Journal of Insect Physiology 139 (2022) 104397

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Proteomic analysis of summer and winter *Apis mellifera* workers shows reduced protein abundance in winter samples

Rachel Ward [a], Mary Coffey [b], Kevin Kavanagh [a,*]

[a] Department of Biology, Maynooth University, Maynooth, Co. Kildare, Ireland
[b] Plant Health Laboratories, Plant Science Division (Bee Health), Department of Agriculture, Celbridge, Co Kildare, Ireland

ARTICLE INFO

Keywords:
Apis mellifera
Seasonal adaptions
Proteomic comparison
Longevity

ABSTRACT

Apis mellifera workers display two stages; short lived summer bees that engage in nursing, hive maintenance and foraging, and long lived winter bees (diutinus bees) which remain within the hive and are essential for thermoregulation and rearing the next generation of bees in spring before dying. Label free quantitative proteomic analysis was conducted on *A. mellifera* workers sampled in June and December to compare the proteomes of summer and winter bees. Proteomic analysis was performed on head, abdominal and venom sac samples and revealed an elevated level of protein abundance in summer bees. Head and abdominal samples displayed an increased abundance in cuticular proteins in summer samples whereas an increase in xenobiotic proteins was observed in winter samples. Several carbohydrate metabolism pathways which have been linked to energy production and longevity in insects were increased in abundance in winter samples in comparison to summer samples. Proteomic analysis of the venom sacs of summer samples showed an increased abundance of bee venom associated proteins in comparison to winter workers. These data provides an insight into the adaptions of *A. mellifera* workers in summer and winter and may aid in future treatment and disease studies on honeybee colonies. Data are available via ProteomeXchange with identifier PXD030483.

1. Introduction

Apis mellifera colonies are composed of three castes; the queen, the drones which are only retained in the colony during summer months, and the workers which make up the largest number of bees within the hive and carry out all house and foraging duties to ensure the survival of the colony, complete the majority of tasks, from feeding larvae, to building and foraging for nutrients. In summer a colony can contain over 50,000 bees with individual workers living on average 6–8 weeks. In winter the population generally reduces to 15,000 to 20,000 bees and is characterised by long-lived winter bees known as diutinus bees, that can live for six months (Omholt and Amdam, 2004; Hooper, 2010; Aurori et al., 2014). In contrast to summer bees diutinus workers do not transition into foragers, instead they remain in the hive until the following spring (Omholt and Amdam, 2004). The emergence of the diutinus bee in the autumn correlates with the onset of unfavourable foraging conditions, reduced temperatures and a reduction in brood rearing (Aurori et al., 2014).

The role of diutinus bees in the hive is different to that of summer in-hive workers as there is no brood present during the winter, instead the main focus of diutinus bees is thermoregulation (Omholt, 1987; Hooper, 2010; Steinmann et al., 2021). Diutinus bees form a cluster between honey stores and empty brood cells, to generate heat through muscular action in the thorax and will occupy empty cells to form a solid cluster (Hooper, 2010). Since honeybees are affected by external temperatures, the cluster reduces heat loss in the colony as workers circulate to various levels providing a slow continuous mass movement and consume honey stores for energy metabolism to fuel muscular action (Omholt, 1987; Hooper, 2010). During the following spring the diutinus bee population will decline as their role transitions from thermoregulation to nursing newly laid summer workers and initiating foraging activities (Steinmann et al., 2021). The emergence of the short-lived summer bee occurs in time for improved foraging conditions and increased brood production (Omholt and Amdam, 2004).

Abbreviations: FDR, False Discovery Rates; GO, gene ontology; SSDA, statistically significant differentially abundant; DEP, differentially expressed proteins; LFQ, Label free quantitative-proteomic; PCA, Principal Component Analysis; RFC, relative fold change; ROS, reactive oxygen species; OXPHOS, oxidative phosphorylation; MRJP, Major Royal Jelly Proteins.

* Corresponding author.
E-mail address: kevin.kavanagh@nuim.ie (K. Kavanagh).

https://doi.org/10.1016/j.jinsphys.2022.104397
Received 6 January 2022; Received in revised form 22 March 2022; Accepted 4 May 2022
Available online 7 May 2022

Summer bees have two stages of senescence, a slow in-hive stage consisting of newly emerged, nurse and guard bees, followed by rapid senescence when workers transition to foragers. In contrast diutinus caste workers have negligible senescence enabling them to survive through the winter (Aurori et al., 2014). Organisms exhibiting long lifespans commonly have a high abundance of antioxidant proteins, which reduce oxidative stresses by breaking down reactive oxygen species (ROS) that can cause oxidative damage to proteins, lipids and nucleic acids (Kodrík et al., 2015). Studies have shown that in-hive workers have a higher number of functional hemocytes and a better ability to deal with ROS presence in comparison to foragers (Münch and Amdam, 2019; Cervoni et al., 2017).

During winter hives are extremely vulnerable to failure due to limited food resources, poor foraging conditions and potential threats of pests and diseases within the confines of the clusters. Despite the myriad of stresses that the diutinus bees faces their life span is significantly longer than short-lived summer bees (Münch and Amdam, 2019). The work presented here characterised proteomes of head, abdomen and venom sacs of late-hive summer bees and diutinus bees and examined the adaptation that may enable the diutinus bees to survive the stresses of winter. The head was chosen as it contains hypopharyngeal glands which play a significant role in the production of major royal jelly and honey metabolism proteins (Deseyn and Billen, 2005). The abdominal cavity is where the fat body cells are located which are essential for the production of vitellogenin and play an important role in the immune system of workers (Amdam and Omholt, 2002). The venom sac is one of the honeybee workers defence strategies therefore a proteomic comparison between winter and summer workers would be useful to gain an insight into potential changes in the venom sac's protein composition. Previous work has investigated the head and abdominal proteome changes in an Italian hybrid of the western honeybee in Korea, using samples collected in January and late February (Lee and Kim, 2017). This work used two-dimensional gel electrophoresis (2-DE) and QPCR to detect changes in protein expression between samples. The present study has employed label free quantitative mass spectrometry techniques to characterise changes in the proteome of *A. mellifera* workers sampled in summer (June) and winter (December).

2. Materials and methods

2.1. A. mellifera sampling regime

All samples of *A. mellifera.* were collected from an apiary managed by a professional beekeeper at Teagasc Oak Park Research Centre (County Carlow, Ireland, coordinates 52°51′53.3″N 6°54′09.7″W). Three hives were used and were sampled in June and December. Samples were stored immediately at −20 °C and transported to be stored at −80 °C within one hour. Obtaining summer bees of known ages was possible through caging the queen within the hive on a single frame. The queen was caged for two days, after which the cage was removed and the queen was released back into the hive. On the 13th day the frame was removed and placed in an incubator at 37 °C for 8 days until all the juvenile bees emerged. Newly emerged bees were marked on the top of the thorax with a coloured bee marker were released back into their respective hives. Summer samples the June laying frame and December samples were taken from the top of the frames.

3. Protein extraction from *A. mellifera*

Protein extraction was completed on single honeybee workers from each hive (n = 3), and analysis was completed on the head, abdomen and venom sac. Workers were decapitated using a sterile disposable scalpel and the heads were collected in a microcentrifuge tube. Bees were pinned to a dissection board and the alimentary canal was removed with care as not to disturb fat body and to keep the venom sac intact. Proteins were extracted via homogenisation using a hand-held motorised pestle and extracted using 300 μl (head), 400 μl (abdomen), and 200 μl (venom sac) 6 M urea, 2 M thiourea and a protease inhibitor tablet (PIC: Complete Series Roche) solution. Cellular debris was pelleted through centrifugation at 10000×*g* for 5 min. All supernatant was precipitated overnight at −20 °C at a ratio of 1:5 with 80% acetone. The acetone was removed, and proteins were re-suspended in 120 μl (head), 200 μl (abdomen), and 50 μl (venom sac) resuspension buffer (6 M urea, 2 M thiourea, 0.1 M tris-HCL, (pH 8.0) dissolved in deionised water). The Qubit™ protein quantification system (Invitrogen) was used to quantify 1 μl aliquots of protein sample. Ammonium bicarbonate (50 mM) was added to 20 μl of protein sample and proteins were reduced with 0.5 M dithiothreitol (DTT) (Sigma-Aldrich) at 56 °C for 20 min and alkylated with 0.5 M iodoacetamide (IAA) (Sigma-Aldrich) in the dark at room temperature for 15 min. Proteins were digested with Sequence Grade Trypsin (0.5μg/ml) (Promega) and incubated overnight at 37 °C. Trifluoroacetic acid (1 μl of 100%) (Sigma-Aldrich) was added to inhibit tryptic digestion during a 5 min incubation. Samples were centrifuged at 13,000×*g* for 10 min. Peptides were purified using C-18 spin columns (Pierce) and dried in a SpeedyVac concentrator (Thermo Scientific Savant DNA120) at 39 °C for 2 h. Samples were resuspended in 2% acetonitrile and 0.05% trifluoroacetic acid followed by sonication in a water bath for 5 min and centrifugation for 5 min at 15,500×*g*. The supernatant was extracted and used for mass spectrometric analysis.

3.1. Mass spectrometry

Digested *Apis mellifera* protein samples (1 μg head, 0.75 μg abdomen, 0.75 μg venom sac) were loaded onto a QExactive Mass Spectrometer (ThermoFisher Scientific) connected to a Dionex Ultimate™ 3000 (RSLCnano) chromatography system. Separation was determined by an acetonitrile gradient in a BioBasic™ C18 PicoFrit™ COLUMN (100 mm in length, 75 mm inner diameter) using a 135 min reverse phase gradient at a flow rate of 250 nL/min. The mass spectrometer was operating in an automatic dependent switching mode to acquire all data. A high-resolution MS scan (300–200 Dalton) was performed using the Orbitrap to select the 15 most intense ions prior to MS/MS. Protein identification and LFQ normalisation of MS/MS data was carried out using the Andromeda search engine MaxQuant version 1.6.6.0 (https://maxquant.org/) to correlate the data against the predicted protein set derived from the *A. mellifera* genome (Amel 4.5 assembly, Honeybee Genome Sequence consortium, 2006).

The following search parameters were used: first search peptide tolerance of 20 ppm, second search peptide tolerance 4.5 ppm, carbamidomethylation of cysteines of N-terminals were set as variable modifications and a maximum of 2 missed cleavage sites allowed. False Discovery Rates (FDR) were set to 1% for both peptides and proteins and the FDR was estimated following searches against a target-decoy database. Peptides with a minimum length of seven amino acids were considered for identification and proteins were only considered identified when more than one unique peptide for each protein was observed. The MS proteomics data and MaxQuant search output files have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD030483.

3.2. Data analysis

Results processing, statistical analysis and graphics were generated using Perseus v. 1.6.15.0. LFQ intensities were $\log_2$ transformed and proteins with a non-existent values (indicative of absence or very low abundance in a sample) were used in statistical analysis following the imputation of representative numbers based on the lowest value for each data set, which was calculated as a 1.8 (standard deviation) downshift from the mean value allowing for 0.3 width in the downshift for the standard deviation. ANOVA significance was calculated between treated and control samples was performed using a p-value of 0.05, only ANOVA significant proteins were used for further analysis. Volcano plots were

generated in Perseus to visualise differentially abundant proteins between control and treated groups and principal component analysis (PCA) was completed on all ANOVA significant proteins. Hierarchical clustering was performed on Z-score normalised intensity values for all statistically significant differentially abundant (SSDA) proteins (proteins with a real fold change > 2) by clustering all samples and proteins using Euclidean distance and complete linkage. The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) v11.0 was used to map known and predicted protein–protein interactions and used to identify protein clusters associated with specific pathways and processes. Pathways were examined further using BLAST Koala which preform KO (KEGG Orthology) to characterise gene functions and construct KEGG pathways (https://www.kegg.jp/blastkoala/).

4. Results

Label-free quantitative (LFQ) proteomics analysis was performed on head, abdomen and venom sac samples of bees collected from three hives in June and December. Data analysis was completed on the head, abdomen and venom sac of each sample and initial data matrices contained 2,609, 1,968, and 1,364 proteins respectively. Post filtration and ANOVA test (p value = 0.05) were completed resulting in 783, 381, and 275 protein matrices respectively.

4.1. Proteomic analysis of head samples

Analysis of head samples revealed 433 and 66 exclusive proteins in summer and winter proteomes respectively, 1074 out of 1573 were common between the samples. Seventeen cuticular and three major royal jelly proteins were identified as exclusive in the summer samples whereas, hexamerin 110 and five cytochrome p450 proteins were identified among the 66 exclusive proteins in the winter samples.

ANOVA (p value = 0.05) significant proteins (783) were analysed in a principal component analysis (PCA) graph (Fig. 1). PCA clustered summer and winter samples separately with a total variance between samples of 93.7%. This provides an initial insight into the distinct differences between winter and summer head proteomes. Volcano plot analysis was conducted to determine the distribution of proteins between the summer and winter samples (Supp Fig. 1). From this analysis, 522 proteins in summer samples and 197 proteins in winter samples were deemed SSDAs with a relative fold change (rfc) of ± 2. Summer samples had a larger rfc range of 2.02–4836.61 in comparison to winter samples (2.01–203.32) indicating reduced proteome abundance in winter bees compared to summer bee samples. Cuticular proteins were increased in abundance in summer samples; cuticle protein 18.7 (rfc 4836), cuticular protein CPF2 isoform 1 precursor (rfc, 733.41), cuticular protein 5 (rfc 512.28) and apidermin 2 precursor (rfc, 134.21) (Table 1). Winter samples had an increased abundance of xenobiotic proteins; Cyp4g11 (rfc, 92.35), Cyp9e2 (rfc, 28.27), Cyp6k1 (rfc, 26.42) and vitellogenin proteins (rfc 17.03, 9.25) (Table 1) (Supp. Fig. 1).

All major royal jelly proteins (MRJP) were significantly increased in abundance (-Log_2) in summer samples in comparison to winter samples (Tukey pairwise comparison 95% confidence). MRJP 6 was the most abundant MRJP identified in summer samples with a rfc of 332.03 (Table 1). Vitellogenin proteins (A0A088ADL8 and A0A088AUT9) were significantly increased in abundance in winter samples (Tukey pairwise comparison 95% confidence). Summer samples had an increased abundance of heat shock and honey production proteins whereas winter samples had an increased abundance of xenobiotic proteins (Table 1). All three honey production enzymes (alpha-amylase, alpha-glucosidase and glucose oxidase (rcf, 1049.77, 340.40, 75.76 respectively)), were identified as significantly increased in abundance in summer samples, only two (alpha-glucosidase 2 precursor (rfc, 26.37), alpha-glucosidase precursor (rfc, 3.02)), were identified in winter samples but at a greatly reduced protein abundance.

SSDAs from both samples were further analysed in BlastKOALA to identify the functional category assignment of proteins. Analysis of summer head proteome samples returned 391 proteins from 522 SSDAs and winter samples returned 147 of 197 SSDAs. Genetic information processing (messenger RNA biosynthesis, chaperones and folding catalysts, and chromosome and associated proteins), Energy metabolism (oxidative phosphorylation), and Environmental information processing (MAPK signalling pathway, Hippo signalling pathway) were increased in abundance in summer samples (Fig. 2). Winter samples had a reduced abundance of proteins (6 vs 31 involved in OXPHOS, 3 vs 7 in glycolysis and 0 vs 9 in TCA cycle). Carbohydrate metabolism was the protein group that had the smallest difference in protein abundance from the two sample groups (Summer 33: Winter 21), a reduction of proteins involved in glycolysis and TCA pathways was observed in winter samples. Three pathways associated with carbohydrate metabolism had a higher abundance of proteins upregulated in winter samples, galactose, ascorbate and aldarate metabolism, and pentose and glucuronate interconversions.

4.2. Proteomic analysis of winter and summer bee abdomen samples

Post filtration analysis of abdominal proteome samples resulted in 1,968 proteins, of which 130 and 27 were identified as exclusive in summer and winter bee samples respectively. Summer bee proteome abdominal samples had several cuticular (7) and defensin and alpha amylase as exclusive proteins. Winter bee proteome samples had four venom-associated proteins and three xenobiotic proteins identified as

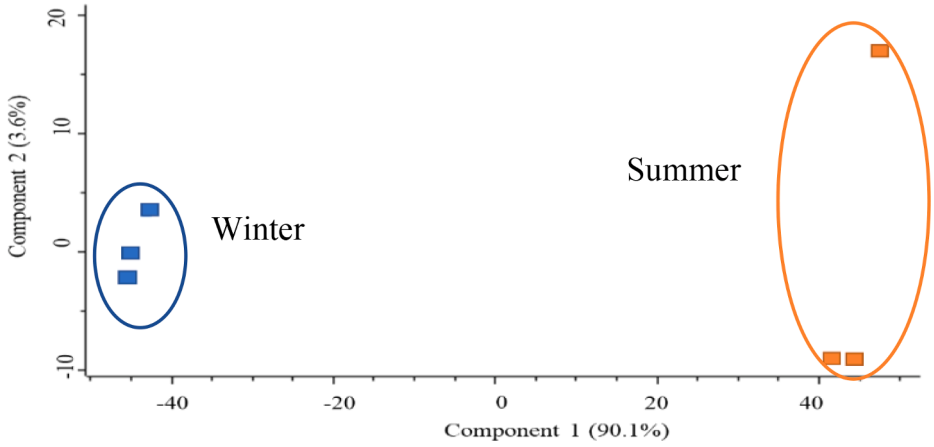

Fig. 1. Principal component analysis on head samples from winter and summer. ANOVA significant head sample proteins (p value = 0.05) plotted on a PCA. Total sample variance on the PCA 93.7%.

Table 1
Statistically significant proteins identified in the head samples of summer and winter bees. Proteins are categorised into three functional categories and labelled according to seasons summer (S), winter (W). The real fold change highlights the variation in protein abundance between the sample sets. Proteins marked with * were identified as exclusively expressed in summer/winter.

Protein Id Code	Protein Name	Functional Category	Sample	Real fold change
A0A088ABR2	Cuticle protein 18.7*	Cuticular proteins	S	4836.61
A0A087ZRG5	Cuticular protein CPF2 isoform 1 precursor*		S	733.41
A0A087ZNJ5	Cuticular protein 5*		S	512.28
A0A088AND4	Apidermin 2 precursor*		S	134.21
A0A088ANC9	Apidermin 3 precursor*		S	71.18
A0A088AKR9	Cuticular protein 28 precursor		S	67.70
A0A087ZR01	Cuticular protein analogous to peritrophins 3-C precursor*		S	34.11
A0A088ABN1	Cuticular protein 4 precursor*		S	4.86
A0A087ZT04	Cuticular protein analogous to peritrophins 3-D precursor*		S	4.37
A0A087ZUS7	Cuticular protein precursor*		S	3.01
A0A088AG34	Cuticular protein 27 precursor		S	2.52
A0A088AU21	Major royal jelly 6 precursor*	Vitellogenin and MRJP	S	332.03
O97432	Major royal jelly 5 precursor		S	49.09
A0A088AU20	Major royal jelly 4 precursor		S	20.73
O18330	Major royal jelly 1 precursor		S	7.01
Q4ZJX1	Major royal jelly 9 precursor*		S	7.15
A0A088AVG8	Major royal jelly protein 1*		S	3.96
A0A088ADL8	Vitellogenin		W	17.03
A0A088AUT9	Vitellogenin		W	9.25
P17722	Defensin-1 preprotein*	Immune response	S	183.61
A0A088AS56	Apolipophorins		W	5.14
A0A088A3R5	Protein lethal(2) essential for life	Heat shock proteins	S	50.76
A0A088A3R7	Protein lethal(2) essential for life		S	32.53
A0A088A3R6	Protein lethal(2) essential for life *		S	20.78
A0A088AGW8	Heat shock 70 kDa protein		S	3.96
A0A087ZTY7	Heat shock protein cognate 5		S	3.24
A0A088AGJ8	Heat shock protein 70Ab-like		S	2.75
A0A088A2L4	Heat shock protein 83		W	3.31
A0A088AV36	Microsomal glutathione S-transferase 1	Antioxidant Proteins	W	17.33
A0A087ZRK9	Catalase isoform X1		W	2.80
A0A088A226	Glutaredoxin-related protein 5 mitochondrial		S	2.49
A0A088AGE7	Thioredoxin peroxidase 3 isoform 2		S	2.04
A0A088AIK9	Cytochrome P450 4G11*	Xenobiotic Enzymes	W	92.35
A0A088A7I0	UDP-glucuronosyltransferase 2C1*		W	51.75
A0A088AVB4	Cytochrome P450 9e2*		W	28.27
A0A088A7G1	Cytochrome P4506k1*		W	26.42
A0A087ZNX2	Beta-ureidopropionase		W	23.63
A0A088A7I2	UDP-glucuronosyltransferase 2A3*		W	21.78
A0A088AKD5	Cytochrome P4506AQ1 isoform X1*		W	11.54
A0A087ZXV8	Cytochrome P450 9e2		W	9.73
A0A088AJN6	beta-glucuronidase*		W	8.52
A0A087ZXU5	Cytochrome P450 9e2*		W	6.65
A0A088AEG8	Alpha-amylase	Other	S	1049.77
Q17058	Alpha-glucosidase		S	340.40
A0A088A031	Glucose oxidase		S	75.76
A0A088A3V5	Yellow-f precursor		S	58.66
A0A087ZVX2	Glucose dehydrogenase		S	6.35
A0A088A030	Glucose dehydrogenase		S	4.45
Q25BT8	Alpha-glucosidase 2 precursor		W	26.37
A0A087ZVX3	Glucose dehydrogenase		W	17.70
A0A0B4J2P5	Alpha-glucosidase precursor		W	3.02
A0A088A4K9	Odorant binding protein 14 precursor	Protein folding and molecular transporters	W	5.44
Q1W633	Odorant binding protein 21 precursor		W	2.25

exclusive. ANOVA significant proteins (381) were analysed in a PCA, which revealed large differences between summer and winter proteomes due to the degree of separation between the sample clusters (Fig. 3). The total sample variance in the PCA equals 92.7%, which demonstrates that the samples do not share a large proportion of their proteome.

ANOVA significant samples were analysed in a volcano plot which resulted in 197 and 104 SSDAs in summer and winter samples respectively. Cuticle protein 18.7 (rfc, 1385.42) and cuticular protein 5 (rfc, 432.82) were the two most abundant proteins in summer bee samples and phospholipase A2 precursor (rfc, 1143.65) and allergen Api m 6 precursor (rfc, 270.42) were the most abundant proteins in winter bee samples (Sup Table 1). Following the same trend as the results from the head proteome analysis, summer abdomen samples had a large abundance of proteins in several protein pathways (Supp. Fig. 2).

Two pathways were increased in abundance in winter abdomen samples, metabolism of cofactors and vitamins and carbohydrate metabolism pathway. As in the head samples three pathways associated with carbohydrate metabolism were increased in abundance in winter workers: galactose, ascorbate and aldarate metabolism, and pentose and glucuronate interconversions. Summer samples had a large abundance of proteins involved in genetic information processing, specifically in messenger RNA biogenesis (10), chromosome and associated proteins (8) and chaperones and folding catalysts (8). Metabolism of cofactors and vitamins were increased in winter samples in, one carbon pool by folate (4) and pantothenate and CoA biosynthesis (2) (Fig. 4).

4.3. Analysis of venom sac proteomes from summer and winter bees

Proteomic analysis on winter and summer bee venom sacs samples revealed significant differences in the proteomic profile of each sample as observed in the PCA graph (Fig. 5). Analysis revealed 106 and 14 exclusive proteins in summer and winter bee proteomes respectively. Several proteins associated with bee venom activity were identified as exclusive in summer samples; major royal jelly proteins (MRJP) 8 and 9, mast cell degranulating peptide, and allergen Api m 6 precursor. No insect allergen proteins were identified in winter venom sac samples.

Volcano plot analysis on venom sac protein samples from winter and summer bees revealed a small cluster of insect allergen related proteins

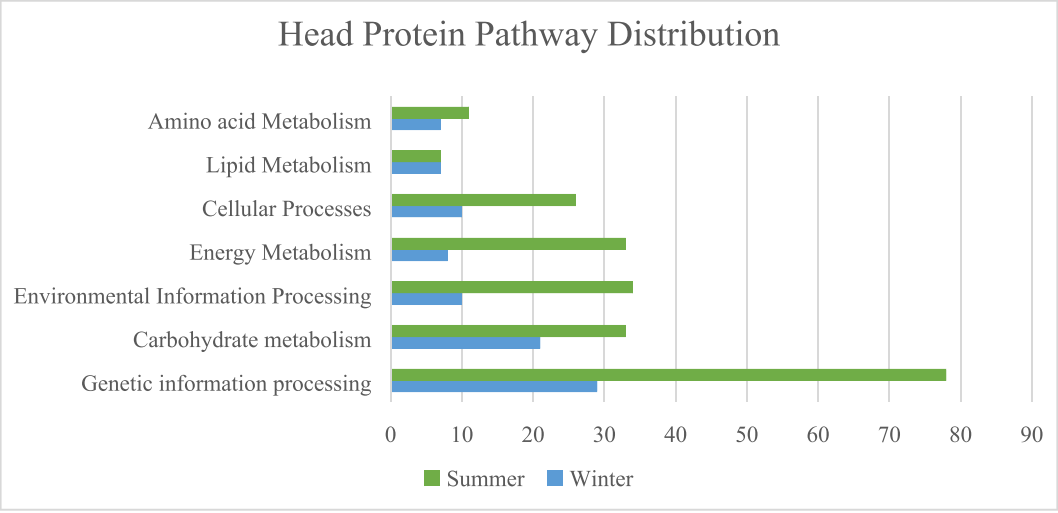

Fig. 2. Pathway analysis on SSDA proteins identified in the summer and winter head samples. 147 of 522 entries in summer and 197 winter proteins of 391 were annotated and used in BlastKOALA analysis.

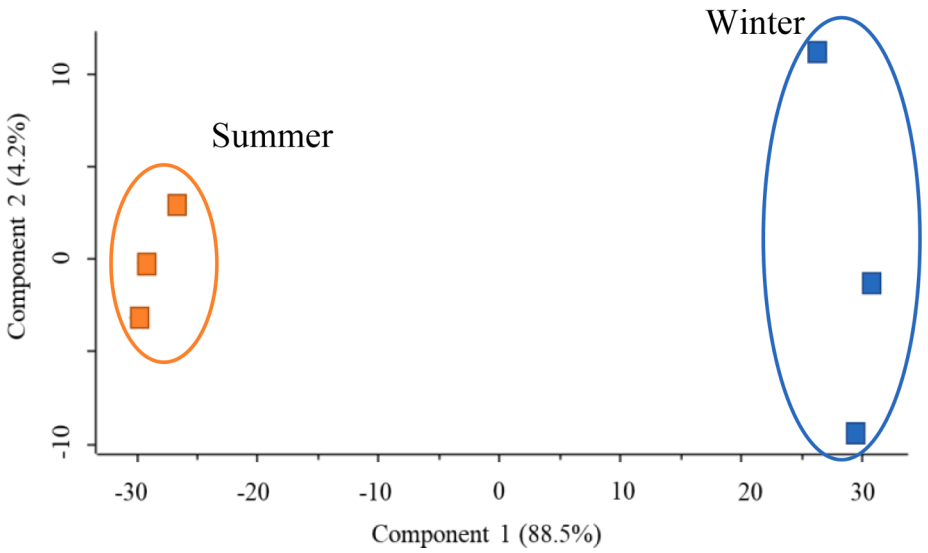

Fig. 3. PCA on summer and winter abdominal samples. ANOVA significant abdomen proteins (p value = 0.05) plotted on a PCA Total sample variation equals 92.7%.

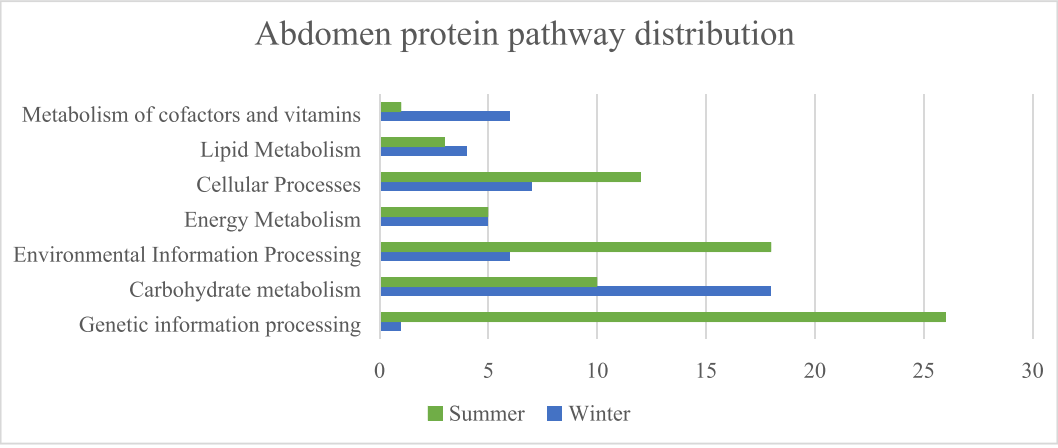

Fig. 4. Pathway analysis on SSDA proteins identified in the Summer and Winter abdomen samples. 143 of 197 entries in summer and 73 winter proteins of 104 were annotated and used in BlastKOALA analysis.

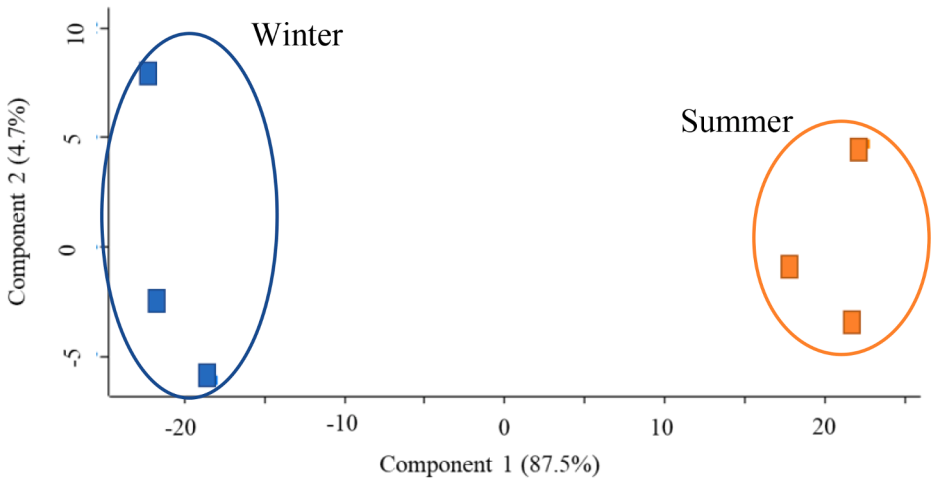

Fig. 5. PCA on summer and winter venom sac samples. ANOVA significant venom sac proteins (p value = 0.05) plotted on a PCA. Total sample variance between samples is 92.2%.

in winter bee samples versus a larger number of insect allergen and venom proteins in summer workers (Table 2, Fig. 6). Several previously identified venom proteins (venom serine proteases, dipeptidyl peptidases, carboxylesterases and acid phosphates) were observed in the data analysis of the samples, although these were not deemed ANOVA significant or SSDA. Summer samples had a higher abundance of venom associated and antioxidant proteins in comparison to winter samples (Table 2). Some MJRP were identified in the analysis, however, MRJP2 and MRJP3 were identified as SSDA proteins in winter samples, these MRJPs have not been associated with the venom sac in previous publications. MRJP9, a known venom sac protein was identified in summer samples. An increase in proteins involved in energy and carbohydrate metabolism was observed in summer samples, in comparison to an abundance of genetic information processing proteins in winter samples (Supp. Fig. 3).

Of the 91 entries identified in summer bee samples 23 are involved in oxidative phosphorylation, no proteins associated with OXPHOS were identified in winter venom sac samples. Both samples had proteins involved in tricarboxylic acid process, summer samples had 8 proteins and winter had 2. Winter samples had an increase in ribosomal proteins (12) in comparison to summer workers (0). Lipid metabolism was the only other pathway that was more abundant in winter samples versus summer samples (7 vs 5).

5. Discussion

Honeybee workers have different roles within the hive depending on the season: brood rearing and honey production in summer and survival in winter (Münch and Amdam, 2019). Summer workers undertake a range of roles as they progress through the hive such as cleaners, nurses, builders, guards, and foragers, and these roles are usually correlated with the worker's age, a process known as age polyethism (Münch and Amdam, 2019). Diutinus workers have a much narrow range of roles in the hive, thermoregulation during the winter and broodless periods, and nursing duties and foraging activities in the following spring (Münch and Amdam, 2019).

Advances in whole cell lysate proteomic extraction techniques enables the identification of a large number of proteins, a higher level of sensitivity and accurate quantification of proteins in a wide range of biological samples. The results presented here highlight a significant difference in the proteomes of workers sampled in the summer (June) and the winter (December). Summer bees have an increased abundance of cuticular proteins in the head and abdomens and a higher number of bee toxin proteins in the venom sac. In contrast bees sampled in winter have a greatly reduced proteome in all three anatomical sites. A reduction in immune protein abundance in winter honeybee workers has been observed previously, which suggests that the reduction may be linked to longevity (Steinmann et al., 2021).

Anatomical components of honeybee workers have been previously investigated to characterise changes/adaptions workers undergo to survive in their environment. Alterations in protein secretion within the hypopharyngeal glands in honeybee worker head samples have been correlated with age related changes (Brouwers, 1983; Ohashi et al., 1999; Kubota et al., 2004; Deseyn and Billen, 2005; Qi et al., 2015; Dobritzsch et al., 2019). Work has also been completed on specific protein changes that occur in workers during age-related changes or in comparisons of summer and winter workers (Amdam et al., 2009; Smedal et al., 2009; Steinmann et al., 2021). A recent study highlighted physiological and immune parameters that change between winter and summer bees, identifying an increase in vitellogenin titres, lipid and a higher level of antibacterial activity in winter bees (Kunc et al., 2019). Similar protein changes were previously identified between workers sampled in January and broodless workers sampled in February (Lee and Kim, 2017). However, the work presented here sought to further explore the proteomic differences that occur in *Apis mellifera* workers between winter (December) and summer (June) using three distinct components of the workers anatomy.

Analysis of the head samples revealed a total of 6 MRJPs, all are more abundant in summer bees rather than in winter bees, including two exclusively expressed MRJPs. This is in contrast to previous findings which identified MRJP 2 and 3 higher in workers sampled in January in comparison to February (Lee and Kim, 2017). Higher levels of MRJPs in summer is expected as the hypopharyngeal glands of winter honeybees despite being hypertrophied, have no secretory cycle is visible in winter bees (Brouwers, 1983; Deseyn and Billen, 2005). Summer bees however, are exposed to brood rearing in the hive and have elevated MRJP levels to produce bee milk for larvae and royal jelly for the queen (Deseyn and Billen, 2005; Ramanathan et al., 2018). It has been suggested that MRJPs that were secreted before workers enter the diutinus phase are stored within diutinus workers and have the potential to be used in the following spring, but little to no production of MRJPs occurs during winter (Deseyn and Billen, 2005). The variation in results between this work and previous findings could be linked to the samples that were used. Workers here were selected from a brood period in June and a broodless period in December, whereas samples in the paper of Lee and Kim (2017) were taken in January and February, a time usually associated with the end of overwintering and the initiation of brood-rearing.

Hexamerin 110 was identified as an exclusively expressed protein in winter workers in the head analysis, and hexamerin 70a was identified as significantly expressed previously (Lee and Kim, 2017). This suggests

Table 2
Statistically significant proteins identified in the venom sac samples of summer and winter bees. Proteins are categorised into three functional categories and labelled according to seasons summer (S), winter (W). The real fold change highlights the variation in protein abundance between the sample sets. Proteins marked with * were identified as exclusively expressed in summer/winter.

Protein Id Code	Protein Name	Functional Category	Sample	Real fold change
A0A088A9M1	Serine Protease 53	Bee venom toxins	W	4.77
P00630	Phospholipase A2 (Api m1)		S	694.82
P01501	Melittin (Api m4)		S	658.29
P83563	Allergen Api m6 (Api m 6)*		S	64
A0A088ARZ8	Arginine kinase isoform X2*		S	10.96
Q4ZJX1	Major royal jelly protein 9*		S	4.14
O61367 x	Arginine kinase		S	2.65
A0A088AVB4	Cytochrome P450 9e2*	Antioxidant systems	W	17.78
A0A088AJN6	Beta glucuronidase*		W	16.13
A0A088A5Z9	Hydroxyacid oxidase 1*		W	14.41
A0A088AIK9	Cytochrome P450 4G11		W	9.09
A0A088AE90	Protein lethal (2) essential for life		W	3.34
A0A088ACF4	Heat shock protein cognate 3		W	2.51
C3VMN1	glutathione S-transferase S1 isoform X1		W	2.34
A0A087ZQD3	Heat shock protein 90		W	2.02
A0A087ZZU9	Cytochrome *c* oxidase 5A		S	70.06
A0A087ZUE7	Glucose dehydrogenase		S	37.16
A0A087ZUZ0	Cytochrome *c* oxidase 5b		S	34.15
A0A088A1K0	Superoxide dismutase 2		S	7.47
A0A088AFL5	Glutathione S-transferase D1 isoform X1		S	5.88
A0A088AQ34	Cytochrome *c* oxidase 6A1		S	3.93
A0A088AAY9	Thioredoxin-2		S	2.34
A0A088A4L3	Odorant binding protein 18	Protein folding and molecular transporters	W	2.68
A0A088A4L0	Odorant binding protein 15		S	15.30
A0A088A635	DnaJ protein homolog 1		S	7.28
A0A088A4L4	Odorant binding protein 19*		S	5.82

that hexamerin is utilised during winter and the start of the brood-rearing period as a storage protein (Döke et al., 2022). However, specific role(s) of hexamerin with regards to physiology and tissue distribution are yet to be fully characterised. Several heat shock proteins were identified to be increased in abundance in summer workers in comparison to winter workers. Lee and Kim (2017) also identified this change in protein expression in their study, with workers sampled in February having higher levels of heat shock 70 kDa protein in comparison to workers sampled in January. Heat shock proteins play a role in cell growth, as molecular chaperones, protein folding and apoptosis (Mayer and Bukau, 2005). Previous work has identified heat shock 70 as higher in foragers in comparison to hive workers, and it was suggested that the increase of heat shock proteins in foragers is attributed to the increase in stress (Elekonich, 2009). An increase in heat shock protein expression has been linked to insects overwintering in harsh environments and those that enter diapause as well as plants that experience dormancy (Denlinger, 2002). The increase in heat shock proteins abundance in workers sampled in December, could give an insight into how workers cope with the stress of their environment and the adaptions that have developed to overwinter within the hives while conditions are unfavourable.

Honeybees lack an adaptive immune system, so rely upon their innate immune system which is composed of four lines of defence: hygienic behaviour, physical barriers, cellular immune and humoral immune responses (Evans et al., 2006; Grzywnowicz et al., 2009). Analysis on the head and abdominal samples revealed an overall increase in cuticular proteins in summer bees in comparison to winter bees, 9 out of 11 and 6 out of 7 cuticular proteins were identified as exclusively expressed in summer head and abdominal samples respectively. Winter samples had a greatly reduced abundance of cuticular/chitin proteins in comparison to summer samples in both the head and abdomen analysis. Winter workers may have less damage to their exoskeleton compared to nurse or forager summer bees as there is a reduction in water evaporation due to lower temperatures, little to no transport of external materials into the hive, no brood and a diminished level of hygienic behaviour involving “licking” or cleaning (Seehuus et al., 2013; Pritchard, 2016).

Several xenobiotic metabolism proteins were identified as increased in abundance in winter worker head and abdomen samples. Several cytochrome P450s were identified as exclusively expressed in winter samples whereas no cytochrome P450 proteins were associated with summer samples. Cytochrome P450s are one of the main enzyme groups responsible for xenobiotic metabolism and catalysing oxidation–reduction reactions in insects, however the stoichiometry of these enzymes has not been experimentally confirmed in insects (Feyereisen, 2012; Xu et al., 2013). Cytochrome P450s can produce reactive oxygen species in their reactions, however, these enzymes are also involved in the production of hormones, pheromones and other enzymes that break down ROSs (Xu et al., 2013). Winter bees displayed a small number of antioxidant proteins upregulated in the head and abdomens - catalase isoform X1, microsomal glutathione S-transferase 1 and peroxiredoxin-like protein which break down hydrogen peroxide (Corona and Robinson, 2006). Winter bee head samples also had an abundance of vitellogenin and apolipophorin-III, a known antioxidant with metal binding capacities and a lipid transporter linked with immune activation and secretion by hemocytes at the site of injury, respectively (Wiesner et al., 1997; Niere et al., 1999; Whitten et al., 2004; Kim et al., 2004) which can preferentially oxidise hemolymph protein over other proteins such as apolipoprotein 1 (Seehuus et al., 2006; Corona and Robinson, 2006). A depletion in vitellogenin levels and subsequently a rise in juvenile hormone titres has been directly linked with the depletion of hemocytes in the hemolymph through nuclear pycnosis (Amdam et al., 2004; Amdam et al., 2005). This can lead to severe health issues with *Varroa* parasitisation as the mites are now known to feed on the fat body of workers (Ramsey et al., 2019). Hemocytes play an important role in cellular immunity with immunological functions in phagocytosis, encapsulation, nodulation, wound repair and production in antibacterial peptides and prophenoloxidase enzymes (Amdam et al., 2005; Negri et al., 2014; Yelkovan et al., 2021). Collectively the increased abundance of xenobiotic metabolism proteins – cytochrome P450s, antioxidant proteins and abundance of hemolymph proteins - vitellogenin and apolipophorins, demonstrate a specialised immune system that aids in reducing stress in cells and aids in longevity.

Carbohydrate metabolism proteins were increased in abundance in the abdomens of winter bees in comparison to summer samples. Galactose, ascorbate and aldarate, along with pentose and glucuronate interconversions all had an increased protein abundance in winter head and abdomen samples. Galactose metabolism is closely linked with energy metabolism (Coelho et al., 2015). Ascorbate and aldarate

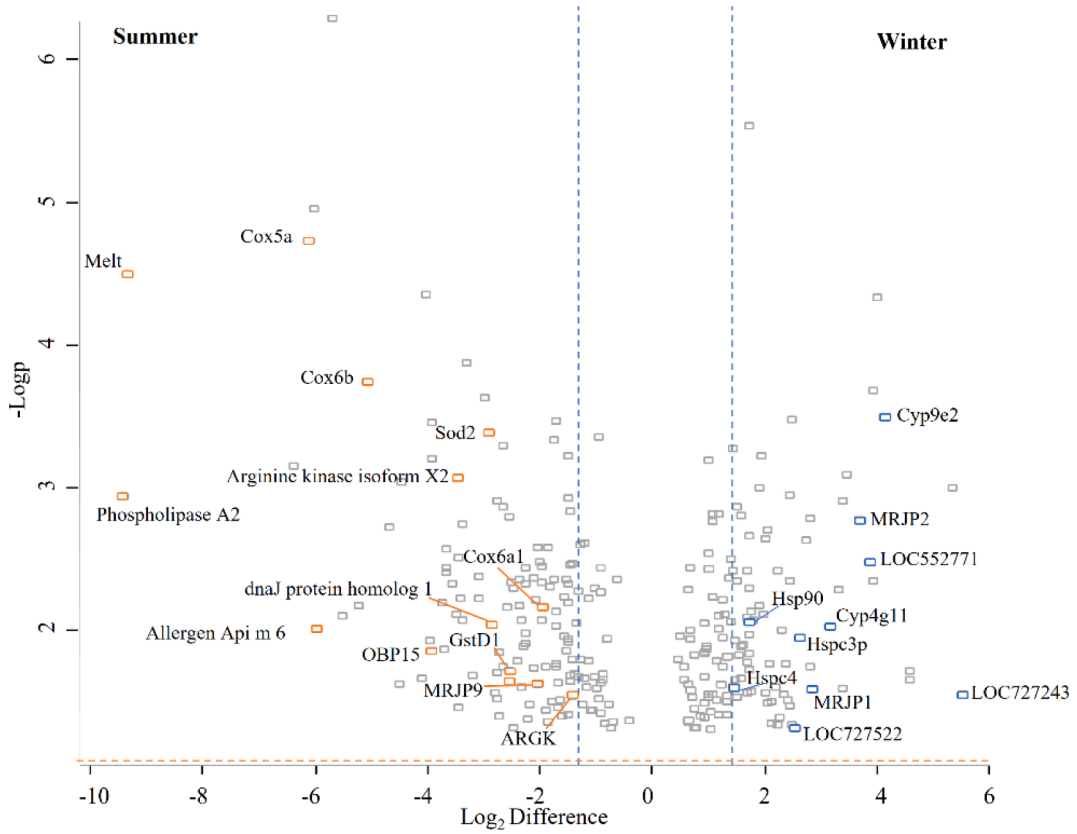

Fig. 6. Volcano plot derived from pairwise comparison between summer and winter venom sac samples. Proteins are distributed according to p-value (-log10 p-value) and fold change (Log_2 difference). Proteins outside the vertical blue lines are considered statistically significant differentially abundant (SSDA).

metabolism forms UDP-glucose which is oxidised to UDP-glucuronic acid and is mainly used for detoxification of toxic compounds through conjugation (Chen et al., 2019; Jiang et al., 2019; Malka et al., 2020). Ascorbate has also been observed to protect cells and organelles from oxidative damage by scavenging ROSs and aids in accumulating energy reserves in the hemolymph (Goggin et al., 2010). Ascorbate can decrease the activity of respiratory enzymes and works to modulate humoral and cellular immune responses (Pristavko and Dovzhenok, 1974; Garg and Mahajan, 1994; Goggin et al., 2010). In blood feeding insects (*Anopheles gambiae*) ascorbate was observed to lower the induction of superoxide radicals in the hemolymph in response to ingestion of iron-rich blood meals (Kumar et al., 2003). Pentose and glucuronate metabolism generate NADPH and pentoses which are mainly used for maintaining/regenerating the cellular detoxifying and antioxidative defence systems, they also function in the synthesis of nucleotides and nucleic acids (Ceddia et al., 2003; Agledal et al., 2010; Malka et al., 2020). An increased protein abundance in the three aforementioned pathways provides an insight into how the winter bees minimise the protein production in cells whilst reserving essential proteins with dual function such as energy metabolism and immune response.

Proteomic analysis on the venom sac samples revealed a significant difference between summer and winter bee samples. Volcano plot analysis highlighted the abundance of bee venom associated proteins expressed in summer samples with a rfc range of 2.65–694.82. The three most abundant proteins associated with summer samples: Api m6, Api m1 and Api m4 may initiate an allergic response in humans, with Api m6 and Api m4 being the two most predominant proteins making up 10–12% and 50% of the dry weight of bee venom respectively (Hider, 1988; Peiren et al., 2005). Several non-toxin proteins; antioxidant, energy metabolism, protein folding and molecular transporter proteins were also identified in high abundance in summer samples in contrast to winter samples. It is hypothesised that proteins involved in these pathways are necessary to protect the secretory cells from the damaging bee venom (Peiren et al., 2008; Li et al., 2013). The high abundance of bee venom proteins in summer bees in contrast has been observed previously (Danneels et al., 2015; Hossen et al., 2017; Scaccabarozzi et al., 2021).

Winter bee samples did not have any bee venom associated proteins identified in the venom sac analysis. Instead, winter bees had a higher abundance of genetic information processing proteins and a relatively high number of proteins involved in carbohydrate metabolism. Interestingly, there were two proteins identified in high abundance in winter samples that have not previously been identified in the venom sac: MRJP 2 (rfc 12.84) and MRJP 1 (rfc 7.17). MRJP9 was identified as SSDA in the summer samples and has been previously identified in bee venom analysis (Blank et al., 2012; Danneels et al., 2015; Ramanathan et al., 2018). Allergen investigations on honey have identified major IgE-binding proteins that correspond to MRJP1 and MRJP2 (Rosmilah et al., 2008). This could suggest that MRJP1 and MRJP2 could have an allergen role in the bee venom of winter workers, however further work would be needed to confirm the role of MRJ1 and 2 in bee venom. Winter and summer bees have different levels of activity within the hive, with winter workers rarely leaving the hive in contrast to summer workers who frequently leave the hive to forage. The different environments also contribute to a change in the type of predator/pests encountered which could lead to the different levels of bee venom associated proteins in the summer and winter workers (Danneels et al., 2015).

6. Conclusion

This study provides an insight into the proteomic composition of honeybee workers in summer and winter. Winter bee samples had a reduced number of proteins identified as SSDAs in comparison to

summer bee samples. This suggests that winter bees have a reduction in the number of proteins synthesised and stored as a means to increase longevity. In all three anatomical components that were analysed, summer bees had higher abundance and number of SSDAs. Summer workers have a wide range of roles to complete within the hive, a varied and plentiful diet, and a constantly changing external environment. Adaption to these changes could be a possible explanation as to why summer workers have a wider range of proteins expressed. In contrast winter bees experience a harsh environment, reduced nutrient availability and their main role is in thermoregulation. This study indicates that the winter bees display reduced protein abundance and expression possibly as a means to limit energy consumption to aid in longevity while ensuring the hives survival until the following spring.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The technical assistance of Mr. Danny Kehoe in bee husbandry is gratefully acknowledged.

Funding

The financial support of Clarins Ireland Ltd. is gratefully acknowledged. Q-exactive mass spectrometer was funded under the SFI Research Infrastructure Call 2012; Grant Number: 12/RI/2346 (3).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2022.104397.

References

Agledal, L., Niere, M., Ziegler, M., 2010. The phosphate makes a difference: cellular functions of NADP. Redox Rep. 15 (1), 2–10.

Amdam, G.V., Ihle, K.E., Page, R.E., 2009. Regulation of honeybee worker (apis mellifera) life histories by vitellogenin. Horm Brain Behav. Online 1003–1027.

Amdam, G.V., Simões, Z.L.P.P., Hagen, A., Norberg, K., Schrøder, K., Mikkelsen, Ø., et al., 2004. Hormonal control of the yolk precursor vitellogenin regulates immune function and longevity in honeybees. Exp Gerontol 39, 767–773. Accessed March 24, 2019. www.elsevier.com/locate/expgero.

Amdam, G. V, Lene, A., Aase, T.O., Seehuus, S.-C., Fondrk, M.K., Norberg, K., and Hartfelder, K. (2005) Social reversal of immunosenescence in honey bee workers. www.elsevier.com/locate/expgero. Accessed March 25, 2020.

Amdam, G.V., Omholt, S.W., 2002. The regulatory anatomy of honeybee lifespan. J Theor Biol 216, 209–228. Accessed June 26, 2019. http://www.ncbi.nlm.nih.gov/pubmed/12079372.

Aurori, C.M., Buttstedt, A., Dezmirean, D.S., Mărghitaş, L.A., Moritz, R.F.A.A., Erler, S., et al., 2014. What Is the Main Driver of Ageing in Long-Lived Winter Honeybees: Antioxidant Enzymes, Innate Immunity, or Vitellogenin? Biol. Sci. Cite J. J. Gerontol. A Biol. Sci. Med. Sci. 69, 633–639. Accessed February 26, 2020. https://academic.oup.com/biomedgerontology/article-abstract/69/6/633/527042.

Blank, S., Bantleon, F.I., McIntyre, M., Ollert, M., Spillner, E., 2012. The major royal jelly proteins 8 and 9 (Api m 11) are glycosylated components of Apis mellifera venom with allergenic potential beyond carbohydrate-based reactivity. Clin. Exp. Allergy 42 (6), 976–985.

Brouwers, E.V.M., 1983. Activation of the hypopharyngeal glands of honeybees in Winter. J. Apic. Res. 22 (3), 137–141.

Ceddia, R.B., Bikopoulos, G.J., Hilliker, A.J., Sweeney, G., 2003. Insulin stimulates glucose metabolism via the pentose phosphate pathway in Drosophila Kc cells. FEBS Lett. 555, 307–310.

Cervoni, M.S., Cardoso-Junior, C.A.M., Craveiro, G., Souza, A.O.D., Alberici, L.C., Hartfelder, K., 2017. Mitochondrial capacity, oxidative damage and hypoxia gene expression are associated with age-related division of labor in honey bee (Apis mellifera L.) workers. J. Exp. Biol. 220, 4035–4046.

Chen, X., Xia, J., Shang, Q., Song, D., Gao, X., 2019. UDP-glucosyltransferases potentially contribute to imidacloprid resistance in Aphis gossypii glover based on transcriptomic and proteomic analyses. Pestic. Biochem. Physiol. 159, 98–106. https://doi.org/10.1016/j.pestbp.2019.06.002.

Coelho, A.I., Berry, G.T., Rubio-Gozalbo, M.E., 2015. Galactose metabolism and health. Curr Opin Clin Nutr Metab Care 18 (4), 422–427.

Corona, M., and Robinson, G.E. (2006) Genes of the antioxidant system of the honey bee: annotation and phylogeny. Wiley-Blackwell, /pmc/articles/PMC1847502/?report=abstract. Accessed January 2, 2021.

Danneels, E.L., Vaerenbergh, M. Van, Debyser, G., Devreese, B., and Graaf, D.C. de (2015) Honeybee Venom Proteome Profile of Queens and Winter Bees as Determined by a Mass Spectrometric Approach. **7** /pmc/articles/PMC4663515/. Accessed September 21, 2021.

Denlinger, D.L., 2002. Regulation of Diapause. Annu. Rev. Entomol. 47 (1), 93–122.

Deseyn, J., Billen, J., 2005. Age-dependent morphology and ultrastructure of the hypop \haryngeal gland of Apis mellifera workers (Hymenoptera, Apidae). Apidologie 36, 49–57. Accessed June 26, 2019. http://www.edpsciences.org/10.1051/apido:2004068.

Dobritzsch, D., Aumer, D., Fuszard, M., Erler, S., Buttstedt, A., 2019. The rise and fall of major royal jelly proteins during a honeybee (Apis mellifera) workers' life. Ecol Evol 9, 8771–8782. Accessed March 3, 2020. https://onlinelibrary.wiley.com/doi/abs/10.1002/ece3.5429.

Döke, M.A., Frazier, M., Grozinger, C.M., 2022. (2015) Overwintering honey bees: biology and management. Curr. Opin. Insect Sci. 10, 185–193. https://doi.org/10.1016/j.cois.2015.05.014. Accessed March 7.

Elekonich, M.M., 2009. Extreme thermotolerance and behavioral induction of 70-kDa heat shock proteins and their encoding genes in honey bees. Cell Stress Chaperones 14 (2), 219–226.

Evans, J.D., Aronstein, K., Chen, Y.P., Hetru, C., Imler, J.-L.J.-L.L., Jiang, H., et al., 2006. Immune pathways and defence mechanisms in honey bees Apis mellifera. Insect Mol Biol 15, 645–656. Accessed March 4, 2019. https://onlinelibrary.wiley.com/doi/full/10.1111/j.1365-2583.2006.00682.x.

Feyereisen, R. (2012) *Insect CYP Genes and P450 Enzymes*. Elsevier B.V., . https://doi.org/10.1016/B978-0-12-384747-8.10008-X.

Garg, S.K., Mahajan, S., 1994. Effect of ascorbic acid on longevity and biochemical alterations in Callosobruchus maculatus F. (Coleoptera: Bruchidae). Arch. Gerontol. Geriatr. 18 (2), 149–157.

Goggin, F.L., Avila, C.A., Lorence, A., 2010. Vitamin C content in plants is modified by insects and influences susceptibility to herbivory. BioEssays 32 (9), 777–790.

Grzywnowicz, K., Ciołek, A., Tabor, A., Jaszek, M., 2009. Profiles of the body-surface proteolytic system of honey bee queens, workers and drones: Ontogenetic and seasonal changes in proteases and their natural inhibitors. Apidologie 40, 4–19. Accessed February 24, 2021. www.apidologie.org.

Hider, R.C., 1988. Honeybee venom: a rich source of pharmacologically active peptides. Endeavour 12 (2), 60–65.

Hooper, T., 2010. Guide to Bees and Honey, 5th ed.

Hossen, M.S., Shapla, U.M., Gan, S.H., Khalil, M.I., 2017. Impact of bee venom enzymes on diseases and immune responses. Molecules 22, 1–16. Accessed September 15, 2021. https://www.mdpi.com/1420-3049/22/1/25/htm.

Jiang, T., Chen, Y., Tan, Z., Li, J., Qian, P., Tang, S., Shen, X., 2019. Expression analysis and functional identification of several genes related to diapause in Bombyx mori. Dev. Growth Differ. 61 (2), 150–157.

Kim, H.J., Je, H.J., Park, S.Y., Lee, I.H., Jin, B.R., Yun, H.K., Yun, C.Y., Han, Y.S., Kang, Y. J., Seo, S.J., 2004. Immune activation of apolipophorin-III and its distribution in hemocyte from Hyphantria cunea. Insect Biochem. Mol. Biol. 34 (10), 1011–1023.

Kodrík, D., Bednářová, A., Zemanová, M., and Krishnan, N. (2015) Hormonal regulation of response to oxidative stress in insects—an update. *Int J Mol Sci* **16**: 25788–25817 /pmc/articles/PMC4632827/?report=abstract. Accessed January 4, 2021.

Kubota, M., Tsuji, M., Nishimoto, M., Wongchawalit, J., Okuyama, M., Mori, H., Matsui, H., Surarit, R., Svasti, J., Kimura, A., Chiba, S., 2004. Localization of α-glucosidases I, II, and III in organs of European honeybees, Apis mellifera L., and the origin of α-glucosidase in honey. Biosci. Biotechnol. Biochem. 68 (11), 2346–2352.

Kumar, S., Christophides, G.K., Cantera, R., Charles, B., Han, Y.S., Meister, S., Dimopoulos, G., Kafatos, F.C., Barillas-Mury, C., 2003. The role of reactive oxygen species on Plasmodium melanotic encapsulation in Anopheles gambiae. Proc Natl Acad Sci U S A 100 (24), 14139–14144.

Kunc, M., Dobeš, P., Hurychová, J., Vojtek, L., Poiani, S.B., Danihlík, J., et al., 2019. The year of the honey bee (Apis mellifera L.) with respect to its physiology and immunity: A search for biochemical markers of longevity. Insects 10.

Lee, S.H., Kim, Y.H., 2017. Comparative proteome analysis of honey bee workers between overwintering and brood-rearing seasons. J Asia Pac Entomol 20 (3), 984–995.

Li, E Al, Li, R., Zhang, L., Fang, Y., Han, B., Lu, X., et al., 2013. Proteome and phosphoproteome analysis of honeybee (Apis mellifera) venom collected from electrical stimulation and manual extraction of the venom gland. BMC Genom. 14, 766. Accessed July 15, 2019. http://bmcgenomics.biomedcentral.com/articles/10.1186/1471-2164-14-766.

Malka, O., Feldmesser, E., Brunschot, S Van, Santos-Garcia, D., Han, W.-H., Seal, S., et al., 2020. The molecular mechanisms that determine different degrees of polyphagy in the Bemisia tabaci species complex. Evol Appl 00, 1–14. Accessed November 23, 2021. http://www.bioin.

Mayer, M.P., Bukau, B., 2005. Hsp70 chaperones: Cellular functions and molecular mechanism. Cell. Mol. Life Sci. 62 (6), 670–684.

Münch, D., Amdam, G.V., 2019. (2010) The curious case of aging plasticity in honey bees. FEBS Lett. 584, 2496–2503. https://doi.org/10.1016/j.febslet.2010.04.007. Accessed December 2.

Negri, P., Quintana, S., Maggi, M., Szawarski, N., Lamattina, L., Eguaras, M., 2014. Apis mellifera hemocytes generate increased amounts of nitric oxide in response to

wounding/encapsulation. Apidologie 45, 610–617. Accessed August 20, 2019. http://link.springer.com/10.1007/s13592-014-0279-0.

Niere, M., Mei, C., Dettlo, M., Weise, C., Ziegler, M., Y, a.w., 1999. Insect immune activation by recombinant Galleria mellonella apolipophorin III 1. Biochim Biophys Acta - Proteins Proteomics 1433, 16–26.

Ohashi, K., Natori, S., Kubo, T., 1999. Expression of amylase and glucose oxidase in the hypopharyngeal gland with an age-dependent role change of the worker honeybee (Apis mellifera L.). Eur J Biochem 265, 127–133. Accessed March 13, 2020. http://doi.wiley.com/10.1046/j.1432-1327.1999.00696.x.

Omholt, S.W., 1987. Thermoregulation in the winter cluster of the honeybee, Apis Mellifera. J. Theor. Biol. 128 (2), 219–231.

Omholt, S.W., Amdam, G. V. (2004) Epigenetic Regulation of Aging in Honeybee Workers. *Sci Aging Knowl Environ* **2004**: pe28–pe28 http://sageke.sciencemag.org/cgi/doi/10.1126/sageke.2004.26.pe28 Accessed February 26, 2020.

Peiren, N., de Graaf, D.C., Vanrobaeys, F., Danneels, E.L., Devreese, B., Beeumen, J Van, Jacobs, F.J., 2008. Proteomic analysis of the honey bee worker venom gland focusing on the mechanisms of protection against tissue damage. Toxicon 52, 72–83. Accessed July 15, 2019. https://linkinghub.elsevier.com/retrieve/pii/S0041010108003577.

Peiren, N., Vanrobaeys, F., de Graaf, D.C., Devreese, B., Van Beeumen, J., Jacobs, F.J., 2005. The protein composition of honeybee venom reconsidered by a proteomic approach. Biochim Biophys Acta - Proteins Proteomics 1752 (1), 1–5.

Pristavko, V.P., Dovzhenok, N.V., 1974. Ascorbic acid influence on larval blood cell number and susceptibility to bacterial and fungal infection in the codling moth, Laspeyresia pomonella (Lepidoptera: Tortricidae). J. Invertebr. Pathol. 24 (2), 165–168.

Pritchard, D.J., 2016. Grooming by honey bees as a component of varroa resistant behavior. J. Apic. Res. 55 (1), 38–48.

Qi, Y., Fan, P., Hao, Y., Han, B., Fang, Y., Feng, M., et al., 2015. Phosphoproteomic analysis of protein phosphorylation networks in the hypopharyngeal gland of honeybee workers (Apis mellifera ligustica). J. Proteome Res.s 14, 4647–4661. Accessed September 6, 2019. https://pubs.acs.org/doi/10.1021/acs.jproteome.5b00530.

Ramanathan, A.N.K.G., Nair, A.J., Sugunan, V.S., 2018. A review on Royal Jelly proteins and peptides. J. Funct. Foods 44, 255–264. https://doi.org/10.1016/j.jff.2018.03.008.

Ramsey, S.D., Ochoa, R., Bauchan, G., Gulbronson, C., Mowery, J.D., Cohen, A., et al., 2019. Varroa destructor feeds primarily on honey bee fat body tissue and not hemolymph. Proc. Natl. Acad. Sci. U.S.A. 116, 1792–1801. Accessed September 12, 2019. http://www.ncbi.nlm.nih.gov/pubmed/30647116.

Rosmilah, M., Shahnaz, M., Patel, G., Lock, J., Rahman, D., Masita, A., Noormalin, A., 2008. Characterization of major allergens of royal jelly Apis mellifera. Trop. Biomed. 25, 243–251.

Scaccabarozzi, D., Dods, K., Le, T.T., Gummer, J.P.A., Lussu, M., Milne, L., Campbell, T., Wafujian, B.P., Priddis, C., Pecoraro, L., 2021. (2021) Factors driving the compositional diversity of Apis mellifera bee venom from a Corymbia calophylla (marri) ecosystem. Southwestern Australia. *PLoS One* 16 (6), e0253838.

Seehuus, S.C., Taylor, S., Petersen, K., Aamodt, R.M., 2013. Somatic maintenance resources in the honeybee worker fat body are distributed to withstand the most life-threatening challenges at each life stage. PLoS One 8. Accessed November 10, 2021. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0069870.

Seehuus, S.C.S.-C.S.C., Norberg, K., Gimsa, U., Krekling, T., Amdam, G.V., 2006. Reproductive protein protects functionally sterile honey bee workers from oxidative stress. Proc. Natl. Acad. Sci. U.S.A. 103, 962–967. Accessed March 24, 2019. http://www.ncbi.nlm.nih.gov/pubmed/16418279.

Smedal, B., Brynem, M., Kreibich, C.D., Amdam, G.V., 2009. Brood pheromone suppresses physiology of extreme longevity in honeybees (Apis mellifera). J. Exp. Biol. 212, 3795–3801.

Steinmann, N., Corona, M., Neumann, P., Dainat, B., Martin, S.J., 2021. (2015) Overwintering is associated with reduced expression of immune genes and higher susceptibility to virus infection in honey bees. PLoS ONE 10 (6), e0129956. https://doi.org/10.1371/journal.pone.0129956. Accessed January 19.

Whitten, M.M.A., Tew, I.F., Lee, B.L., Ratcliffe, A., Ratcliffe, N.A., Ratcliffe, A., 2004. A Novel Role for an Insect Apolipoprotein (Apolipophorin III) in β-1,3-Glucan Pattern Recognition and Cellular Encapsulation Reactions. J. Immunol. 172, 2177–2185.

Wiesner, Andreas, Losen, Susanne, Kopáček, Petr, Weise, Christoph, Götz, Peter, 1997. Isolated Apolipophorin III from Galleria mellonella stimulates the immune reactions of this insect. J. Insect Physiol. 43 (4), 383–391.

Xu, J., Strange, J.P., Welker, D.L., James, R.R., 2013. Detoxification and stress response genes expressed in a western North American bumble bee, Bombus huntii (Hymenoptera: Apidae). BMC Genomics 14 (1).

Yelkovan, S., Arıkan, H., Çakıcı, Ö., 2021. Caste and age-related changes in circulatory hemocytes of honey bee, Apis mellifera anatolica (Hymenoptera: Apidae). J. Apic. Res. 60, 512–521. https://doi.org/10.1080/00218839.2020.1834768.