Does the pollen diet influence the expression and production of antimicrobial peptides in honey bee body parts?

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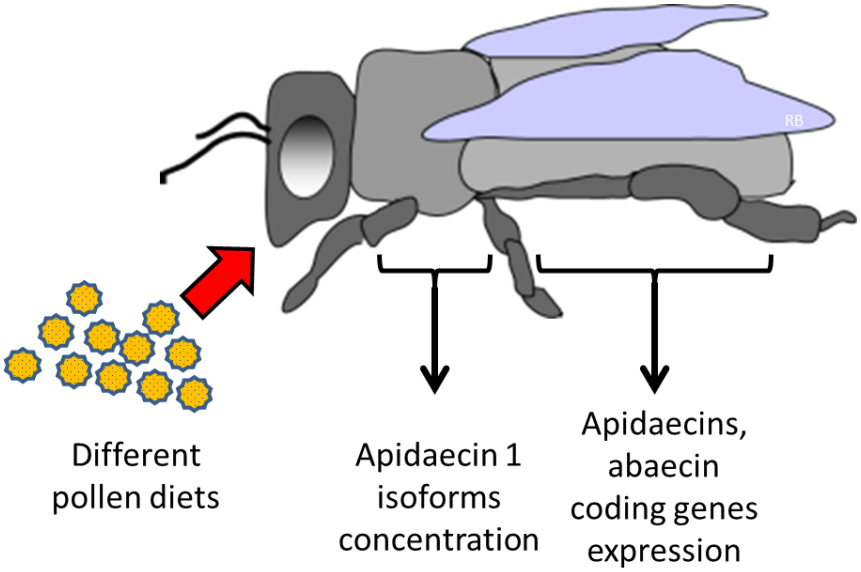
Abstract

In our study we investigated the importance of protein nutrition for honey bee immunity. Different protein diets (pollen substitute Feedbee™, monofloral pollens of *Helianthus spp.*, *Sinapis spp.*, *Asparagus spp.*, *Castanea spp.* and a pollen mixture of the four different pollens) were fed to honey bees in cages *ad libitum*. After 18 days of the feeding, thorax weight and apidaecin 1 isoforms concentration in the thorax were measured using nLC-MS. Expression levels of apidaecins and abaecin coding genes in the abdomen were determined using quantitative PCR. The results indicate that protein-containing nutrition in adult worker honey bees can trigger certain metabolic responses. Bees without dietary protein showed a reduced thorax weight and lower apidaecin 1 isoforms concentration. We also detected differences among the effects of the investigated protein diets. Expression levels of the respective genes were also affected by the protein diets and different expression levels of these two antimicrobial peptides were found. The significance of feeding bees with different protein diets as well as the importance of pollen nutrition for honey bee immunity is demonstrated a discussed.

Graphical abstract



Highlights

- this is the first insight into the level of apidaecin 1 in individual honey bees
- protein nutrition is important for thorax development and apidaecin 1 production
- abaecin and apidaecins gene expression levels are influenced by protein diets

Keywords

Apis mellifera, apidaecin, abaecin, gene expression, diet proteins, pollen

Abbreviations

AMP: antimicrobial peptide

Cq: quantification cycle

FA: formic acid

GITC: guanidium isothiocyanate

MALDI-TOF: matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry

nLC-MS: nanoflow liquid chromatography coupled with mass spectrometry

qPCR: quantitive polymerase chain reaction

RP-HPLC: reversed-phase high performance liquid chromatography

TFA: trifluoroacetic acid

UHR Q-TOF: ultra-high resolution quadrupole-time-of-flight mass spectrometry

WCX: weak cation exchange

Introduction

Honey bees are important for pollination of agricultural crops (Gallai et al., 2009). Plentiful biotic and abiotic stressors have been studied as potential causes for bee colony losses (vanEngelsdorp et al., 2009). *Varroa* mites and their associated viruses are very often responsible for the weakening or collapsing of colonies. Moreover, interactions with secondary virus infections can be damaging for bee colonies (de Miranda and Genersch, 2010). Honey bee colonies are also endangered by many pathogens such as bacterial or fungal diseases, e. g. mostly American foulbrood or chalkbrood (Aronstein and Murray, 2010; Genersch, 2010).

Poor nutrition of honey bee colonies can result in the reduction in larval number or in a weakened vitality of adult bees (Brodschneider and Crailsheim, 2010). Sufficient nectar, pollen and water sources are important for the development of bee larvae, which are required for the growth of colonies. The most needed nutrients are sugars, proteins and lipids contained in nectar and pollen. Adult bees need an appropriate diet during their life, because they are confronted with various functions which are changing in lifetime. Different carbohydrate diets that are fed to colonies as winter food elicit different expressions of various genes in fat body (Wheeler and Robinson, 2014). The lack of important nutrients may lead to a malnutrition of adults or larvae (Brodschneider and Crailsheim, 2010). The nutritional quality of pollen differs among plant species, with a significant variability in the total content of proteins, lipids, sugars and amino acids and also in the antioxidant capacity (Di Pasquale et al., 2013).

Social insects in general possess a lower number of immune-related genes compared to solitary insects (Evans et al., 2006; Barribeau et al., 2015). There are three levels of bee immunity reflected in interactions with pathogens – physical barriers, cellular immunity and humoral immunity, and moreover, bees as social insects evolved a special type of immunity – the social immunity (Wilson-Rich et al., 2009; Evans and Spivak, 2010). The nutritional intake of honey bees and effectiveness of individual immune response appear in a possible relationship (DeGrandi-Hoffman and Chen, 2015). The humoral part of the worker bee immunity consists of enzymes, lectins and antimicrobial peptides (AMP). Winter and summer generations of worker bees are present in the colony during the year. Gätschenberger et al. (2013) showed that the overall immune power of summer and winter bees remains at the same level. However, when bees are highly infected with deformed wing virus, cellular immunity genes are downregulated, whereas the expression of humoral immune genes increases (Steinmann et al., 2015).

Several AMP, namely apidaecins, abaecin, defensins and hymenoptaecin, were detected in the honey bee hemolymph (Casteels et al., 1989; Casteels et al., 1990; Fujiwara et al., 1990; Casteels et al., 1993; Klaudiny et al., 2005). Focused on apidaecins, the analysis of cDNA displayed the occurrence of 3 mRNAs of apidaecins. They are secreted as preproteins: apidaecin type 73 (Q06602, UniProtKB), apidaecin type 22 (P35581, UniProtKB), apidaecin type 14 (Q06601, UniProtKB), these preproteins are coded by genes *Apid73* (Gene ID 406115), *Apid22* (Gene ID 494510) and *Apid14* (Gene ID 406140). The preproteins are finally spliced into three active isoforms detected on the peptide level: apidaecin 1 (1A and 1B) and apidaecin 2, one apidaecin isoform sequence is only predicted from cDNA library (Casteels et al., 1989; Casteels-Josson et al., 1993).

AMPs are produced in fat bodies presented in bee abdomen into hemolymph, which is occurring in whole bee body (Vilmos and Kurucz, 1998). AMPs expression is triggered by a microbial challenge through Toll, IMD-JNK or JAK/STAT signalling pathways (Evans et al., 2006; Danihlík et al., *in press*). The expression of hymenoptaecin, a peptide present in the hemolymph of honey bees and bumble bees, can dramatically increase by an inoculation of bees with bacteria, e.g. *E. coli* (Kucharski and Maleszka, 2003). AMPs show a broad spectrum of antimicrobial or antifungal activity. However, the effectiveness towards target microorganisms varies for individual AMPs. Defensin-1 is more effective towards Gram-positive bacteria, on the other hand, apidaecin isoforms and hymenoptaecin display higher activity against Gram-negative bacteria (Casteels et al., 1989; Casteels et al., 1993).

The research work dealing with changes in the expression of genes for AMPs as a response to specific stimuli is mainly based on quantitative PCR analyses of bee tissues or whole bodies (Chaimanee et al., 2012; Siede et al., 2012; Jefferson et al., 2013). In addition, several other methods such as polyacrylamide gel electrophoresis, RP-HPLC and MALDI-TOF mass spectrometry have been used for detection of bee antimicrobial peptides (Casteels et al., 1990; Bilikova et al., 2002; Baracchi et al., 2011). Studies on bee AMPs concern mostly changes in the expression of the corresponding genes as only a few reports on the quantification of AMP peptide levels have been published. The reason resides in the fact that numerous bees immunized with bacterial pathogens have to be processed for the analysis. A new analytical method has recently been developed for the quantification of apidaecin 1 isoforms by nLC-MS method in the hemolymph or body parts of individual bees, which is promising for accurate quantification (Danihlík et al., 2014).

Here we attempted to detect changes in selected parameters of the humoral immune system in individual bees fed with different pollen diets. We analyzed the relative expression of genes

coding for antimicrobial peptides abaecin and apidaecins in honey bee abdomens, where they are synthetized in fat bodies. The level of the active apidaecin 1 isoforms was quantified in bee thoraces, which contain hemolymph with active apidaecins. Their quantity has not been investigated at individual level of bees yet. This first insight into the concentration level of apidaecin 1 isoforms in bee thoraces was obtained using the recently developed nLC-MS method.

Material and methods

Bee rearing and feeding

We incubated sealed brood combs from several colonies of *Apis mellifera carnica* from the Institute of Zoology, Graz at 34.5 °C under standard conditions to obtain newly emerged honey bee workers younger than 24 hours (Williams et al., 2013). Bees were chosen randomly and mixed before they were put into experimental cages consisting of clear plastic cups. Cages were also supplied with a wax bar and experiments were maintained for 18 days.

Preparation of pollen diets and nutrition factors

All bees were provided with 50% (w/v) sucrose solution *ad libitum*. All cages except one were additionally fed one of the following protein diets: Feedbee^{TM}, supplementary protein diet available on a market without any bee products (Saffari et al., 2010), corbicular pollens containing 94.8 % of sunflower (*Helianthus spp.*), 91.2 % of mustard (*Sinapis sp.*p), 70.6 % of asparagus (*Asparagus spp.*) or 87.6 % of chesnut (*Castanea sativa*). The monofloral pollens were collected by bees in Austria and kept frozen until use. The mixed pollen group received a 25 % (w/w) mixture of each of the four different pollens. Each pollen was palynologically analyzed at AGES – SPB, Abteilung, Bienenkunde und Bienenschutz, Lunz am See, Austria and kneaded into a dough that was provided to caged bees in one half of a cylindrical 10 ml plastic tube (Williams et al., 2013). All diets were daily renewed.

Sample pretreatment

Experimental bees were stored at -80 °C until use. Before processing, individual bees were dissected to whole thoraces, used for the quantification of apidaecin 1 isoforms, and abdomens, used for the quantification of gene expression of AMPs apidaecins and abaecin. Samples of particular body parts of individual bees were marked and analyzed.

Quantification of apidaecin 1 isoforms

Bee thoraces were weighed without legs and wings before processing. Afterwards they were homogenized with 600 µl of 0.1% v/v trifluoroacetic acid (TFA) using ceramic balls in 2-ml microtubes. The next steps were in accordance with the protocol by Danihlík et al. (2014). Briefly, the homogenized bee thoraces were centrifuged, heated at 100 °C for 10 min to further denature proteins and centrifuged again. The aliquot of 180 µl of clarified supernatant was lyophilized and stored at -80 °C until further processing. The lyophilized sample was dissolved in 100 µl of 5% v/v formic acid (FA) and then 33 µl were loaded in technical duplicates onto WCX-Tip microcolumns filled with 5 mg of Oasis® WCX 30 µm sorbent (Waters, USA). The final enriched fraction of apidaecin 1 isoforms was evaporated and dissolved in 5% FA before a desalting step, which included its purification by reversed-phase liquid chromatography using C_8-Stage Tips. The purified and desalted sample was diluted in 30 µl 0.1% (v/v) TFA before nLC-MS analysis. The quantification of apidaecin 1 isoforms was performed on a nanoflow liquid chromatograph (nanoEASY) coupled via electrospray ion source with an ultra-high resolution Q-TOF mass spectrometer (UHR-QTOF maXis) controlled by CompassQTOF v 1.4 (Bruker Daltonics, Bremen, Germany). The concentration of apidaecin 1 in thoraces was calculated from a linear calibration curve constructed using a synthetic external standard of increased apidaecin 1A concentration and constant 1 pmol isotopically labelled [$^{13}C_6{}^{15}N_4$] apidaecin 1A diluted in the purified homogenate of a freshly emerged bee using WCX-Tip microcolumns. For detailed information about the set up of nLC-MS method, see Supplementary material.

Protein assay

Bradford assay was applied in a microarray layout for protein quantification in samples. Bovine serum albumin served as a protein standard (Bradford, 1976).

RNA isolation and cDNA preparation

Individual abdomens of bees were homogenized in the GITC buffer (300 µl per abdomen) (Evans et al., 2013). The RNeasy Plant mini kit (Qiagen) was used for RNA isolation. The homogenate (100 µl) was mixed with 350 µl of RTL buffer from the kit and processed following manufacturer's instructions. Finally, the RNA concentration was quantified by

absorbance at 260 nm using a BioSpec-nano micro-volume spectrophotometer (Shimadzu, Tokyo, Japan).

Contaminating DNA was digested with Turbo DNAse (Ambion). The sample was incubated 30 min at 37 °C with 2U of the enzyme. This procedure was repeated two times. Then the RNA was immediately purified using paramagnetic beads Agercourt RNAclean XP (Beckman Coulter) with a slight modification of manufacturer's protocol. Briefly, 50 µl of magnetic particles and 40 µl of isopropanol were mixed with approximately 100 µl of an RNA sample. The mixture was incubated at laboratory temperature for 10 min and washed 3 times with 70% (v/v) ethanol. Finally, the RNA was eluted by 32 µl of RNAse-free water. Its integrity was checked by gel electrophoresis on 1.1% (w/v) agarose gels containing ethidium bromide.

cDNA synthesis and endpoint PCR

The Transcriptor High Fidelity kit (Roche) was used for cDNA synthesis from purified RNA. All working steps followed manufacturer's instructions. The quality of cDNA and presence of a possible genomic DNA contamination were tested in a PCR reaction, where GoTaq® Polymerase Green Master Mix (Promega) was used: 4.75 µl of water, 0.5 µl of 10 µM of forward primer, 0.5 µl of 10 µM of reverse primer, 0.5 µL of template, 6.25 µl of GoTaq® Green Master Mix 2×. PCR cycling was programmed as follows: 95 °C - 2 min, 35×[95 °C - 40 s, 60 °C - 30 s, 72 °C - min], 72 °C - 5 min, 4 °C. Gel electrophoresis in 3% (w/v) agarose gels with ethidium bromide was used as a detection method with 50-1000 bp PCR Marker (Promega) as a standard.

Quantitative PCR reaction

Quantitative PCR (qPCR) reaction was performed on a CFX96 TouchTM Real-Time PCR Detection System (Bio Rad) with SyberSelect® Master Mix (Life Technologies) in a total reaction volume of 5 µL: 2.5 µl of 2× SyberSelect® Master Mix, 0.45 µl of 3.3 µM of forward primer, 0.45 µl of 3.3 µM reverse primer, 1.6 µl of the template, templates were used in concentration range recommended by manufacturer's condition. The qPCR reaction set up was as follows: denaturation at 95 °C for 10 min, 40 × [95 °C 15 s, 60 °C 1 min], followed by a melting curve analysis where the dissociation curve was assessed for a product size confirmation. Used primers were applied for both PCR and qPCR (Table 1). Primers

employed for the gene *Apid1* (*Apid14* is a synonym name) are universal for all three apidaecin genes *Apid14* (Gene ID 406140), *Apid22* (Gene ID 494510) and *Apid73* (Gene ID 406115).

Table 1: Primer sequences and gene names used for PCR reactions.

Peptid/protein	Gene name	Gene ID	Amplicon size (bp)		Primer 5′→ 3′	T_m (°C)	Reference
Abaecin	*LOC406144*	406144	72	F	CAGCATTCGCATACGTACCA	64.6	(Evans, 2006)
				R	GACCAGGAAACGTTGGAAAC	63.3	
Apidaecin type 14	*Apid1*	406140	80	F	TTTTGCCTTAGCAATTCTTGTTG	60.0	(Simone et al., 2009)
				R	GTAGGTCGAGTAGGCGGATCT	63.4	
Actin related protein	*Arp1*	406122	155	F	TGCCAACACTGTCCTTTCTG	64.0	(Lourenço et al., 2008)
				R	AGAATTGACCCACCAATCCA	64.1	
EF-1a	*EF1a-F2*	544670	153	F	GGAGATGCTGCCATCGTTAT	63.9	(Lourenço et al., 2008)
				R	CAGCAGCGTCCTTGAAAGTT	64.4	

Assessment of RNA quality and purity and PCR reaction

The gene expression was quantified in abdomens of collected honey bees. The total extracted RNA from bee abdomens was measured and quantified by absorbance at 260 nm after the purification step and its quality monitored by agarose gel electrophoresis. Poorly concentrated or disintegrated RNA samples were excluded from the further analysis. The purity of RNA samples after the DNase treatment was tested by gel electrophoresis after an endpoint PCR. The specificity of each qPCR gene expression assay was evaluated by the corresponding dissociation melting curve.

Validation of reference genes

The primers for the reference genes (*Arp1* and *EF1a-F2*) were validated for developmental stages of bee brood or tissues (brain, ovary, fat body and hemocytes of queens) (Lourenço et al., 2008). Here, these reference genes (RGs) were validated also for the whole bee abdomens. The stability of RGs were calculated with BestKeeper© application, version 1 (Pfaffl et al., 2004). This validated software uses both raw data of quantification cycle (Cq) and PCR efficacy to calculate the best stable RGs. The descriptive statistics for RGs is calculated what allows to choose the most appropriate RG.

Quantification of relative gene expression

The relative gene expression was calculated using the REST 2009 program based on a randomization and bootstrapping technique computed from raw Cq data (Pfaffl et al., 2002).

Both selected RGs, *Arp1* and *EF1a-F2*, were used for the normalization of the genes coding for peptides apidaecins and abaecin.

Statistical analysis

The statistical analysis for thorax weight and apidaecin 1 isoforms concentrations in bee thoraces was performed using SPSS version 21 (IBM SPSS Statistics) with Kruskal-Wallis and *post hoc* multiple median comparison tests because the data were not normally distributed. The expression stabilities of genes coding for *Arp1* and *EF1a-F2* were evaluated in BestKeeper (Pfaffl et al., 2004) and the relative expressions of the genes for apidaecins and abaecin were calculated by REST 2009 (Pfaffl et al., 2002). These programs are freely available for academic use at http://www.gene-quantification.de.

Results

Effect of pollen diet on thorax weight

The median weights of thoraces were significantly different among studied groups (The Independent-Samples Kruskal-Wallis Test, $p < 0.05$). The no pollen diet resulted in a median thorax weight of 30.0 mg (95% confidence interval, CI: 26.3 - 30.7 mg), whereas all bees fed with protein diets had higher thorax weights (median 33.0 mg, 95% CI: 32.4 - 33.6 mg) (Fig. 1, $p < 0.05$). The highest thorax weights were observed in bees fed *Helianthus* pollen, mix pollen diet and Feedbee^{TM}.

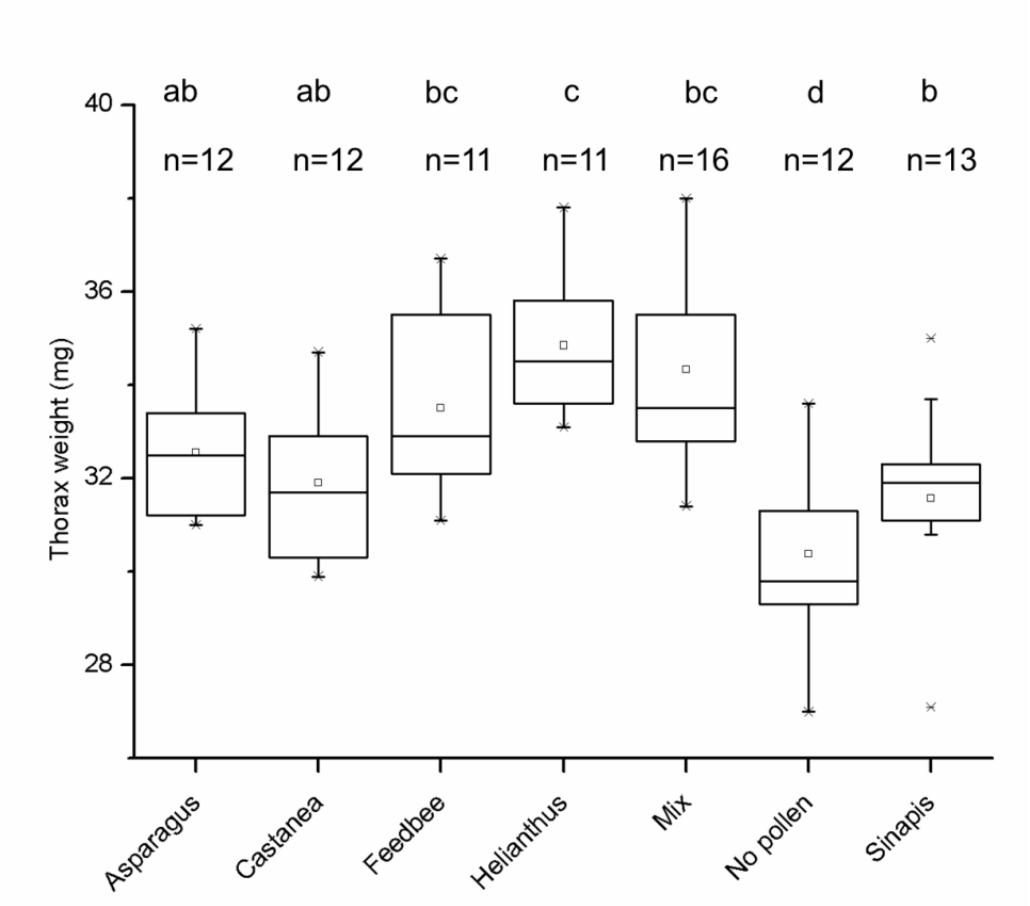


Fig. 1: Weight of thoraces of caged bees fed different diets. Groups are labeled by small case letters, different letters denote significant differences between groups (Independent Samples Kruskal-Wallis Test, $p < 0.05$). Boxes show 1st and 3rd interquartile range, the median is denoted with a line. Stars are outliers.

There was no significant difference registered in the protein concentration in homogenates of bee thoraces between bees fed no pollen or any of the protein diets (Independent-Samples Kruskal-Wallis Test, $p > 0.05$). Therefore, no pairwise comparisons were performed. The grand median of protein concentration was 0.07 mg protein per mg of homogenized thorax (95% CI: 0.06 – 0.08 mg/mg) (data not shown).

Effect of pollen diet on isoforms of apidaecin 1 concentrations in thorax tissues

When bees were fed with no protein in their diet, apidaecin 1 concentration was 0.2 ng/mg, whereas all bees fed with protein-containing diets during the first 18 days after their emergence showed higher apidaecin 1 concentrations (Independent-Samples Kruskal-Wallis Test, $p < 0.05$); Fig. 2. The overall median concentration of apidaecin 1 in bees fed any of

studied protein-containing diets was 2.2 ng/mg (95% CI: 1.8 – 2.6 ng/mg). The only pairwise significant difference found *post hoc* was between bees fed *Castanea* pollen which had higher apidaecin 1 concentration than bees fed the mixed pollen diet (median: 31.7 ng/mg, 33.8 ng/mg respectively) (Independent-Samples Kruskal-Wallis Test, $p < 0.05$); Fig. 2. The amount of the apidaecin 1 isoform was also expressed per a unit (i.e. thorax) to allow a comparison with data published in Danihlík et al. (2014) (data not shown). Bees fed with no pollen diet had a median amount of apidaecin 1 of 4.5 ng per thorax, whereas bees fed the investigated protein diets contained from 25.6 to 96.4 ng of the peptide isoforms.

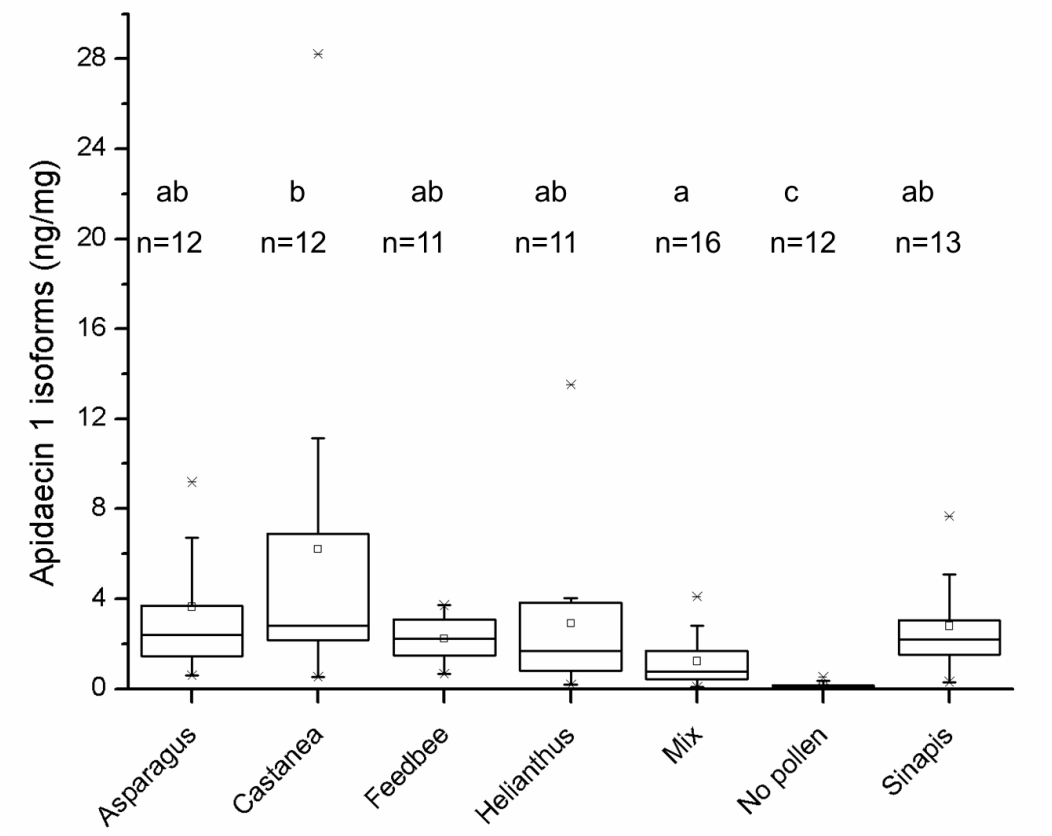


Fig. 2: Concentration of apidaecin 1 isoforms in thorax tissue of caged bees fed different diets. Groups are labeled by small case letters, different letters denote significant differences between groups (Independent-Samples Kruskal-Wallis Test, $p < 0.05$). Boxes show 1st and 3rd interquartile range with line, median is denoted. Stars represent outliers.

Validation of reference genes

Two reference genes, *Arp1* and *EF1a-F2*, were utilized for the normalization and calculation of the relative gene expression level for all samples. These x-fold expression results for RGs were corrected using the determined PCR efficiency. Neither *Arp1* nor *EF1a-F2* showed

minimum or maximum x-fold values higher than 3. This limit is used for integrity analysis of a single sample (Pfaffl et al., 2004; Lourenço et al., 2008). The maximum x-fold values were 2.42 and 2.64 for *EF1a-F2* and *Arp1*, respectively. The geometric mean values for Cqs were 19.67 ± 0.49 and 19.87 ± 0.56 for *EF1a-F2* and for *Arp1*, respectively. A pair-wise correlation analysis of RGs provided a value of 0.87 (p = 0.001) for *EF1a-F2* and 0.91 for *Arp1*. The regression analysis of data for RGs and validation found power values of 1.87 for *EF1a-F2* and 2.16 for *Arp1*. Based on this, both analyzed genes could be considered suitable RGs.

Effect of pollen diet on expression of the studied bee genes

Those bees, which were fed the sugar-containing diet only, were considered a control (untreated) group. The relative gene expression values for the studied AMP-coding genes were calculated either using reference data for each individual RG or when combining data for both RGs. The results are summarized in Tables 2 and 3. There was no significant difference observed when the two approaches of data normalization were used.

When compared to the group of bees fed on protein free diet, the *Apid1* transcript level was lower in bees fed with the mixed pollen whereas exactly the opposite trend was observed for the other groups with protein diets. On the other hand, different trends were measured for the abaecin coding gene expression. The expression of gene of the peptide abaecin was significantly upregulated only in bees fed the pollens of *Asparagus* or *Castanea*. Otherwise it remained stable when compared to the control.

	No. of	*Arp1*			*EF1a-F2*			Both RGs		
	individuals	Expression	p-value	Result	Expression	p-value	Result	Expression	p-value	Result
Mix	14	0.032	0.001	DOWN	0.028	0.000	DOWN	0.030	0.001	DOWN
Feed Bee	12	5.189	0.000	UP	5.161	0.000	UP	5.180	0.000	UP
Sinapis	12	6.995	0.000	UP	6.952	0.000	UP	6.969	0.000	UP
Asparagus	11	10.296	0.001	UP	8.430	0.003	UP	9.298	0.001	UP
Helianthus	11	4.221	0.000	UP	5.637	0.001	UP	4.880	0.000	UP
Castanea	10	11.212	0.000	UP	9.902	0.001	UP	10.537	0.000	UP

Tab. 2: Relative expression of the apidaecins genes calculated using RGs and compared to the control group kept on sugar diet.

	No. of individuals	*Arp1*			*EF1a-F2*			Both RGs		
		Expression	p-value	Result	Expression	p-value	Result	Expression	p-value	Result
Mix	14	7.031	0.173	STABLE	6.027	0.201	STABLE	6.514	0.174	STABLE
Feed Bee	12	0.771	0.835	STABLE	0.766	0.834	STABLE	0.769	0.835	STABLE
Sinapis	12	4.614	0.297	STABLE	4.586	0.310	STABLE	4.597	0.294	STABLE
Asparagus	11	55.700	0.008	UP	45.603	0.012	UP	50.301	0.011	UP
Helianthus	11	0.424	0.582	STABLE	0.567	0.710	STABLE	0.491	0.637	STABLE
Castanea	10	27.700	0.018	UP	24.464	0.022	UP	26.033	0.017	UP

Tab. 3: Relative abaecin coding gene expression calculated using RGs compared to the control group kept on sugar diet.

Discussion

When fed on protein diet, adult honey bees showed increased weight of thoraces. Thorax weight is considered a measure for the development of flight muscles and flight performance (Brodschneider et al., 2009). Adult honey bees undergo specific physiological changes in their flight musculature after emergence (Herold, 1965; Hersch et al., 1978). The highest thorax weights were found in bees fed on a mixture of different pollens, which is similar to their natural conditions in a diverse environment, or when bees were fed Feedbee™ or the monofloral pollen of sunflower (*Helianthus*). Monofloral mustard plant (*Sinapis*) pollen did not influence the thorax weight to the extent provided by the other three pollen diets investigated in this study. This is the first result bringing some evidence that a different quality of protein diet could affect thorax development of adult honey bees. On the other hand, the protein concentration of thorax was not influenced by the protein-containing diets. According to our measurements, the thorax contains of about 7 % of extractable protein per mg of its´ weight. But it should be noted at this point, that thorax was weighed including the exoskeleton without muscle tissue extraction and our samples were diluted in 0.1% TFA which causes a precipitation of proteins (Tantipaiboonwong et al., 2005). Denaturation and precipitation of proteins might have tent to decrease the protein content of sample.

In general, the dietary protein is a prerequisite for protein synthesis, including the formation of the antimicrobial peptide apidaecin 1 isoforms (Fig. 2). Almost any protein-containing diet, which was fed in this study, resulted in higher or similar apidaecins 1 concentrations in the thorax compared to the control containing exclusively sugar. Only chestnut (*Castanea*) pollen resulted in higher values of apidaecins 1 compared to the mixed pollen diet. We did not find any significant differences in its content among the other monofloral pollen diets or the

artificial protein diet Feedbee^{TM}. To our knowledge, there is only one previous report where apidaecins were measured in the hemolymph of honey bees (Casteels et al., 1989). Casteels et al. (1989) detected and defined three isoforms of apidaecin (1A 1B, 2) using a HPLC-based method with UV-Vis detection. They measured a concentration of apidaecin isoforms in the hemolymph of about 100 µg/ml, when the bees had been treated *in vitro* with *E. coli*. A ratio of 1:20 between the isoforms 1A and 1B has been reported. The recent validated method based on nLC-MS (Danihlík et al., 2014) allowed to measure 10 µg/ml apidaecin 1 isoforms in hemolymph of honey bees, what is indeed ten times lower than the previously measured number. But it must be taken into account, that the bees investigated in the study from 2014 were not immunized and showed no clinical signs of infection (Casteels et al., 1989; Danihlík et al., 2014).

Here the apidaecins 1 concentration in homogenates of thorax tissues appeared at 2.2 ng per mg of the material. Bees from colonies have been reported to contain 18-54 ng of apidaecins 1 in whole thoraces (Danihlík et al., 2014), which is higher compared to the bees in this study that did not feed on pollen and similar to those fed different protein diets. Bees fed with the mixed pollen diet contained an amount of 25.6 ng, which is similar to previous data from a feral colony (Danihlík et al., 2014). However, caged bees fed *Asparagus* or *Castanea* pollen showed even higher apidaecin 1 amounts, although the differences were not statistically significant.

A majority of experimental research studies published on AMPs in bee tissues or whole bees were based on determining the expression levels of the coding genes by quantitative PCR (Chaimanee et al., 2012; Siede et al., 2012; Jefferson et al., 2013). It is widely known, that the level of gene expression does not necessarily correlate with the concentration of active peptide molecules. This may occur e.g. when microRNAs regulate the translation (Selbach et al., 2008; Asgari, 2013). AMPs alone could also inhibit nucleic acids and protein synthesis or inhibit enzyme activity (Brogden, 2005). Protein-containing diet can influence gene expression profile of bees. A comparison of bees on non-pollen diet versus those on pollen previously showed that a high number of genes could be differentially expressed, especially in older bees (Corby-Harris et al., 2014).

The normalization of relative gene expression to *Arp1*or *EF1a-F2* as housekeeping genes provided the same trend for both studied AMPs-coding genes (Tables 2 and 3). In this investigation, protein diets triggered an up-regulation of apidaecins coding expression. Recent findings of Siede et al. (2012) demonstrated that wounding or injections of immuno-stimulating substances in adult workers generally resulted in an increased expression of genes

for AMPs. Other studies showed inconsistent evidences of down- or upregulations of apidaecins coding genes in the worker bees orally inoculated with *Nosema ceranae* spores (Chaimanee et al., 2014) or in larvae immunized with spores of *Paenibacillus larvae* or *Ascosphaera apis* (Aronstein et al., 2010; Cornman et al., 2013).

The expression of the abaecin-coding gene in bees on protein diets mostly did not differ from the control group in this study. Only the bees on *Asparagus* and *Castanea* pollen showed an up-regulation. Abaecin coding gene expression was inconsistently reported in different studies to be down-regulated or up-regulated in adult bees infected with *Nosema* (Chaimanee et al., 2012; Jefferson et al., 2013). In bumble bees, a short term lack of pollen diet resulted in reduced immune responses and changes in the expression of a number of genes (Brunner et al. (2014). Moreover, in bees fed a pollen-containing diet, the upregulation of abaecin-coding gene was detected only when diet were using a *Castanea* or *Asparagus* pollen, what is a similar trend like after an infection, but the expression remained stable when the pollen was limited. According to the conclusions of Siede et al. (2012), this up-regulation can be expected as a result of detrimental treatments, but it was not observed in adults infected with deformed-wing virus, where a down-regulation was observed (Flenniken and Andino (2013). When considering the effect of the treatment of varroacides, Garrido et al. (2013) found an either increase or decrease in abaecin-coding gene expression depending on the use of different acaricides. In our study, we could not determine equivocally, whether the observed up-regulation of the abaecin-coding gene in bees fed *Asparagus* or *Castanea* pollen, was attributed to the nutritional value of the diet or evoked by antigens or other chemical components in it. Together with other stress factors reducing the immunocompetence of honey bees (e.g. pesticides), malnutrition may act synergistically influencing negatively bee immune pathways and the ability of defence against pathogens (Boncristiani et al., 2012; Schmehl et al., 2014; Johnson, 2015).

Dietary proteins are necessary for the proper function of multiple immune pathways of honey bees (Alaux et al., 2011). Pollen diet, which is composed of pollen grains of many different botanical species, is often considered the most natural and nutritive diet for honey bees (Campana and Moeller, 1977; Schmidt et al., 1987; Höcherl et al., 2012). It is also important for the maintenance of their immunocompetence (Alaux et al., 2010). In contrast to this, the level of gene expression for another AMP, hymenoptaecin, did not differ between mononutritions on maize pollen or a mixed pollen (Höcherl et al., 2012). Di Pasquale et al. (2013) measured diameters of the hypopharyngeal glands acini and the expression of genes for transferrin and vitellogenin in bees fed one of four different monofloral pollen diets or

their mixture. They also confirmed the importance of dietary proteins compared to a non-protein control and some differences between monofloral pollen diets. In contrast to our findings, the pollen mixture was not found to cause significant modulations of gene expression compared to the monofloral diets. Similarly, apidaecin 1 isoforms concentration and thorax weights were also not different in bees fed the pollen mixture, suggesting a variable importance of diverse pollen nutrition. Optimal protein nutrition should provide a balanced content of essential amino acids required for honey bees (de Groot, 1953; Paoli et al., 2014). The quality of monofloral diets is dependent on how they fulfil this biochemical requirement. Each particular pollen, including that of *Helianthus*, can lack several amino acids, which are essential for honey bee (Nicolson and Human (2013).

In this work, the expression of genes coding for apidaecins and abaecin in caged bees responded differentially to the various pollen diet. From the observation that all diets except the mixed pollen resulted in up-regulation of apidaecin coding gene, we infer that unnatural or monofloral diets may impose a nutritional stress and weaken the honey bee immune system, similar to effects reported to be induced by other detrimental treatments. On the other hand, interpretation of these results is not evident in the light of the findings of Siede et al. (2012). Even the mixed pollen diet did not cause any difference compared to the non-protein diet. Nevertheless, we clearly demonstrate the importance of protein containing diet for the production of active apidaecin 1 isoforms. To asses qualitative differences of different monofloral pollen diets and completely determine their nutritional value, higher sample amounts would be necessary for a better understanding of gene expression related to the production of AMPs. Previous studies on the suitability of artificial protein diets for honey bees brought controversial results depending on the investigated trait of honey bee vitality (De Jong et al., 2009; Höcherl et al., 2012). Compared to different pollen diets used in our experiment, no striking advantages or disadvantages of Feedbee^{TM} could be detected based on evaluation of thorax weight, apidaecin 1 isoforms concentration and expression of the genes coding for apidaecins and abaecin.

Conclusions

In our study we investigated the importance of protein containing nutrition for honey bee immunity. Our findings demonstrate the general importance of protein (originating e.g. from a natural pollen diet) in adult honey bee nutrition, and for some physiological (thorax weight) or immunological parameters (apidaecin 1 isoforms concentration and gene expression of apidaecins and coding genes). Previous studies have dealt with determination of the

expression profiles of genes coding for AMPs and not with their actual levels in bee tissues. This work shows that analyzing the levels of active AMPs in dependence on dietary factors may reveal interesting findings related to the physiology of bees. We can state that the apidaecin 1 isoforms concentration is significantly influenced by pollen dietary protein. This phenomenon would not be revealed by gene expression analysis alone.

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Supplementary material

nLC-MS method details

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