What is the main driver of ageing in long-lived winter honeybees – antioxidant enzymes, innate immunity or vitellogenin?

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Running headline: Ageing in long-lived winter honeybees

Abstract

Senescence or ageing in invertebrates is only partly unscrambled. Up to now five different theories deal with explaining the biology of ageing. Most likely physiology, genetic predestination and the impact of the environment form the image of ageing in individuals and groups. Social insects, especially the honeybee *Apis mellifera*, present the best model system to study developmentally related ageing, because high phenotypic plasticity makes the worker caste useful to dissolve remaining questions. Here, we used long-lived winter honeybee workers and measured transcriptional changes of 14 antioxidative enzymes, immunity and ageing-related (Insulin/insulin-like growth factor signalling-pathway) genes at two time points during hibernation. Additionally, the bees received a bacterial infection to see ageing and infection associated immunity changes. Gene expression levels for each group of target genes revealed that ageing had a much higher impact than the bacterial infections, notably for immunity related genes. Antimicrobial peptide and antioxidative enzyme genes were significantly up-regulated in aged worker honeybees independent of bacterial infections. *Vitellogenin* and *IlP-1*, known ageing markers, were contrary regulated with increasing *vitellogenin* levels during ageing. The increased antioxidative enzyme and antimicrobial peptide gene expression may have a positive and also protective effect during ageing in hibernating worker honeybees.

Introduction

Throughout history, and probably even before, humans try to understand the mystery of ageing. In 1513, Juan Ponce de León depicted the fountain of youth to be in Florida (1) to overcome ageing, though up to now this has never been verified. As a matter of fact the research field eventually took a different track, and recently even social insects took the stage in ageing research joining the classical model systems *Drosophila melanogaster*, *Caenorhabditis elegans* and various rodent model systems (2-5) Indeed social insects are

particularly suited for ageing research: In contrast to other insects, queens of eusocial species show extreme longevity, low levels of extrinsic mortality as adult and increasing fertility with age (6). Evolution of eusociality is associated with a 100-fold lifespan increase of the eusocial insect queens (ant, termite, honeybee, 10.1 ± 6.4 years) compared to the life span of a female in solitary insect species (eight orders, 0.1 ± 0.2 years) (6). However, the within species variance in life span is much more important for understanding processes of ageing. In the honeybee, *Apis mellifera*, workers and queens develop from the same genome, but queen lifespan is about 10 times longer (longevity queen: 3-5 years, summer worker bees: 4-6 weeks, winter worker bees: 22-24 weeks) (7,8). In temperate zones, honeybee workers are classified as either short-lived summer bees or long-lived winter bees (*diutinus*) showing distinct pattern of age-related division of labour (9). In summer the worker caste is associated with rapid senescence (guarding and foraging - collecting water, pollen and nectar) after stages of slow senescence (nursing - brood care). In winter bees, senescence is almost negligible. Flight activity associated with foraging has been claimed to be responsible for the very short lifespan of summer workers also including increased parasite pressure (4,10). The single genotype of the honeybee can produce exceptionally different phenotypes through exposure to environmental variation between but also within castes (phenotypic plasticity).

The winter bees emerge during a restricted period in late summer and autumn and survive the cold season (e.g. November to February in the northern hemisphere) forming the winter cluster without brood rearing (11). Instead of becoming foragers, young workers enter the stress resistant survival form '*diutinus*' stage which is characterized by oxidative stress tolerance and longevity ((12,13) and references therein). Omholt and Amdam (13) showed that individuals' storage protein content is linked to the transition to the long-lived stage. Winter bees are not quiescent or in diapause. Their main activities are heating and thermoregulation, which keeps the colony core between 12-35 °C even when ambient temperatures drop below 0 °C (14,15).

In the last decades several evolutionary theories of ageing ('mutation accumulation', 'antagonistic pleiotropy', 'disposable soma') were used to explain ageing in social insects (reviewed and discussed for the honeybee (16) and ants (3)). However, the principal determinant in the evolution of longevity is the level of extrinsic mortality. None of these theories was designed to address ageing as a facultative trait of sexually non-reproducing worker honeybees. The disposable soma theory of ageing (17) most likely explains ageing in honeybees. More recently, Lee (18) developed the 'social transfer' theory aiming that selection for survival can act on the level of resources embodied by the individual.

Irrespective of the suite of theories on ageing, various basic genetical and physiological changes are known to correlate with ageing. The genetically ageing-related adaptations like telomerase activity seem to be less relevant in social insects and both telomerase activity and telomere lengths showed no age effects in honeybees and insects in general (19). In contrast, somatic tissues of short-lived males had dramatically shorter telomeres than those of the much longer-lived queens and workers in the ant *Lasius niger* which only shows sex-specific telomere dynamics (20).

The 'oxidative stress' theory of ageing (21) which constitutes senescence related loss of function (because of degenerative diseases and ageing) by the progressive and irreversible accrual of molecular oxidative damage might be more relevant for social insects. Long-lived organisms produce less ROS (reactive oxygen species produced by changes in the regulation of mitochondrial genes) or have increased expression of anti-oxidative enzymes. However, longevity in social insects is not directly coupled to higher levels of enzymatic and non-enzymatic antioxidants (22). Increased expression of SOD1 (superoxide dismutase) is not required for an extreme lifespan in ants (23) and SOD1 rather shows sex-specific dynamics as shown for telomeres. Insects possess a suite of antioxidant enzymes and small molecular weight antioxidants that may form a concatenated response to an onslaught of dietary and endogenously produced oxidants (24). The honeybee genome revealed the presence of 38

antioxidant genes (major components of the enzymatic antioxidant system) (25). Nevertheless, even the extreme longevity of the honeybee queen appears to have evolved via mechanisms other than enhanced antioxidant gene expression (26).

Worker bees' age-associated transition from hive (nurse) to more risky foraging bees is linked to a dramatic decline in immunity, the programmed cell-death of hemocytes through nuclear pycnosis (27-29) in correlation to the juvenile hormone (JH) titre profile (28). But not only in honeybees systemic immunity is negatively associated to JH (30). If honeybee workers are experimentally forced to revert from foraging to nurse tasks, they also show reversal of immunosenescence, i.e., a recovery of immunity with age and a rapid recruitment of new hemocytes (28). The same effect can also be observed for recovery-related brain plasticity (olfactory associative learning ability and changes in the proteomic composition of brains) of honeybees when senesced foragers revert to nursing tasks (31). Hence, immunosenescence in honeybee workers is not a simple function of chronological age but it is rather linked to the workers' behavioral role and its function in the colony. Nevertheless, Schmid *et al.* (32) showed in the early life of all adult phenotypes (queens, workers and drones) that the reduction of hemocytes which is age- but not task-dependent, and maybe independent of JH and vitellogenin. Furthermore, they found that the dynamics of polyphenol oxidase (PO)-activity levels have sex- and caste-specific characteristics. Honeybee queen and over-aged nurse bee PO levels continuously increased with age whereas in age right workers PO activity reached a plateau within the first week of adult life. These results might reflect another example of a trade-off between PO and cellular immunity. This is probably a common trait in social insects since also bumblebees show cellular age related immunosenescence (33) and active PO was increased in older workers of the leaf-cutting ant *Acromyrmex octospinosus* (34).

The role of the protein status as major determinant of honeybee lifespan will not be discussed here in detail (reviewed in (16)) except for proteins and hormones (juvenile

hormone, JH) involved in Insulin-Insulin-like growth factor (IGF)-1 signalling (IIS). IIS is a key integrative pathway regulating ageing, fertility and other important biological processes in vertebrates and invertebrates. Down-regulation of IIS is associated with increased longevity and decreased fertility in *C. elegans* and *D. melanogaster* (35). Ageing summer bees showed an increasing titre of JH whereas winter bees kept a constant low level. Only, at the end of winter JH titres rose to high levels, similar to those of one month old summer bees (11,36). The same studies also showed that winter bees had higher levels of total proteins and vitellogenin than hive bees in summer.

Vitellogenin (180-kD monomer; (37)) is the major circulating zinc binding protein of adult bees and zinc has a positive effect on worker hemocyte viability *in vitro* (27). The hypothesized regulatory chain appears as follows: JH → Vitellogenin → Zinc → Immune cell integrity (27). This vitellogenin-hemocyte association was experimentally confirmed using RNA interference (38). Furthermore, vitellogenin has antioxidant functions in workers (38) and is tissue specifically expressed in an age-dependent manner, with old queens showing much higher expression than workers. Since queens are more resistant to oxidative stress than workers caste-specific difference in vitellogenin expression is a key factor in queen longevity (39).

We here aim to characterize immunity and ageing specific gene expression during the slowed process of ageing within a single caste, long-lived winter honeybee workers. Indeed it would be highly adaptive if the immune system of winter bees were highly efficient. They rarely leave the hive for many months and the high density of workers as well as the high nest temperature provides ideal conditions for both transmission and growth of bacterial pathogens. So we test if there can be ageing without compromising the cellular immune system of the winter bees and how this relates to the antioxidant enzymes and vitellogenin.

Material and Methods

Honeybee samples

Sister worker honeybees (*Apis mellifera*) produced by a single queen, from one colony at the University apiary, were sampled at the beginning of winter (November 2010, approximately 6 weeks old) and in the middle (January 2011, approximately 12 weeks old). We used sister worker offspring of a single queen to reduce genetic variance among the tested bees, to prevent genetic variance among the workers to interfere with the physiological variance related to ageing effects. Eggs and brood were absent throughout the sampling period ensuring that the sampled worker winter bees (40) and that workers sampled in January were indeed six weeks older than those sampled in November. Ageing related innate immune reactions were studied in response to a bacterial challenge, to see if ageing affects or interacts with the regulation if the immune system compared to non-infected control workers. The abdomen of 30 bees for each group was pricked with a 0.3 mm sterile needle dipped in bacteria solution of *E. coli* (strain FV755 serogroup O139 Spain) suspended in physiological ringer (OD_{600} = 2.6). Bees from the control group were pricked with physiological ringer (0.9% NaCl) only. Post-treatment bees (infected and non-infected) were kept separately in standard cages for 24 hours, fed *ad libitum* with a mixture of honey and physiological ringer solution, killed by freezing in liquid nitrogen, and stored at -80 °C until further processing. Hence we had four groups of 30 worker bees, a treatment and a control group for each sampling time.

Candidate gene expression

Total RNA was extracted from the whole abdomen of five randomly chosen individuals of each group using 1 mL QIAzol (Qiagen, Hilden, Germany) according to the manufactures protocol. Quality and quantity of the total RNA were photometrically determined with a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). cDNA

and qPCR (quantitative real-time PCR) amplifications were processed using Bioline kits (cDNA synthesis: cDNA Synthesis Kit, qPCR: SensiMix SYBR No-ROX Kit).

Several key genes known to be involved in ageing and the immune response were chosen to determine the interactions between ageing and the immune system - references for primer sequences are given in brackets: 1) Antioxidative enzymes (key components of the antioxidant defence system): *catalase* (41), *glutathione peroxidase* [*Gtpx-1*] (26), *glutathione S-transferase* [*GstD-1*] (26), *CuZn superoxide dismutase 1* [*SOD1*] (41) and two mitochondrial markers – *Mn superoxide dismutase 2* [*mtSOD2*] (26), *thioredoxin peroxidase* [*mtTpx-3*] (26), 2) Innate immunity: *apidaecin 1* (42), *defensin 1* (43), *hymenoptaecin* (43) and the transcription factor of the Imd-pathway *relish* (44); and 3) Insulin/insulin-like growth factor signalling-pathway: *insulin-like peptide* [*IlP-1*] (39), *insulin receptor B* [*InR-1*] (39), *insulin-like receptor-like* [*InR-2*] (39) and *vitellogenin* [*Vg*] (42). *Ribosomal protein S5* [*RPS5*] was chosen in order to standardize expression levels between individuals and groups (43).

We used the same qPCR protocol for all primer pairs. An initial denaturation step of 10 min at 95 °C was followed by 40 amplification cycles (95 °C, 15 sec; 56 °C, 15 sec, an elongation step at 72 °C for 15 sec), and a subsequent melting curve analysis between 56 °C and 95 °C, reading the fluorescence at 1 °C increments. Two technical replicates were run for each sample using the LightCycler® 480 Real-Time PCR System (Roche, Mannheim, Germany).

Protein extraction

In order to confirm the winter bee phenotype, the amount of the storage protein vitellogenin was compared between winter and summer bees. Proteins were extracted from the thorax of three bees from November 2010 as well as January and July 2011. The thorax was homogenized in 400 µL 100 mM Tris/HCl - pH 7.0, 1 mM EDTA. For disruption of the

cell membranes, samples were frozen for 15 min at -20 °C and afterwards heated to 90 °C for 10 min. 6 µL of each sample were mixed with 3 µL Lane Marker Reducing Sample Buffer (Thermo Scientific, Wilmington, DE, USA) and loaded onto an 8% SDS polyacrylamide gel. SDS-PAGE was performed as described (45).

Statistics

LightCycler® 480 Software (Roche, Mannheim, Germany) was used to determine the C_t values after baseline subtraction. Relative target gene expression was estimated according to Pfaffl (46), using *RPS5* (43) as honeybee reference gene and the real PCR efficiency for each target gene (range: 1.79-2.14) determined by serial dilution qPCR.

Data were analysed with regard to normal distribution by Shapiro-Wilk test and log-transformed if they did not match the criteria for normality and homoscedasticity. *t*-test was used for single comparisons of two groups: (1) infected *vs.* control for November and January, (2) January *vs.* November for control and infected bees and (3) pooled data sets to see the effect of ageing (January *vs.* November) and infection (infected *vs.* control) for each single target gene. The general effects of treatment and/or ageing on antioxidative enzyme, innate immune system gene and Insulin/insulin-like growth factor signalling-pathway gene expression was determined using MANOVA. All statistical analyses were done using STATISTICA 8.0 (StatSoft, Tulsa, Oklahoma, USA).

Results

Verification of winter bee phenotype

In addition to the typical colony characteristics (absence of brood), the typical winter bee phenotype in the November 2010 and January 2011 sample was confirmed by the high amounts of the storage protein vitellogenin using SDS polyacrylamide gel electrophoresis; see protein amounts at 180 kDa (Figure 1). All winter bees had the typical high vitellogenin

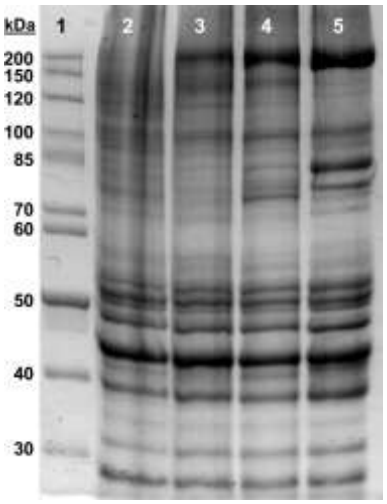

Figure 1. An 8% SDS polyacrylamide gel of honeybee worker homogenate proteins. Equal amounts of 6 µL were loaded in each lane; 1: PageRuler Unstained Protein Ladder (Thermo Scientific, Wilmington, DE, USA), 2: summer bee (forager), 3: summer bee (nurse), 4: November bee, 5: January bee. The monomeric protein between 150 kDa and 200 kDa represents the glycolipoprotein vitellogenin with an expected size of 180 kDa (37).

concentrations, which were less in summer nurses and could not be detected in summer foragers (reviewed in (2,13)). All replicates (data not shown) yielded the same protein pattern, confirming that we were studying physiologically true winter bees.

The effects of age and infection

We compared the effect of age with the bacterial infection on the classical ageing genes with those of the innate immune pathways. In general, ageing had a much higher impact on gene expression levels of antioxidative enzyme, innate immune system genes and Insulin/insulin-like growth factor signalling-pathway genes than the bacterial infections (Table 1). Only the expression levels of the immune genes were significantly affected by the infection ($P < 0.005$), but even there the effect of the age of the bees was significantly stronger ($P < 0.0001$) (Table 1) with elevated transcript abundances in the older bee group. None of the analyzed groups of target genes showed significant interactions between infection and ageing on mRNA levels. The details on the single comparisons of gene expression

Table 1. MANOVA results of testing the impact of infection and ageing on relative antioxidative enzyme, innate immune system and Insulin/insulin-like growth factor signalling-pathway (IlS-pathway) gene expression levels

	Effect	Wilks' λ	*F*	*df*	*P*
Antioxidative enzymes	**Infection**	0.430	2.426	6	0.096
	Ageing	0.066	25.961	6	**< 0.0001**
	Infection × Ageing	0.524	1.673	6	0.217
Innate immunity	**Infection**	0.34	6.316	4	**0.005**
	Ageing	0.123	23.487	4	**< 0.0001**
	Infection × Ageing	0.508	3.148	4	0.051
IIS-pathway	**Infection**	0.53	2.884	4	0.065
	Ageing	0.36	5.796	4	**0.007**
	Infection × Ageing	0.835	0.641	4	0.643

Notes: Significant values are shown in bold letters.

differences for all groups, infected *vs.* control for November and January bees and January *vs.* November for control and infected bees, are summarized in Supplementary Table S1.

Antioxidative enzyme genes

Except for *GstD-1* and *mtSOD2* all transcripts of genes coding for cytological and mitochondrial antioxidative enzymes were significantly more abundant in older winter bees. The transcript abundance in the January workers was up to twice as high as in the November bees, (Figure 2, Supplementary Table S2). The bacterial infection did not significantly affect the transcript abundance in any of the antioxidative enzymes genes (Supplementary Table S1, S2).

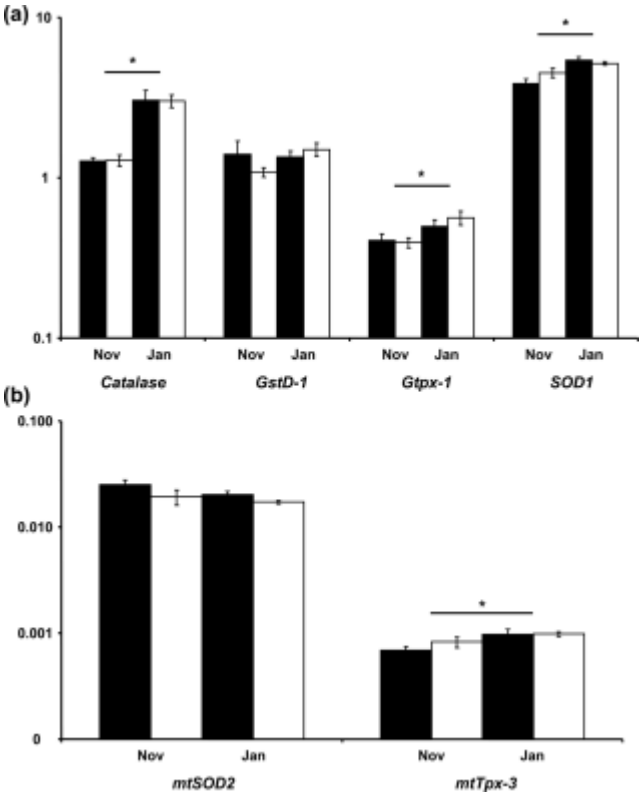

Figure 2. Analyses of mRNA levels in winter worker honeybees. The y axis (log-scaled) indicates the relative levels of mRNA expression for (a) cytological and (b) mitochondrial antioxidative enzymes. Infected worker are indicated by black bars and control bees by white bars. Significant differences between November and January bees on candidate gene expression are marked by an asterisk.

Antimicrobial peptide genes

The antimicrobial peptide genes *apidaecin 1* and *defensin 1* were significantly up-regulated in infected January bees whereas *hymenoptaecin* showed such effects only in November bees (Figure 3, Supplementary Table S1). However all of them revealed much higher (2-3 times) mRNA levels in the older January bees in both the control and the infected bees (Supplementary Table S1, S2). The transcription factor *relish*, usually activated by gram-negative bacterial infections, was significantly up-regulated after infection in November bees (Figure 3). By comparing the November and January bees, the mRNA level was significantly lower in 'older' bees (Supplementary Table S1).

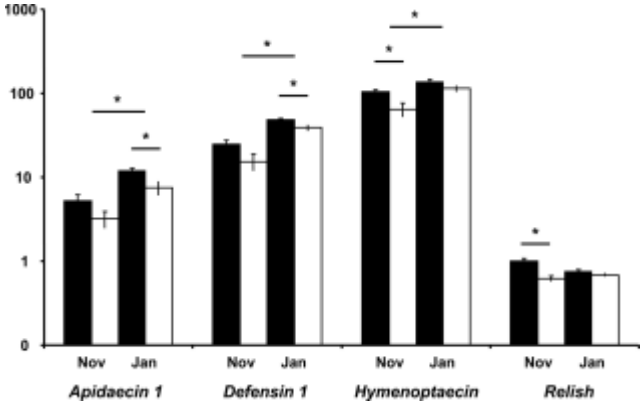

Figure 3. Analyses of mRNA levels in winter worker honeybees. The y axis (log-scaled) indicates the relative levels of mRNA expression for *apidaecin 1*, *defensin 1*, *hymenoptaecin* and *relish*. Infected worker are indicated by black bars and control by white bars. Significant differences between non-infected and infected bees for each month, and the effect of ageing on candidate gene expression in general, are marked by an asterisk.

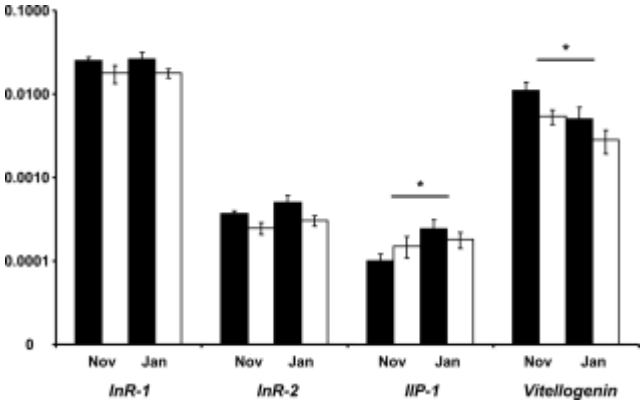

Figure 4. Analyses of mRNA levels in winter worker honeybees. The y axis (log-scaled) indicates the relative levels of mRNA expression for key members of the Insulin/insulin-like growth factor signalling-pathway. Infected worker are indicated by black bars and control bees by white bars. Significant effects of ageing on candidate gene expression are marked by an asterisk.

'Ageing' genes

The different ages had significant impact on mRNA levels of *IlP-1* and *vitellogenin*. *Ilp-1* was up-regulated in the older winter bees whereas *vitellogenin* revealed much lower mRNA levels in the January bees compared to November bees (Figure 4, Supplementary

Table S2). Both putative insulin receptors were not regulated in an age-dependent way. Infection did not affect the expression levels of any of the tested ageing related genes (Figure 4, Supplementary Table S1).

Discussion

The gene expression in all three mechanisms, the antioxidative enzymes, the innate immune system and the insulin/insulin-like growth factor signalling-pathway is significantly affected by ageing in long-lived winter bees. A decrease of antioxidant gene expression was shown in long-lived honeybee and ant queens but not in short-lived workers nor in this study on long-lived worker bees, where expression levels increased with age or did not change (23,26). Also in various cross-species analyses of vertebrates high antioxidant levels and longevity were not necessarily positively correlated (47). In general, gene expression influenced by ageing has to be regarded with caution in honeybees when analyzing ageing between castes or within a specific honeybee caste. Since the 'oxidative stress' theory of ageing (21) cannot explain the system of ageing in winter honeybees (they rarely fly), the elevated levels of antioxidative enzyme genes expression in the old winter bees in this study, might serve to protect over-wintering workers against damages through reactive oxygen species (oxygen ions and peroxides) (ROS). In contrast, results from foraging workers (old summer bees) showed the highest levels of antioxidant gene expression in response to an increase in ROS production that was caused by flight-related increases in metabolism (26). On the other side, Aamodt (48) found distinct down-regulation patterns of genes involved in prevention and repair of oxidative damage to the DNA.

An alternative explanation for this phenomenon can be the cell membrane composition. Cell membranes of queens contain more monounsaturated and less poly-unsaturated fatty acids than those of worker bees and are thus probably more resistant to

oxidative damage, because a lower level of peroxidisable fatty acids in membranes is linked with a longer maximum lifespan (3,49).

Molecular networks seem to play the key role in regulating ageing in insects. Hence, the role of transcription factors will be very prominent. For example, forkhead (FOXO) is known to regulate detoxification/stress responses and immunity genes (50,51) but also down-regulating specific life-shortening genes (51). RNAi directed against FOXO resulted in suppression of both *catalase* and *SOD-2* (52). Antimicrobial peptide activation can be achieved independently of immunoregulatory pathways by nuclear FOXO activity when induced by starvation using insulin signalling (50). In our study control and infected workers bees showed up-regulated AMP levels and AMP gene expression was much more affected by ageing than infection. An up-regulation of FOXO might explain the treatment independent up-regulation of antimicrobial peptide genes in 'older' winter bees. The down-regulation of relish in January bees can be the result of negative feedback loop regulation of the Imd-pathway as suggested from a study on bumblebees (53). Bull *et al.* (54) confirmed our findings that immunocompetence in social insects is not independent of ageing, and older long-lived bees can be less susceptible to parasite infections than younger bees. Furthermore, phenoloxidase activity is positively linked with ageing in honeybees (32). We cannot exclude that immune senescence (55) can occur on other levels of the innate immune system than antimicrobial peptides in honeybees (e.g., cellular immune response, hemocytes). Results on non-social insect immunity and cold temperature stress offer additional models of immunity regulation in winter bees. The activation of the immune pathways in response to cold stress might be a sign for similar cellular responses to cold and infection stress or individuals pre-emptively up-regulate the immune response in the cold season (56).

Fluri *et al.* (36) described two categories of honeybees with high (winter bees) and low (summer bees) proportions of dmL (normal leucocytes with a dense cell membrane) and drastic differences in their JH titre. Years later, Corona *et al.* (39) could show JH effects on

vitellogenin and *IlP-1* expression in opposite directions and they suggested the possibility of a regulatory feedback loop between these elements. Ageing in winter bees also affects *vitellogenin* and *IlP-1* gene expression in our study (Figure 3). The up-regulation of *IlP-1,* the down-regulation of *vitellogenin* and the lack of any ageing-related regulation of the two putative insulin receptors is perfectly in line with previous models (39). As winter bees reveal much higher levels of the storage protein Vitellogenin (13) it is not advantageous to produce much more during hibernation. Vitellogenin might also contribute to the total antioxidative capacity of winter bees (38). Nevertheless, expression profiles of *IlP-1* and its putative receptors are age dependent in queens and workers (39).

Analogous to mammals, longevity of queens and winter bees might also increase associated with caloric restriction (57) or induction of diapause-like states post-reproductively could also induce longevity (35). To complete the picture of the role of ageing in long-lived workers, our results need to be carefully interpreted because the protein and metabolite content can provide more information on the active state of cells or tissues than transcript abundance. Nonetheless this study showed the first time ageing-related transcriptional changes in three groups of genes associated with ageing in long-lived winter honeybees. Additional studies on metabolic changes, DNA methylation (epigenetic control of longevity by imprinting through DNA methylation) and including newly emerged theories of ageing, as the recent spermidine/mitophagy theory (58), will complete the concept of ageing in honeybees and other social insects, but has to be considered separately in ageing within or between several castes.

Funding

This work was supported by the project RoBeeTech (grant POS CCE 206/20.07.2010 SMIS code 618/12460 to L.A. Mărghitaş, D.S. Dezmirean and R.F.A. Moritz).

Acknowledgements

We would like to thank Cristian R. Sisea (USAMV Cluj-Napoca) for providing the LightCycler® 480 Real-Time PCR System, Flore Chirilă (USAMV Cluj-Napoca) for providing the bacteria strain and Helge Schlüns for assistance with lab work.

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Supplementary material

Table S1 Statistical overview of relative antioxidative enzyme, innate immune system and Insulin/insulin-like growth factor signalling-pathway gene expression levels between infected and non-infected honeybee workers of each month, and between January and November bees of each type of treatment (*t*-test, N = 10, significant values are marked bold).

	Infection *vs.* Control						**January *vs.* November**					
	November			**January**			**Control**			**Infection**		
	RGE	***t*-value**	***P***	**RGE**	***t*-value**	***P***	**RGE**	***t*-value**	***P***	**RGE**	***t*-value**	***P***
Catalase	1.02±0.038	0.063	0.952	1.04±0.080	-0.177	0.864	2.42±0.124	-6.946	**<0.001**	2.40±0.168	-4.666	**0.002**
GstD-1	1.32±0.120	0.929	0.380	0.93±0.052	-0.776	0.460	1.41±0.066	-2.724	**0.026**	1.11±0.088	-0.106	0.918
Gtpx-1	1.05±0.052	0.259	0.802	0.92±0.054	-0.868	0.411	1.45±0.074	-2.616	**0.031**	1.27±0.072	-1.411	0.196
SOD1	0.87±0.032	-1.667	0.134	1.05±0.024	0.805	0.444	1.16±0.032	-1.981	0.083	1.42±0.052	-4.152	**0.003**
mtSOD2	1.44±0.108	1.463	0.182	1.18±0.042	1.715	0.125	0.99±0.062	0.640	0.540	0.85±0.056	1.625	0.143
mtTpx-3	0.87±0.052	-1.222	0.256	1.01±0.056	-0.037	0.971	1.25±0.050	-1.410	0.196	1.47±0.096	-2.071	0.072
Apidaecin 1	2.00±0.244	1.614	0.145	1.89±0.190	2.600	**0.032**	2.89±0.348	-2.714	**0.027**	2.61±0.196	-4.982	**0.001**
Defensin 1	2.12±0.298	1.944	0.088	1.28±0.050	2.555	**0.034**	3.37±0.446	-4.979	**0.001**	2.11±0.110	-6.596	**<0.001**
Hymenoptaecin	2.09±0.272	2.884	**0.020**	1.25±0.064	1.556	0.158	2.30±0.308	-3.141	**0.014**	1.34±0.056	-2.656	**0.029**
Relish	1.66±0.070	4.766	**0.001**	1.10±0.034	1.235	0.252	1.13±0.042	-1.222	0.257	0.76±0.026	3.205	**0.013**
InR-1	1.80±0.170	1.515	0.168	1.58±0.160	1.465	0.181	1.26±0.126	-0.015	0.989	1.08±0.102	-0.171	0.869
InR-2	1.71±0.160	2.183	0.061	1.78±0.176	1.952	0.087	1.43±0.150	-1.043	0.328	1.41±0.122	-1.222	0.256
IIP-1	0.91±0.132	-0.958	0.366	1.67±0.274	0.707	0.500	1.65±0.242	-0.638	0.541	3.16±0.578	-2.330	**0.048**
Vitellogenin	2.50±0.368	2.286	0.052	2.56±0.520	0.608	0.560	0.63±0.106	1.982	0.083	0.55±0.096	1.991	0.082

RGE: normalized relative gene expression ratio as mean ± SE (standard error)

Table S2 Statistical overview of relative antioxidative enzyme, innate immune system and Insulin/insulin-like growth factor signalling-pathway gene expression levels between infected and non-infected honeybee workers, and between January and November bees; pooled data sets for each sub-set (*t*-test, N = 20, significant values are marked bold).

	January *vs.* November			**Infection *vs.* Control**		
	RGE	***t*-value**	***P***	**RGE**	***t*-value**	***P***
Catalase	2,41±0,073	-8,276	**<0.001**	1,23±0,089	-0,064	0,950
GstD-1	1,26±0,041	-1,408	0,176	1,12±0,046	0,318	0,754
Gtpx-1	1,36±0,037	-2,927	**0,009**	1,00±0,033	-0,498	0,624
SOD1	1,29±0,024	-4,198	**0,001**	0,97±0,023	-0,582	0,568
mtSOD2	0,93±0,034	1,490	0,154	1,31±0,041	1,966	0,065
mtTpx-3	1,36±0,041	-2,553	**0,020**	0,96±0,036	-0,733	0,473
Apidaecin 1	2,93±0,183	-4,348	**<0.001**	2,32±0,190	1,925	0,070
Defensin 1	2,84±0,201	-6,669	**<0.001**	2,06±0,202	1,480	0,156
Hymenoptaecin	1,88±0,137	-3,395	**0,003**	1,76±0,134	2,276	**0,035**
Relish	0,95±0,029	1,214	0,240	1,37±0,034	3,553	**0,002**
InR-1	1,23±0,082	-0,133	0,896	1,69±0,086	2,224	**0,039**
InR-2	1,51±0,101	-1,368	0,188	1,78±0,101	2,861	**0,010**
IIP-1	2,34±0,210	-2,124	**0,048**	1,36±0,136	-0,208	0,838
Vitellogenin	0,65±0,071	2,699	**0,015**	2,96±0,325	1,391	0,181

RGE: normalized relative gene expression ratio as mean ± SE (standard error)