Article

Revealing Changes in Ovarian and Hemolymphatic Metabolites Using Widely Targeted Metabolomics between Newly Emerged and Laying Queens of Honeybee (*Apis mellifera*)

Shiqing Zhong [1,2,†]**, Luxia Pan** [1,2,†] **, Zilong Wang** [1,2] **and Zhijiang Zeng** [1,2,*]

[1] Honeybee Research Institute, Jiangxi Agricultural University, Nanchang 330045, China; shiqing_zhong@163.com (S.Z.); luxia_pan@163.com (L.P.); wzlcqbb@126.com (Z.W.)
[2] Jiangxi Province Key Laboratory of Honeybee Biology and Beekeeping, Nanchang 330045, China
[*] Correspondence: bees@jxau.edu.cn
[†] These authors contributed equally to this work.

Simple Summary: The queen bee specializes in colony reproduction and undergoes behavioral and physical changes following egg-laying. Metabolomics is a high-throughput technique that can unveil the intricate connection between biological phenotypic states and an organism's small molecules. We are intrigued to determine the characteristic substances present in the hemolymph and ovary, as well as to understand how their rapid metabolism contributes to the process of egg-laying by queens. In this study, we reared *Apis mellifera* queens from three distinct time periods: newly emerged queen (NEQ), newly laying queen (NLQ), and old laying queen (OLQ). Using widely targeted metabolomics, we found that NLQ and OLQ exhibited significant metabolite alterations compared to NEQ, such as up-regulated expression of carnitines, fatty acids, and some antioxidants, and significantly down-regulated expression of amino acids, etc. The results of this study provide a novel perspective for analyzing the oviposition mechanism of queens.

Abstract: The queen bee is a central and pivotal figure within the colony, serving as the sole fertile female responsible for its reproduction. The queen possesses an open circulatory system, with her ovaries immersed in hemolymph. A continuous and intricate transportation and interchange of substances exist between the ovaries and hemolymph of queen bees. To determine the characteristic metabolites in the hemolymph and ovary, as well as understand how their rapid metabolism contributes to the process of egg-laying by queens, we reared *Apis mellifera* queens from three different age groups: newly emerged queen (NEQ), newly laying queen (NLQ), and old laying queen (OLQ). Using widely targeted metabolomics, our study revealed that the laying queen (NLQ and OLQ) exhibited faster fatty acid metabolism, up-regulated expression of antioxidants, and significant depletion of amino acids compared to the NEQ. This study revealed that the levels of carnitine and antioxidants (GSH, 2-O-α-D-glucopyranosyl-L-ascorbic acid, L-ascorbic acid 2-phosphate, etc.) in the NLQ and OLQ were significantly higher compared to NEQ. However, most of the differentially expressed amino acids, such as L-tryptophan, L-tyrosine, L-aspartic acid, etc., detected in NLQ and OLQ were down-regulated compared to the NEQ. Following egg-laying, pathways in the queens change significantly, e.g., Tryptophan metabolism, Tyrosine metabolism, cAMP signaling pathway, etc. Our results suggest that carnitine and antioxidants work together to maintain the redox balance of the queen. Additionally, various amino acids are responsible for maintaining the queen's egg production.

Keywords: queen bee; ovary; hemolymph; metabolomics

1. Introduction

The colony's reproduction and growth depend on the queen, who must maintain high rates of egg production. *Apis mellifera* queens have the capacity to lay 1500–2000 eggs daily [1–3]. The queens' ovaries have a rapid metabolism, allowing them to lay more eggs in 24 h than their own body weight. However, in the practice of beekeeping, the queen's egg-laying ability is influenced by various factors, including rearing methods [4,5], rearing season [6], and comb age [7,8], with the primary factor being the queens themselves, their genome [9,10], age [11], health [11,12], etc.

The queen bee possesses a highly developed reproductive system. The fully developed ovaries of the queen consist of a pair of large, pear-shaped ovarioles. The estimated number of ovarioles per ovary in *Apis mellifera* (*A. mellifera*) is generally between 100 and 180 [1,13,14]. The ovary is essential for the queen; the weight of the ovaries and the number of ovarioles are common indicators of the reproductive capacity of honeybee queens [15,16]. Additionally, the developmental and reproductive performance of queens is usually measured by the use of genes highly expressed in the ovaries, such as *Hexamerin* (*Hex*) *110* [17–19], *Hex 70a* [20,21], and *Vitellogenin* (*Vg*) [22,23].

The honeybee has an open circulatory system with a fluid similar to blood, called hemolymph, and its ovary is immersed in it [24,25]. The hemolymph is essential to honeybee immunity and is widely used as a research medium for investigating viral, bacterial, and parasitic infections in honeybees [26–30]. It is also used to transfer micro- and macro-elements and has been employed in the assessment of metal pollution [31]. The main components of honeybee hemolymph are proteins, lipids, carbohydrates, and amino acids, as well as water [32]. Therefore, the hemolymph facilitates the transportation of various nutrients to the queen's ovaries. For example, vitellogenin (Vg), synthesized by the honeybee's fat body, plays a role in oocyte growth and embryo energy supply [33,34] and is taken up by oocytes in the honeybee ovary [35,36].

Metabolomics is a high-throughput technique that can unveil the intricate connection between biological phenotypic states and an organism's small molecules. In honeybee metabolomics research, commonly employed experimental samples include the brain, head part, gut, and hemolymph [37]. These studies have primarily focused on investigating the impacts of insecticide and herbicide exposure [38–41], infections [42–44], dietary changes [45,46], and other factors on honeybees. Previous studies have also analyzed the metabolic profiles of the spermatheca [47] and ovaries [48] in the laying queen (LQ). However, a lack of research exists into the metabolite alterations in ovaries and hemolymph between newly emerged queen (NEQ) and LQ (*A. mellifera*).

There is a continuous and intricate transportation and interchange of substances between the ovaries and hemolymph of queens, which is intimately linked with the process of oviposition. Therefore, we are intrigued to determine the characteristic substances present in the hemolymph and ovary, as well as to understand how their rapid metabolism contributes to the process of egg-laying by queens. In this study, we reared *A. mellifera* queens from three distinct time periods. We aimed to identify differentially expressed metabolites (DEMs) and important pathways between NEQ and LQ through metabolomic analysis. This provides a novel perspective for analyzing the oviposition mechanism of queens.

2. Materials and Methods

2.1. Acquisition of Experimental Samples

The honeybees (*A. mellifera ligustica*) were maintained at the Honeybee Research Institute of Jiangxi Agricultural University (28.46° N, 115.49° E). The queens were collected from three different age groups: newly emerged queen (NEQ, zero days old), newly laying queen (NLQ, approximately one month old), and old laying queen (OLQ, approximately one year old). The NEQ was obtained through the standard artificial queen rearing method [2]. Each age group had six replicates. The NLQ and OLQ were natural

mating. Six NEQs, three NLQs, or three OLQs were chosen for each ovary sample. Six queens were selected for every hemolymph sample.

2.2. Gathering the Ovary and Hemolymph of the Queen Bee

The queens were attached to a beeswax dish with insect pins after the wings and legs were removed. Subsequently, their ovaries were then promptly preserved in liquid nitrogen with freezing tubes after being stripped using sterile scissors and tweezers. For hemolymph, after comparing the various methods [49–51], we chose the most effective method, i.e., dorsal sinus with a capillary hemolymph sampling (DCHS). Firstly, the queens were anesthetized at −20 °C for 10 min. Then, the connecting membrane between the third and fourth dorsal segments was pierced using a capillary. The hemolymph was introduced into the capillary and subsequently extruded into a freezing tube before being stored in liquid nitrogen. All processes are carried out on an ultra-clean table.

2.3. Widely Targeted Metabolomics Profiling Methods and Conditions

T3 UPLC Conditions: The LC-ESI-MS/MS system (UPLC, ExionLC AD, https://sciex.com.cn/, accessed on 17 July 2022; MS, QTRAP® System, https://sciex.com/, accessed on 17 July 2022) was utilized for the analysis of sample extracts. The experimental parameters were set as follows: UPLC included a Waters ACQUITY UPLC HSS T3 C18 column (1.8 μm, 2.1 mm × 100 mm) maintained at a temperature of 40 °C with a flow rate of 0.4 mL/min. The sample was injected either at a volume of 2 μL or 5 μL using a solvent system consisting of water with 0.1% formic acid and acetonitrile with 0.1% formic acid. The gradient program included the following steps: 95:5 *v/v* at 0 min, 10:90 *v/v* at 10.0 min, 10:90 *v/v* at 11.0 min, 95:5 *v/v* at 11.1 min, 95:5 *v/v* at 14.0 min.

Considering it could acquire MS/MS spectra in an information-dependent manner (IDA) during an LC/MS experiment, the Triple TOF mass spectrometer was employed. In this mode, the MS/MS spectra acquisition is triggered by certain conditions, and the acquisition software (TripleTOF 6600, AB SCIEX) continually assesses the full scan survey MS data as it is being collected. In this mode, the acquisition software (TripleTOF 6600, AB SCIEX) continuously evaluates the full scan survey MS data while collecting it and triggers the acquisition of MS/MS spectra based on preselected criteria. Twelve precursor ions with intensities higher than 100 were chosen for fragmentation at a collision energy (CE) of 30 V during each cycle. This produced 12 MS/MS events with product ion accumulation times of 50 msec each. The following parameters were configured for the ESI source: Ion source gas 1 at 50 Psi, Ion source gas 2 at 50 Psi, Curtain gas at 25 Psi, source temperature at 500 °C, and Ion Spray Voltage Floating (ISVF) set to either 5500 V or −4500 V in positive or negative modes, respectively.

The QTRAP® LC-MS/MS System, functioning as a triple quadrupole-linear ion trap mass spectrometer with an ESI Turbo Ion-Spray interface, was used to acquire LIT and triple quadrupole (QQQ) scans. Under the software control of Analyst 1.6.3 (Sciex), the apparatus functioned in both positive and negative ion modes. The 500 °C source temperature, 5500 V (positive) and −4500 V (negative) ion spray voltage (IS), 50, 50, and 25.0 psi for ion source gas I (GSI), gas II (GSII), and curtain gas (CUR), respectively, and high collision gas (CAD) were the settings for the ESI source. In QQQ and LIT modes, 10 and 100 μmol/L polypropylene glycol solutions were used for instrument tuning and mass calibration, respectively. Specific MRM transitions were monitored for each period based on the eluted metabolites.

2.4. Data analysis and Statistics

DEMs were identified based on criteria, including |Log$_2$FC| > 1.0 and VIP ≥ 1, with VIP values obtained from the OPLS-DA results utilizing the R package MetaboAnalystR. Analyzing data and plotting ring and PCA plots using the Metware Cloud platform (https://cloud.metware.cn/, accessed on 21 December 2023).The Kyoto Encyclopedia of

Genes and Genomes (KEGG) Compound database (http://www.kegg.jp/kegg/compound/, accessed on 7 January 2024) was used to annotate the identified metabolites. Pathways containing significantly regulated metabolites were subjected to metabolite set enrichment analysis (MSEA), with significance assessed using the *p*-values derived from the hypergeometric test.

3. Results

3.1. Identification of Metabolites and Widely Targeted Metabolomic Analysis

The widely targeted metabolomics analysis of the 36 samples from the ovary and hemolymph revealed a total of 3312 metabolites. These included 24 different classes of metabolites (Figure 1A), such as "Amino acid and its metabolites", "Benzene and substituted derivatives", "Heterocyclic compounds", "Organic acid and its derivatives", "Aldehyde, Ketones, and Esters", etc. After performing principal component analysis (PCA) on ovary and hemolymph, we found that there was a clear difference between the metabolites of LQ and NEQ, but the difference between NLQ and OLQ is relatively small (Figure 1B). We conducted an orthogonal partial least squares discriminant analysis (OPLS-DA) concurrently (Figure S1). These findings imply that the OPLS-DA models are dependable ($Q2 \geq 0.9810$) and can be utilized for additional research on differentially expressed metabolites (DEMs).
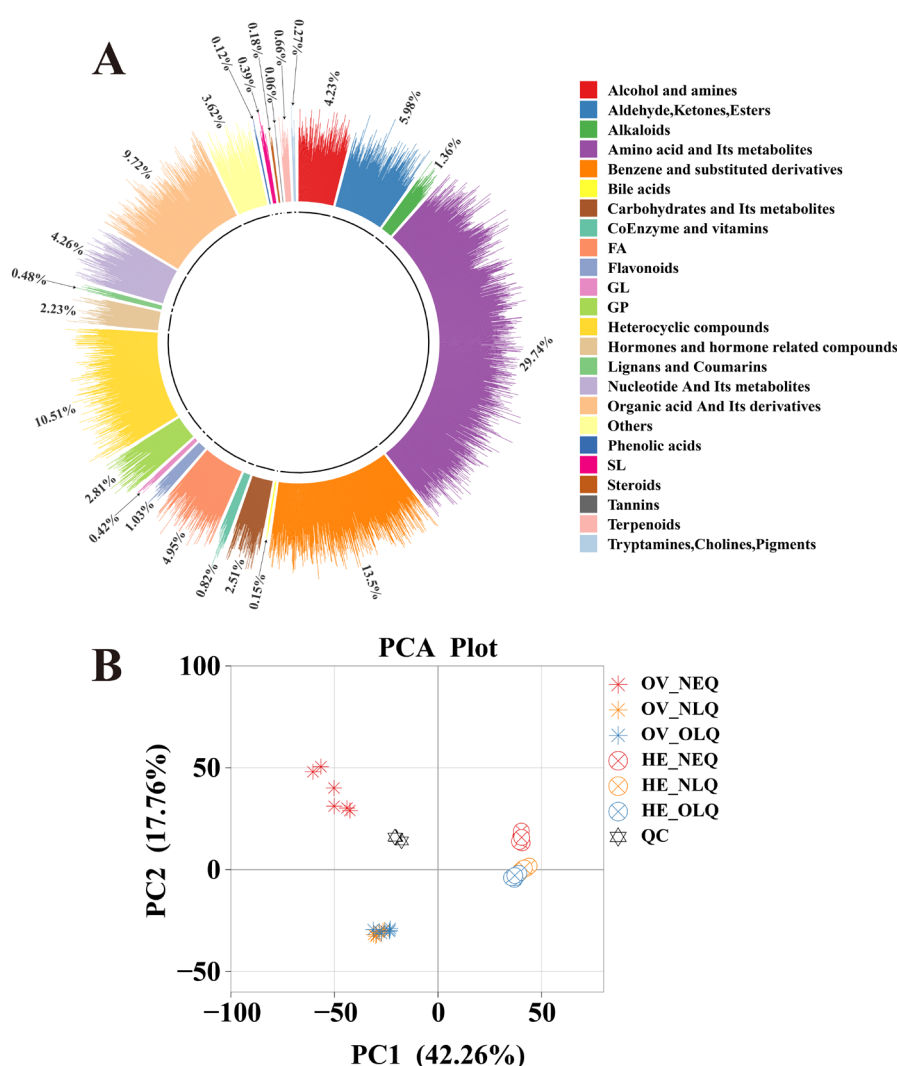

Figure 1. Ring plot and PCA plots in all groups. (**A**) Ring plot of metabolites in queens' ovary and hemolymph. (**B**) PCA plots in all groups of queens' ovary and hemolymph. "OV": Ovary; "HE": Hemolymph.

3.2. DEMs in Ovary and Hemolymph

The number of DEMs in the *A. mellifera* ovary (OV) in the NLQ vs. NEQ and OLQ vs. NEQ groups was 1202 and 1209, respectively. In the *A. mellifera* hemolymph (HE), the number of DEMs in the NLQ vs. NEQ and OLQ vs. NEQ groups was 803 and 817, respectively (Figure 2A). To identify the common key metabolites in NLQ vs. NEQ and OLQ vs. NEQ, we utilized Venn diagrams to classify the up-regulated and down-regulated metabolites. In comparison to NEQ, a total of 248 metabolites were found to be up-regulated in OV from NLQ and OLQ (Figure 2B, OV_Up). However, a total of 817 metabolites were down-regulated in OV from NLQ and OLQ compared to NEQ (Figure 2B, OV_Down). Furthermore, the results showed an upregulation of 144 metabolites in HE from NLQ and OLQ when compared to NEQ (Figure 2B, HE_Up). In addition, 447 down-regulated metabolites were found in HE from NLQ and OLQ compared to NEQ (Figure 2B, HE_Down).
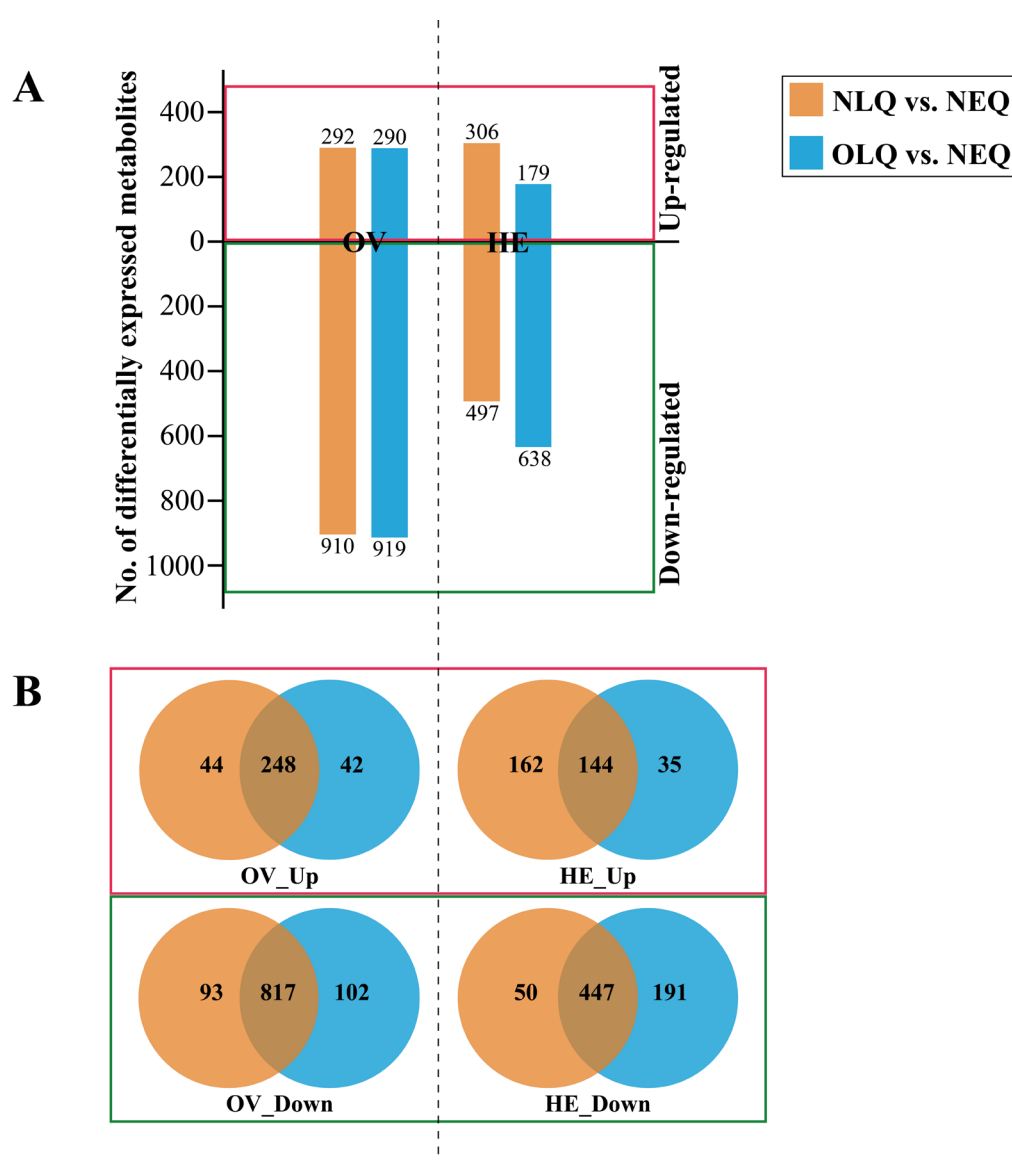

Figure 2. Analysis of DEMs. (**A**) Number of DEMs in all groups. (**B**) The Venn diagram shows the common DEMs between NLQ vs. NEQ and OLQ vs. NEQ. "OV_Up": The metabolites that expressed higher in the OV of NLQ and OLQ than in the OV of NEQ; "HE_Up": The metabolites that expressed higher in the HE of NLQ and OLQ than in the MH of NEQ; "OV_Down" and "HE_Down": The down-regulated metabolites that showed a lower expression level in the OV or HE of NLQ and OLQ compared to those in NEQ.

3.3. Analysis of Key Metabolites with Consistent Trends

To better understand the trends in the expression of honeybee queen ovary and hemolymph metabolites, we selected some of the critical DEMs to create heatmaps. In the ovaries of NLQ and OLQ, the expression of the fatty acyls (FA) and glycerophospholipid (GP) classes was much higher than NEQ (Table S1), including stearoyl-L-carnitine, carnitine C2:0, carnitine C3:0, FFA (12:1), PC (8:0/8:0), PC (16:0/2:0), LPC (0:0/18:2), LPE (16:1/0:0), LPI (14:0/0:0), etc. Moreover, the levels of glutathione reduced form, glutathione oxidized, oxiglutatione, and L-ascorbic acid 2-phosphate were significantly increased in the ovaries of LQ compared to NEQ (Figure 3A). The metabolites highly expressed in the NEQ ovary are mainly concentrated in amino acid classes. In particular, DEMs such as N1-acetyl-5-methoxykynuramine (AMK), 6-hydroxymelatonin, 2-hydroxymelatonin, (+)-fluprostenol, and testosterone glucuronide were exclusively detected in the ovaries of NEQ (Figure 3B).
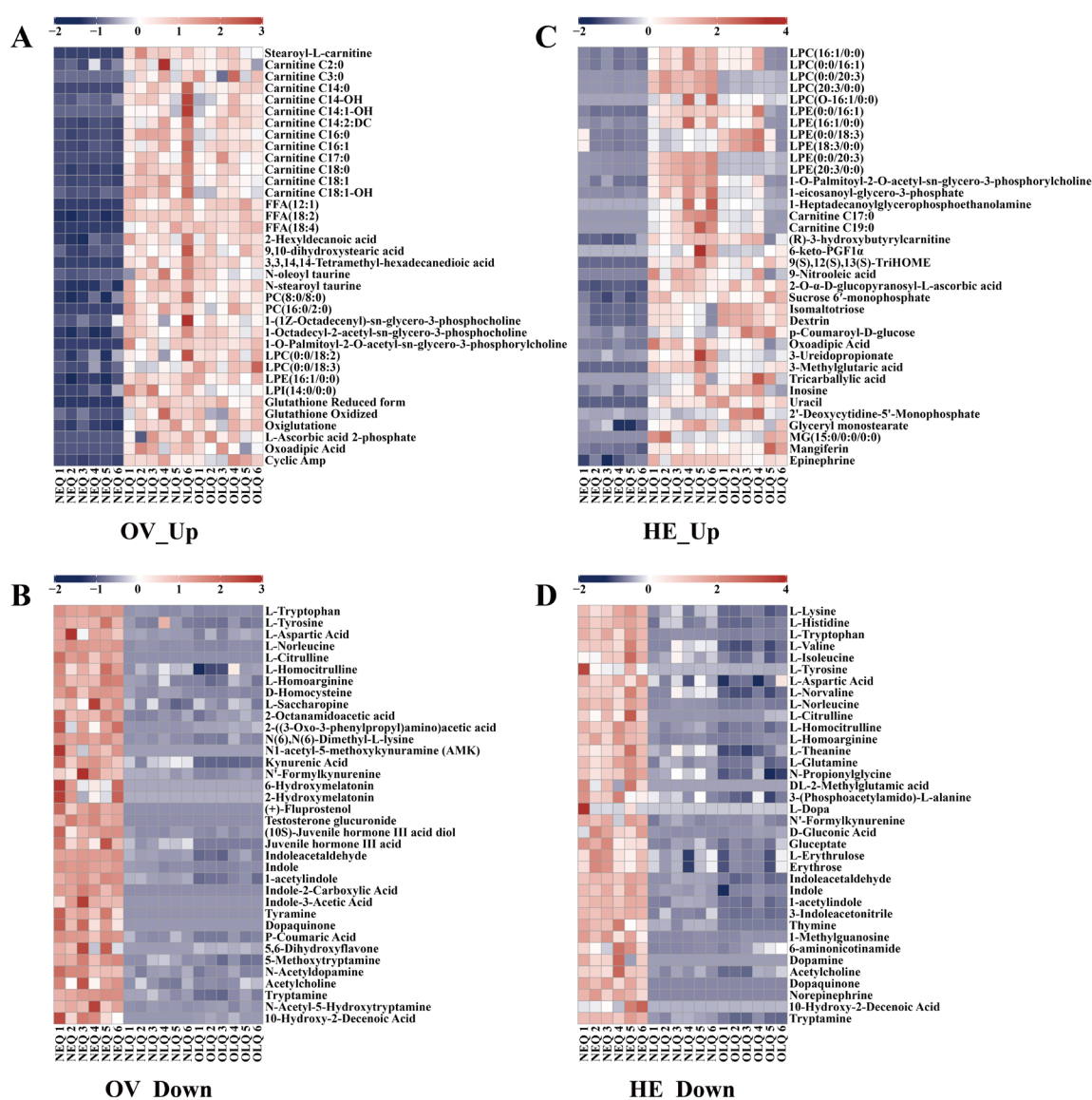

Figure 3. The heatmaps show the representatively metabolites in the ovary and hemolymph. (**A**) The DEMs that showed higher expression levels in the ovary of NLQ and OLQ compared to that of NEQ. (**B**) The DEMs that showed lower expression levels in the ovary of NLQ and OLQ compared to that of NEQ. (**C**) The DEMs that showed higher expression levels in the hemolymph of NLQ and OLQ compared to that of NEQ. (**D**) The DEMs that showed lower expression levels in the hemolymph of NLQ and OLQ compared to that of NEQ.

In hemolymph, the up-regulated metabolites were primarily FA and GP (Table S2), in addition to the usual nucleotides, carbohydrates, and their metabolites. The metabolites highly expressed in the NEQ hemolymph are also mainly concentrated in amino acid classes. Furthermore, dopamine, L-dopa, DL-2-methylglutamic acid, and norepinephrine were only found in the hemolymph of NEQ (Figure 3D).

3.4. Important Metabolic Pathways Affected by Metabolite Variations

We employed two sets of intersecting metabolites in the Venn diagram, NLQ vs. NEQ and OLQ vs. NEQ (Table S3), to perform KEGG analysis. The results demonstrated that, following egg-laying, pathways in the ovary and hemolymph of queens change significantly ($p < 0.05$), such as Tryptophan metabolism, Tyrosine metabolism, cAMP signaling pathway, Thyroid hormone synthesis, Phosphonate and phosphinate metabolism, Pantothenate and CoA biosynthesis, and Aminoacyl-tRNA biosynthesis (Figure 4).
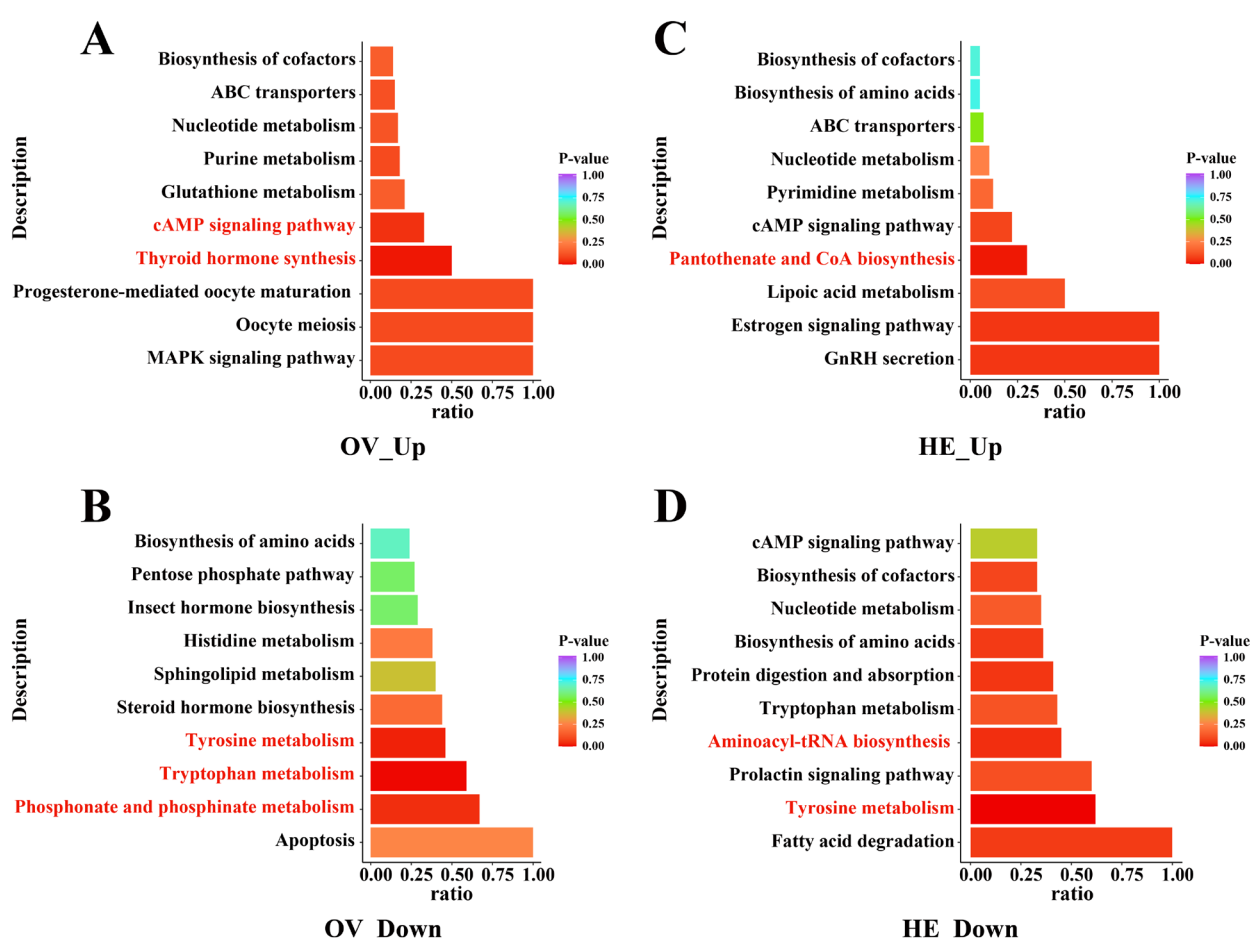

Figure 4. The bar plot of KEGG metabolic pathways commonly shared by NLQ vs. NEQ and OLQ vs. NEQ. (**A**) Up-regulated metabolic pathways in the ovary. (**B**) Down-regulated metabolic pathways in the ovary. (**C**) Up-regulated metabolic pathways in the hemolymph. (**D**) Down-regulated metabolic pathways in the hemolymph. The redder colors of the bar indicate pathways that are more important. Pathways marked in red font indicate significant differences. $p < 0.05$ was considered statistically significant.

4. Discussion

In this study, it was observed that the levels of carnitine in the ovaries of LQ were significantly higher compared to NEQ. The trend of free fatty acids, such as FFA (18:2), FFA (18:4), FFA (12:1), etc., in queen bee ovaries was consistent with carnitine. Carnitine acts as a carrier molecule for activated fatty acids, promoting fatty acid oxidation within

the mitochondria [52,53]. L-carnitine has been extensively studied compared to other carnitines, with numerous investigations demonstrating its efficacy in increasing oocyte quality and expediting blastocyst formation during in vitro oocyte maturation across various species, including pigs [54,55], cattle [56,57], mice [58,59], etc. Notably, Li et al. conducted single-cell metabolomic sequencing on mouse oocytes at three different developmental stages and confirmed the heightened levels of carnitines during oocyte meiosis [60]. Furthermore, DL-carnitine has been identified as a stimulant for oviposition in *Drosophila melanogaster* (*D. melanogaster*) by Geer and Dolph [61]. Therefore, it is postulated that carnitine plays a crucial role in promoting fatty acid metabolism during queen oviposition, ensuring a continuous supply of fatty acids for rapid oocyte development. Additionally, owing to its antioxidant properties [62,63], carnitine may protect against oxidative stress within the ovary.

Oxidative stress occurs when the body produces an excessive amount of reactive oxygen species (ROS) or fails to effectively eliminate the excess ROS. Antioxidants can regulate ROS levels and maintain redox balance in the body. In mammals, antioxidants are regarded as pivotal elements in ovarian physiological metabolism. Enzymatic antioxidants, including catalase (CAT) [64,65], superoxide dismutase (SOD) [66,67], glutathione-S-transferase (GST) [68,69], etc., have been demonstrated to contribute significantly to the development of oocytes. Glutathione (GSH) [70,71], ascorbic acid (AA) [72,73], melatonin (MLT) [74–76], etc., which are non-enzymatic antioxidants, safeguard oocytes against damage caused by ROS and enhance the quality of oocytes. In honeybees, the activities of the queen ovary CAT, SOD, and GST transcripts increase with the reproductive maturity of the queen [77]. Weirich et al. conducted assays and found no significant differences in the activities of CAT, SOD, and GST in the hemolymph between NEQ and LQ [78]. Non-enzymatic antioxidants in honeybees have received limited attention, despite their significance. Notably, GSH has been exclusively identified in the ovaries of LQ. Consequently, we propose a hypothesis that LQ produces substantial amounts of ROS and necessitates elevated levels of GSH to maintain homeostasis. Honeybees have the ability to produce AA [79]. Supplementation of honeybee colonies with AA during the winter and early spring has been shown to enhance honeybees' resistance to oxidative stress, resulting in a reduced infestation rate by *Varroa destructor* [80,81]. We only detected L-AA in the hemolymph of queens, and there was a significant difference between NLQ vs. NEQ groups, but not between OLQ vs. NEQ groups. 2-O-α-D-glucopyranosyl-L-ascorbic acid (AA-2G), the stable AA derivative, exhibits increased levels in LQ hemolymph compared to NEQ. AA-2G possesses inherent resistance against oxidative stress and can also be enzymatically converted into the highly potent antioxidant AA [82,83]. In the LQ ovary, we found another AA derivative, L-ascorbic acid 2-phosphate (AA-2P). AA-2P is highly stable under normal cell culture conditions and exhibits a longer duration of vitamin C activity compared to AA, thereby effectively preventing oxidative DNA damage [84–86]. MLT is mostly found in the head of honeybees. This study revealed the absence of MLT in the ovaries and hemolymph of queens. Interestingly, three distinct MLT metabolites, namely AMK, 6-hydroxymelatonin, and 2-hydroxymelatonin, were exclusively identified within the ovaries of NEQ. This phenomenon is likely to be attributed to the LQ's uninterrupted oviposition and their absence of diel rhythmicity, while the NEQ demonstrates free-running circadian rhythms [87]. MLT can enhance honeybees' ability to resist cold tolerance [88] and imidacloprid [89]. Further study is needed to determine whether MLT also affects egg production in queens, similar to its effects on *D. melanogaster* [90].

The antioxidant capacity is closely associated with the queen's high egg-laying performance, while the amino acid requirement also reflects the speed of the queen's high egg-laying. Amino acids play a crucial role in facilitating ovarian functions through their contribution to protein synthesis, hormone production, immune regulation, and other essential processes [91–95]. Honeybees, similar to other animals, have 10 essential amino acids (EAAs) that need to be supplemented in order to maintain normal growth,

development, and reproduction: lysine (Lys), histidine (His), tryptophan (Try), valine (Val), arginine (Arg), isoleucine (Ile), leucine (Leu), methionine (Met), phenylalanine (Phe), and threonine (Thr) [96,97]. In our study, we found that most of the differentially expressed amino acids detected in LQ were down-regulated compared to NEQ. The EAAs that were down-regulated in hemolymph were L-Lys, L-His, L-Try, L-Val, and L-Ile. The down-regulated amino acids shared in the ovary and hemolymph include L-Try, L-tyrosine (L-Tyr), L-aspartic acid (L-Asp), L-citrulline (L-Cit), L-homocitrulline (L-HC), L-homoarginine (L-HA), and L-norleucine (L-NLE). Hrassnigg et al. also found that free amino acids without proline and some free EAAs in hemolymph were decreased in LQ compared to NEQ, e.g., Tyr, Arg, Ile, Leu, Lys, and His [98]. Egg-laying by queens can require substantial amounts of amino acids, resulting in a down-regulation of most amino acids compared to NEQ. Sang and King discovered that egg production in *D. melanogaster* requires all 10 EAAs and that non-essential amino acids also maintain egg production [99]. Alves et al. showed that a diet deficient in a single amino acid (Arg, Ile, Leu, Lys, Phe, Thr, and Try) resulted in a sharp drop in egg production in *D. melanogaster*, whereas deficiencies in Met, His, and Val declined more gradually in egg production [100]. The queen can acquire EAAs from royal jelly [101,102], enabling her to sustain her egg-laying capacity for an extended period of time.

The metabolism of Try and Tyr shows a great impact after egg-laying in queens. Metabolites in the tryptophan and tyrosine metabolic pathways are predominantly down-regulated in LQ compared to NEQ. This is most likely to be due to the sustained consumption of Try, Tyr, and their metabolites by the queen in her reproductive role. Try is an EAA for all insects, playing crucial roles in various physiological processes, including reproduction. Try can promote yolk formation [103], induce and promote ovarian development [104,105], and regulate queen ovary metabolism [48]. Tyr is a non-EAA that is synthesized via Phe. Tyr is the key precursor for tanning *Aedes aegypti* eggs [106]. Tyr deficiency significantly reduces insect egg production and affects egg hatchability [107–109]. Tyr in royal jelly may promote workers' ovarian development in queenless colonies [110]. It is also likely that queens laying eggs require large amounts of Tyr from royal jelly. L-Lys, L-His, L-Val, L-Ile, L-aspartic acid, etc., may also have a close correlation with queens laying eggs, but additional verification is required.

5. Conclusions

The high rate of fatty acid metabolism and the high expression of antioxidants in the ovary and hemolymph provide material security and stress resistance for laying queens. Various amino acids are responsible for maintaining the queen's egg production; oviposition significantly depletes amino acid reserves in queens. Following egg-laying, tryptophan and tyrosine metabolic pathways are significantly affected in queens' ovary and hemolymph, underscoring the pivotal role of tryptophan and tyrosine in facilitating the queen bee's reproductive capacity.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/insects15040263/s1; Figure S1: The PCA plots, the OPLS-DA score plots, and the OPLS-DA permutation test plots are in each group. (A) The group NLQ vs. NEQ in the ovary. (B) The group OLQ vs. NEQ in the ovary. (C) The group NLQ vs. NEQ in the hemolymph. (D) The group OLQ vs. NEQ in the hemolymph; Table S1: The up-regulated differential metabolites FA and GP in the ovary of NLQ and OLQ compared to that of NEQ; Table S2: The up-regulated differential metabolites FA and GP in the hemolymph of NLQ and OLQ compared to that of NEQ; Table S3: Table S3.1 The metabolites expressed higher in the ovary of NLQ and OLQ than in the ovary of NEQ; Table S3.2 The down-regulated metabolites showed a lower expression level in the ovary of NLQ and OLQ compared to those in NEQ; Table S3.3 The metabolites expressed higher in the hemolymph of NLQ and OLQ than in the hemolymph of NEQ; Table S3.4 The down-regulated metabolites showed a lower expression level in the hemolymph of NLQ and OLQ compared to those in NEQ.

References

1. Snodgrass, R.E. *Anatomy of the Honey Bee*; Cornell University Press: Ithaca, NY, USA, 1956.
2. Büchler, R.; Andonov, S.; Bienefeld, K.; Costa, C.; Hatjina, F.; Kezic, N.; Kryger, P.; Spivak, M.; Uzunov, A.; Wilde, J. Standard methods for rearing and selection of *Apis mellifera* queens. *J. Apic. Res.* **2013**, *52*, 1–30.
3. Kumar, N. Evaluation of larval grafted queen and natural reared queen of Italian honey bees (*Apis mellifera* L.). *J. Pharmacogn. Phytochem.* **2018**, *7*, 3181–3183.
4. Woyke, J. Correlations between the age at which honeybee brood was grafted, characteristics of the resultant queens, and results of insemination. *J. Apic. Res.* **1971**, *10*, 45–55.
5. Yi, Y.; Liu, Y.-B.; Barron, A.B.; Zeng, Z.-J. Effects of commercial queen rearing methods on queen fecundity and genome methylation. *Apidologie* **2021**, *52*, 282–291.
6. Al-Ghzawi, A.A.M.; Zaitoun, S. Origin and rearing season of honeybee queens affect some of their physiological and reproductive characteristics. *Entomol. Res.* **2008**, *38*, 139–148.
7. Taha, E.-K.A.; Al-Kahtani, S.N. The relationship between comb age and performance of honey bee (*Apis mellifera*) colonies. *Saudi J. Biol. Sci.* **2020**, *27*, 30–34.
8. Taha, E.-K.A.; Shawer, M.B.; Taha, R.; Elashmawy, A.; Gaber, S.; Mousa, K. Comb age significantly influences the emergency queen rearing, morphometric and reproductive characteristics of the queens. *J. Apic. Res.* **2024**. https://doi.org/10.1080/00218839.2024.2336376.
9. Tarpy, D.R. Genetic diversity within honeybee colonies prevents severe infections and promotes colony growth. *Proc. R. Soc. Lond. Ser. B Biol. Sci.* **2003**, *270*, 99–103.
10. Mattila, H.R.; Seeley, T.D. Genetic diversity in honey bee colonies enhances productivity and fitness. *Science* **2007**, *317*, 362–364.
11. Simeunovic, P.; Stevanovic, J.; Cirkovic, D.; Radojicic, S.; Lakic, N.; Stanisic, L.; Stanimirovic, Z. *Nosema ceranae* and queen age influence the reproduction and productivity of the honey bee colony. *J. Apic. Res.* **2014**, *53*, 545–554.
12. Amiri, E.; Strand, M.K.; Rueppell, O.; Tarpy, D.R. Queen quality and the impact of honey bee diseases on queen health: Potential for interactions between two major threats to colony health. *Insects* **2017**, *8*, 48.
13. Jackson, J.T.; Tarpy, D.R.; Fahrbach, S.E. Histological estimates of ovariole number in honey bee queens, *Apis mellifera*, reveal lack of correlation with other queen quality measures. *J. Insect Sci.* **2011**, *11*, 82.
14. Alqarni, A.S.; Balhareth, H.M.; Owayss, A.A. Queen morphometric and reproductive characters of *Apis mellifera jemenitica*, a native honey bee to Saudi Arabia. *Bull. Insectology* **2013**, *66*, 239–244.
15. Gilley, D.C.; Tarpy, D.R.; Land, B.B. Effect of queen quality on interactions between workers and dueling queens in honeybee (*Apis mellifera* L.) colonies. *Behav. Ecol. Sociobiol.* **2003**, *55*, 190–196.
16. Gregorc, A.; Škerl, M.I.S. Characteristics of honey bee (*Apis mellifera carnica*, Pollman 1879) queens reared in Slovenian commercial breeding stations. *J. Apic. Sci.* **2015**, *59*, 5–12.
17. Martins, J.R.; Nunes, F.M.; Cristino, A.S.; Simões, Z.L.; Bitondi, M.M. The four hexamerin genes in the honey bee: Structure, molecular evolution and function deduced from expression patterns in queens, workers and drones. *BMC Mol. Biol.* **2010**, *11*, 23.
18. Martins, J.R.; Bitondi, M.M.G. The *HEX 110* hexamerin is a cytoplasmic and nucleolar protein in the ovaries of *Apis mellifera*. *PLoS ONE* **2016**, *11*, e0151035.
19. Tian, Y.; Qu, Y.; Dong, K.; He, S.; Jie, W.; Huang, J. Characterization and developmental expression patterns of four hexamerin genes in the bumble bee, *Bombus terrestris* (Hymenoptera: Apidae). *J. Insect Sci.* **2021**, *21*, 13.

20. Martins, J.R.; Nunes, F.M.F.; Simões, Z.L.P.; Bitondi, M.M.G. A honeybee storage protein gene, *hex 70a*, expressed in developing gonads and nutritionally regulated in adult fat body. *J. Insect Physiol.* **2008**, *54*, 867–877.

21. Martins, J.R.; Anhezini, L.; Dallacqua, R.P.; Simoes, Z.L.; Bitondi, M.M. A honey bee hexamerin, *HEX 70a*, is likely to play an intranuclear role in developing and mature ovarioles and testioles. *PLoS ONE* **2011**, *6*, e29006.

22. Amdam, G.V.; Simões, Z.L.; Hagen, A.; Norberg, K.; Schrøder, K.; Mikkelsen, Ø.; Kirkwood, T.B.; Omholt, S.W. Hormonal control of the yolk precursor vitellogenin regulates immune function and longevity in honeybees. *Exp. Gerontol.* **2004**, *39*, 767–773.

23. Corona, M.; Velarde, R.A.; Remolina, S.; Moran-Lauter, A.; Wang, Y.; Hughes, K.A.; Robinson, G.E. Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 7128–7133.

24. Jones, J.C. *The Circulatory System of Insects*; Charles C. Thomas: Springfield, IL, USA, 1977.

25. Hillyer, J.F.; Pass, G. The insect circulatory system: Structure, function, and evolution. *Annu. Rev. Entomol.* **2020**, *65*, 121–143.

26. Wang, D.-I.; Moeller, F. Comparison of the free amino acid composition in the hemolymph of healthy and *Nosema*-infected female honey bees. *J. Invertebr. Pathol.* **1970**, *15*, 202–206.

27. Mayack, C.; Naug, D. Parasitic infection leads to decline in hemolymph sugar levels in honeybee foragers. *J. Insect Physiol.* **2010**, *56*, 1572–1575.

28. Aliferis, K.A.; Copley, T.; Jabaji, S. Gas chromatography–mass spectrometry metabolite profiling of worker honey bee (*Apis mellifera* L.) hemolymph for the study of *Nosema ceranae* infection. *J. Insect Physiol.* **2012**, *58*, 1349–1359.

29. Burritt, N.L.; Foss, N.J.; Neeno-Eckwall, E.C.; Church, J.O.; Hilger, A.M.; Hildebrand, J.A.; Warshauer, D.M.; Perna, N.T.; Burritt, J.B. Sepsis and hemocyte loss in honey bees (*Apis mellifera*) infected with *Serratia marcescens* strain sicaria. *PLoS ONE* **2016**, *11*, e0167752.

30. Huang, S.; Li, J.; Zhang, Y.; Li, Z.; Evans, J.D.; Rose, R.; Gilligan, T.M.; LeBrun, A.; He, N.; Zheng, T. A novel method for the detection and diagnosis of virus infections in honey bees. *J. Virol. Methods* **2021**, *293*, 114163.

31. Ilijević, K.; Vujanović, D.; Orčić, S.; Purać, J.; Kojić, D.; Zarić, N.; Gržetić, I.; Blagojević, D.P.; Čelić, T.V. Anthropogenic influence on seasonal and spatial variation in bioelements and non-essential elements in honeybees and their hemolymph. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **2021**, *239*, 108852.

32. Horvatinec, J.; Svečnjak, L. Infrared (FTIR) spectral features of honey bee (*Apis mellifera* L.) hemolymph. *J. Cent. Eur. Agric.* **2020**, *21*, 37–41.

33. Sappington, T.W.; Raikhel, A.S. Molecular characteristics of insect vitellogenins and vitellogenin receptors. *Insect Biochem. Mol. Biol.* **1998**, *28*, 277–300.

34. Guidugli, K.R.; Piulachs, M.D.; Bellés, X.; Lourenço, A.P.; Simões, Z.L. Vitellogenin expression in queen ovaries and in larvae of both sexes of *Apis mellifera*. *Arch. Insect Biochem. Physiol. Publ. Collab. Entomol. Soc. Am.* **2005**, *59*, 211–218.

35. Excels, W. Occurrence and significance of vitellogenins in female castes of social Hymenoptera. *Am. Zool.* **1974**, *14*, 1229–1237.

36. Fleig, R. Role of the follicle cells for yolk uptake in ovarian follicles of the honey bee *Apis mellifera* L. (Hymenoptera: Apidae). *Int. J. Insect Morphol. Embryol.* **1995**, *24*, 427–433.

37. Jung, J. Metabolomic Studies in *Apis mellifera*. *J. Apic.* **2023**, *38*, 151–162.

38. Shi, T.; Burton, S.; Wang, Y.; Xu, S.; Zhang, W.; Yu, L. Metabolomic analysis of honey bee, *Apis mellifera* L. response to thiacloprid. *Pestic. Biochem. Physiol.* **2018**, *152*, 17–23.

39. Gao, J.; Yang, Y.; Ma, S.; Liu, F.; Wang, Q.; Wang, X.; Wu, Y.; Zhang, L.; Liu, Y.; Diao, Q. Combined transcriptome and metabolite profiling analyses provide insights into the chronic toxicity of carbaryl and acetamiprid to *Apis mellifera* larvae. *Sci. Rep.* **2022**, *12*, 16898.

40. Wang, B.; Habermehl, C.; Jiang, L. Metabolomic analysis of honey bee (*Apis mellifera* L.) response to glyphosate exposure. *Mol. Omics* **2022**, *18*, 635–642.

41. Shi, X.; Shi, J.; Yu, L.; Wu, X. Metabolic profiling of *Apis mellifera* larvae treated with sublethal acetamiprid doses. *Ecotoxicol. Environ. Saf.* **2023**, *254*, 114716.

42. Wu, J.-L.; Zhou, C.-X.; Wu, P.-J.; Xu, J.; Guo, Y.-Q.; Xue, F.; Getachew, A.; Xu, S.-F. Brain metabolomic profiling of eastern honey bee (*Apis cerana*) infested with the mite *Varroa destructor*. *PLoS ONE* **2017**, *12*, e0175573.

43. Jousse, C.; Dalle, C.; Abila, A.; Traïkia, M.; Diogon, M.; Lyan, B.; El Alaoui, H.; Vidau, C.; Delbac, F. A combined LC-MS and NMR approach to reveal metabolic changes in the hemolymph of honeybees infected by the gut parasite *Nosema ceranae*. *J. Invertebr. Pathol.* **2020**, *176*, 107478.

44. Li, Z.; Hou, M.; Qiu, Y.; Zhao, B.; Nie, H.; Su, S. Changes in antioxidant enzymes activity and metabolomic profiles in the guts of honey bee (*Apis mellifera*) larvae infected with *Ascosphaera apis*. *Insects* **2020**, *11*, 419.

45. Ricigliano, V.A.; Cank, K.B.; Todd, D.A.; Knowles, S.L.; Oberlies, N.H. Metabolomics-guided comparison of pollen and microalgae-based artificial diets in honey bees. *J. Agric. Food Chem.* **2022**, *70*, 9790–9801.

46. Chang, H.; Ding, G.; Jia, G.; Feng, M.; Huang, J. Hemolymph Metabolism Analysis of Honey Bee (*Apis mellifera* L.) Response to Different Bee Pollens. *Insects* **2023**, *14*, 37.

47. Liu, Z.; Liu, F.; Li, G.; Chi, X.; Wang, Y.; Wang, H.; Ma, L.; Han, K.; Zhao, G.; Guo, X. Metabolite support of long-term storage of sperm in the spermatheca of honeybee (*Apis mellifera*) queens. *Front. Physiol.* **2020**, *11*, 574856.

48. Li, W.-L.; Huang, Q.; Li, J.-L.; Wu, P.; Wei, B.; Li, X.-J.; Tang, Q.-H.; Dong, Z.-X.; Xiong, J.; Tang, H. Gut microbiota-driven regulation of queen bee ovarian metabolism. *Microbiol. Spectr.* **2023**, *11*, e02145-23.

49. Kostecki, R. Investigation on the Haemocytes and Haemolymph of Honeybees. *J. Apic. Res.* **1965**, *4*, 49–54.

50. Garrido, P.M.; Martin, M.L.; Negri, P.; Eguaras, M.J. A standardized method to extract and store haemolymph from *Apis mellifera* and the ectoparasite *Varroa destructor* for protein analysis. *J. Apic. Res.* **2013**, *52*, 67–68.

51. Borsuk, G.; Ptaszyńska, A.A.; Olszewski, K.; Domaciuk, M.; Krutmuang, P.; Paleolog, J. A new method for quick and easy hemolymph collection from apidae adults. *PLoS ONE* **2017**, *12*, e0170487.
52. Fritz, I.B. Carnitine and its role in fatty acid metabolism. *Adv. Lipid Res.* **1963**, *1*, 285–334.
53. Bremer, J. Carnitine--metabolism and functions. *Physiol. Rev.* **1983**, *63*, 1420–1480.
54. Wu, G.-Q.; Jia, B.-Y.; Li, J.-J.; Fu, X.-W.; Zhou, G.-B.; Hou, Y.-P.; Zhu, S.-E. L-carnitine enhances oocyte maturation and development of parthenogenetic embryos in pigs. *Theriogenology* **2011**, *76*, 785–793.
55. You, J.; Lee, J.; Hyun, S.-H.; Lee, E. L-carnitine treatment during oocyte maturation improves in vitro development of cloned pig embryos by influencing intracellular glutathione synthesis and embryonic gene expression. *Theriogenology* **2012**, *78*, 235–243.
56. Phongnimitr, T.; Liang, Y.; Srirattana, K.; Panyawai, K.; Sripunya, N.; Treetampinich, C.; Parnpai, R. Effect of L-carnitine on maturation, cryo-tolerance and embryo developmental competence of bovine oocytes. *Anim. Sci. J.* **2013**, *84*, 719–725.
57. Knitlova, D.; Hulinska, P.; Jeseta, M.; Hanzalova, K.; Kempisty, B.; Machatkova, M. Supplementation of l-carnitine during in vitro maturation improves embryo development from less competent bovine oocytes. *Theriogenology* **2017**, *102*, 16–22.
58. Dunning, K.R.; Akison, L.K.; Russell, D.L.; Norman, R.J.; Robker, R.L. Increased beta-oxidation and improved oocyte developmental competence in response to l-carnitine during ovarian in vitro follicle development in mice. *Biol. Reprod.* **2011**, *85*, 548–555.
59. Moawad, A.R.; Xu, B.; Tan, S.L.; Taketo, T. L-carnitine supplementation during vitrification of mouse germinal vesicle stage–oocytes and their subsequent in vitro maturation improves meiotic spindle configuration and mitochondrial distribution in metaphase II oocytes. *Hum. Reprod.* **2014**, *29*, 2256–2268.
60. Li, L.; Zhu, S.; Shu, W.; Guo, Y.; Guan, Y.; Zeng, J.; Wang, H.; Han, L.; Zhang, J.; Liu, X. Characterization of metabolic patterns in mouse oocytes during meiotic maturation. *Mol. Cell* **2020**, *80*, 525–540.e529.
61. Geer, B.; Dolph, W. A dietary choline requirement for egg production in *Drosophila melanogaster*. *Reproduction* **1970**, *21*, 9–15.
62. Gülçin, I. Antioxidant and antiradical activities of L-carnitine. *Life Sci.* **2006**, *78*, 803–811.
63. Solarska, K.; Lewińska, A.; Karowicz-Bilińska, A.; Bartosz, G. The antioxidant properties of carnitine in vitro. *Cell. Mol. Biol. Lett.* **2010**, *15*, 90–97.
64. Gupta, S.; Choi, A.; Hope, Y.Y.; Czerniak, S.M.; Holick, E.A.; Paolella, L.J.; Agarwal, A.; Combelles, C.M. Fluctuations in total antioxidant capacity, catalase activity and hydrogen peroxide levels of follicular fluid during bovine folliculogenesis. *Reprod. Fertil. Dev.* **2011**, *23*, 673–680.
65. Park, Y.S.; You, S.Y.; Cho, S.; Jeon, H.-J.; Lee, S.; Cho, D.-H.; Kim, J.-S.; Oh, J.S. Eccentric localization of catalase to protect chromosomes from oxidative damages during meiotic maturation in mouse oocytes. *Histochem. Cell Biol.* **2016**, *146*, 281–288.
66. Corona de Lau, C. Correlation between follicle levels of superoxide dismutase and oocyte quality, fertilization rates and embryo development. *Ginecol. Obstet. Mex.* **2004**, *72*, 335–344.
67. Tatemoto, H.; Muto, N.; Sunagawa, I.; Shinjo, A.; Nakada, T. Protection of porcine oocytes against cell damage caused by oxidative stress during in vitro maturation: Role of superoxide dismutase activity in porcine follicular fluid. *Biol. Reprod.* **2004**, *71*, 1150–1157.
68. Meijide, S.; Hernández, M.L.; Navarro, R.; Larreategui, Z.; Ferrando, M.; Ruiz-Sanz, J.I.; Ruiz-Larrea, M.B. Glutathione S-transferase activity in follicular fluid from women undergoing ovarian stimulation: Role in maturation. *Free Radic. Biol. Med.* **2014**, *75*, S41.
69. Thaker, R.; Mishra, V.; Gor, M.; Agarwal, R.; Sheth, H.; Kapadia, P.; Kumar, S. The role of stimulation protocol, number of oocytes retrieved with respect to follicular fluid oxidative stress and IVF outcome. *Hum. Fertil.* **2020**, *23*, 23–31.
70. Barros, F.D.d.A.; Adona, P.R.; Guemra, S.; Damião, B.C.M. Oxidative homeostasis in oocyte competence for in vitro embryo development. *Anim. Sci. J.* **2019**, *90*, 1343–1349.
71. Ren, J.; Hao, Y.; Liu, Z.; Li, S.; Wang, C.; Wang, B.; Liu, Y.; Liu, G.; Dai, Y. Effect of exogenous glutathione supplementation on the in vitro developmental competence of ovine oocytes. *Theriogenology* **2021**, *173*, 144–155.
72. Kere, M.; Siriboon, C.; Lo, N.-W.; Nguyen, N.T.; Ju, J.-C. Ascorbic acid improves the developmental competence of porcine oocytes after parthenogenetic activation and somatic cell nuclear transplantation. *J. Reprod. Dev.* **2013**, *59*, 78–84.
73. Yu, X.-X.; Liu, Y.-H.; Liu, X.-M.; Wang, P.-C.; Liu, S.; Miao, J.-K.; Du, Z.-Q.; Yang, C.-X. Ascorbic acid induces global epigenetic reprogramming to promote meiotic maturation and developmental competence of porcine oocytes. *Sci. Rep.* **2018**, *8*, 6132.
74. Tamura, H.; Takasaki, A.; Miwa, I.; Taniguchi, K.; Maekawa, R.; Asada, H.; Taketani, T.; Matsuoka, A.; Yamagata, Y.; Shimamura, K. Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. *J. Pineal Res.* **2008**, *44*, 280–287.
75. Tamura, H.; Takasaki, A.; Taketani, T.; Tanabe, M.; Kizuka, F.; Lee, L.; Tamura, I.; Maekawa, R.; Aasada, H.; Yamagata, Y. The role of melatonin as an antioxidant in the follicle. *J. Ovarian Res.* **2012**, *5*, 5.
76. Sakaguchi, K.; Itoh, M.T.; Takahashi, N.; Tarumi, W.; Ishizuka, B. The rat oocyte synthesises melatonin. *Reprod. Fertil. Dev.* **2013**, *25*, 674–682.
77. Collins, A.; Williams, V.; Evans, J. Sperm storage and antioxidative enzyme expression in the honey bee, *Apis mellifera*. *Insect Mol. Biol.* **2004**, *13*, 141–146.
78. Weirich, G.F.; Collins, A.M.; Williams, V.P. Antioxidant enzymes in the honey bee, *Apis mellifera*. *Apidologie* **2002**, *33*, 3–14.
79. Herbert, E., Jr.; Vanderslice, J.; Higgs, D. Effect of dietary vitamin C levels on the rate of brood production of free-flying and confined colonies of honey bees. *Apidologie* **1985**, *16*, 385–394.
80. Farjan, M.; Dmitryjuk, M.; Lipiński, Z.; Biernat-Łopieńska, E.; Żółtowska, K. Supplementation of the honey bee diet with vitamin C: The effect on the antioxidative system of *Apis mellifera carnica* brood at different stages. *J. Apic. Res.* **2012**, *51*, 263–270.

81. Farjan, M.; Łopieńska-Biernat, E.; Lipiński, Z.; Dmitryjuk, M.; Żółtowska, K. Supplementing with vitamin C the diet of honeybees (*Apis mellifera carnica*) parasitized with *Varroa destructor*: Effects on antioxidative status. *Parasitology* **2014**, *141*, 770–776.

82. Taniguchi, M.; Arai, N.; Kohno, K.; Ushio, S.; Fukuda, S. Anti-oxidative and anti-aging activities of 2-O-α-glucopyranosyl-L-ascorbic acid on human dermal fibroblasts. *Eur. J. Pharmacol.* **2012**, *674*, 126–131.

83. Hanada, Y.; Iomori, A.; Ishii, R.; Gohda, E.; Tai, A. Protection of free radical-induced cytotoxicity by 2-O-α-d-glucopyranosyl-l-ascorbic acid in human dermal fibroblasts. *Biosci. Biotechnol. Biochem.* **2014**, *78*, 301–306.

84. Hata, R.I.; Senoo, H. L-ascorbic acid 2-phosphate stimulates collagen accumulation, cell proliferation, and formation of a three-dimensional tissuelike substance by skin fibroblasts. *J. Cell. Physiol.* **1989**, *138*, 8–16.

85. Saika, S.; Kanagawa, R.; Uenoyama, K.; Hiroi, K.; Hiraoka, J.-I. L-ascorbic acid 2-phosphate, a phosphate derivative of L-ascorbic acid, enhances the growth of cultured rabbit keratocytes. *Graefe's Arch. Clin. Exp. Ophthalmol.* **1991**, *229*, 79–83.

86. Shima, N.; Kimoto, M.; Yamaguchi, M.; Yamagami, S. Increased proliferation and replicative lifespan of isolated human corneal endothelial cells with L-ascorbic acid 2-phosphate. *Investig. Ophthalmol. Vis. Sci.* **2011**, *52*, 8711–8717.

87. Johnson, J.N.; Hardgrave, E.; Gill, C.; Moore, D. Absence of consistent diel rhythmicity in mated honey bee queen behavior. *J. Insect Physiol.* **2010**, *56*, 761–773.

88. Fan, W.; Li, G.; Zhang, X.; Wang, Y.; Wang, C.; Xu, B.; Guo, X.; Li, H. The role of melatonin and Tryptophan-5-hydroxylase-1 in different abiotic stressors in *Apis cerana cerana*. *J. Insect Physiol.* **2021**, *128*, 104180.

89. Li, Z.; Duan, J.; Chen, L.; Wang, Y.; Qin, Q.; Dang, X.; Zhou, Z. Melatonin enhances the antioxidant capacity to rescue the honey bee *Apis mellifera* from the ecotoxicological effects caused by environmental imidacloprid. *Ecotoxicol. Environ. Saf.* **2022**, *239*, 113622.

90. Finocchiaro, L.; Callebert, J.; Launay, J.; Jallon, J. Melatonin biosynthesis in *Drosophila: Its* nature and its effects. *J. Neurochem.* **1988**, *50*, 382–387.

91. Estienne, M.; Barb, C. The control of adenohypophysial hormone secretion by amino acids and peptides in swine. *Domest. Anim. Endocrinol.* **2005**, *29*, 34–42.

92. Li, P.; Yin, Y.-L.; Li, D.; Kim, S.W.; Wu, G. Amino acids and immune function. *Br. J. Nutr.* **2007**, *98*, 237–252.

93. Wu, G. Amino acids: Metabolism, functions, and nutrition. *Amino Acids* **2009**, *37*, 1–17.

94. Grohmann, U.; Bronte, V. Control of immune response by amino acid metabolism. *Immunol. Rev.* **2010**, *236*, 243–264.

95. Wu, G.; Bazer, F.W.; Dai, Z.; Li, D.; Wang, J.; Wu, Z. Amino acid nutrition in animals: Protein synthesis and beyond. *Annu. Rev. Anim. Biosci.* **2014**, *2*, 387–417.

96. De Groot, A. Amino acid requirements for growth of the honeybee (*Apis mellifica* L.). *Experientia* **1952**, *8*, 192–194.

97. de Groot, A.P. Protein and amino acid requirements of the honeybee (*Apis mellifica* L.). *Physiol. Comp. Oecol.* **1953**, *3*, 197–285.

98. Hrassnigg, N.; Leonhard, B.; Crailsheim, K. Free amino acids in the haemolymph of honey bee queens (*Apis mellifera* L.). *Amino Acids* **2003**, *24*, 205–212.

99. Sang, J.H.; King, R.C. Nutritional requirements of axenically cultured *Drosophila melanogaster* adults. *J. Exp. Biol.* **1961**, *38*, 793–809.

100. Alves, A.N.; Sgrò, C.M.; Piper, M.D.; Mirth, C.K. Target of Rapamycin Drives Unequal Responses to Essential Amino Acid Depletion for Egg Laying in *Drosophila Melanogaster*. *Front. Cell Dev. Biol.* **2022**, *10*, 822685.

101. Boselli, E.; Caboni, M.F.; Sabatini, A.G.; Marcazzan, G.L.; Lercker, G. Determination and changes of free amino acids in royal jelly during storage. *Apidologie* **2003**, *34*, 129–137.

102. Xue, X.; Wu, L.; Wang, K. Chemical Composition of Royal Jelly. In *Bee Products—Chemical and Biological Properties*; Springer: Cham, Switzerland, 2017; pp. 181–190.

103. Harlow, P.M. A study of ovarial development and its relation to adult nutrition in the blowfly *Protophormia terrae-novae* (RD). *J. Exp. Biol.* **1956**, *33*, 777–797.

104. Kobayashi, K.; Maezawa, T.; Tanaka, H.; Onuki, H.; Horiguchi, Y.; Hirota, H.; Ishida, T.; Horiike, K.; Agata, Y.; Aoki, M. The identification of d-tryptophan as a bioactive substance for postembryonic ovarian development in the planarian *Dugesia ryukyuensis*. *Sci. Rep.* **2017**, *7*, 45175.

105. Maezawa, T.; Ishikawa, M.; Sekii, K.; Nagamatsu, G.; Furukawa, R.; Kobayashi, K. D-Tryptophan enhances the reproductive organ-specific expression of the amino acid transporter homolog *Dr-SLC38A9* involved in the sexual induction of planarian *Dugesia ryukyuensis*. *Zool. Lett.* **2021**, *7*, 4.

106. Li, J.; Christensen, B.M. Involvement of l-tyrosine and phenol oxidase in the tanning of *Aedes aegypti* eggs. *Insect Biochem. Mol. Biol.* **1993**, *23*, 739–748.

107. Zografou, E.; Tsiropoulos, G.; Margaritis, L. Effect of phenylalanine and tyrosine analogues on *Bactrocera oleae* Gmelin (Dipt., Tephritidae) reproduction. *J. Appl. Entomol.* **2001**, *125*, 365–369.

108. Fuchs, S.; Behrends, V.; Bundy, J.G.; Crisanti, A.; Nolan, T. Phenylalanine metabolism regulates reproduction and parasite melanization in the malaria mosquito. *PLoS ONE* **2014**, *9*, e84865.

109. Sterkel, M.; Oliveira, P.L. Developmental roles of tyrosine metabolism enzymes in the blood-sucking insect *Rhodnius prolixus*. *Proc. R. Soc. B Biol. Sci.* **2017**, *284*, 20162607.

110. Matsuyama, S.; Nagao, T.; Sasaki, K. Consumption of tyrosine in royal jelly increases brain levels of dopamine and tyramine and promotes transition from normal to reproductive workers in queenless honey bee colonies. *Gen. Comp. Endocrinol.* **2015**, *211*, 1–8.