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RESEARCH ARTICLE

Winter honeybee (*Apis mellifera*) populations show greater potential to induce immune responses than summer populations after immune stimuli

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ABSTRACT

In the temperate climates of central Europe and North America, two distinct honeybee (*Apis mellifera*) populations are found in colonies: short-living summer bees emerge in spring and survive until summer, whereas long-living winter bees emerge in late August and overwinter. Besides the difference in their life spans, each of these populations fulfils a different role in the colonies and individual bees have distinct physiological and immunological adaptations depending on their roles. For instance, winter worker bees have higher vitellogenin levels and larger reserves of nutrients in the fat body than summer bees. The differences between the immune systems of both populations are well described at the constitutive level; however, our knowledge of its inducibility is still very limited. In this study, we focus on the response of 10-day-old honeybee workers to immune challenges triggered *in vivo* by injecting heat-killed bacteria, with particular focus on honeybees that emerge and live under hive conditions. Responses to bacterial injections differed between summer and winter bees. Winter bees exhibited a more intense response, including higher expression of antimicrobial genes and antimicrobial activity, as well as a significant decrease in vitellogenin gene expression and its concentration in the hemolymph. The intense immune response observed in winter honeybees may contribute to our understanding of the relationships between colony fitness and infection with pathogens, as well as its association with successful overwintering.

KEY WORDS: Antimicrobial peptides, Honeybee, Humoral immunity, Immune system, Longevity

INTRODUCTION

The Western honeybee, *Apis mellifera*, is one of the most important pollinators worldwide. Its contribution to the agricultural production of numerous high-value crops is essential. Honeybees also play an important role in many natural ecosystems. Over the past decade, honeybee health has been much emphasized due to negative factors damaging managed colonies (Gallai et al., 2009; Klein et al., 2007), such as pests, pathogens, pesticides, malnutrition and poor beekeeping practices (Dolezal and Toth, 2018; Jacques et al., 2017; Steinhauer et al., 2018; Straub et al., 2019).

One of the main drivers of colony losses are honeybee viruses, such as deformed wing virus, acute bee paralysis virus, slow bee paralysis virus, and others, of which several are vectored by mite parasites, e.g. *Varroa destructor* (Beaurepaire et al., 2020; DeGrandi-Hoffman and Chen, 2015), and typically affect all stages of honeybee development. American and European foulbrood, well-known bacterial diseases of bee brood, also cause serious problems in beekeeping practices worldwide (Ebeling et al., 2016; Forsgren, 2010; Morrissey et al., 2015). Moreover, several other microbial pathogens cause diseases affecting adult honeybees, e.g. microsporidia *Nosema apis* and *Nosema ceranae* colonize the bee mid-gut and cause digestive problems (Fries, 2010; Higes et al., 2013). Other microorganisms found in honeybee hemocoel, such as *Serratia marcescens*, *Spiroplasma* spp., *Crithidia* spp. and *Lotmaria* spp. have been newly recognized as potentially pathogenic to honeybees (Fünfhaus et al., 2018; Schwarz et al., 2015; Stevanovic et al., 2016). Taken together, *V. destructor* parasitism, viral infections, trypanosomatids, nosemosis and bacteriosis have already been correlated to colony losses (Fünfhaus et al., 2018).

The honeybee immune system protects against these pathogens. Social insects rely on two key levels of defense: (1) social immunity, which is based on behavioral cooperation among individuals in the colony, and (2) immunity of individual bees (Evans et al., 2006). Social immunity can manifest in various behavioral reactions, such as removal of dead adults (known as undertaking or necrophoric behavior) and diseased or parasitized brood (hygienic behavior) from the nest by workers, thermoregulation in the nest (Simone-Finstrom et al., 2014) or collecting antimicrobial resins such as propolis (Simone-Finstrom and Spivak, 2010; Simone-Finstrom et al., 2017); individuals groom themselves or other bees, thereby removing foreign particles or mites from the body (auto-grooming and allo-grooming, respectively) (Evans and Spivak, 2010).

The first line of defense in individual immunity consists of physical barriers (e.g. cuticle, peritrophic membrane). If pathogens breach these barriers, a complex network of cellular and humoral immune reactions is triggered as a second line of individual defense (DeGrandi-Hoffman and Chen, 2015; Evans and Spivak, 2010). Hemolymph contains numerous immune cells called hemocytes, which are responsible for defense reactions of cellular immunity, including phagocytosis, nodulation, encapsulation and cytotoxicity (Lavine and Strand, 2002; Negri et al., 2015). Furthermore, immune reactions initiate the synthesis of specific immune proteins with strong antibacterial activity (e.g. antimicrobial peptides) in the fat body and hemocytes. The fat body is an important immunological

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List of symbols and abbreviations

AmPs	antimicrobial peptides
HKG	housekeeping gene
JH	juvenile hormone
JHE	juvenile hormone esterase
JHEH	juvenile hormone epoxide hydrolase
MVApp	multivariate analysis application for streamlined data analysis and curation
PBS	phosphate buffered saline
PTU	phenylthiourea
TFA	trifluoracetic acid
Vg	vitellogenin

tissue present in the bee abdomen that produces antimicrobial peptides (AmPs), lysozymes and other proteins in the hemolymph (Danihlík et al., 2016; Gliński and Jarosz, 2001). To date, several antimicrobial peptides (e.g. apidaecins, abaecin, defensin-1 and hymenoptaecin) have been detected in the honeybee hemolymph when bacteria were present (Danihlík et al., 2016). Distinct age and caste influence the response of the honeybee immune system (Bull et al., 2012; Gätschenberger et al., 2013; Lourenço et al., 2019; Randolt et al., 2008). Drones and workers induce the immune system in different ways to counter bacterial challenge (Laughton et al., 2011).

Two main populations of honeybees emerge during the year in temperate climates. The short-living population of bees emerges from early spring to late summer; these so-called 'summer bees' usually live for 30–40 days and mainly take part in foraging and brood rearing in the nest (Fukuda and Sekiguchi, 1966). The population of winter bees emerges in late summer and early autumn; this population has to survive the coldest months, regulate nest temperature during winter, and initiate brood rearing to produce the new population after winter. Therefore, these bees live markedly longer (~80–160 days) than summer bees (Fukuda and Sekiguchi, 1966; Mattila et al., 2001; Maurizio and Hodges, 1950).

It has been previously found that winter and summer bee populations differ in protein content, mainly in the abundance of the glycolipoprotein vitellogenin, which has anti-oxidant and nutritional functions (Fluri et al., 1982, 1977; Seehuus et al., 2006). Vitellogenin (Vg) is synthesized in the fat body and released into the hemolymph. Honeybee Vg is a 180 kDa protein monomer (Wheeler and Kawooya, 1990) with varying levels in honeybee hemolymph, depending on the developmental stage and caste of the bees (Engels et al., 1990). Newly emerged bees have very low levels of Vg, while Vg levels peak in nurse bees. When nurse bees develop into foragers, Vg levels decline again (Amdam et al., 2003). Higher levels of Vg are also associated with the extended lifespan of the winter bee population and the longevity of honeybee queens, which live for 3–5 years (Corona et al., 2007; Engels et al., 1990).

Vg synthesis is moderated by juvenile hormone (JH) titers. In most insects, JH and Vg are positively correlated and increased JH titer triggers Vg production (Raikhel and Dhadialla, 1992). However, in honeybees, there is an inverse relationship between JH titer and Vg production: increased JH titers cause cessation of Vg production (Amdam et al., 2005; Pinto et al., 2000). Consequently, JH levels are low in nurse bees and higher in newly emerged and foraging bees (Huang and Robinson, 1995; Steinmann et al., 2015). Therefore, JH affects development, maturation and social behavior (Wang et al., 2012). These interactions between JH and Vg regulate important physiological processes such as reproduction, division of labor and longevity (Steinmann et al., 2015; Wang et al., 2012). To modulate the interaction between JH and Vg, JH titer in hemolymph is regulated during synthesis in corpora allata and degradation in hemolymph by specific enzymes (Bomtorin et al., 2014). The main degrading enzymes are juvenile hormone esterase (JHE) and juvenile hormone epoxide hydrolase (JHEH). JHE is primarily involved in the JH degradation pathway in honeybees (Mackert et al., 2010).

When comparing immune reactions between the summer and winter populations, no differences were observed between their immune susceptibility to *Escherichia coli* infection in *in vitro*-reared bees (Gätschenberger et al., 2013). Steinmann et al. (2015) focused on the impact of deformed wing virus infection on the immune response and physiological activity of summer and winter workers living in the hive. Results indicated that reduced cellular immunity, physiological activity and pronounced gene expression of antibacterial factors are characteristic features of the winter population. The present study investigated the immune response of 10-day-old nursing worker bees living in the hive because they represent a developmental stage characterized by the pronounced in-hive role and the main link between brood and foragers directly exposed to environmental conditions. Our research focused on short-living summer bees and long-living winter bees and their simultaneous physiological and immunological responses to experimental challenges, emphasizing microbial infection. Specifically, we compared the expression of AmP genes, the antimicrobial activity of hemolymph, the concentration of apidaecin 1, hemocyte counts, and total lipid, carbohydrate and protein concentrations in the hemolymph of these two populations of adult workers.

Our data show that the winter population of bees had a stronger immune response, especially the humoral part, than the short-lived summer bees. Immune stimulation led to an increase in antimicrobial activity in the hemolymph, with a significantly higher response observed in winter bees. This research demonstrates that the winter bee population induces a stronger immune response after bacterial infection and provides evidence that immune activation could be a serious factor influencing longevity-related molecules such as Vg.

MATERIALS AND METHODS

Experimental bees

Honeybees (*Apis mellifera* Linnaeus 1758) were obtained from three colonies kept at the Kývalka apiary near Brno, Czech Republic (49.1886747°N, 16.4513211°E). Experimental colonies were managed by a professional beekeeper according to usual beekeeping practices, with a flumethrin treatment for *V. destructor* in August. Colonies were fed every autumn with approximately 15 kg of sucrose solution (3:2 w/v sucrose:water); no other supplements were added. Samples for analyses were collected from the selected colonies that did not show any clinical symptoms of diseases or *V. destructor* infestation. The first bee sampling was performed in 2017 and was repeated in 2018. In total, four series of independent samplings were performed during two beekeeping seasons. Bees from the summer population were collected in June; this month is considered as the peak of the beekeeping season. Winter bee population was sampled in late August when queens usually stop laying eggs after the summer season and newly emerged bees are supposed to survive until the next spring.

Bees of the same age were prepared for the experiment as follows: three selected frames (39×24 cm) containing a capped brood (1–3 days before emergence) were placed in frame cages and kept in the three source hives until the bees emerged (Williams et al., 2015). Newly emerged bees were gently brushed from isolated frames to a swarm box and marked with a color on their thorax. They

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were then returned to their respective source colonies. After 10 days of life in hives, marked bees were recollected from all three colonies, pooled and transferred to the laboratory for immunization. Bees were transported in a swarm box and the transport from the apiary to the laboratory took approximately 20 min. Bees were processed within 3 h of delivery to the laboratory.

Bee treatment

Collected and pooled bees from the three colonies were randomly divided into three groups and transferred into laboratory cages. Before treatment, bees were narcotized for approximately 2 min by gaseous carbon dioxide. The control group of bees was narcotized without any further treatment ('control'), whereas the injection control group ('PBS') was injected with 5 µl of sterile phosphate buffered saline (Dulbecco's PBS; Sigma-Aldrich, USA) using a 5 µl Hamilton microliter syringe. In the third group ('bacteria'), the bees were immunized with 5 µl of a bacterial mixture containing 10^5 heat-killed bacterial cells of both gram-negative *E. coli* (CCM 3954; Czech Collection of Microorganisms, Brno, Czech Republic) and gram-positive *Paenibacillus larvae* (a field isolate, Czech Republic, 2016) in 0.01 mol l^{-1} PBS (pH 7.0) and at a ratio of 1:1. PBS or bacterial mixture was injected into the dorsal part of the bee abdomen. After injection, bees were kept in plastic cages at 30°C for 24 h and fed *ad libitum* with 50% sucrose solution; the bees were then processed for further analyses. Each experimental group contained three to four replicates, consisting of one cage with 100 individual bees.

Hemolymph collection

Hemolymph samples were collected from bee thoraces to avoid contamination from the digestive tract. Bee abdomens were cut off and pure hemolymph drops were obtained by gently pressing the thorax (Kunc et al., 2019). Two microliters of the hemolymph per individual bee were immediately collected with an automatic pipette. The hemolymph samples used for the determination of total carbohydrates and lipid concentration, vitellogenin and antimicrobial activity were diluted 1.25× with phenylthiourea solution (PTU, 1 mg ml^{-1} in PBS) to prevent coagulation and melanization. The hemolymph samples used for apidaecin quantification were diluted 10× with 0.1% trifluoracetic acid (TFA). Fresh hemolymph samples (without PTU) were kept on ice to prevent melanization and were used immediately after collection to determine hemocyte and total protein concentrations.

Determination of total proteins, lipids and carbohydrates in the bee hemolymph

The concentration of total proteins in the hemolymph was measured according to the Lowry method using a commercial kit (DC Protein Assay kit, Bio-Rad, USA). Hemolymph samples from five bees were pooled into one sample in which the total protein concentration was determined. Five mixed hemolymph samples were measured for each group. Bovine serum albumin (Sigma-Aldrich) was used as a standard for the calibration curve. The absorbance of the samples was measured with a Multiscan GO spectrophotometer (Thermo Fisher Scientific, USA) at 700 nm.

The concentration of total lipids was determined by the sulpho-phospho-vanillin method according to Zöllner and Kirsch (1962) with modifications by Kodrik et al. (2000). One microliter of pooled hemolymph sample prepared from five bees was used for the analysis; at least three samples were measured within each group. Oleic acid (Penta, Prague, Czech Republic) was used as a standard for a calibration curve. Absorbance was measured at 546 nm with a Sunrise spectrophotometer (Tecan, Männedorf, Switzerland).

The concentration of total carbohydrates was determined by the anthrone method (Carroll et al., 1956). Specifically, 50 µl of 200× diluted samples of pooled hemolymph were used per reaction. Absorbance was measured at 620 nm with a Sense spectrophotometer (Hidex, Turku, Finland); at least three samples were measured within each group. The concentration of total carbohydrates was calculated according to a calibration curve prepared from glucose used as a standard (Sigma-Aldrich).

Vitellogenin levels in hemolymph were determined as described by Kunc et al. (2019). Briefly, denaturing SDS polyacrylamide gel electrophoresis was used according to Gätschenberger et al. (2012) with some modifications using the Miniprotean II apparatus (Bio-Rad). The hemolymph samples were diluted 50× in 0.125 mol l^{-1} Tris buffer (pH 6.8) and 10 µl of it was mixed with 10 µl of sample buffer (0.06 mol l^{-1} Tris-HCl, pH 6.8; 10% glycerol; 2% SDS; 0.1% Bromophenol Blue), 7 µl of β-mercaptoethanol (Sigma-Aldrich) and 73 µl of 0.125 mol l^{-1} Tris buffer (pH 6.8). After 5 min incubation at 90°C, the samples were loaded to 2.5% focusing gel, which was followed by 7.5% separation polyacrylamide gel. After separation, the gels were transferred to Coomassie Blue staining solution (1.2 mmol l^{-1} Coomassie Blue R250, Bio-Rad) and subsequently destained in a solution of 2 mmol l^{-1} acetic acid in 45% methanol until the protein bands were clearly visible. Gels were scanned and the density of Vg bands was evaluated in ImageJ version 1.52a software (National Institutes of Health, USA). The total Vg concentration was estimated according to a calibration curve prepared using bands of bovine serum albumin (66.5 kDa; Sigma-Aldrich) present in each gel.

Quantification of hemocytes in the hemolymph

To determine hemocyte counts, 10 µl of pooled hemolymph sample prepared from five bees was mixed with 2.5 µl of PTU in PBS (1 mg ml^{-1}). The total number of hemocytes was counted with a hemocytometer under a CX31 light microscope (Olympus, Japan) using phase contrast. The results were expressed as the number of hemocytes per microliter of hemolymph.

Determination of antimicrobial activity

The antimicrobial activity of hemolymph was determined by a radial diffusion assay. Antimicrobial activity of samples was tested against the gram-positive bacterium *Micrococcus luteus* (CCM 169). Bacterial suspensions were cultured overnight in LB liquid medium (MO BIO, Czech Republic) on a rotary shaker (100 rpm, 25°C). After cultivation, the bacterial suspension was diluted to an OD_{600} (optical density at 600 nm) value of 1.5 and mixed in the ratio 1:500 (v/v) with melted 4% LB agar (MO BIO) previously cooled below 50°C, and poured onto Petri dishes. After agar solidification, wells of 2 mm diameter were cut out in each plate. Into the wells, 5 µl of hemolymph samples and calibration prepared from a serial dilution of lysozyme (EC 3.2.1.17; Sigma-Aldrich) were loaded. After incubation at 30°C for 24 h, the diameter of inhibition zones around the wells was measured and the antimicrobial activity of samples was expressed as the concentration of lysozyme with equivalent bacterial growth-inhibiting activity.

Quantification of relative gene expression

To quantify relative gene expression, pooled samples of 10 bee abdomens were used for RNA extraction. The abdomens were placed in plastic mesh bags (Bioreba, Reinach, Switzerland), 2 ml of guanidinium isothiocyanate (GITC) lysis buffer (Evans et al., 2015) containing 1% β-mercaptoethanol (Sigma-Aldrich) was added and samples were homogenized using a pestle. Total RNA was extracted

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from 300 µl of abdomen homogenates in GITC buffer using the NucleoSpin RNA Plus kit according to the manufacturer's instructions (Macherey-Nagel, Düren, Germany). RNA was eluted in 60 µl of RNase-free water, then the concentration and quality of RNA samples were quantified by a Synergy HT microplate reader (BioTek, Bad Friedrichshall, Germany). RNA integrity was confirmed by electrophoresis in 1.1% (w/v) agarose gel stained by GelRed (Biotium, Fremont, CA, USA). Reverse transcription was performed with a Transcriptor High Fidelity kit (Roche, Basel, Switzerland) in 20 µl reaction volumes according to the manufacturer's protocol. The expected PCR product size was tested by gel electrophoresis in 3% (w/v) agarose gel with detection by GelRed and with 50–1000 bp PCR Marker (Promega, Madison, WI, USA). Relative gene expression was determined by the SYBR Green detection method (SYBR Select Master Mix, Applied Biosystems, Beverly, MA, USA) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Product quality and contamination were tested by measuring the melting curves of PCR products (60–95°C, using increments of 0.5°C for 0.1 min). The relative expression of genes of interest to housekeeping genes (actin, EF-1 alpha) was calculated according to Pfaffl (2001). For primer sequences, see Table S1.

Quantification of apidaecin 1 by LC-MS analysis

Hemolymph samples were collected from experimental bees (2 µl of hemolymph per bee; 10 bees in one sample) and diluted in 200 µl of 0.1% (v/v) TFA. Samples were lyophilized and then dissolved in 5% formic acid prior to UHPLC-MS analysis. Identification and quantification of apidaecin 1 were performed on a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific) coupled with a Compact qTOF mass spectrometer (Bruker Daltonics, Bremen, Germany) with electrospray ionization (ESI). The system was controlled by OTOF CONTROL, data were processed using DataAnalysis version 4.4, and apidaecin 1 was quantified using TASQ version 1.4 (Bruker Daltonics). Apidaecin 1 was quantified using an isotopically [$^{13}C_6^{15}N_4$] labeled internal standard of apidaecin 1A (purity >98%; Clonestar Peptide Services, Brno, Czech Republic) according to Danihlík et al. (2014) with some modifications. UHPLC separation was performed on Kinetex 1.7 µm EVO C_{18}, 100 Å, 150×2.1 mm, C_{18} pre-column was connected to the analytical column with oven temperature 40°C. The mobile phases were (A) 0.5% formic acid in water and (B) 0.5% formic acid in acetonitrile. The separation was performed at 0.2 ml min^{-1} flow with gradient: 0–3 min 2% B, 3–12 min 2–45% B, 12–14 min 45–95% B, 14–17 min 95% B, 17–18 min 95–2% B, 19–20 min 2% B. The mass data were collected under this set-up: mass range 100–2800 Da, spectra rate 4.0 Hz, end plate offset 500 V, capillary 4500 V, nebulizer 2.5 bar, dry gas 6.0 l min^{-1}, dry temperature 200°C, HPC internal calibration with sodium formate.

Statistical analysis

Plots were drawn and analyses were performed using OriginPro 2019 software (OriginLab Corporation, Northampton, MA, USA). Data were tested for normality by the Kolmogorov–Smirnov test. A logarithmic transformation of relative gene expression data was applied before the two-way ANOVA and multivariate analysis application for streamlined data analysis and curation (MVApp) statistical analysis. Two-way ANOVA was used to test the significance of differences among the experimental groups for summer and winter populations, and immune challenge (control, PBS, bacteria). Tests were performed at a α=0.05 significance level with Bonferroni *post hoc* test.

MVApp was used to calculate and visualize Pearson correlation coefficients between transcript abundance of genes related to bee life longevity markers (Vg, JHE) and antimicrobial peptides (Julkowska et al., 2019).

RESULTS

Survival rate

Approximately 1800 freshly emerged bees were marked for each experimental batch of summer or winter bee populations and returned to their respective colonies to continue with their natural development. Approximately 60% of marked bees were found in the colonies after 10 days of performing in-hive duties; they were collected and transported to the laboratory. The mean mortality rate 24 h after immunization, during which the bees were maintained in plastic cages, was lowest in the control group (1.5%), whereas the PBS and bacteria groups showed mortality rates of 24 and 18%, respectively. Therefore, mortality after aseptic injury in the PBS groups was comparable to that in bees injected aseptically with heat-killed bacteria.

Immune challenge has a negligible effect on the concentration of nutrients in the hemolymph

Biochemical characterization of the hemolymph was performed by analysing total protein, lipid and carbohydrate concentrations (Fig. 1). The average values of carbohydrates and lipids were 59.3±25.5 and 2.0±1.1 µg µl^{-1}, respectively. We did not observe any significant

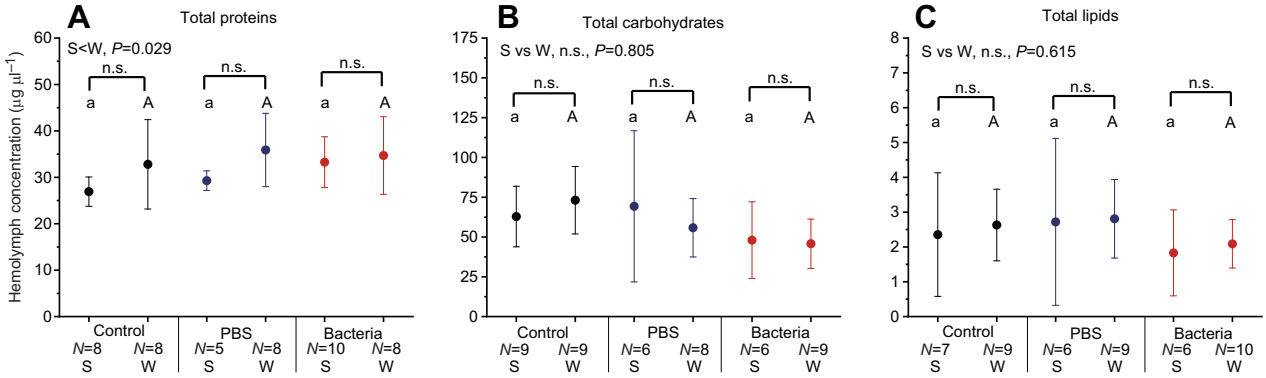

Fig. 1. Effect of immune challenge on nutrients in the honeybee hemolymph. Concentrations of (A) total proteins, (B) carbohydrates and (C) lipids in honeybee hemolymph. The dots represent the means, with ranges representing standard deviations. The number of samples is noted as *N*. Significant differences (P<0.05) among the treatment groups are indicated by different lowercase and uppercase letters above the means of the summer and winter populations, respectively. Clamps denote statistically significant differences between summer and winter populations. Differences in the population levels are noted in the top left corner of each graph. n.s., not significant; S, summer population; W, winter population.

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differences in the concentrations of these nutrients between summer and winter bee populations. However, the winter population displayed higher total protein concentration in the hemolymph than the summer population: 34.47±8.38 and 30.20±4.99 µg µl^{-1}, respectively (two-way ANOVA, d.f.=1, P=0.029, F=5.13; Table S2). The immune challenge had no significant effect on concentrations of proteins, lipids and carbohydrates (two-way ANOVA, d.f.=2, P=0.224, P=0.283, P=0.059, F=1.55, 1.3, 3.04; Table S2) (Table 1).

Immunostimulation results in vitellogenin and JHE downregulation in winter and summer bee populations

We analysed the specific effects of experimental treatments on *Vg* gene expression and Vg level. Generally, the relative gene expression of *Vg* was significantly higher in winter bees than in summer bees (two-way ANOVA, d.f.=1, P=4.82×10^{-4}, F=13.0; Table S2) (Fig. 2A). When focusing on the response of winter and summer populations together, the reaction to PBS injection or heat-killed bacteria experimental treatments decreased *Vg* gene expression (two-way ANOVA, d.f.=2, P=4.35×10^{-13}, F=38.6; Table S2) (Fig. 2A). There was no significant interaction between bee populations and experimental challenges (Table 1).

Quantification of Vg protein (Fig. 2B) confirmed higher Vg concentrations in winter bee populations than summer populations, i.e. 12.52±8.00 and 3.49±1.25 µg µl^{-1}, respectively (two-way ANOVA, d.f.=1, P=1.085×10^{-6}F=37.7; Table S2). Vg levels were influenced by immunostimulation; we specifically observed that Vg concentrations decreased in winter bees after immune challenge (two-way ANOVA, d.f.=2, P=0.012, F=5.22 for immune challenge; two-way ANOVA, d.f.=2, P=0.004, F=6.79 for interaction; Table S2) (Table 1).

To determine the differences in Vg concentration levels, all groups were normalized to the control group of the summer bee population (Fig. 2C). The control group of the winter population had a significantly higher Vg concentration (5.00×) than the others. There was a decline in Vg concentration in Bacteria group (2.46× for winter population) compared with the control group, indicating that the Vg concentration in winter bees decreased drastically after bacterial infection. Immunization had no observable effect on the summer population.

Both populations in the experimental groups reacted similarly at the level of *JHE* gene expression (two-way ANOVA, d.f.=1, P=0.883, F=0.022; Table S2). Immunostimulation with bacteria yielded a lower *JHE* gene expression than the control in both populations (two-way ANOVA, d.f.=2, P=1.479×10^{-9}, F=25.2; Table S2). No interaction between bee populations and experimental treatment was recorded (two-way ANOVA, d.f.=2, P=0.557, F=0.588; Table S2) (Fig. 3, Table 1).

Winter bee population is characterized by higher antimicrobial activity of the hemolymph and its strong inducibility by an immune challenge

The total hemocyte count was the same in both populations (two-way ANOVA, d.f.=1, P=0.586, F=0.303) (Fig. 4A). Moreover, we did not detect any significant differences in hemocyte count among treatments in both winter and summer bee populations (two-way ANOVA, d.f.=2, P=0.074, F=2.8) (Table 1).

The measurement of antimicrobial activity showed significant differences between summer and winter bee populations (two-way ANOVA, d.f.=1, P=5.659×10^{-13}, F=93.9; Table S2) (Fig. 4B). Winter bees were characterized by their hemolymph having higher antimicrobial activity than that of the summer bees. The antimicrobial activity of winter bees increased in the PBS and bacteria groups compared with the control (two-way ANOVA, d.f.=2, P=4.082×10^{-10}, F=34.7; Table S2) (Fig. 4B, Table 1). Specifically, the bacterial treatment increased the antimicrobial activity by a factor of 1.8 (Fig. 4C).

Apidaecin 1 concentration increased after immunostimulation regardless of the bee population

Antimicrobial peptide apidaecin 1 concentration in bee hemolymph samples was quantified by the highly sensitive LC-MS method (Danihlík et al., 2014). No significant differences between the winter and summer bee populations were detected (two-way ANOVA, d.f.=1, P=0.101, F=2.83; Table S2) (Fig. 5). PBS or bacteria injection induced significantly higher levels of this antimicrobial peptide in the hemolymph compared with the control. Peptide concentrations in the hemolymph were, on average, 2.70±1.84, 17.54±6.55 and 21.88±6.23 ng µl^{-1} in the control, PBS and bacteria groups, respectively (two-way ANOVA, d.f.=2, P=7.515×10^{-11}, F=47.7; Table S2). The interaction between the bee population and experimental treatment was not significant (two-way ANOVA, d.f.=2, P=0.924, F=0.079; Table S2) (Table 1).

Summer and winter bees exhibited differences in *AmP* gene expression intensity after immunostimulation

All studied *AmP* genes, including *apidaecin*, *abaecin*, *hymenoptaec*in, *defensin-1* and *defensin-2*, displayed upregulated gene expression after injury or immunostimulation in both summer and winter populations. There was a significantly different response at the population level in gene expression of *apidaecin* and *defensin-1*: *apidaecin* exhibited lower expression in winter bees (two-way ANOVA, d.f.=1, P=0.004, F=8.766; Table S2) whereas *defensin-1* exhibited higher expression in the winter population (two-way ANOVA, d.f.=1, P=4.167×10^{-4}, F=13.3; Table S2) (Fig. 6A and I, respectively).

Table 1. Overview of the significant differences in all measured parameters at the levels of the bee population (summer versus winter) and experimental groups (control, PBS, bacteria), and the interaction between them

		Differences between bee populations (summer versus winter)	
		Significant	Non-significant
Differences among experimental groups (control, PBS, bacteria)	Significant	*Vitellogenin* (gene expression) Vitellogenin (concentration)* Antimicrobial activity* *Apidaecin* (gene expression)* *Defensin-1* (gene expression)	Apidaecin (concentration) *Abaecin* (gene expression)* *Hymenoptaecin* (gene expression)* *Defensin-2* (gene expression)* *JHE* (gene expression)
	Non-significant	Total proteins	Total carbohydrates Total lipids Hemocyte count

Significance was determined by two-way ANOVA; detailed results are in Table S2. *Interaction.

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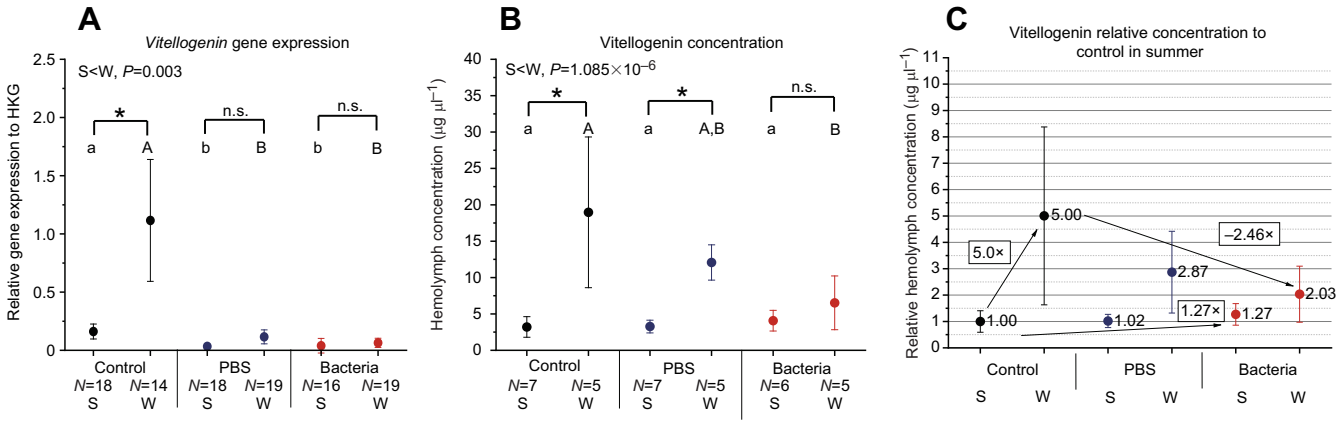

Fig. 2. *Vitellogenin* gene expression and protein concentration is modulated differently in summer and winter populations after immune stimuli. (A) Gene expression of *Vg* normalized to housekeeping genes (HKG), (B) concentration of Vg in hemolymph and (C) concentration of Vg normalized to the control group in summer. The dots are means with ranges representing 95% confidence intervals (A,C) or standard deviations (B). The ratios between groups are illustrated by arrows and values (C). The number of samples is noted as *N*. Significant differences ($P<0.05$) among the treatment groups are indicated by different lowercase and uppercase letters above the means of the summer and winter populations, respectively. Clamps display statistical differences between summer and winter populations; asterisks represent a significant difference (*$P<0.05$). Differences in the population levels are noted in the top left corner of each graph. n.s., not significant; S, summer population; W, winter population.

To further analyse these results, the gene expression ratios were normalized to the control group of the summer population (Fig. 6, right-hand panels). The expressions of *apidaecin* and *abaecin* were significantly lower in the winter bee than summer bee control populations. Only the gene expression ratio of *hymenoptaecin* was higher in the control of winter bees. The results compare the strength of the AmP-mediated humoral response between summer and winter bee populations. *Apidaecin* and *abaecin* gene expressions increased only slightly after immunostimulation (summer population: 5.21× for *apidaecin* and 3.99× for *abaecin*; winter population: 10.14× for *apidaecin* and 13.98× for *abaecin*). However, *hymenoptaecin*, *defensin-2* and *defensin-1* were strongly upregulated after injection with heat-killed bacteria (summer population: 35.07×, 90.9× and 15.81×; winter population: 27.10×, 399.78× and 34.3×, respectively). Interestingly, the upregulation of gene expression was the strongest for the *defensin-2* gene, i.e. 90.9× for the summer and 399.78× for the winter population. The interactions between the bee populations and experimental treatments were significant for all AmP genes except *defensin-1* (Table 1; Table S2).

Pairwise correlation of the monitored genes

Correlation analysis of gene expression levels in summer and winter bee populations showed a positive correlation among all studied AmPs, with an especially strong positive correlation for all *AmP* genes in the winter bees (Fig. 7). In the summer population, *hymenoptaecin–defensin-1*, *hymenoptaecin–defensin-2* and *defensin-1–defensin-2* had the strongest positive correlations. Interestingly, *Vg* expression was negatively correlated with all tested genes except *JHE*. There was a strong positive correlation between *JHE* and *Vg* in the winter population. Similarly, *JHE* expression was negatively correlated with those of all *AmP* genes in the winter bee population. *JHE* gene expression in summer bees was not significantly correlated with *AmP* genes or *Vg*.

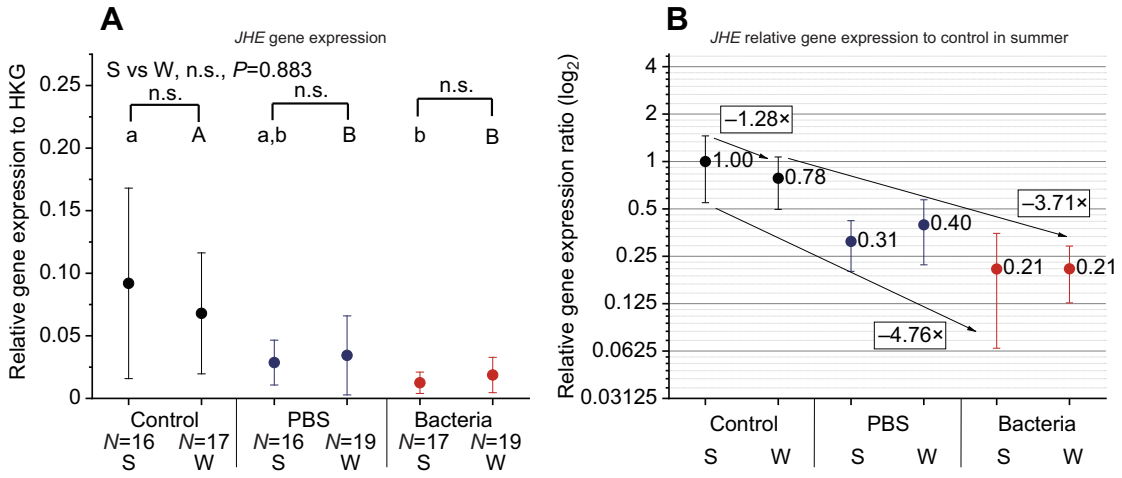

Fig. 3. Immune stimuli decreases gene expression of *JHE* in both summer and winter populations. (A) Gene expression of *JHE* normalized to HKG and (B) relative gene expression normalized to the control group in summer. The dots are means, with ranges representing 95% confidence intervals. The ratios between groups are illustrated by arrows and values (B). The number of samples is noted as *N*. Significant differences ($P<0.05$) among the treatment groups are indicated by different lowercase and uppercase letters above the means of the summer and winter population, respectively. Clamps display statistical differences between summer and winter populations. Differences in the population levels are noted in the top left corner of each graph. n.s., not significant; S, summer population; W, winter population.

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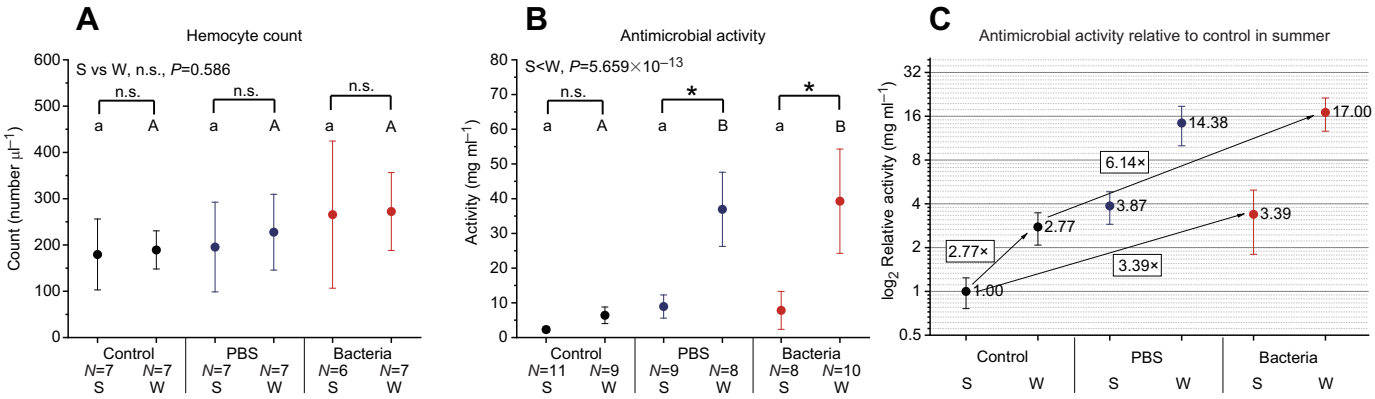

Fig. 4. The effect of immune stimuli on hemocyte count and antimicrobial activity of honeybee hemolymph. (A) Hemocyte count in the hemolymph, (B) total antimicrobial activity of hemolymph against gram-positive bacteria *M. luteus* in the hemolymph and (C) relative antimicrobial activity normalized to the control group in summer. The dots are means with ranges representing standard deviations (A,B) or 95% confidence intervals (C). The ratios between groups are illustrated by arrows and values (C). The number of samples is noted as *N*. Significant differences ($P<0.05$) among the treatment groups are indicated by different lowercase and uppercase letters above the means of the summer and winter population, respectively. Clamps display statistical differences between summer and winter populations; asterisks represent a significant difference (*$P<0.05$). Differences in the population levels are noted in the top left corner of each graph. n.s., not significant; S, summer population; W, winter population.

DISCUSSION

Two populations of honeybees with different life spans and roles in the colony emerge during the year: a summer and a winter population (Fluri et al., 1982, 1977; Fukuda and Sekiguchi, 1966; Mattila et al., 2001). Long-living winter bees have been previously characterized by higher concentrations of proteins (Fluri et al., 1982; Kunc et al., 2019; Kunert and Crailsheim, 1988). In general, the present study confirmed that winter bees had a significantly higher concentration of total proteins in the hemolymph than summer bees (Fig. 1A). The carbohydrate and lipid levels in the bee hemolymph were previously described to be stable throughout the year, without any changes correlated to seasonal development (Kunert and Crailsheim, 1988). Here, we sampled 10-day-old bees that spent their lives under typical hive conditions with sufficient food at two time points. Based on our results, we did not observe malnutrition, which could influence the basic biochemical parameters, occurring prior to or during the sampling periods of June and August.

In agreement with previous studies (Aurori et al., 2014; Corona et al., 2007; Fluri et al., 1982; Kunc et al., 2019), we detected low levels of Vg in 10-day-old bees from the summer population and higher Vg concentrations in winter bees collected in late August (Fig. 2B). Similarly, a higher concentration of total hemolymph proteins was detected (Fig. 1A) in bees collected in late August. Therefore, we concluded that bees collected in late August belonged to the winter population.

Vg concentration was not influenced by immunostimulation with *S. marcescens* after 5 h (Lourenço et al., 2019). Similarly to Lourenço et al. (2019), we did not detect changes in Vg concentration in the summer bee population after immunostimulation, but observed a significant decrease in Vg concentration in winter.

Vg expression in summer and winter bees 24 h after the injection (Fig. 2A) decreased in both populations. Our experiments support the findings of Goblirsch et al. (2013), who observed the same trend in bees orally administered with *N. ceranae*, or Lourenço et al.

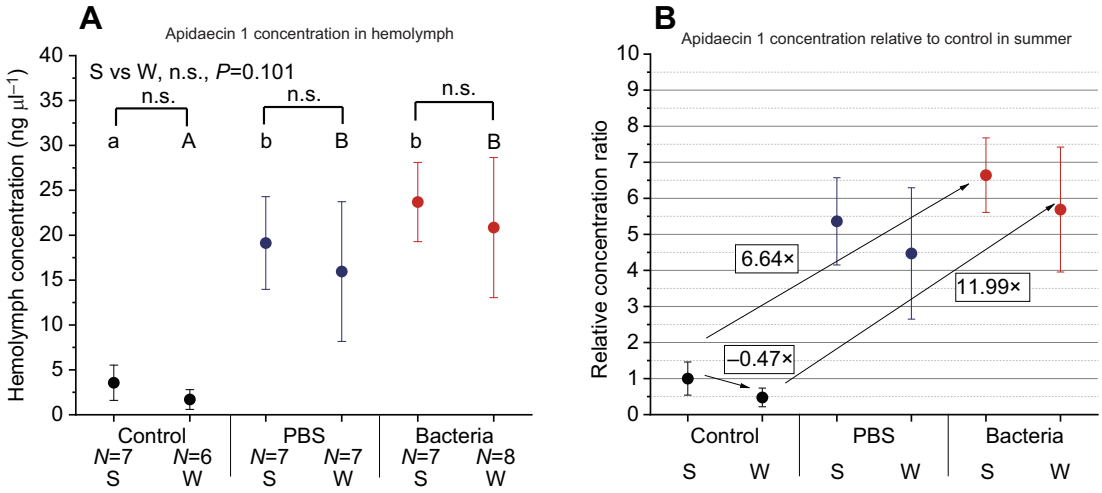

Fig. 5. Changes of apidaecin 1 in summer and winter honey bee populations after immune stimuli. (A) Concentration of apidaecin 1 in the honeybee hemolymph and (B) relative apidaecin 1 concentration normalized to the control group in summer. The dots are means with ranges representing standard deviations. The number of samples is noted as *N*. Significant differences ($P<0.05$) among the treatment groups are indicated by different lowercase and uppercase letters above the means of summer and winter population, respectively. Clamps display statistical differences between summer and winter populations. Differences in the population levels are noted in the top left corner of each graph. n.s., not significant; S, summer population; W, winter population.

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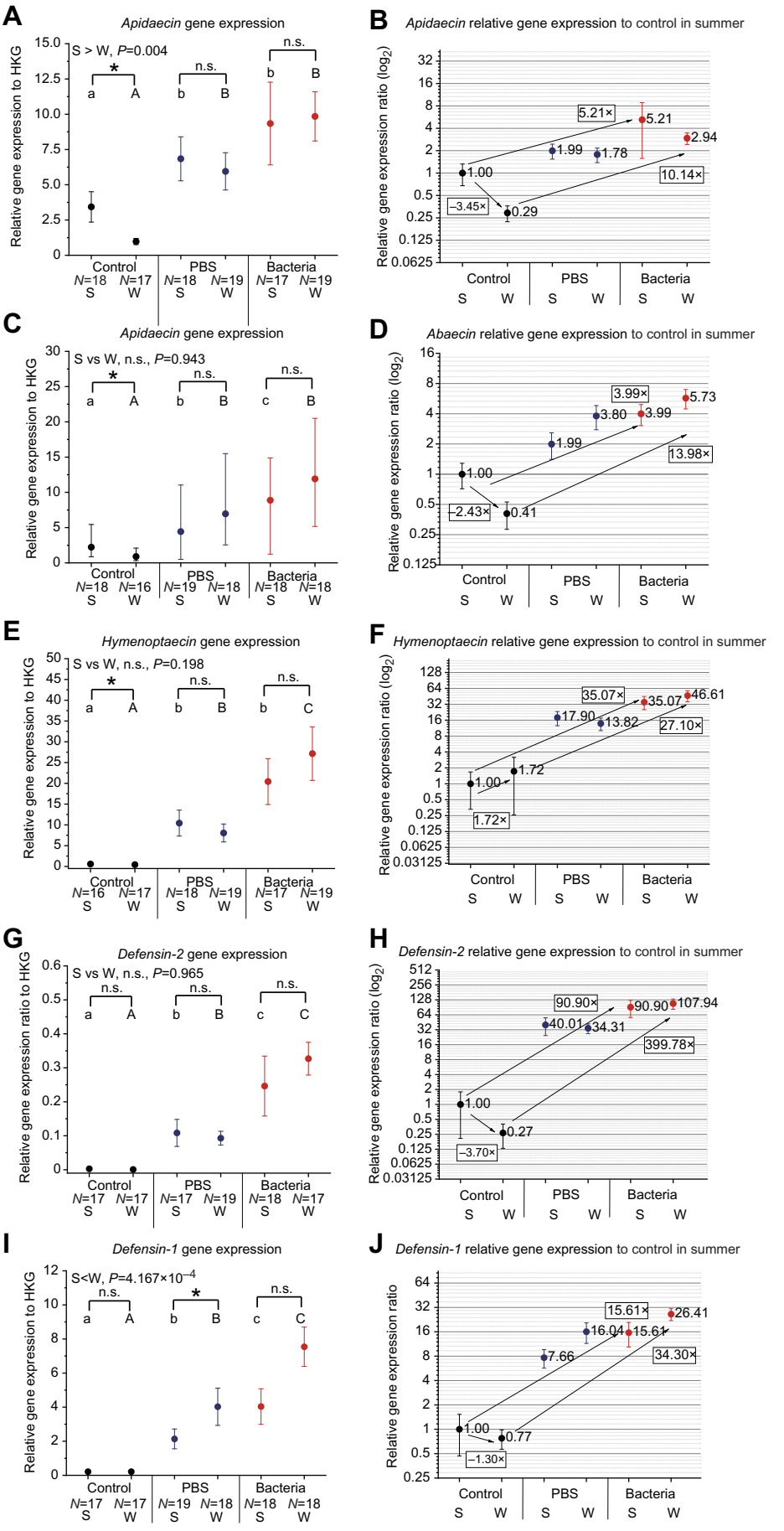

Fig. 6. Changes in gene expression of individual AmPs after immune stimuli in summer and winter honey bee populations. Relative gene expression of (A) *apidaecin*, (C) *abaecin*, (E) *hymenoptaecin*, (G) *defensin-2* and (I) *defensin-1* normalized to HKG and (B) *apidaecin*, (D) *abaecin*, (F) *hymenoptaecin*, (H) *defensin-2* and (J) *defensin-1* normalized to the control group in summer. The dots are means, with ranges representing 95% confidence intervals. The ratios between groups are illustrated by arrows and values (right column). The number of samples is noted as *N*. Significant differences ($P<0.05$) among the treatment groups are indicated by different lowercase and uppercase letters above the means of summer and winter population, respectively. Clamps display statistical differences between summer and winter populations; asterisks represent a significant difference (*$P<0.05$). Differences in the population levels are noted in the top left corner of each graph. n.s., not significant; S, summer population; W, winter population.

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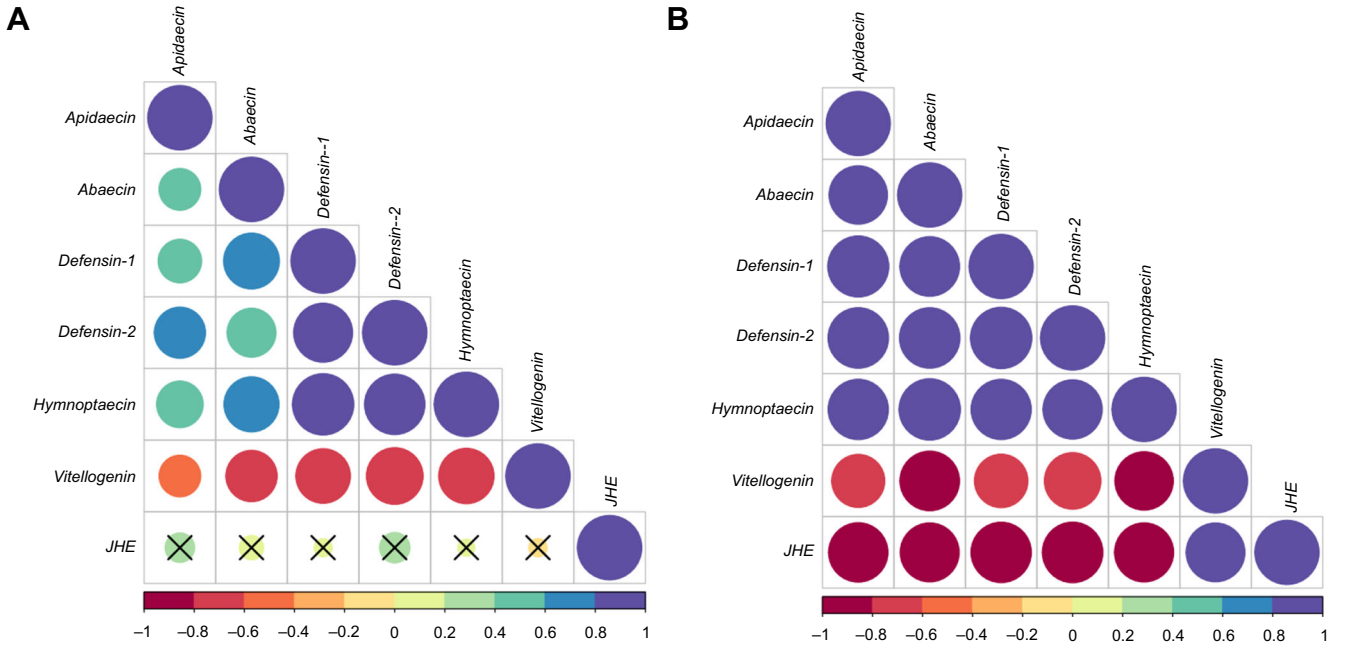

Fig. 7. Pearson correlation coefficients between transcript abundances of analysed genes of bee longevity markers and bee antimicrobial peptides. (A) Summer population; (B) winter population. The color and size of the circle reflect the strength of the correlation. Red represents a negative correlation and blue a positive correlation. Non-significant correlations with a *P*-value above 0.05 are indicated with crosses in individual cells. Correlation coefficients were calculated using $\log_{10}$-transformed data without outliers using 58 samples (A) and 55 samples (B). Values of Pearson correlations are available in Table S3. JHE, juvenile hormone esterase.

(2012) who inoculated bees orally with *S. marcescens*. Both studies report downregulation of *Vg* expression after oral inoculation; however, the mechanism of immunostimulation by injection or oral inoculation can be different.

Vg plays multiple roles in honeybee health and reproduction. Its essential roles in egg development and as a nutrient reserve have been widely documented; moreover, Vg functions are connected to the immune response in workers and Vg is also believed to possess anti-oxidant properties (Amdam et al., 2003; Corona et al., 2007; Kodrik et al., 2019; Munch et al., 2008; Seehuus et al., 2006). Thus, we hypothesize that strong modulation of Vg levels in immune-challenged bees can affect their amino acid and energy reserves, and thus their susceptibility to stress conditions, e.g. by utilizing Vg as an amino acid reserve for AmP production or an energy source for basal metabolism. Two previous studies by Aronstein et al. (2010) on bee larvae and Lourenço et al. (2013) on summer adult bees observed a significant downregulation of *Vg* in response to microbial infection, which is in agreement with the decreased Vg levels detected in our study. Moreover, pairwise correlation analysis showed that the expression of the *Vg* gene is strongly and negatively correlated with *AmP* expression in honeybees, especially within the winter population. Our results support the findings that there is an association between Vg and colony fitness, and that Vg is an important source of protein reserves during winter (Dainat et al., 2012; Lourenço et al., 2019; Ricigliano et al., 2018; Smart et al., 2016; Tufail and Takeda, 2012).

Vg gene expression is closely regulated by the JH signaling pathway. *Vg* synthesis is suppressed by an elevated JH titer in the hemolymph (Cardoso-Junior et al., 2018; Hartfelder and Engels, 1998). Analysis of the expression of *JHE*, a key degrading enzyme controlling JH titers, showed the same trend as *Vg* expression after experimental treatment, which supports the proposed role of JHE in JH degradation and crosstalk between JH signaling and immune activation (Mackert et al., 2010). The pairwise correlation of *Vg* and *JHE* genes also strengthens the hypothesis described above, as *JHE* is the only gene in our study that is positively correlated with *Vg* in the winter population (Fig. 7).

The cellular immune response of individual bees is mediated by specialized cells called hemocytes. Cellular immune mechanisms (e.g. nodule formation and encapsulation) are stronger in summer than in winter bees (Gatschenberger et al., 2013; Steinmann et al., 2015); moreover, hemocyte counts differ across life stages and castes of bees, with nurse bees and foragers having the lowest number of hemocytes (Wilson-Rich et al., 2008). Kunc et al. (2019) measured the total number of hemocytes in hive bees across the entire year and found no significant differences between the summer and winter populations. Our results confirm this observation; furthermore, PBS injection or stimulation by heat-killed bacteria did not have any significant effect on hemocyte count in the hemolymph (Fig. 4A). Control and treated bees were kept in cages for only 24 h and this time interval may not have been enough to produce new hemocytes in response to PBS or bacterial injection. However, insect hemocytes can be released to the circulation from sessile compartments and quickly increase their numbers (Hillyer, 2016). As this was not observed in our experiments, we can speculate that the induction of honeybee immune system by heat-killed bacteria is not as strong a stimulus as natural bacterial infections.

Antimicrobial activity of bee hemolymph has been previously studied in response to infections by pathogens (Azzami et al., 2012; Laughton et al., 2011; Steinmann et al., 2015). Gatschenberger et al. (2013) studied the immune responses of summer and winter bee populations maintained under laboratory conditions and found that winter bees reduced the number of viable *E. coli* bacteria injected into the bee hemocoel faster than summer bees. However, the antimicrobial activity of the collected hemolymph samples tested by the zone inhibition assay did not detect any significant difference between summer and winter bees. Our findings showed an almost two-fold increase in antimicrobial activity in the winter bee population after 24 h of exposure to heat-killed bacteria.

Levels of the antimicrobial peptide apidaecin 1, detected in the hemolymph within this study, confirmed the stimulation of the humoral immune system in immune-challenged bees. Apidaecins can be detected in the bee hemolymph early after infection and the concentration was found to have peaked at 36 h post-infection (Casteels et al., 1989; Casteels-Josson et al., 1993). Values measured for apidaecin 1 in the present study were on average 2.70±1.84 ng μl^{-1} for the controls in 10-day-old bees. Apidaecin 1 concentration increased to 21.88±6.23 ng μl^{-1} 24 h after bacterial infection; therefore, the concentration of apidaecin 1 increased 8.1× after immune stimuli. Higher levels of apidaecin 1 after immune stimuli correspond with the gene expression pattern of the *apidaecin* gene within the bacteria-injected group. This positive trend between apidaecin 1 concentration and *apidaecin* gene expression has been described previously (Danihlík et al., 2018).

When comparing the antimicrobial activity and levels of apidaecin 1 with the relative expression of AmP genes, it is evident that bee humoral immunity is stimulated after injury or bacterial infection in both populations. As previously described, the relative gene expression of AmP genes increases in response to the chalkbrood fungus in honeybee larvae (Aronstein et al., 2010), after bacteria injection in adult bees (Evans et al., 2006; Yang and Cox-Foster, 2005), and even in winter bees (Evans et al., 2006). A similar trend was detected in bumblebees infected with *E. coli* suspension (Erler et al., 2011). Our study follows previously published results and broadens the knowledge on immune responses and inducibility of summer and winter populations, with a focus on 10-day-old bees living under field conditions.

Not surprisingly, mechanical wounding and bacterial infection had an impact on all tested gene expressions, i.e. *apidaecin*, *abaecin*, *defensin-1*, *defensin-2* and *hymenoptaecin*, in both summer and winter populations with a similar pattern. The same trends were previously described in honeybees by Evans et al. (2006) (*apidaecin*, *abaecin*, *defensin-1, defensin-2* and *hymenoptaecin*), Yang and Cox-Foster (2005) (*defensin-1* and *hymenoptaecin*), or by Lourenço et al. (2019) (*defensin-1*), as reviewed by Danihlík et al. (2016).

In this study, we found that the induced *defensin-2* expression was most intense in bee abdomens; however, *defensin-2* peptide has not been identified in the hemolymph like other AmPs (Danihlík et al., 2016). A trend was observed in control winter bees (Fig. 6B,D), in which the expressions of *apidaecin* and *abaecin* were lower than in the summer population. A strong increase in AmP expression after the immune stimuli occurred, where AmP expression increased to the same or higher levels compared with the summer bees (Fig. 6B,D,F,H,J). This indicates a more intense immune response in winter bees. Pairwise correlation analysis of monitored genes showed a positive correlation among all studied AmPs, confirming that the extensive activation of AmP peptides and their consequent actions are key components of bee humoral immune responses (Danihlík et al., 2016; Evans et al., 2006). To our knowledge, the dynamics of expression or production of AmPs has been limited in previously published studies to a few time points after immunostimulation (Gatschenberger et al., 2013, 2012; Randolt et al., 2008), thus the dynamics of AmP production should be clarified in future studies.

Gene expression of humoral factors was also stimulated by mechanical injury due to the injection of PBS alone. This treatment could imitate certain natural conditions, e.g. cuticle damage caused by bites from *Varroa destructor* mites. Our findings suggest that activation of humoral immunity can be triggered early after *Varroa destructor* mites penetrate the external barriers of the bee body. This is supported by a recent study that found many defense genes to be upregulated in bees highly infested with *Varroa destructor* mites, including genes for AmPs *apidaecin* and *hymenoptaecin* (Zanni et al., 2017). Thus, injury or damage to the cuticle triggers humoral responses in honeybees, including bees bitten by mites and transmission of *Varroa destructor*-vector pathogens, e.g. deformed wing virus (de Miranda, 2012; Kanbar and Engels, 2003; Steinmann et al., 2015).

The honeybee immune system consists of a complex of interlinked reactions that are mostly activated by microbial pathogens (viruses, fungi and bacteria). As social insects, honeybees can also employ mechanisms of social immunity on top of the individual immune response, thereby protecting the colony in a coordinated way. Despite this sophisticated protection network in honeybee colonies, honeybee survival in temperate climates depends primarily on the vitality and responsiveness of the long-living winter bee population, whose presence in the colony is crucial for successful overwintering.

In this study, we subjected 10-day-old short-living summer bees and long-living winter bees, developed naturally under colony conditions, to an immune challenge and compared their physiological and immune responses. In accordance with our expectations, the winter honeybee population showed hallmarks of longevity, i.e. higher *Vg* gene expression, higher Vg concentration in the hemolymph, and higher concentration of total proteins compared with summer bees; however, the most pronounced differences between summer and winter bees were found in their responses to immune stimuli. Injury alone stimulates a humoral immune response that is comparable to the immune reaction following immunostimulation with pathogens. Results of this study provide evidence that the immune system undergoes extensive activation in the winter bee population and this is supported by a clear positive correlation among all studied AmPs in the winter population.

The long-living population was characterized by higher inducibility of the antimicrobial response, accompanied by a significant decrease in the Vg protein and gene expression levels within 24 h. Considering the pluripotent role of Vg in honeybees, a microbial infection can significantly impair honeybee overwintering. Of note is the fact that the levels of total nutrients in the hemolymph were not affected by immune challenge, and therefore future research focused on Vg and its metabolism could add novel insight into the crosstalk between immunity and nutrition.

More pronounced insight into activation of the immune system of winter bees could be obtained with experiments on bees collected in the late autumn (e.g. October, November). However, the design of our study is limited due to the fact that queens stop laying eggs in August–September. Afterwards it is not possible to get the bees of the same age for both populations, i.e. the 10 days used in our study. We consider the age to be a critical factor in comparing both summer and winter populations, so the winter population for this study was sampled in late August. Future research should also consider the dynamics of stress responses. Here we looked only at one time point, i.e. 24 h post-immune stimulation, and thus the information on the peak of the response remains unknown. Results of this study will also serve as a basis for further research focused on monitoring the inducibility of immune systems immediately after bee immunization and could be supplemented with research focused on forager workers or drones.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: S.D., P.D., M.K., J. Hurychova, D.T., J. Havlik, J.D.; Methodology: P.D., D.T.; Validation: M.S.; Formal analysis: S.D., M.K., J. Hurychova; Investigation: S.D., P.D., M.P., D.T.; Data curation: M.S., J.D.; Writing - original draft: S.D., J.D.; Writing - review & editing: P.D., M.P., J. Havlik, P.H., J.D.; Visualization: S.D., M.S.; Supervision: M.P., P.H., J.D.; Project administration: P.H., J.D.; Funding acquisition: P.H., J.D.

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Data availability
The original data are available upon request from the corresponding author.

Supplementary information
Supplementary information available online at
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