Original article

Proteomic characterisation of the summer–winter transition in *Apis mellifera*

Rachel WARD[1], Mary COFFEY[2], and Kevin KAVANAGH[1]

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

Abstract – The aim of the work presented here was to characterise the proteomic shifts that occur in worker honeybees as they transition from summer to winter. Label-free quantitative proteomic analysis was performed on the heads of *Apis mellifera* collected from July to October to compare the proteomes of bees as the colony progressed from summer to autumn. Analysis highlighted differential protein expression between emerged bees and hive (early and late) and forager bees, highlighting the process of age polyethism within honeybees. A shift in protein expression in bees sampled in September and October compared to those sampled in July and August was also characterised. Bees sampled in September and October had a higher abundance of proteins associated with oxidative phosphorylation and storage proteins such as hexamerin and a decreased abundance of major royal jelly proteins which could be an indication to the change that occurs in the hypopharyngeal gland in the winter bee. Results of this study indicate that bees emerging in September and October display significant proteomic differences compared to those that emerged in July and August, and that these changes may enable the bees to withstand the stresses of winter and perform their function of thermoregulation in the hive.

Apis mellifera / seasonal adaptations / proteomic comparison / longevity

Abbreviations

FDR	False discovery rates
GO	Gene Ontology
SSDA	Statistically significant differentially abundant
LFQ	Label-free quantitative-proteomic
PCA	Principal component analysis
RFC	Relative Fold Change
ROS	Reactive Oxygen Species
OXPHOS	Oxidative phosphorylation
TCA	Tricarboxylic acid cycle
MRJP	Major royal jelly proteins

Corresponding author: K. Kavanagh,
Kevin.Kavanagh@mu.ie
Manuscript editor: Klaus Hartfelder

1. INTRODUCTION

Honeybee (*Apis mellifera*) colonies are highly structured eusocial societies, defined by an overlap of generations, cooperative brood care and reproductive division of labour (Wilson 1971). Colonies are structured into three castes: a single queen, tens of thousands of sterile female workers and a small number of fertile males, called drones. In temperate climates, bees can be subdivided into two groups which are present within the colony at different times of the year. Short-lived summer bees, with a life span of approximately 6 weeks, make up the worker population in the summer months, undertaking brood rearing, building and foraging (Johnson 2010; Hooper 2010). Long-lived winter bees (also known as diutinus bees) with a life span of up to 6 months engage in

thermoregulation over the winter (Maurizio and Hodges 1950; Omholt and Amdam 2004). Winter bees first emerge in the autumn and reside in the colony throughout the winter months and are replaced by summer bees in early spring (Omholt and Amdam 2004). The diutinus caste of workers allows *A. mellifera* to be one of only a few insect species to survive winter conditions without becoming dormant or migrating to warmer areas (Seeley and Visscher 1985; Knoll et al. 2020).

Summer bees are found within the colony from spring to early autumn and diutinus bees are thought to emerge in the colony between August and October (Free and Spencer-Booth 1959). The main role of diutinus bees is to generate heat and maintain colony temperature between 20 and 35 °C through the process of thermoregulation, via muscle activation in the thorax (Watmough and Camazine 1995; Stabentheiner et al. 2003; Amdam et al. 2009; Stabentheiner et al. 2010). Winter bees also rear the next generation of summer bees in the late winter/early spring, when the queen recommences laying (Seeley and Visscher 1985). It is thought that the absence of brood and associated pheromones trigger the emergence of the diutinus phenotype as when broodless conditions were applied to summer colonies, bees were observed to live for over 200 days (Yang et al. 2017).

There are several physiological attributes that distinguish diutinus bees from summer bees such as hypopharyngeal gland size and activity (Brouwers 1983; Deseyn and Billen 2005), organ composition, fat body activity (Fluri et al. 1977) and levels of vitellogenin and hexamerin within the haemolymph (Amdam and Omholt 2002; Lee and Kim 2017). The exact time of the emergence of diutinus bees is still undefined. However, previous work has observed bees that emerge in the first week of October have a greater longevity than those emerging in August and September (Free and Spencer-Booth 1959). Several conditions are thought to trigger the transition of summer to diutinus bees within the colony such as the absence of brood/brood pheromones, photoperiod, temperature and lack of nutritional

availability (Free and Spencer-Booth 1959; Döke et al. 2015).

The work presented here utilised label-free quantitative proteomics to analyse variations in protein abundance from bees sampled over the course of a four month period (July to October) in order to identify the time of the appearance of the diutinus bee population. Several time points throughout bees' lives were selected for analysis in order to identify shifts in protein abundance as bees' age. The utilisation of whole cell lysate on honeybee head samples of various ages across 4 months provides a novel insight into the adaptions honeybee workers undergo as the colony approaches winter. This study sought to further characterise the potential alterations in protein abundance in head associated glands such as the hypopharyngeal and mandibular glands whilst including other protein changes that occur in the head such as antenna associated proteins (Hu et al. 2019; Iovinella et al. 2011, 2018). Previous work was completed using samples from three anatomical sites from honeybee workers (head, abdomen and venom sac) and a similar proteomic profile was seen in abdominal and head samples (Ward et al. 2022b). The information presented here will be of importance to beekeepers for their autumn checks and in the application of treatments and feeds for colonies.

2. METHODS

2.1. *A. mellifera* sampling regime

All samples of *A. mellifera* were collected from an apiary in Teagasc Research Centre, Oak Park, Co. Carlow, Ireland (coordinates 52°51′53.3″N 6°54′09.7″W) which was under the care of a professional beekeeper. Colonies were monitored on a weekly basis to complete routine hive checks — checking queens, laying patterns and presence of disease. Mean, max and minimum temperature were measured for the months sampled (Table S1). Obtaining bees of known ages was possible through caging the queen within the hive on a single frame for 2 days, after which the cage was removed, and the queen was released back into

the hive. The frame was kept within the hive for 13 days, allowing the eggs to develop in sealed brood. On the 13th day, the frame containing the sealed brood was removed and placed in an incubator at 37 °C (similar to in-hive brood conditions (Jones and Oldroyd 2017) for 8 days until all the bees emerged. Newly emerged bees (approximately 150) (day 1) were marked on the top of the thorax with a bee marker and released back into their respective hives, immediately after all were marked. Workers were sampled from the selected hives at specific time points in their development: newly emerged bees (day 1), early hive bees (days 5–11), late hive bees (days 19–22) and forager bees (days 29–36). Each time point consisted of 10 worker bees. Once collected, samples were stored at −20 °C and transported on ice to Maynooth University where they were stored at −80 °C.

2.2. Protein extraction from *A. mellifera*

Protein extraction was completed on a single bee from each of three hives each month. Bees were decapitated using a sterile disposable scalpel and the heads placed in a microcentrifuge tube. Head samples were selected to detect proteomic changes in the glands and antenna proteins as the workers age. Proteins were extracted via homogenisation using a hand-held motorised pestle and extracted using 300 μl (head) 6 M urea, 2 M thiourea and a protease inhibitor tablet (PIC: Complete Series Roche) solution. Cellular debris was pelleted through centrifugation at $10,000 \times g$ 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) resuspension buffer (6 M urea, 2 M thiourea, 0.1 M tris–HCL, (pH 8.0) dissolved in deionised water). Protein digestion, quantification, cleanup and mass spectrometry search parameters are described in previous work (Ward et al. 2022a).

2.3. 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 non-existent values (indicative of absence or very low abundance in a sample) were used following the imputation of representative numbers, based on the lowest value for each data set. This 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. Multiple sample *t* test (ANOVA) was used to filter proteins. Volcano plots were generated in Perseus to visualise differentially abundant proteins between different worker ages and principal component analysis (PCA) was completed on 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), calculated by clustering all samples and proteins using Euclidean distance and complete linkage. Protein abundance in pathways were examined using g:Profiler (Raudvere et al. 2019). The mass spectrometry proteomics data and MaxQuant search output files have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD032755.

3. RESULTS

3.1. Individual month analysis

Bees were colour marked upon emergence from cells to identify date of emergence and returned to their respective hives. Bees were collected from three hives at four specific time points each month from July to October as described in Table I. Proteins were extracted from head samples and analysed using LFQ mass spectrometry. Data from each month was analysed individually in Perseus. The data matrix was first filtered for contaminants and peptides were identified. Normalised LFQ intensities were used to quantify protein abundances and were assigned to corresponding groups i.e. newly emerged worker bees. Proteins were filtered based on valid values — proteins

Table I Relative fold change values for cytochrome P450 and cytochrome c oxidase proteins identified as SSDAs in October Early and Late relative in volcano plot analysis to July and August Early and Late hive samples respectively

Relative fold change	July early	July late	August early	August late
CYP9Q2	2.25	3.14	2.68	
CYP (A0A088AVB4)	2.09		2.01	
CYP6AQ1		5.49		
CYP6AS17		4.94	2.18	6.21
COX4I1	2.29	4.87	2.74	3.92
Cox6a1		3.86	2.61	3.64
Cox6c				2.52

not found in three out of three replicates in at least one group were omitted from the analysis. LFQ intensity values were then subjected to ANOVA t test (p value $= 0.1$) which identifies proteins as significant using the difference between means at the defined p value.

Principal component analysis (PCA) were completed on ANOVA significant (p value $= 0.1$) samples from all months. July's PCA demonstrated a pattern of age polyethism with newly emerged bees in one cluster, hive (early and late) bees were in a second and foragers in the third cluster (Figure 1). Interestingly, August and September's PCAs had a different arrangement of samples as clusters were arranged as follows, emerged bees, early hive, late hive and foragers (Figure 1). Another shift of sample arrangement was observed in the October's PCA- emerged bees are in one cluster and early, late and forager bees are together in a second distinct cluster.
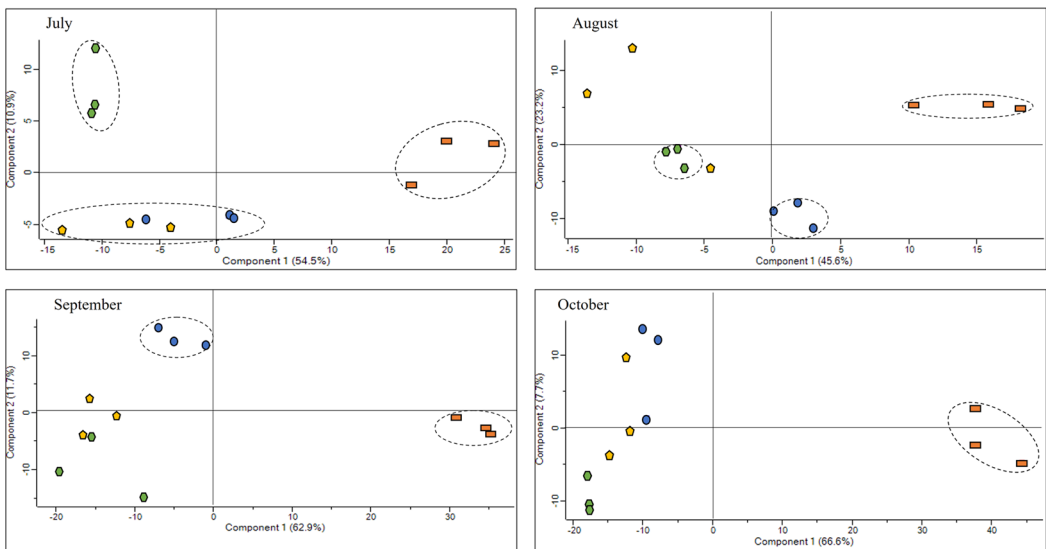

Figure 1. Principal component analysis of worker bee head samples collected in July to October. Each sample is grouped by age in each month: orange–emerged, blue–early, yellow–late, green–forager. Each PCA has an overall degree of separation value ranging between 65.4 and 76%.

The arrangement of the samples in July clearly grouped bees based on similar ages and their subsequent roles within the colony. In contrast, in August and September, it appears that the proteome of the forager and the late hive bees are similar in contrast to forager and late hive bee proteomes in July. The PCA from October samples clearly highlighted a similarity in protein expression involving all hive and forager samples as they are clustered close together. Each PCA had an overall degree of separation value ranging between 65.4 and 76%, which demonstrated the large differences in protein profiles between emerged bee samples compared to hive and forager bee samples, as these samples are consistently clustered separately. The high overall degree of separation in the October PCA highlights how similar the proteomes of hive and forager bee samples are to each other.

3.2. Age group analysis

Proteomic pathway analysis was conducted collectively and the data matrix contained 3819 proteins. Post filtration and ANOVA tests (p value = 0.05) were completed resulting in a matrix of 1295 proteins. Samples were grouped based on month collected and age of bees. Volcano plot comparisons were completed on samples of the same age (i.e. emerged bees sampled from July to October) and between each age group within each month (i.e. all July samples were compared to each other). Proteins with a $\log_2$ fold change greater than 1 and a $-\log p$ value greater than 1.3 in the volcano plots were deemed SSDAs and were isolated for further analysis. In total, 984 SSDAs were identified from the volcano plot comparisons.

3.3. Emerged Bees

Comparisons of the proteomes of newly emerged bee samples revealed a significant shift in protein abundance from July and August samples compared to September and October samples. July and August emerged samples had a higher number of SSDAs generated in volcano plot analysis in comparison to September and October emerged bee samples (Figure S1A).

Some interesting protein expression patterns were observed in the volcano plot comparisons between the emerged bee samples from the different months (Figure S2). Hexamerin 70c (A6YLP8) was identified as increased in abundance in October emerged bee samples when compared with July (rfc 75.6) and August (rfc 37.7) emerged bee samples (Figure S2). LFQ intensity values of both hexamerin 70c and 110 were observed to be higher in September and October compared with July and August.

A number of cuticular proteins were increased in abundance in September samples in all comparisons with the emerged samples (July, August and October) (Figure S2). LFQ intensity values showed a higher abundance of cuticular proteins in September emerged bees compared to July, August and October samples; however, no significant change in protein abundance was observed.

Major royal jelly proteins (MRJPs) were consistently identified as highly expressed in both July samples in comparison to September and October samples (rfc range: 8.04–672.10 and 2.83–755.51 respectively) and August samples in comparison to September and October samples (rfc range: 14.36–1145.23 and 2.17–1287.34 respectively). LFQ protein intensity values for MRJPs were also higher in July and August emerged bee samples in comparison to September and October samples. Protein intensity values for MRJPs were averaged and plotted on a boxplot, which shows the significant increase in MRJP intensity values in July and August versus intensity values in September and October (Figure 2). ANOVA test also highlighted a further decrease in protein abundance was observed in October in comparison to September.

3.4. Early and late hive bees

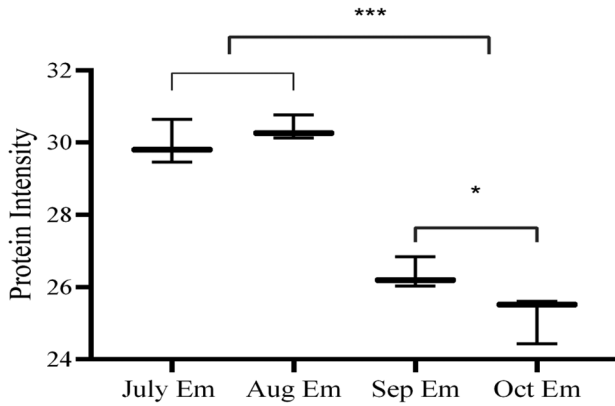Overall, there was a reduced number of SSDAs generated from early and late hive bee

Figure 2. Averaged major royal jelly protein LFQ values from the head samples of newly emerged bees. Significance is represented by 0 '***', 0.001'**', 0.1'*'.

volcano plot comparisons demonstrating a similarity in proteome composition and expression of hive bees across the months sampled (Figure S1B, C). The highest number of SSDAs occurred in October early and late hive bee samples when compared to July and August samples, indicating a shift in protein expression in October workers.

Several mitochondrial and xenobiotic metabolism proteins were observed to be SSDAs in September and October early and late hive samples. Specifically, cytochrome P450 proteins (subunits: 9e2 and 6AQ1) and cytochrome c oxidase (4I1, 6a1, 6b1, 6c) were identified as SSDAs in October early and late hive bee samples in comparison to July and August samples (Table I). Cytochrome c oxidase subunit 6c1 was observed as increase in abundance in September samples

in comparison to July and August late hive bee samples (rfc, 2.27 and 2.41 respectively).

Protocadherin Fat 4 isoform X1 (LOC552546) was amongst the top five most abundant SSDAs associated with July and August early and late hive bee samples when plotted against September and October workers (Table II). Overall, protocadherin Fat 4 isoform X1 LFQ values were significantly higher in abundance in July and August samples in comparison to September and October (Figure S3).

Several odorant-binding proteins (OBPs) were identified in the early and late hive bee comparisons from July to October. July and August early comparison identified OPB 13 and 3 in July early hive samples (rfc, 8.66 and 3.88) and OBP 21 in August early hive sample (rfc, 2.08). August and September early comparison found

Table II Relative fold change values for protocadherin Fat 4 isoform X1 identified as an SSDA in July and August early and late hive bee samples in comparison to September and October early and late hive bee samples respectively

Relative fold change	September early	September late	October early
July early	67.87		103.24
July late		113.59	
August early	54.57		83
August late		73.05	

OBP13, OBP3 and OBP21 (rfc, 18.24, 5.2, 2.19) as SSDAs associated with September early hive samples. Late hive bee comparisons between July and September identified OBP2 and OBP17 (rfc, 31.81 and 2.19) as SSDAs associated with September samples. OBP16 was identified as an SSDA in both July and August late hive bee samples when compared to October late hive bee samples (rfc, 15.39, 32.94 respectively). Finally, volcano plot comparison between September and October late hive bee samples resulted in OBP16 (rfc, 23.78), OBP2 (rfc, 20.99) and OBP17 (rfc, 2.73) as SSDAs in September late hive bee samples. No OBPs were identified as SSDAs in October early or late hive bee samples.

3.5. Forager bees

Similar to early and late hive bee samples, a large number of SSDAs were generated when September and October forager samples were compared with July and August forager samples (Figure S1D).

Volcano plot analysis of October samples to July, August and September samples revealed an increased abundance in cytochrome P450 proteins and cytochrome c oxidase proteins associated with October forager bees. Analysis with July revealed two cytochrome c oxidase proteins: Cox4I1 (rfc,4.17) and Cox6a1 (rfc,3.42) and two cytochrome P450 proteins: CYP9Q2 (rfc, 4.96) and CYP6a14 (rfc, 4.79). Comparison to August samples identified three cytochrome c oxidase (COX): Cox4I1 (rfc, 4.40), Cox6a (rfc, 3.51) and Coxfa4 (rfc, 2.81) and one cytochrome P450, CYP9Q2 (rfc, 2.35) as SSDAs in October. Finally, volcano plot analysis to September forager bees revealed one cytochrome P450: CYP6a14 (rfc, 2.99), and two cytochrome c oxidase proteins: COXfa4 (rfc, 2.15), COX (rfc, 2.15).

Protocadherin Fat 4 isoform X1 was the most abundant protein associated with September and August foragers in comparison to October forager samples (rfc, 63.09 and 77.06 respectively). It was not identified in other volcano plot analysis of forager samples. Storage protein

hexamerin 110 was only identified in the comparison between September and October and was one of the top 5 most abundantly expressed proteins in October forager samples (rfc, 6.89).

SSDAs from forager volcano plot analysis were further analysed in g:Profiler for functional enrichment analysis (*p*-value of 0.05), and KEGG terms were used to identify enrichment in pathways (Table III). No enrichment terms were identified as significant in the August forager samples when compared to September samples. Mitochondrial proteins were observed to be increased in relative abundance in September and October forager samples (Table III).

3.6. Pathway analysis

Hierarchical clustering was performed on the *z*-scored normalised LFQ intensity values for the 984 SSDA proteins identified by the volcano plot comparisons (Figure 3). Samples were resolved into three distinct groups determined by Euclidean distance analysis; July and August emerged bee samples, all hive (early and late) and forager bee samples and September and October emerged bee samples. Three (A–C) protein clusters were identified based on protein abundance profile similarities (Figure 3). Gene Ontology (GO), Pfam and KEGG term enrichment analyses were performed on all clusters, of which cluster A and B showed protein enrichment (Table IV). This included proteins associated with tricarboxylic acid cycle (TCA), major royal jelly (MRJ) proteins and extracellular region (cluster A). Integral component of membrane in cluster B. Details of all clusters are within (Supplementary Dataset 2). TCA and MRJ protein abundance was reduced in September and October emerged bee samples, which corresponded with what was observed in the volcano plot analysis. Curiously, the integral component of membrane protein abundance was high in September and October emerged samples and in October hive (early and late) and forager samples.

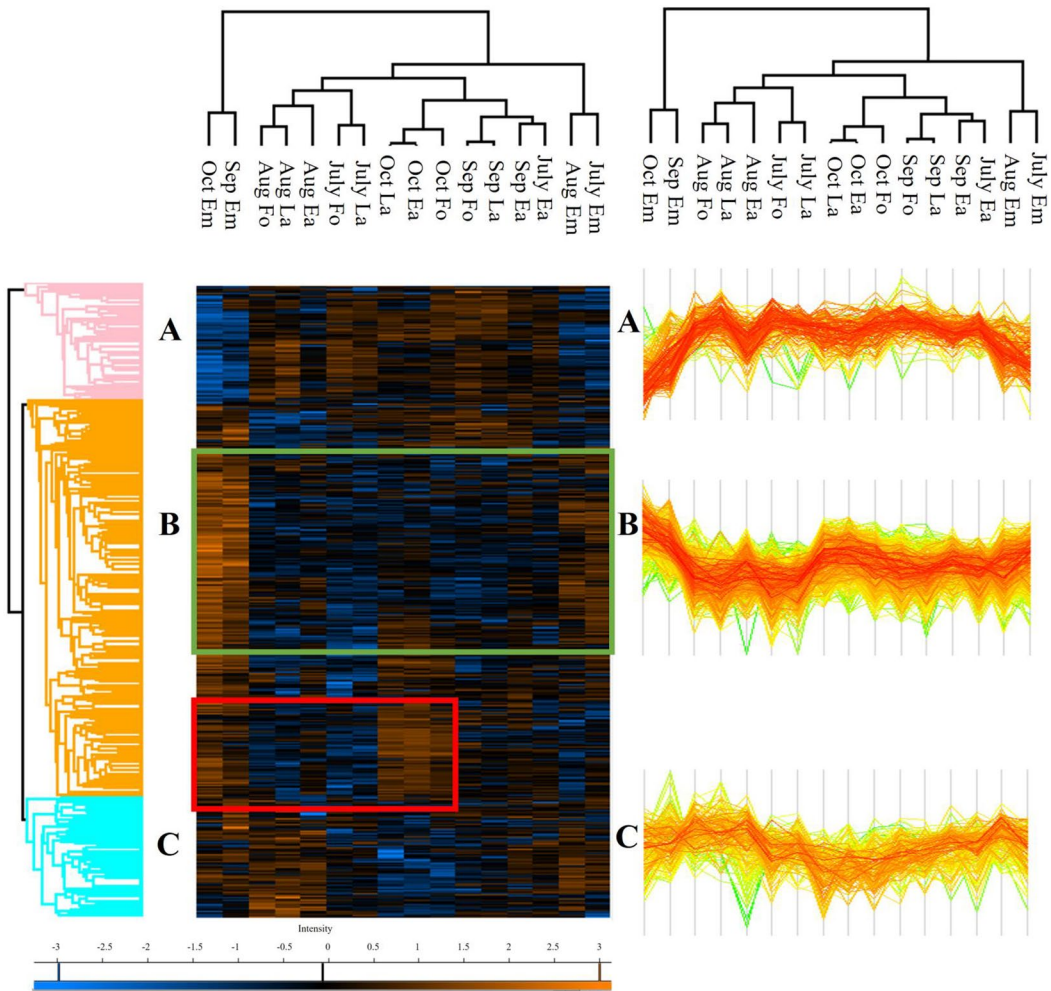Within cluster B, a group of 333 proteins were identified as upregulated in all newly emerged

Figure 3. Hierarchical clustering analysis on all ANOVA SSDA proteins from head samples taken between July to October. Proteins were grouped via unsupervised clustering and subsequently divided into three groups (A–C). Clusters A and B both had protein enrichment pathways, whereas cluster C did not have any. The green box highlights a group of proteins ($n = 333$) that represent proteins upregulated in all emerged bee samples in this analysis. The red box highlights a group of proteins ($n = 157$) that are only upregulated in October hive and forager bee samples but are downregulated in the remaining samples in the analysis.

samples from July to October (Figure 3, green box) and g:Profiler analysis identified proteins involved in the spliceosome and involvement in the basement membrane, indicating a developmental proteome of emerged workers in comparison to older bees such as hive and foragers (Figure S4).

Also within cluster B, a group of 157 proteins were identified as upregulated in October hive and forager bee samples, but downregulated in July, August and September (Figure 3, red box). These proteins were analysed using g:Profiler to investigate the protein pathway distribution. A large number of proteins were identified to be associated with the mitochondrion and to have a role in ion transmembrane transporter activity, there was one single KEGG term identified as significant in protein abundance from the 157 proteins, oxidative phosphorylation (Figure 4).

Table III g:Profiler functional analysis term results. Months with the same comparison number are a result of the direct comparison between those months. Term size is the number of genes that have been annotated to the term. Intersection size is the number of genes in the input query that are annotated to the corresponding term in the order of the input queries

Comparison	Month	Gene Ontology source	Term name	Term ID	-log10 adjusted *p*-value	Term size	Intersection size
1	July	GOMF	MAP kinase activity	0004707	3.01	5	2
1	September	KEGG	Protein processing in endoplasmic reticulum	04141	1.79	70	6
2	September	KEGG	OXPHOS	00190	3.32	73	8
3	July	GOCC	Cytoplasm	0005737	2.36	1309	18
3	October	KEGG	Ribosome	03010	1.83	97	5
4	August	KEGG	Arginine and proline metabolism	00330	1.52	14	2
4	October	KEGG	OXPHOS	00190	3.46	71	8
5	September	KEGG	Arginine and proline metabolism	00330	0.35	14	1
5	October	KEGG	OXPHOS	00190	2.05	73	5

No enrichment terms were identified for cluster C in Perseus analysis (Figure 3). Protein IDs from the cluster were analysed in g:Profiler, which identified proteins involved in translation, protein folding and the cytoplasm. Proteins in cluster C were mostly upregulated in August hive and forager samples, with a slight degree of upregulation in July and August emerged samples also.

4. DISCUSSION

The time of the emergence of the diutinus or winter bee in *A. mellifera* populations is poorly characterised and this may be partly attributed to the lack of physical differences between summer and winter bees within colonies. Research has indicated that the chemical and environmental signals such as shorter days, reduced nutrient availability, brood absence and temperature decline may trigger the transition (Döke et al. 2015; Mattila and Otis 2007). Previous studies have utilised immunohistochemistry and 'omic' methods such as genomics, proteomics and metabolomics to investigate the molecular composition of bees in order to better understand the physiological differences between summer and winter bees (Amdam and Omholt 2002; Aurori et al. 2014; Kunc et al. 2019; Omholt and

Table IV Statistically enriched gene ontology terms identified in hierarchical clustering (Figure 3). Cluster A and B are protein groups identified by calculating the Euclidean distance using Z-scored LFQ intensity values of SSDA proteins

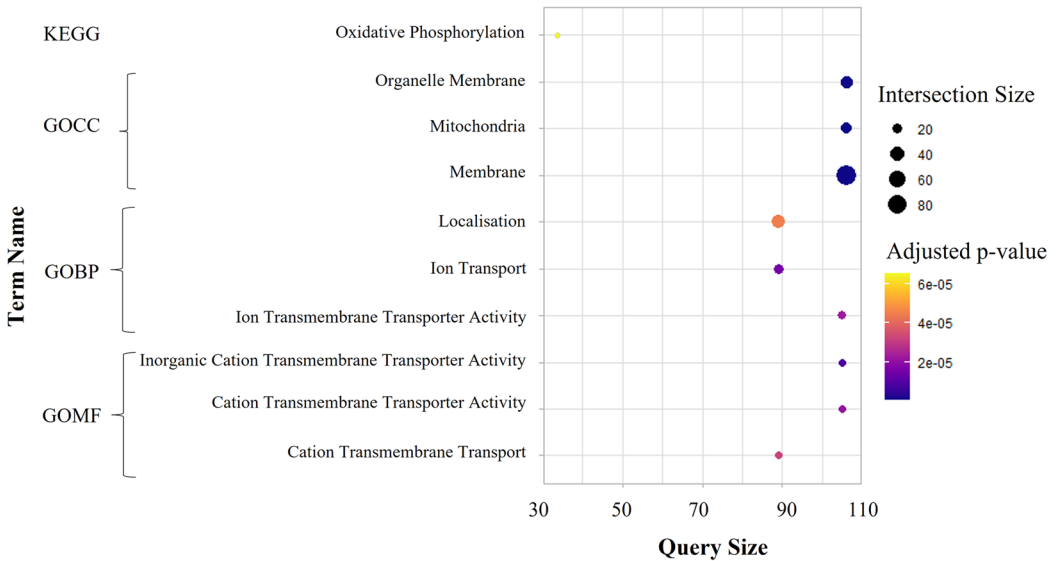| Cluster	Name	P value	Enrichment
	Tricarboxylic acid cycle	2.26E − 07	5.38
A	MRJP	3.22E − 07	4.3
	Extracellular region	1.17E − 04	2.93
	HSP20	4.59E − 05	4.7
B	Integral component of membrane	2.52E − 07	1.16

Figure 4.   g:Profiler gene ontology analysis on red cluster. Colours highlight the gene ontology group identified from the 157 proteins analysed. Yellow-GOMF, green-GOBP, blue, GOCC and Orange-Kegg.

Amdam 2004; Seehuus et al. 2006). This study aimed to characterise the emergence of winter bees using whole-cell proteomic techniques on head samples from workers sampled from colonies between July and October.

The first step in this analysis was to investigate how bees of known age within each month could be differentiated using label-free proteomics using worker bee head samples. PCAs were constructed on samples taken from hives in July to October. Figure 1 demonstrates a progression of the proteomic expression from newly emerged bees through to foragers within each month. In July, samples can be divided into three groups: emerged bees, hive bees (early and late) and foragers. July PCA shows the best example of the known progression of summer bees within the colony: emerged bees to early hive bees to late hive to foragers (Johnson 2010; Seeley 1982). However, in August and September, the orientation of samples on the PCAs changed as the groups are now arranged into emerged bee cluster, early hive bee cluster, late bee and forager cluster. A final change in sample orientation occurred in October with two

distinct groups arising: emerged bees and then hive bees (early and late) and forager samples. These shifts in sample arrangement within the PCAs from July to October could suggest the transitional period of summer to winter workers in the hives is approximately September as identified in previous work (Döke et al. 2015; Knoll et al. 2020; Kunc et al. 2019; Maurizio and Hodges 1950).

Volcano plot analysis was performed to investigate the shifts in proteomic expression from samples of the same age taken in different months. This analysis identified an increase in MRJ proteins in July and August emerged bee samples in comparison to September and October emerged samples, with no MRJ proteins identified as SSDAs in September or October. MRJ proteins make up 80–90% w/w of the composition of royal jelly and are produced and secreted by the hypopharyngeal glands of worker bees (Jianke et al. 2010; Ramanathan et al. 2018). Newly emerged bees (day 1) showed reduced production and secretion of MRJ proteins in comparison to both day 3 and nurse bees (days 5–7) (Qi et al. 2015), demonstrating that the development

and activity of the glands is directly related to the age of the worker. Hypopharyngeal glands in summer bees can become hypertrophied in response to broodless periods, much like the condition experience by those of winter bees (Brouwers 1983; Hrassnigg and Crailsheim 1998). There is also a low rate of protein synthesis that occurs in the hypopharyngeal glands in both broodless summer and winter conditions (Amdam and Omholt 2002; Brouwers 1983; Huang et al. 1989). Analysis on the hypopharyngeal glands has previously identified the presence of MRJ proteins within winter bees (Li et al. 2008). The current study did not find any MRJ proteins classified as SSDA in newly emerged September and October bees but instead identified an abundance of MRJ proteins in newly emerged bees in July and August samples. This could provide some insight into what triggers the emergence of winter bees, as newly emerged bees sampled closer to summer months have a higher relative abundance of MRJ proteins than those sampled closer to winter months.

Protocadherin fat isoform X1 was increased in abundance in July and August samples in comparison to September and October samples. Protocadherins are members of the cadherins superfamily which are usually transmembrane proteins (Takeichi 2006). The extracellular domain of these transmembrane proteins has cadherin repeats which contain sequences that are associated with calcium binding (Takeichi 2006). In *Drosophila* protocadherin, fat plays a role in regulating growth and for normal planar cell polarity of several *Drosophila* tissues including hair orientation (Bosch et al. 2014; Casal et al. 2002; Clark et al. 1995; Hogan et al. 2011; Matakatsu and Blair, 2012). Currently, there is limited information about protocadherin fat protein function in bees and none associated with the emergence of winter bees. Cadherin proteins localised in the nuclear and cytoplasm of the gonads of both sexes and castes of bees, thereby suggesting alternative function of these proteins besides their characterised roles in cellular adhesion (Florecki and Hartfelder 2012). Results presented here highlight the abundance of protocadherin fat isoform X1 in July and August hive and forager samples in comparison to September and October samples.

Protocadherin fat isoform X1 may influence the development of bees through its role in the Hippo pathway and therefore a reduced expression in protocadherin fat isoform X1 may trigger the emergence of the winter bee phenotype, which are known to have a different structures and functions to summer workers in specific organs and glands such as hypopharyngeal glands and fat body (Brouwers 1983; Hrassnigg and Crailsheim 1998; Snodgrass 1910).

Hexamerin 70c and 110 proteins were in higher relative abundance in September and October emerged and October forager samples. Hexamerins are well-studied proteins which are abundantly expressed in larval fat body used for amino acid storage, insect development and JH binding (Lee and Kim 2017). Hexamerin 70c was present at high levels in larval stages, but progressively decline in abundance in pupal development, but can still be detected on the 7th day of an adult (Cunha et al. 2005). Hexamerin 110 was found to be high in bee larvae, to decrease in pupae, only to increase up to fivefold in 6-day-old bees (Bitondi et al. 2006). Hexamerins are in greater abundance in the diapausing *Anthonomus grandis* (boll weevil), functioning as a storage protein (Lewis et al. 2002), and hexamerin has also been identified in the adults of several ant species during the period of brood rearing (Wheeler and Martinez 1995). Hexamerin protein levels decrease as bees age, with the abundance being highest in newly emerged bees and lowest in foragers, and it has been suggested that the hexamerin levels are nutrient dependent (Bitondi et al. 2006; Hu et al. 2019; Martins et al. 2010). This suggests that the abundance of hexamerin proteins in winter bees has a dual function, to act as a storage protein in times of limited nutrient availability and to be used in late winter/early spring when brood rearing resumes.

The presence of odorant-binding proteins (OPBs) in early and late hive worker bee head samples in July, August and September, but the absence in October indicate a specific role of the proteins in the transition of summer to winter. OBPs recognise chemical stimuli and have been revealed to have a role as pheromone carriers (Chertemps 2017; Forêt and

Maleszka 2006; Iovinella et al. 2011). Previous work using 2DGE and mass spectrometry on worker bee hemolymph has identified a shift in odorant-binding proteins between summer and winter workers, with summer worker bees expressing OBP13, OBP1314 and OBP1315 whereas OBP14 was only identified in winter workers (Erban et al. 2013). This analysis did not identify OBP14 as an ANOVA significant SSDA in the comparative analysis between July and October. OBPs have different functions regarding cell signalling or pheromone detection for example: OBP13 binds to oleic acid with good specificity, oleic acid and β-ocimene have been linked to hygienic behaviour with brood removal (Iovinella et al. 2011; McAfee et al. 2018), whereas OBP21 binds with some affinity to components of the queen's mandibular pheromones- 9-oxo-2-decenoic acid and methyl p-hydroxybenzoate, which are used to inhibit the activation of ovaries and follicle development in workers and aid in retaining the hierarchical structure within colonies (Hartfelder 2000; Iovinella et al. 2011). Work has also been completed on the change of protein expression levels of OBPs within of worker bee according to their cast and age (Iovinella et al. 2018). Some OBPs such as OBP 2 and 16 were intensely expressed in foragers antennae whereas OBP13, OBP17 and OBP21 are expressed more intensely within the mandibular glands of foragers (Iovinella et al. 2011, 2018). The presence of OBP2, OBP3, OBP13, OBP16, OBP17 and OBP 21 in July, August and September early and late hive bees and absence in October might be an indicator into the changes that occur within colonies as the seasons change from autumn to winter.

Hierarchical clustering provided an overview of the relationship between the proteomes of bees of all ages from July to October. Interestingly, emerged bees from September and October samples were clustered separately from July and August emerged bee samples. This indicates a significant shift in proteome expression in September and October emerged bees and potentially the emergence of the winter bee phenotype within the hive. However, 333 proteins were upregulated in all emerged bee samples, which

were characterised as being associated with the spliceosome and the basement membrane. The spliceosome and basement membrane are both essential pathways in the development and growth of organisms, responsible for removing introns from pre-messenger RNAs and the ligation of remaining exons (Kahlscheuer et al. 2015; Stegeman et al. 2018), and the cellular polarity of cells ensuring cells are the correct size and shape in order to function normally (Khalilgharibi and Mao 2021). The presence of these upregulated proteins in emerged bee samples could be indicative of the developmental process all emerged bees undergo as they mature to fully formed workers. Previous work has identified an increase in ribosomal proteins in newly emerged worker bees (Liu et al. 2014) and suggests that the increase in genes associated with development in newly emerged bees could be linked with the rapid growth phase of the hypopharyngeal glands.

A cluster of 157 proteins was upregulated in only the October samples. Pathway characterisation of these proteins highlighted functionality in ion transport and mitochondrial activity, specifically oxidative phosphorylation. Organisms with an increased abundance of oxidative phosphorylation (OXPHOS) proteins have an increased tolerance to oxidative stress which can aid in the longevity of an organism (Cervoni et al. 2017; Harman 1956). An increased tolerance of oxidative stress in winter bees has been characterised through increased levels of vitellogenin, OXPHOS and xenobiotic metabolism proteins (Amdam and Page 2005; Münch and Amdam 2010; Omholt and Amdam 2004). The increased presence of mitochondrial proteins in the October samples in comparison to July–September samples could identify the presence of the winter bees in the colonies. Typically foragers have a reduced OXPHOS capacity than nurse bees (Cervoni et al. 2017). However, hive and forager samples in October have an increased presence of OXPHOS proteins in comparison to hive and forager samples in July to September. This provides further evidence to the time frame when winter workers appear in the colonies, as it seems that October 'foragers' are not typical summer foragers, but instead belong to the diutinus caste.

Previous work has identified autumn as the time for the emergence of winter workers, however the time frame can vary up to 4 weeks depending on the colony strength, nutrient availability and weather conditions (Mattila and Otis 2007; Maurizio and Hodges 1950; Omholt 1988). Pollen stores can affect the production of long-lived winter bees relative to control colonies as an increase in pollen stores in colonies can delay the emergence of diutinus workers (Mattila and Otis 2007). However, as the pollen and nectar vary in both availability and quality, the effects of nutrition on the transition of hives from summer to winter remains to be fully determined (Döke et al. 2015).

Weather data collected from the apiary location, identified a drop in temperature from July to September and subsequentially September to October. The drop in temperature could be a contributing factor to the change in proteome expression. However, future work would need to specifically investigate the changes in temperature as a factor to proteomic changes in worker bees.

5. CONCLUSION

The proteomic analysis conducted here on worker bee head samples demonstrates the pattern of age polyethism in July and August months with the division of bee proteomes between newly emerged bees through to foragers, and the emergence of winter bee phenotypes in colonies in late autumn — as observed in October's PCA. Specific changes in protein expression within samples taken between July and October provide evidence of how *A. mellifera* has adapted to unfavourable conditions in temperate climates. The increase in proteins associated with reducing oxidative stress (OXPHOS proteins) and of storage protein hexamerin in September and October samples, demonstrate some of the adaptions bees undergo to survive the winter months. The shift of protein expression in newly emerged bees observed in the hierarchical clustering analysis, was particularly interesting as it indicates that bees emerging later in the year have a different protein profile to those that emerge in the summer or early autumn months. This work provides proteomic evidence of the time frame when winter bees emerge.

DATA AVAILABILITY

The mass spectrometry proteomics data and MaxQuant search output files have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD032755. Excel datasets containing the volcano plot and hierarchical clustering information have been submitted with the journal.

CODE AVAILABILITY

Not applicable.

DECLARATIONS

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent to publish Not applicable.

Conflict of interest The authors declare no competing interests.

REFERENCES

Amdam GV, Ihle KE, Page RE (2009) Regulation of honeybee worker (*Apis mellifera*) life histories by vitellogenin. Horm Brain Behav Online 1003–1027

Amdam GV, Omholt SW (2002) The regulatory anatomy of honeybee lifespan. J Theor Biol 216(2):209–228. https://doi.org/10.1006/jtbi.2002. 2545

Amdam GV, Page RE (2005) Intergenerational transfers may have decoupled physiological and chronological age in a eusocial insect. Ageing Res Rev 4(3):398–408. https://doi.org/10.1016/j.arr.2005. 03.007

Aurori CM, Buttstedt A, Dezmirean DS, Mărghitaş LA, Moritz RFAA, Erler S, Mărghitaş LA, Moritz RFAA, Erler S (2014) What is the main driver of ageing in long-lived winter honeybees: antioxidant enzymes, innate immunity, or vitellogenin? BIOLOGICAL SCIENCES Cite Journal as: J Gerontol A Biol Sci Med Sci 69(6):633–639. https://doi.org/ 10.1093/gerona/glt134

Bitondi MMG, Nascimento AM, Cunha AD, Guidugli KR, Nunes FMF, Simões ZLP (2006) Characterization and expression of the Hex 110 gene encoding a glutamine-rich hexamerin in the honey bee, Apis mellifera. Arch Insect Biochem Physiol 63(2):57–72. https://doi.org/10.1002/arch.20142

Bosch JA, Sumabat TM, Hafezi Y, Pellock BJ, Gandhi KD, Hariharan IK (2014) The drosophila F-box protein Fbxl7 binds to the protocadherin fat and regulates Dachs localization and Hippo signaling. Elife 3(2014):1–25. https://doi.org/10.7554/eLife.03383

Brouwers EVM (1983) Activation of the hypopharyngeal glands of honeybees in Winter. J Apic Res 22(3):137–141. https://doi.org/10.1080/00218839. 1983.11100576

Casal J, Struhl G, Lawrence PA (2002) Developmental compartments and planar polarity in drosophila. Current Biology : CB 12(14):1189–1198. https:// doi.org/10.1016/S0960-9822(02)00974-0

Cervoni MS, Cardoso-Junior CAM, Craveiro G, De Souza AO, Alberici LC, 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(21):4035–4046. https:// doi.org/10.1242/jeb.161844

Chertemps T (2017) Molecular basis of pheromone detection in insects. Ref Module Life Sci (2016). https://doi.org/10.1016/b978-0-12-809633-8. 04038-3

Clark HF, Brentrup D, Schneitz K, Bieber A, Goodman C, Noll M (1995) Dachsous encodes a member of the cadherin superfamily that controls imaginal disc morphogenesis in Drosophila. Genes Dev 9(12):1530–1542. https://doi.org/10.1101/GAD.9.12.1530

Cunha AD, Nascimento AM, Guidugli KR, Simo L, Bitondi, rcia M. (2005) Molecular cloning and expression of a hexamerin cDNA from the honey bee, Apis mellifera. J Insect Physiol 51:1135–1147. https://doi.org/10.1016/j.jinsphys.2005.06. 004

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

Döke MA, Frazier M, Grozinger CM (2015) Overwintering honey bees: biology and management. Current Opinion in Insect Science 10:185–193. https:// doi.org/10.1016/J.COIS.2015.05.014

Erban T, Jedelsky PL, Titera D (2013) Two-dimensional proteomic analysis of honeybee, Apis mellifera, winter worker hemolymph. Apidologie 44(4):404–418. https://doi.org/10.1007/s13592-012-0190-5

Florecki MM, Hartfelder K (2012) Unconventional cadherin localization in honey bee gonads revealed through domain-specific Apis mellifera E- and N-cadherin antibodies indicates alternative functions. Insects 3(4):1200–1219. https://doi.org/10. 3390/INSECTS3041200

Fluri P, Wille H, Gerig L, Lüscher M (1977) Juvenile hormone, vitellogenin and haemocyte composition in winter worker honeybees (*Apis mellifera*). Experientia 33:1240–1241. http://link.springer.com/10. 1007/BF01922354. Accessed 25 Feb 2019

Forêt S, Maleszka R (2006) Function and evolution of a gene family encoding odorant binding-like proteins in a social insect, the honey bee (Apis mellifera). Genome Res 16(11):1404. https://doi.org/10.1101/ gr.5075706

Free JB, Spencer-Booth Y (1959) The longevity of worker honey bees (*Apis mellifera*). Proc R Entomol Soc London 34A:141–150. http://doi. wiley.com/10.1111/j.1365-3032.1959.tb00230.x. Accessed 17 Mar 2020

Harman D (1956) Aging: a theory based on free radical and radiation chemistry. J Gerontol 11(3):298–300. https://doi.org/10.1093/geronj/11.3.298

Hartfelder K (2000) Insect juvenile hormone: From "status quo" to high society. Braz J Med Biol Res 33(2):157–177. https://doi.org/10.1590/S0100-879X2000000200003

Hogan J, Valentine M, Cox C, Doyle K, Collier S (2011) Two frizzled planar cell polarity signals in the Drosophila wing are differentially organized by the Fat/Dachsous pathway. PLoS Genetics *7*(2). https://doi. org/10.1371/JOURNAL.PGEN.1001305

Hooper T (2010) Guide to bees and honey, 5th edn.

Hrassnigg N, Crailsheim K (1998) Adaptation of hypopharyngeal gland development to the brood status of honeybee (Apis mellifera L.) colonies. J Insect Physiol 44(10):929–939. https://doi.org/10. 1016/S0022-1910(98)00058-4

Hu H, Bezabih G, Feng M, Wei Q, Zhang X, Wu F, Meng L, Fang Y, Han B, Ma C, Li J (2019) In-depth proteome of the hypopharyngeal glands of honeybee workers reveals highly activated protein

and energy metabolism in priming the secretion of royal jelly. Mol Cell Proteomics 18(4):606–621. https://doi.org/10.1074/mcp.RA118.001257

Huang Z-Y, Otis GW, Teal PEA (1989) Nature of brood signal activating the protein synthesis of hypopharyngeal gland in honey bees, Apis mellifera (Apidae : Hymenoptera). Apidologie 20(6):455–464. https://doi.org/10.1051/apido:19890601

Iovinella I, Cappa F, Cini A, Petrocelli I, Cervo R, Turillazzi S, Dani FR (2018) Antennal protein profile in honeybees: caste and task matter more than age. Front Physiol 9(748). https://doi.org/10.3389/fphys.2018.00748

Iovinella I, Dani FR, Niccolini A, Sagona S, Michelucci E, Gazzano A, Turillazzi S, Felicioli A, Pelosi P (2011) Differential expression of odorant-binding proteins in the mandibular glands of the honey bee according to caste and age. J Proteome Res 10(8):3439–3449. https://doi.org/10.1021/pr2000754

Jianke L, Mao F, Begna D, Yu F, Aijuan Z (2010) Proteome comparison of hypopharyngeal gland development between Italian and royal jelly producing worker honeybees (Apis mellifera L.). J Proteome Res 9(12):6578–6594. https://doi.org/10.1021/pr100768t

Johnson BR (2010) Division of labor in honeybees: form, function, and proximate mechanisms. Behav Ecol Sociobiol 64(3):305–316. https://doi.org/10.1007/s00265-009-0874-7

Jones JC, Oldroyd BP (2017) Nest Thermoregulation in social insects nest thermoregulation in social insects 2806(2006). https://doi.org/10.1016/S0065-2806(06)33003-2

Kahlscheuer ML, Widom J, Walter NG (2015) Single-molecule pull-down FRET to dissect the mechanisms of biomolecular machines. Meth Enzymol 558:539–570

Khalilgharibi N, Mao Y (2021) To form and function: on the role of basement membrane mechanics in tissue development, homeostasis and disease. Open Biol 11. https://royalsocietypublishing.org/doi/abs/10.1098/rsob.200360. Accessed 23 Feb 2022

Knoll S, Pinna W, Varcasia A, Scala A, Cappai MG (2020) The honey bee (Apis mellifera L., 1758) and the seasonal adaptation of productions. Highlights on summer to winter transition and back to summer metabolic activity. A review. Lives Sci 235(2019):104011. https://doi.org/10.1016/j.livsci.2020.104011

Kunc M, Dobeš P, Hurychová J, Vojtek L, Poiani SB, Danihlík J, Havlík J, Titěra D, Hyršl P (2019) The year of the honey bee (Apis mellifera L.) Supplymentaty Materials. Insects 10(8). https://doi.org/10.3390/INSECTS10080244

Lee SH, Kim YH (2017) Comparative proteome analysis of honey bee workers between overwintering and brood-rearing seasons. Journal of Asia-Pacific Entomology 20(3):984–995. https://doi.org/10.1016/J.ASPEN.2017.07.011

Lewis DK, Spurgeon D, Sappington TW, Keeley LL (2002) A hexamerin protein, AgSP-1, is associated with diapause in the boll weevil. J Insect Physiol 48(9):887–901. https://doi.org/10.1016/S0022-1910(02)00158-0

Li J, Feng M, Zhang Z, Pan Y (2008) Identification of the proteome complement of hypopharyngeal glands from two strains of honeybees (Apis mellifera). Apidologie 39(2):199–214. https://doi.org/10.1051/APIDO:2007059

Liu H, Wang ZL, Tian LQ, Qin QH, Wu XB, Yan WY, Zeng ZJ (2014) Transcriptome differences in the hypopharyngeal gland between Western honeybees (Apis mellifera) and Eastern Honeybees (Apis cerana). BMC Genomics 15(1):1–12. https://doi.org/10.1186/1471-2164-15-744/FIGURES/6

Martins JR, Nunes FM, Cristino AS, Simões ZL, Bitondi MM (2010) 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 11(1):1–20. https://doi.org/10.1186/1471-2199-11-23

Matakatsu H, Blair SS (2012) Separating planar cell polarity and hippo pathway activities of the protocadherins Fat and Dachsous. Development 139(8):1498–1508. https://doi.org/10.1242/DEV.070367/-/DC1

Mattila HR, Otis GW (2007) Dwindling pollen resources trigger the transition to broodless populations of long-lived honeybees each autumn. Ecological Entomology 32(5):496–505. https://doi.org/10.1111/j.1365-2311.2007.00904.x

Maurizio A, Hodges FED (1950) The influence of pollen feeding and brood rearing on the length of life and physiological condition of the honeybee preliminary report. Bee World 31(2):9–12. https://doi.org/10.1080/0005772x.1950.11094617

McAfee A, Chapman A, Iovinella I, Gallagher-Kurtzke Y, Collins TF, Higo H, Madilao LL, Pelosi P, Foster LJ (2018) A death pheromone, oleic acid, triggers hygienic behavior in honey bees (Apis mellifera L.). Sci Rep 8(1):1–13. https://doi.org/10.1038/s41598-018-24054-2

Münch D, Amdam GV (2010) The curious case of aging plasticity in honey bees. FEBS Lett 584(12):2496–2503. https://doi.org/10.1016/j.febslet.2010.04.007

Omholt SW (1988) Relationships between worker longevity and the intracolonial population dynamics of the honeybee. J Theor Biol 130(3):275–284. https://doi.org/10.1016/S0022-5193(88)80030-4

Omholt SW, Amdam GV (2004) Epigenetic Regulation of aging in honeybee workers. Sci Aging Knowledge Environ 2004(26):pe28–pe28. https://doi.org/10.1126/sageke.2004.26.pe28

Qi Y, Fan P, Hao Y, Han B, Fang Y, Feng M, Cui Z, Li J (2015) Phosphoproteomic analysis of protein phosphorylation networks in the hypopharyngeal gland of honeybee workers (*Apis mellifera ligustica*). J Proteome Res 14(11):4647–4661. https://doi.org/10.1021/acs.jproteome.5b00530

Ramanathan ANKG, Nair AJ, Sugunan VS (2018) A review on royal jelly proteins and peptides. J Funct Foods 44(2017):255–264. https://doi.org/10.1016/j.jff.2018.03.008

Raudvere U, Kolberg L, Kuzmin I, Arak T, Adler P, Peterson H, Vilo J (2019) g:Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). Nucleic Acids Res 47(W1):W191–W198. https://doi.org/10.1093/NAR/GKZ369

Seehuus SC, Krekling T, Amdam GV (2006) Cellular senescence in honey bee brain is largely independent of chronological age. Exp Gerontol 41(11):1117. https://doi.org/10.1016/J.EXGER.2006.08.004

Seeley TD (1982) Adaptive significance of the age polyethism schedule in honeybee colonies. Behav Ecol Sociobiol 11(4):287–293. https://doi.org/10.1007/BF00299306

Seeley TD, Visscher PK (1985) Survival of honeybees in cold climates: the critical timing of colony growth and reproduction. Ecol Entomol 10:81–88. https://onlinelibrary.wiley.com/doi/full/10.1111/j.1365-2311.1985.tb00537.x. Accessed 6 Mar 2022

Snodgrass RE (1910) The anatomy of the honey bee, 1st edn. Government Printing Office, Washington

Stabentheiner A, Kovac H, Brodschneider R (2010) Honeybee colony thermoregulation - Regulatory mechanisms and contribution of individuals in dependence on age, location and thermal stress. PLoS One 5:8967. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2813292/?report=abstract. Accessed 19 Jan 2021

Stabentheiner A, Pressl H, Papst T, Hrassnigg N, Crailsheim K (2003) Endothermic heat production in honeybee winter clusters. J Exp Biol 206:353–358. https://journals.biologists.com/jeb/article/206/2/353/13914/Endothermic-heat-production-in-honeybee-winter. Accessed 20 Jan 2021

Stegeman R, Hall H, Escobedo SE, Chang HC, Weake VM (2018) Proper splicing contributes to visual function in the aging Drosophila eye. Aging Cell 17:e12817. https://onlinelibrary.wiley.com/doi/full/10.1111/acel.12817. Accessed 25 Feb 2022

Takeichi M (2006) The cadherin superfamily in neuronal connections and interactions. Nat Rev Neurosci 8(1):11–20. https://doi.org/10.1038/nrn2043

Ward R, Coffey MF, Kavanagh K (2022a) Exposure of Apis mellifera to anti-Varroa destructor formic acid treatment induces significant proteomic alterations. 1–9. https://doi.org/10.1080/00218839.2022a.2038055

Ward R, Coffey M, Kavanagh K (2022b) Proteomic analysis of summer and winter Apis mellifera workers shows reduced protein abundance in winter samples. J Insect Physiol 139:104397. https://doi.org/10.1016/J.JINSPHYS.2022.104397

Watmough J, Camazine S (1995) Self-organized thermoregulation of honeybee clusters. J Theor Biol 176:391–402

Wheeler DE, Martinez T (1995) Storage proteins in ants (Hymenoptera:Formicidae). Comp Biochem Physiol B: Biochem Mol Biol 112(1):15–19. https://doi.org/10.1016/0305-0491(95)00035-7

Wilson EO (1971) The insect societies. Belknap Press of Harvard University Press

Yang W, Tian Y, Han M, Miao X (2017) Longevity extension of worker honey bees (*Apis mellifera*) by royal jelly: Optimal dose and active ingredient. PeerJ 2017:e3118