen collection (Han et al. 2015). Similarly, in RJB nurses, the enhanced level of phosphatidylinositol signaling and arachidonic acid metabolism contributed for stronger olfaction sense to respond to larval pheromone stimulation, and in RJB foragers, enriched pathways related to signal processing resulted in higher pollen collection efficiency via enhancing

nerve sensitivity (Han et al. 2017). This forms the basis for the success of decades of selection for enhanced RJ yields. Moreover, immunostaining of the brain and the HGs have shown differential expression of MRJPs in different brain regions of honeybee castes and subcastes (Peixoto et al. 2009), suggesting that the activity level of the brain might be another factor contributing to enhanced RJ production in RJBs.

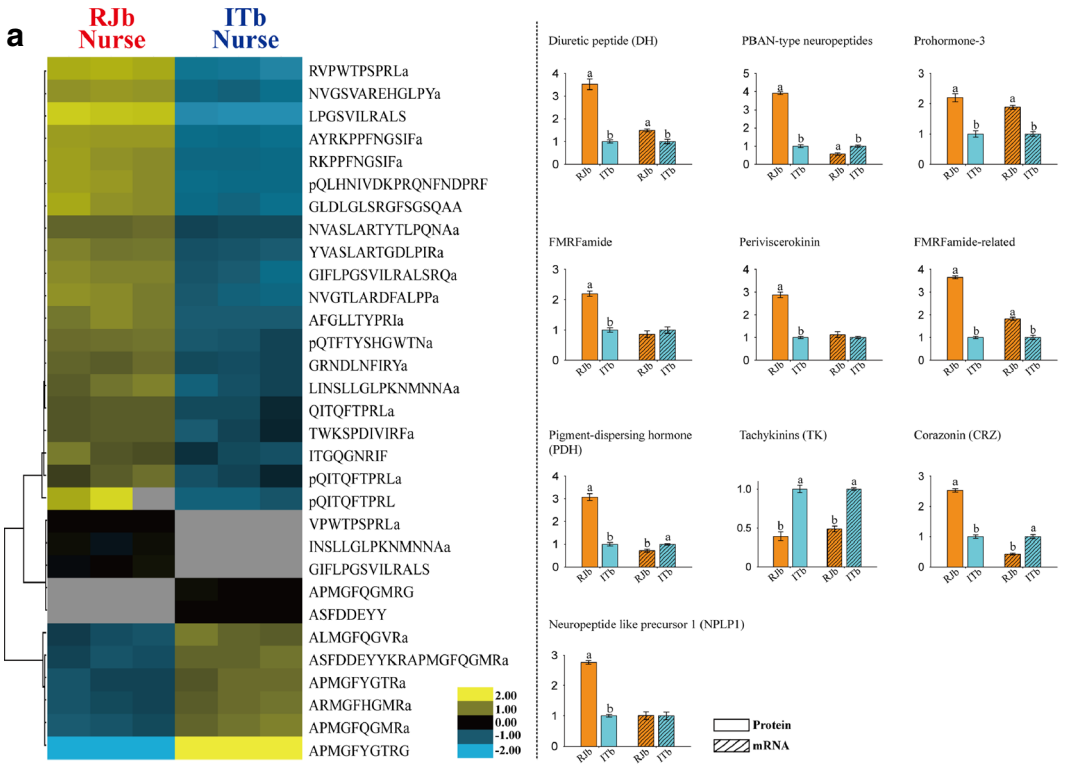
5.4. Comparison of hemolymph proteome of RJBs and ITBs

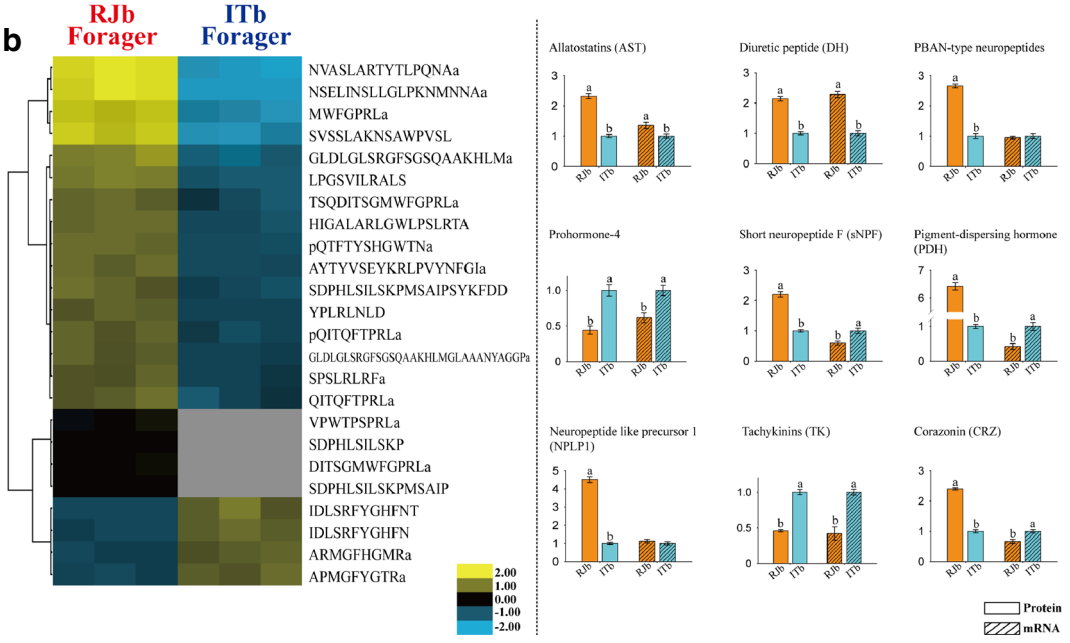
Hemolymph plays a crucial role in the investigation of various aspects of the honeybee's phenotype and physiology (Chan et al. 2006; Feng et al. 2014; Erban et al. 2016). The honeybee hemolymph comprises a wide range of organic and inorganic substances such as proteins, lipids, carbohydrates, nucleic acid macrophage-like cells, salts, hormones, and degradation products of these compositions (Chan et al. 2006; Bogaerts et al. 2009; Erban et al. 2013). The hemolymph protein components, such as enzymes, nutrient and pheromone transporters, structural proteins, immune response proteins, and MRJPs, vary between developmental stages and physiological conditions and is used to study caste differences as well as development (Chan et al. 2006; Randolt et al. 2008). The hemolymph of the honeybee serves in transportation of biological molecules, nutrients, and hormones, and in immune defense as a strategy to develop and match specific physiology (Feng et al. 2014).

To better understand the mechanism of enhanced RJ production in RJBs, one of the promising strategies is to compare the hemolymph proteome between honeybee species and/or within lines of sub-species. For instance, the comparison between RJB and ITB larval and adult samples shows that both beelines use distinct hemolymph proteome in driving their physiology (Ararso et al. 2018). Particularly, in day 4 larvae of the RJBs, hemolymph proteome consolidates amino acid and protein synthesis to support the development and immune responses, which is the selective pressure that favors high RJ production, and the NBs of RJBs reinforce energy metabolism, protein synthesis, and cellular homeostasis to respond to the increased RJ yields. This implies that the RJBs reprogram their hemolymph proteome to follow a different developmental path and efficient RJ secretion. Moreover, the metabolomic difference in protein synthesis and energy metabolism, for instance, has a dramatic effect on the morphology and physiology of the HGs through participating in proper cell divisions (Ararso et al. 2018).

5.5. Proteome, phosphoproteome, and glycoproteome comparisons of RJ from different honeybee species or lines

To better understand the biological properties of RJ from RJBs and other honeybee species, comparative investigations on proteome, phosphoproteome, and glycoproteome are conducted. For instance, there is a significant difference in RJ protein complements between Carnica bees and RJBs or ITBs, but there is no significant difference found between RJBs and ITBs (Li et al. 2007a, b). However, controversial results were found on the 10-HDA content of RJ. One study showed a lower 10-HDA content of RJ from high RJB colonies relative to that from ITB colonies (Chen 2005), whereas, in another, a non-significant difference in 10-HDA content between the two lines was reported (Huo et al. 2016). The RJ proteome and function from the two bee species of *Apis mellifera ligustica* (*Aml*) and *Acc* show a profound difference (Fang et al. 2010). Although the protein types are the same, in *Aml*-RJ there is a significantly higher level of MRJPs than in *Acc*-RJ (Fang et al. 2010). Proteins such as peroxiredoxin 2540, glutathione S-transferase S1, and MRJP5 are identified only in *Aml*-RJ, and MRJP1 is the most abundant MRJP in *Aml*-RJ. In *Acc*-RJ, the only protein found is MRJP7, and, similarly to *Aml*-RJ, MRJP1 is the most highly abundant MRJP. In addition, a significantly higher protein level of MRJP1-5 is found in the *Aml*-RJ than in *Acc*-RJ (Fang et al. 2010), whereas the phosphorylated proteins abundance level shows the opposite, with phosphorylated peptides from *Acc*-RJ showing stronger anti-microbial and anti-fungal activity (Han et al. 2014). The RJBs and *Acc* employ unique phosphorylation strategies that


a
RJb Nurse
ITb Nurse
RVPWTPSPRLa
NVGSVAREHGLPYa
LPGSVILRALS
AYRKPPFNGSIFa
RKPPFNGSIFa
pQLHNIVDKPRQNFNDPRF
GLDLGLSRGFSGSQAA
NVASLARTYTLPQNAa
YVASLARTGDLPIRa
GIFLPGSVILRALSRQa
NVGTLARDFALPPa
AFGLLTYPRIa
pQTFTYSHGWTNa
GRNDLNFIRYa
LINSLLGLPKNMNNAa
QITQFTPRLa
TWKSPDIVIRFa
ITGQGNRIF
pQITQFTPRLa
pQITQFTPRL
VPWTPSPRLa
INSLLGLPKNMNNAa
GIFLPGSVILRALS
APMGFQGMRG
ASFDDEYY
ALMGFQGVRa
ASFDDEYYKRAPMGFQGMRa
APMGFYGTRa
ARMGFHGMRa
APMGFQGMRa
APMGFYGTRG
2.00
1.00
0.00
-1.00
-2.00
Diuretic peptide (DH)
PBAN-type neuropeptides
Prohormone-3
FMRFamide
Perivisceroki nin
FMRFamide-related
Pigment-dispersing hormone (PDH)
Tachykinins (TK)
Corazonin (CRZ)
Neuropeptide like precursor 1 (NPLP1)
RJb
ITb
Protein
mRNA


b
RJb Forager
ITb Forager
NVASLARTYTLPQNAa
NSELINSLLGLPKNMNNAa
MWFGPRLa
SVSSLAKNSAWPVSL
GLDLGLSRGFSGSQAAKHLMa
LPGSVILRALS
TSQDITSGMWFGPRLa
HIGALARLGWLPSLRTA
pQTFTYSHGWTNa
AYTYVSEYKRLPVYNFGIa
SDPHLSILSKPMSAIPSYKFDD
YPLRLNLD
pQITQFTPRLa
GLDLGLSRGFSGSQAAKHLMGLAAANYAGGPa
SPSLRLRFa
QITQFTPRLa
VPWTPSPRLa
SDPHLSILSKP
DITSGMWFGPRLa
SDPHLSILSKPMSAIP
IDLSRFYGHFNT
IDLSRFYGHFN
ARMGFHGMRa
APMGFYGTRa
2.00
1.00
0.00
-1.00
-2.00
Allatostatins (AST)
Diuretic peptide (DH)
PBAN-type neuropeptides
Prohormone-4
Short neuropeptide F (sNPF)
Pigment-dispersing hormone (PDH)
Neuropeptide like precursor 1 (NPLP1)
Tachykinins (TK)
Corazonin (CRZ)
RJb
ITb
Protein
mRNA

◀ **Figure 6.** Brain membrane of RJBs showed unique proteome and phosphoproteome settings to consolidate nursing and foraging behaviors. **a** In RJB nurse, the highly abundant proteins, diuretic hormones (DHs), periviscerokinin (PVK), and tachykinins (TK)), are implicated in water and ion homeostasis and brood pheromone recognition, respectively, to underline the elevated physiology of RJ secretion compared with ITBs. **b** In RJB foragers, the highly abundant proteins: (prohormone-4 and TK) and (PDH, sNPF, FMRFamide, Corazonin (CRZ)) enhanced pollen collection and foraging capacity, which supports the required amount of food and protein supply to enhance the level of RJ production compared with ITBs. Reprinted with permission from Han et al. (2015).

support their diverse biological characteristics (Han et al. 2014). The abundance level of MRJPs in the two species could be explained better in terms of their biological requirements for survival and development. In *Aml* MRJPs, abundance is related with supporting their large body size, whereas in *Acc*, high abundance of phosphorylated peptides is to balance the low level of MRJPs by ensuring survival and development (Han et al. 2014).

Phosphoproteome comparison of RJ between the honeybee species also shows a distinct variation. For instance, the RJBs and *Acc* employ unique phosphorylation strategies that support their diverse biological characteristics (Han et al. 2014). These two bee species have evolved significant variation in phosphosites, peptide abundance, and antimicrobial activity of the phosphorylated RJ proteins. In *Am*-RJ, 16 phosphoproteins carrying 67 phosphorylation sites are identified, while in *Acc*-RJ, nine proteins phosphorylated on 71 sites are found. In both RJ samples, eight phosphorylated proteins are common, and the same motif ([S-x-E]) is extracted, indicating that in both honeybee species the function of MRJPs is evolutionarily reserved to act as nutrients and immune agents(Fang et al. 2014). In addition, Zhang and his colleagues (2014) identified 25 N-glycosylated proteins including 53 N-glycosylation sites, of which 42 N-linked glycosylation sites were positioned as novel RJ protein. Of the 42 non-redundant RJ proteins found in *Aml*-RJ, 13 are novel proteins whose activity is mainly related to metabolic processes and heal improvement (Zhang et al. 2014). In addition to extending the RJ proteome coverage, the newly identified protein provides new information to our knowledge on the biochemical property of RJ, as the identification and characterization of unknown RJ proteins is potentially useful for pharmacokinetic and biological activity (Zhang et al. 2014). Future research should be done to identify possible proteins for such uses.

In RJ, glycosylation modulates many important biological processes that have a vital function for both honeybees and humans. For instance, several N-glycosylated proteins found in RJ are associated with MRJPs, developmental regulation, metabolism processes, and immunity activities (Feng et al. 2015). As a result of species-specific glycosylation of RJ, the two bee species' RJ is endowed with different functional properties (Feng et al. 2015). For instance, in comparison to *Acc*-RJ, the *Aml*-RJ lacked antibacterial associated glycosylated apidaecin, hymenoptaecin, and peritrophic matrix, and low inhibitory efficiency of N-glycosylated MRJP2 against *Paenibacillus larvae* (*P. larvae*), due to which the *Aml* larvae were susceptible to *P. larvae* (Feng et al. 2015). Furthermore, a stronger antihypertensive activity of N-glycosylated MRJP1 in *Acc* than in *Aml* was found, which depicts the purpose of specific RJ protein and their modification for the treatment of hypertension for humans (Ramadan and Al-Ghamdi 2012; Feng et al. 2015). Generally, the two honeybee species have evolved species-specific strategies of glycosylation to tune protein activity to acting/serving as nutrients and immune agents, which benefits both the honeybee and adds health-promoting activity for humans. This evidence adds a valuable resource for the investigation of the biological functions of RJ proteins for honeybee and medical communities.

6. CONCLUSION

Due to the genetic selection of RJBs from ITBs, both stocks have shaped distinct genetic architecture in RJ production and acceptance of the larvae in queen cells. The differences in behavioral adaptations and biological systems of both bee stocks manifest in their proteome. With the advancement of proteomics technologies, the mechanistic insight into the enhanced RJ secretion

by RJBs reaches a new depth. However, the genes and proteins that are involved in regulating the stronger performance of RJ yields in RJBs still remain to be discovered. Moreover, despite a large number of new proteins that have been identified at this point, future identification and isolation of individual proteins, such as has been done for MRJP1-5, is required.

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OPEN ACCESS

Aperçu moléculaire dans l'amélioration des performances de la sécrétion de gelée royale par un stock d'abeilles domestiques (*Apis mellifera ligustica*) sélectionnées pour augmenter la production de gelée royale

Abeilles italiennes / abeilles de gelée royale / production de gelée royale / base moléculaire protéome

Molecular Einblicke in die verbesserte Leistung von Gelée royale-Sekretion durch einen Vorrat an Honigbiene (*Apis mellifera ligustica*) ausgewählt zur Erhöhung Gelee royale Produktions

Italienische Bienen/Gelée Royale Bienen/Gelée Royale Produktion/molekulare Basis Proteom

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