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Low food stores affect dance communication and health-related gene expression in honey bees

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Honey bees, *Apis mellifera*, are important pollinators, and they face many natural and anthropogenic challenges that affect their ability to collect the resources needed to maintain the colony. Foragers can make use of a remarkable repertoire of communication behaviours that help colonies to exploit their environment successfully. Food source availability is a key factor for colony success and, therefore, survival and reproduction. Few studies have investigated how food stores impact forager communication strategies and bee physiology. We experimentally manipulated honey stores and (1) quantified the production and following of waggle dances, (2) quantified the expression of immune-related genes using qPCR and (3) analysed fatty acids from bee abdomens using GC-MS 6 days after the experimental manipulation. We found that the number of waggle dances increased by about 60% when honey bees were starved of honey. The number of followers per dance, however, decreased, which may be due to a switch to proactive, solitary foraging or to the occurrence of more waggle dances. Waggle dance duration, the number of waggle phase followers that were followed and foraging distances were not affected by the treatments. Bees in starved colonies showed a higher expression of the gene *defensin 1*, which is an important predictor of overwinter survival, but there was no treatment effect on fatty acid content. Our results show that the amount of honey stored in hives affects communication behaviours and the investment in immunocompetence of bees, possibly to counter the negative health effects of nutritional stress. However, fat content does not seem to be affected in the time span of the study.

Bees play a critical role in pollinating agricultural crops (Hristov et al., 2020; van der Sluijs & Vaage, 2016) as well as wild flowers (Garibaldi et al., 2013; Hung et al., 2018) but their health is under pressure in many human-modified areas (Ollerton, 2021; van Engelsdorp & Meixner, 2010) due to a cocktail of stressors, including pesticides, pathogens, climate change and habitat loss (Outhwaite et al., 2022; Soroye et al., 2020; Goulson & Nicholls, 2022). The conversion of natural habitat into urban or intensively managed agricultural land, in particular, is thought to negatively impact the health of honey bee, *Apis mellifera*, colonies and contribute to the decline of wild bees in some areas (Branchiccela et al., 2019; Liang et al., 2023; Naug, 2009). These changes in land use can lead to an inadequate and unbalanced nutrition, with negative effects on the growth and development of honey bee colonies and individual bees (Brodschneider & Crailsheim, 2010; Di Pasquale et al., 2013). For example, insufficient pollen nutrition can weaken the bees' immune system (Alaux et al., 2010), affect their pesticide tolerance (Barascou et al., 2021) and increase the risk of colony death (Goulson et al., 2015). While the effects of pollen shortages have been relatively well studied, research on the effects of low honey stores remains scarce. We aimed to explore the consequences of low honey stores on three aspects: (1) foraging behaviour, specifically the waggle dance communication, (2) immune-relevant gene expression and (3) bee physiology, measured as their fatty acid stores.

Honey bees use the unique waggle dance behaviour to convey information about the presence, smell and location of important resources (von Frisch, 1967; Dyer, 2002; Grüter & Farina, 2009; Couvillon, 2012). Studies have suggested that the characteristics and value of waggle dance communication depend on ecological factors, such as the availability and distribution of food sources (Dornhaus et al., 2006; Couvillon, 2012; Couvillon et al., 2014; I'Anson Price & Grüter, 2015). I'Anson Price et al. (2019), for example, found that dance following increased over time in an environment with few food sources. However, even under constant

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environmental conditions, dance followers vary greatly in their interest in dances, that is, the number of waggle phases they follow, depending on whether they seek spatial information to locate the advertised food source or whether they primarily seek olfactory information that helps foragers decide whether to resume foraging at previously exploited food sources (von Frisch, 1967; Grüter et al., 2008, 2013; Grüter & Farina, 2009). Rinderer (1982) found that dance frequency and dance follower number also depended on the amount of empty comb space, with bees dancing more in colonies with more empty comb area. Based on these different observations, we hypothesized that waggle dance frequency and follower number will increase in honey-starved colonies as foragers seek more information about high-quality food sources. We also tested whether follower interest, measured as the duration of dance following, and foraging distances, measured as waggle phase duration, are also affected by honey store depletion.

Nutritional stress and diet can shape the immunocompetence of bees, including immune-related gene expression (Alaux et al., 2010; Corona et al., 2023). Innate immunity is an important line of defence against pathogens, which includes humoral immune responses associated with antimicrobial peptides such as defensin 1 (Casteels-Josson et al., 1994), hymenoptaecin (Casteels et al., 1993) and abaecin (Casteels et al., 1990) and cellular responses (Strand & Pech, 1995). Several studies show that nutritional stress affects both the expression of immune genes (Alaux et al., 2010; Corby-Harris et al., 2014) and susceptibility to different pathogens (DeGrandi-Hoffman et al., 2010; Di Pasquale et al., 2013; Tritschler et al., 2017). Moreover, defensin 1 was found to be upregulated in old bees in pollen-restricted colonies, which are likely to be exposed to greater health risks (Corona et al., 2023). We predicted that genes known to be important for immunocompetence will be expressed more when colonies experience an acute honey shortage. Fatty acid stores in the bee's fat body, a tissue with an essential role in energy storage, metabolism and immunity, can also be an indicator of the bee's nutritional condition (Stanley-Samuelson et al., 1988). Nutritional stress can significantly reduce the fatty acid stores, while lipids in pollen or commercial supplement will increase the bees' lipid and essential fatty acids stores (Arien et al., 2020). Dolezal et al. (2019) found that bees inhabiting intensively farmed monocultures experienced a reduction in fat stores and colony weight when food sources became scarce, both of which affect survival (Dolezal et al., 2019). We explored whether a reduction in honey stores leads to a reduction in fat stores in the short to medium term, that is, within a few days.

METHODS

Ethical Note

No licences or permits were required for this research. The colonies were placed into observation hives, and bees foraged in the natural environment. After the experiment, the colonies were carefully returned to their 'Deutsch Normal' hives and provided with sufficient bee syrup to ensure they had enough food to survive the winter.

Study Site and Study Animals

The study was conducted between May and August 2020 on three pairs of observation hives (OH1–OH6) with *A. m. carnica* honey bees, each comprising 3000–4000 workers, food stores, brood and a naturally mated queen. The study took place in the apiary of the Johannes Gutenberg University in Mainz, Germany. All observation hives were kept in a wooden shed. Bees could reach the outside of the shed and visit natural food sources by passing through a plastic tube that led to the outside.

Experimental Procedure

We used a paired design with two observation hives being tested at a time. We created each hive a few days before the experiment began and designed them to have three 'Deutsch Normal' frames: a honey store frame at the top, a brood frame in the middle and a mixed frame (containing honey, pollen and empty space in similar proportions) at the bottom. The two hives of a pair were first left unchanged for 6 days. The two treatments were as follows: (1) we replaced the top honey frame in one of the paired hives with an empty frame (EH treatment); (2) we selected a full honey frame (FH treatment) from a colony in the apiary to replace the existing top honey frame in the other hive. This ensured both hives experienced the opening and replacement of a frame. Subsequently, for 6 days one hive experienced the EH treatment while the other experienced the FH treatment. The 6-day treatment period was short enough to ensure that EH colonies would not die from starvation. After another 6-day recovery period with a full honey frame in both hives, the treatments were reversed for another 6 days (Fig. A1). We filmed the 'dance floor' near the entrance (i.e. the area where dancing is most intense; von Frisch, 1967) on both sides of the observation hives for 2 h in the morning and 2 h in the afternoon throughout the treatment period using digital cameras (Panasonic video HC-V180). For the last pair (OH5 and OH6), we recorded only 90 min in the morning and 90 min in the afternoon because the shorter daytime and cooler temperatures in August reduced the duration of foraging activity. In the event of short periods of rain, we adjusted the recording schedule to maintain the filming duration. At the end of each treatment period (day 6), we collected nine returning foraging bees from the entrance of the observation hive, and immediately placed them into liquid nitrogen. They were then stored at −80 °C until we analysed the expression of immune-related genes and fatty acid content in the fat body.

Behavioural Observations

To determine the overall dancing activity of a colony, we used a scan sampling approach and counted the waggle dances that occurred during 2 min every 20 min of video recording. For each of the counted waggle dances, we also counted the waggle phase number as a measure of waggle dance duration. To assess dance follower interest in a standardized way, we selected the first five waggle dances with at least five waggle phases from scans each day and counted the followers and waggle phases that these bees followed. We identified followers as bees that (1) faced the dancer, (2) were within an antenna's length during the waggle phase and (3) followed the dancer's movements during at least one waggle phase of the dance (Al Toufailia, Couvillon, et al., 2013). Data on the number of dance followers were collected starting from the third waggle phase from the start of a dance (W3) for three waggle phases (W3-5; I'Anson Price et al., 2019). The follower number per dance was calculated as the average number of followers across these three waggle phases. We counted the waggle runs followed per follower present at W3 to assess the motivation of bees to follow an individual dance (I'Anson Price et al., 2019). In addition, we quantified the distance of the food sources visited by the dancers by examining the average waggle phase time frame by

frame, from the second to fifth waggle phase, based on the universal calibration curve (Couvillon et al., 2012; Schürch et al., 2019).

Immune Genes Expression

The expression of several antimicrobial peptide (AMP) genes, namely *abaecin, apidaecin, hymenoptaecin* and *lysozyme*, has been shown to be related to pollen nutrition stress in adult bees (Castelli et al., 2020; Danihlík et al., 2018). *Defensin 1* and *apimisin* are also AMPs and were found to be highly expressed in the nectar-processing tissues, hypopharyngeal gland and mandibular glands of forager bees, which may help protect them against microorganisms and xenobiotic compounds acquired while foraging (Vannette et al., 2015). *Relish* was found to be involved in the regulation of these AMPs (Brutscher et al., 2015; Schlüns & Crozier, 2007).

We used real-time quantitative PCR (qPCR) to investigate whether the expression of these seven immune genes (*abaecin, apidaecin, apimisin, relish, hymenoptaecin, defensin 1* and *lysozyme*) increases in foragers, captured on day 6 of the treatment period, when colonies experience a depletion in honey stores. We used four bees per colony and treatment ($N = 24$ from the EH treatment; $N = 24$ from the FH treatment). Total RNA was extracted from whole bees using RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA quality and quantity were assessed using a Qubit spectrophotometer (Thermo-Fisher Scientific, Foster City, CA, U.S.A.); 0.8 μg total RNA for each reaction were used. cDNA was synthesized using a Quanti Tect Reverse Transcription Kit (Qiagen) following the manufacturer's instructions.

We performed qPCR on a mic qPCR cycler (Bio Molecular Systems, Upper Coomera, QLD, Australia) using the Blue S'Green qPCR mix Separate ROX (BioZyme, St Joseph, MO, U.S.A.). Each reaction volume of 10 μl contained 5 μl Blue S'Green qPCR mix, 0.25 μM of each primer, 1 μl cDNA and DNase/RNase free distilled water. The following cycling parameters were used: 95 °C for 2 min; 40 cycles of 95 °C for 5 s and 60 °C for 20 s. The fluorescence signal was measured at the end of each extension step at 60 °C. Quantification cycle (Cq) values were determined at the same fluorescent threshold for each gene by the micPCR Version 2.6 software (Bio Molecular Systems). Gene primers of tested immune genes were based on published sequences (Table 1). The transcript levels of the target genes were expressed as normalized transcript abundance using *GAPDH* and *β-actin* as internal reference genes (Chen et al., 2005; Peng et al., 2021; Reim et al., 2013). We found that combining the two reference genes was more stable than using a single reference gene.

Table 1
The primers of the selected immune genes

Immune genes	Sequence (5′-3′)	Source
Abaecin	ATCTTCGCACTACTCGCCAC AGCCTTGAGGCCATTTAATTTTCG	Zhao et al. (2019)
Apidaecin	GGCACGAGAAGAATTTTGCCT GAAGGCGCGTAGGTCGAGTA	Zhao et al. (2019)
Hymenoptaecin	CTCTTCTGTGCCGTTGCATA GCGTCTCCTGTCATTCCATT	Zhao et al. (2019)
Defensin 1	TGCGCTGCTAACTGTCTCAG AATGGCACTTAACCGAAACG	Zhao et al. (2019)
Apisimin	TGAGCAAAATCGTTGCTGTC AACGACATCCACGTTCGATT	Evans (2006)
Lysozyme	GGAGGCGAGGATTCTGACTCAATG TGTTGCATATCCCTCCGCTGTG	Aronstein (2010)
Relish	GCAGTGTTGAAGGAGCTGAA CCAATTCTGAAAAGCGTCCA	Evans (2006)
GAPDH	ACCTTCTGCAAAATTATGGCGA CACCTTTGCCAAGTCTAACTGTTAAG	Reim et al. (2013)
β actin	TGCCAACACTGTCCTTTCTGGAGGT TTCATGGTGGATGGTGCTAGGGCAG	Francis et al. (2013)

Fatty Acids Extraction

Fatty acid contents were extracted from the abdomen of bees using 1 ml of a chloroform:methanol mixture, 2:1 (v/v), over a period of 24 h (Folch et al., 1957). The samples were evaporated to dryness under gentle nitrogen flow and then redissolved in 250 μl of a 2:1 dichloromethane:methanol (v/v) mixture. We added 2 μg of nonadecanoic acid (dissolved in 10 μl DCM/MeOH) as internal standard. After vortexing, we moved 15 μl of this solution into a new glass vial and evaporated it to dryness under a gentle nitrogen flow. Finally, we added 20 μl trimethylsulphonium hydroxide (TMSH; 0.25 M in MeOH, Sigma-Aldrich, Munich, Germany) to samples to derivatize to fatty acid methyl esters (FAMEs) and analysed them with a 7890A gas chromatograph (Agilent) coupled to a 5975C mass-selective detector (Agilent, GC/MS). Helium was used as the carrier gas at a flow rate of 1.2 ml/min. The temperature of the GC oven started at 60 °C for 1 min, then increased by 15 °C/min to 150 °C, followed by an increase to 200 °C with a heating rate 3 °C/ min, and finally increased by 10 °C/min to 320 °C, where it was held constant for 10 min. The separated FAMEs were transferred to the MS and electron ionization mass spectra were recorded at 70 EV from m/z 40 to 650. Resulting peak areas were integrated manually using the software MSD Chem Station G1701EA E.02.02.1431 (Agilent) and identified from diagnostic ions, retention time and the molecular peak. Only fatty acids with abundance >1% were included in our analyses (Rosumek et al., 2017). This method can detect fatty acids between C10 and C20, but only chain lengths of C16 to C19 were found.

Statistical Analyses

Behaviour: waggle dances and followers

The data were analysed in R 4.2.2 (R Core Team, 2022). We used general and generalized linear mixed-models (LMEs and GLMMs), with hive ID and hive pair ID included as random effects to control for the nonindependence of data from the same colony (Zuur et al., 2009). Our fixed effects were honey storage (EH versus FH) and day of treatment (2–6, the 1st treatment day was not considered and allowed bees to adapt to the new honey storage condition). Dance frequency data were zero-inflated, so we ran a zero-inflated model with a Poisson distribution to perform the GLMMs. We used the 'lme4' and 'lmerTest' packages to calculate *P* values for fixed effects (Bolker et al., 2009). We tested whether our fixed effects affected the (1) waggle dance frequency, (2) waggle dance duration, (3) average number of waggle dance followers per dance, (4) average number of waggle phases followed per dance and (5) waggle phase duration. We first used likelihood ratio tests (LRT) to determine whether to retain both random effects in the model. To simplify the model structure, pair ID was removed from the random effects if it was not significant, while colony ID was always retained as a random effect to account for nonindependence of observations due to the shared hive environment. Significance of fixed effects and their interaction was tested by comparing models with and without the fixed effects of interest using LRTs. The 'DHARMa' (Hartig, 2022) package was used to check whether model assumptions were met (Zuur, Alain F et al., 2009). If necessary, we log-transformed response variables to achieve a Gaussian distribution of the model residuals. We used *Z* scores to check for outliers in the data (Shiffler, 1988).

Gene expression

The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and the following formula: normalized target gene $= 2^{-(CqTarget-CqReference)}$ (Schmittgen & Livak, 2008). PCR efficiency (E) values were calculated by the software micPCR Version 2.6 (Bio Molecular Systems) for each gene from the slope following the running standard curves and the formula: $E = 2^{-1/slope}-1$ (Taylor et al., 2010). We used LMEs with gene expression as the response variable. The fixed effect was the honey storage condition (EH versus FH). Random effects were colony ID and pair ID, as described above.

Fatty acids

We tested for differences in the absolute quantity of fatty acids as well as the proportions of saturated and di-unsaturated fatty acids (tri-unsaturated acids were not detected) by normalizing the values using the quantity of the internal standard. The remaining fatty acids, the monounsaturated fatty acids, are equal to 1 − (saturated + double unsaturated fatty acids). We used LMEs to compare the fatty acid quantities in bees from different treatments (EH versus FH), again including colony ID and pair ID as a random effect, following the procedure described above. Pair ID was removed as a random effect if it was not significant. To compare the relative proportions of different types of fatty acids, we used nonmetric multidimensional scaling (NMDS; command metaMDS, package 'vegan'; Oksanen et al., 2022). The permutational multivariate analysis of variance (PERMANOVA) used the 'adonis' function (package 'vegan') to randomly rearrange the proportions of different types of fatty acids within the levels of the 'Treatment' variable while keeping the structure of the 'Colony' grouping intact to assess the significance of the results by permutations.

Since treatment effects on physiological traits could also depend on the treatment order (first EH versus first FH), we tested whether treatment order interacted with honey storage condition to affect gene expression and fatty acid quantities. However, we found no significant interaction between treatment order and honey storage condition on any of the physiological and immune-related traits ($P > 0.05$). Since we had no a priori interest in treatment order effects and to avoid unnecessarily complex models, we did not include treatment order as a predictor to assess whether honey storage condition affected gene expression and fatty acid quantities.

RESULTS

Communication Behaviour in Relation to Honey Stores

Waggle dance frequency

We performed 743 scans and observed 1400 dances and 8581 waggle phases. We found that the waggle dance frequency was significantly higher in honey-depleted hives than in hives with full honey stores (GLMMs: zero-inflation model; EH mean (95% confidence interval, CI) = 2.33 (2.00–2.65); FH = 1.45 (1.21–1.68); $Z = 2.66$, $P = 0.008$; Fig. 1). In addition, there was an increase in dance frequency from second to last treatment day (second day of treatment (95% CI) = 1.47 (1.02–1.91); last day of treatment = 2.56 (2.03–3.09); $Z = -4.120$, $P < 0.001$). No interaction was found between treatment day and treatment ($Z = 0.74$, $P = 0.46$).

We found no significant differences in the dance duration ($N = 58$) depending on the treatment or treatment day (LME; treatment: 6.09 waggle phase per dance (5.04–7.13) versus FH = 6.47 (5.13–7.81); $F_{1,49.22} = 0.873$, $P = 0.35$; treatment day: 6.09 waggle phase per dance (5.04–7.13) versus FH = 6.47 (5.13–7.81); $F_{1,49.13} = 0.008$, $P = 0.93$), and no interaction was found between days and treatments ($F_{1,49.13} = 1.50$, $P = 0.23$).

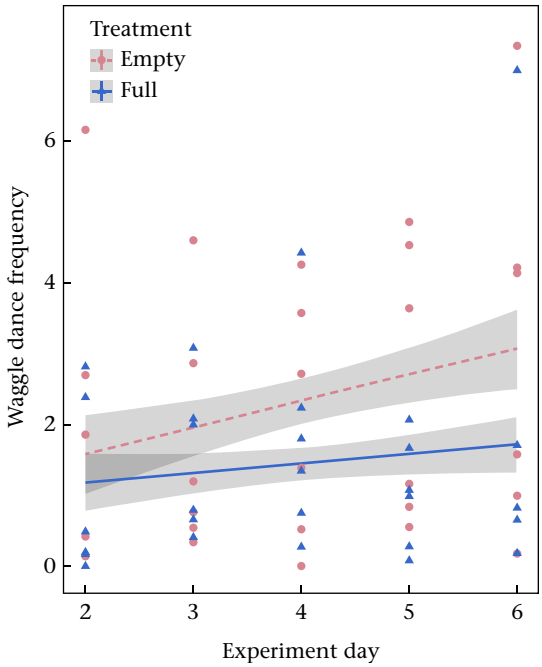

Figure 1. Number of waggle dances counted for 2 min every 20 min of video recording in relation to treatment from the second to sixth day of the treatment period. Treatments were empty and full honey store hives. Dots represent the average waggle dance number of each colony. The lines depict the predicted linear trend of each treatment (FH and EH). Grey areas delimit 95% confidence intervals.

Waggle dance follower behaviour

The number of dance followers was lower when the honey stores were low (LME: EH = 2.82 (2.58–3.06) versus FH = 3.43 (3.21–3.66); $F_{1,190.65} = 17.3$, $P < 0.001$; Fig. 2). Treatment day had no effect (first day of video recording, day 2 = 3.00 (2.62–3.38) versus last day of video recording, day 6 = 2.96 (2.55–3.37); $F_{1,192.16} = 0.32$, $P = 0.58$) and no interaction was found between days and treatments ($F_{1,188.76} = 2.47$, $P = 0.12$).

We also analysed the interest of the followers to follow a waggle dance but found no difference in the number of the waggle phases followed by dance followers in FH hives compared to EH hives (LME: EH = 4.23 waggle phase followers followed (3.67–4.79) versus FH = 4.22 (3.82–4.61); $F_{1,184.57} = 0.05$, $P = 0.83$). The number of waggle phases followed by followers was not affected by the treatment day (FD = 4.73 (4.03–5.44) versus LD = 4.45 (3.70–5.20); $F_{1,186.55} = 0.49$, $P = 0.48$). No interaction was found between treatment day and treatment ($F_{1,183.90} = 0.16$, $P = 0.69$).

We found no difference in foraging distance, measured as the waggle phase duration, between FH and EH hives (LME: EH = 1.05 s (0.93–1.17 s) versus FH = 1.21 s (1.07–1.35 s); this corresponds to ~0.76 km versus ~0.88 km; $F_{1,182.68} = 1.87$, $P = 0.17$). We found a borderline nonsignificant trend of treatment day to affect the foraging distance (FD = 1.18 s (0.88–1.48 s) versus LD = 1.22 s (1.04–1.40 s); 0.86 km versus 0.88 km; $F_{1,185.44} = 3.42$, $P = 0.066$). No interaction was found between treatment day and treatment ($F_{1,181.53} = 2.40$, $P = 0.12$).

Immune Gene Expression

We found that the expression of *defensin 1* was significantly higher in honey-depleted hives (LME: $F_{1,46} = 4.77$, P=0.035; Fig. 3), while the other six genes (*abaecin, apidaecin, apimisin, relish, hymenoptaecin* and *lysozyme*) showed no change in expression due to the removal of honey stores (*abaecin*: $F_{1,46} = 1.16$, $P = 0.29$;

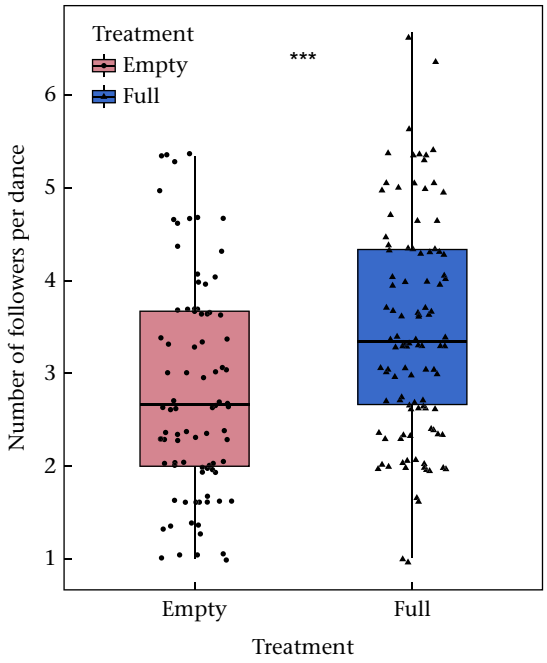

Figure 2. Number of waggle dance followers per dance during the experimental period in hives with empty and full honey stores. Box plots show the median, 25th and 75th quartiles and the 5th and 95th percentiles. ***$P < 0.001$. Individual data points are represented by the black points and triangles.

apidaecin: $F_{1,46} = 0.21$, $P = 0.65$; *apimisin*: $F_{1,46} = 0.86$, $P = 0.36$; *relish*: $F_{1,46} = 0.07$, $P = 0.79$; *hymenoptaecin*: $F_{1,46} = 0.25$, $P = 0.62$; *lysozyme*: $F_{1,46} = 2.26$, $P = 0.14$; Fig. 3), compared to hives with a full honey frame (FH).

Fatty Acids

We identified five main fatty acids: palmitic acid (C16:0), a monounsaturated C16 acid (probably palmitoleic acid, C16:1), stearic acid (C18:0), oleic acid (C18:1) and a di-unsaturated C18 acid (probably linoleic acid, C18:2). Palmitic acid, stearic acid and oleic acid can be biosynthesized by the bees and are most abundant in their bodies. Palmitoleic acid can be converted from palmitic acid in the fat body but is only present in small amounts. Linoleic acid, on the other hand, has to be acquired from the diet (Stanley-Samuelson et al., 1988). Rosumek et al. (2017) found that linoleic acid (C18:2) can quickly accumulate through dietary supplement.

We found no effect of our treatment on the absolute quantity of fatty acids (LME: FH versus EH: $\chi^2_1 = 0.009$, $P = 0.92$; Fig. 4). When we assessed the proportions of different types of fatty acids, saturated fatty acids and di-unsaturated fatty acids, we again found no difference between treatments in saturated fatty acids ($\chi^2_1 = 0.05$, $P = 0.83$) or di-unsaturated fatty acids ($\chi^2_1 = 0.006$, $P = 0.93$).

DISCUSSION

We found that the waggle dance frequency increased by ~60% when colonies had very little honey compared to colonies with full

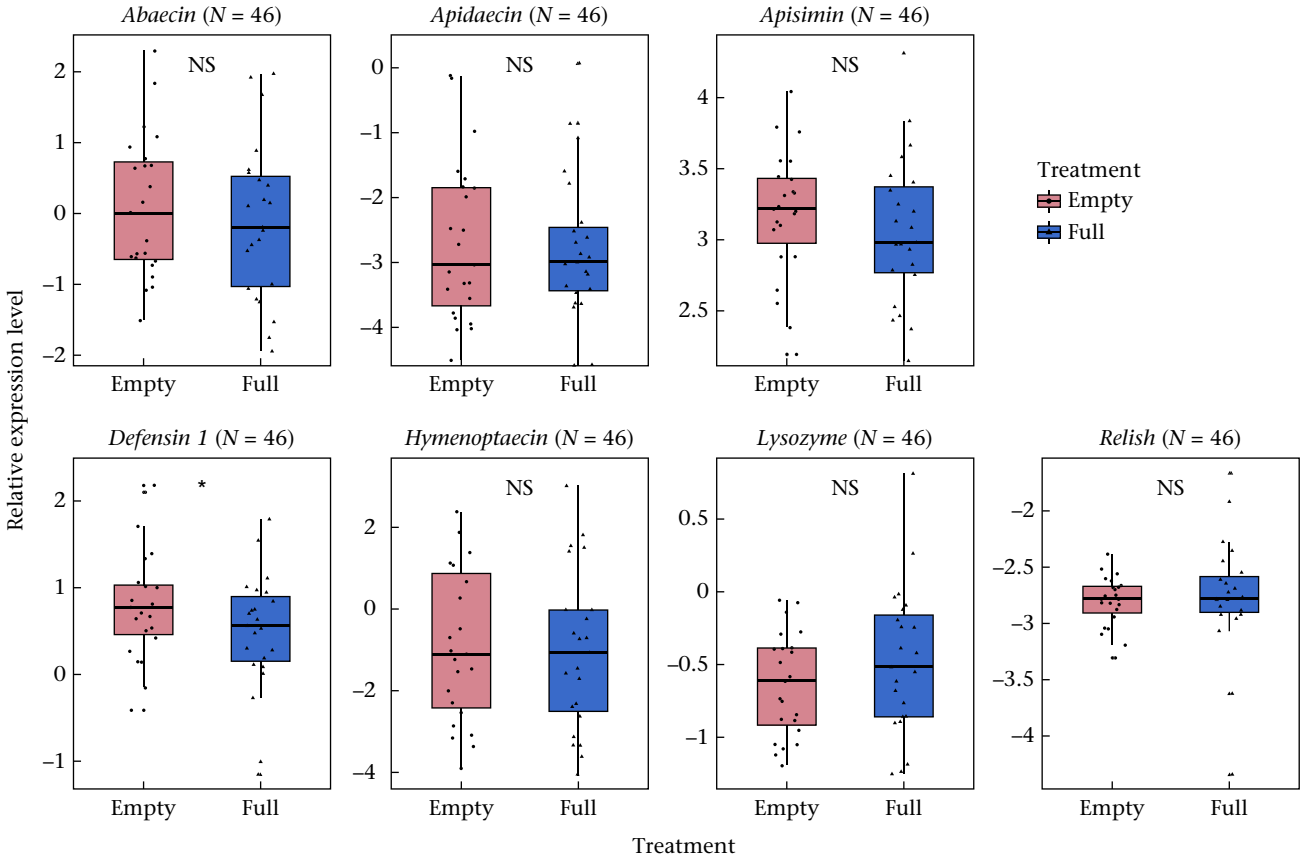

Figure 3. Expression of the seven tested immune genes in hives with empty and full honey stores. Box plots show the median, 25th and 75th quartiles and the 5th and 95th percentiles. *$P \leq 0.05$.

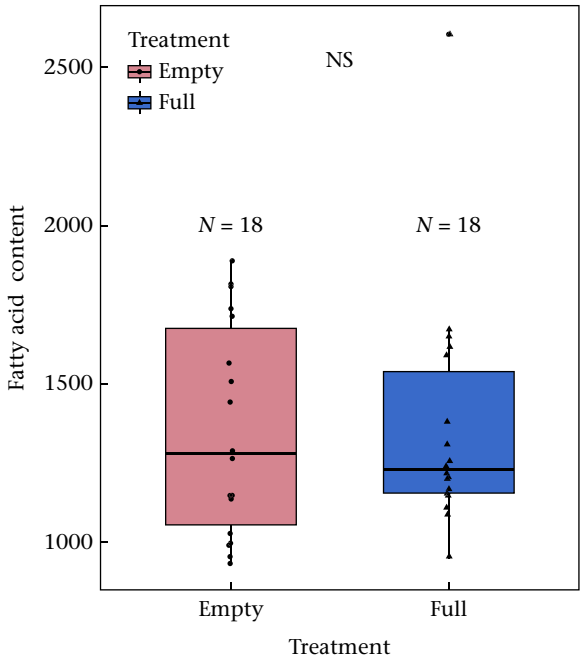

Figure 4. Absolute quantity (μg) of fatty acids in bee abdomens in hives with empty and full honey stores. Box plots show the median, 25th and 75th quartiles and the 5th and 95th percentiles.

honey stores (Fig. 1); thus, workers were providing more information about foraging opportunities to nestmates under these conditions. Furthermore, dancing increased during our experimental period. Several reasons could explain this finding. First, starvation causes bees to start foraging at a younger age, which may temporarily increase the forager pool (Schulz et al., 1998, 2002). Furthermore, foraging activity increased after honey removal in both stingless bees (*Melipona fasciculata*) and honey bees (Gostinski et al., 2017; Schulz et al., 1998, 2002), suggesting that foragers may have switched to a more proactive foraging strategy when honey stores are depleted. In addition, increased storage space reduced the production of stop signals, which inhibit waggle dancing (Kietzman & Visscher, 2021). These processes, possibly acting in concert, could explain why we found more dances after the experimental reduction in honey stores. Rinderer (1982) found that empty cells could be used as an information cue by foragers to assess the nutritional state of the colony and thereby affect the number of dances. At a physiological level, the perception of low honey stores is likely to modulate the sucrose response threshold of foragers, which is linked to a range of foraging- and communication-related behaviours, such as learning(Scheiner et al., 2003, 2004), foraging division of labour (Pankiw & Page Jr, 2000) and dancing (George et al., 2020).

We did not find a difference in the average duration of waggle dances, which often correlates with dance motivation (von Frisch, 1967; Seeley et al., 2000). This might indicate that our treatment did not increase average dance motivation. However, if low food stores caused bees that would not normally dance to perform short dances, average duration of all dances might remain constant despite a general increase in dance motivation in the forager population. Overall, our results indicate that starved colonies are likely to become less selective than colonies with full honey stores. We found no effect of honey removal on foraging distances indicated by dancing bees; colonies foraged at an average distance of ~0.76 km (starved) to ~0.88 km (full). Colonies will often collect nectar and pollen at greater distances if food sources are scarce (Couvillon et al., 2014; Ohlinger et al., 2022), but since our treatment did not affect food source abundance and bees will often return to the same food sources for several days (Butler et al., 1943; von Frisch, 1967; Al Toufailia, Gruter et al., 2013), a lack of an effect of honey stores on foraging distances is not surprising.

The number of followers per dance decreased by ~20% when colonies were honey depleted, suggesting that fewer foragers relied on spatial information about resources, which is consistent with foragers switching to a proactive, solitary foraging strategy (I'Anson Price et al., 2019). An alternative explanation is that forager interest in dances remained constant, but individual bees had more options to follow waggle dances since there were more dances overall, leading to a reduction in dance followers per dance. As a result of this reduction in dance follower number and the concurrent increase in dance number, starved colonies might spread out and exploit a larger number of different food sources. Our finding contrasts somewhat with Rinderer (1982) who found that dancers had more followers in colonies with more available storage space. However, Rinderer (1982) manipulated storage space rather than honey stores, which could explain the different outcomes.

Food stores had no effect on the number of waggle phases that followers followed. The number of waggle phases a bee follows is an indication of how a bee uses the dance information. Longer dance following suggests bees try to decode the vector information, whereas shorter dance following indicates that bees are using it for reactivation or confirmation that a previously visited food source is still available (Biesmeijer & Seeley 2005; Grüter & Ratnieks 2011; Grüter et al., 2013). On average, interest in spatial information seems to have remained constant in those bees that did follow dances.

We found that the expression of the immune-related gene *defensin 1* increased when colonies had low honey stores, while the other tested genes did not respond to our treatment in the time window we studied. *Defensin 1* plays an important role in social immunity, it regulates AMPs expression and affects antimicrobial and antifungal activity (Ilyasov et al., 2013). Previous studies found that low pollen reserves in hives increased the expression of *defensin 1* in honey bees, and increased expression was a reliable predictor for colony survival over winter (Barroso-Arévalo et al., 2019; Corona et al., 2023). An increased *defensin 1* expression suggests an increased investment in immunity in response to nutritional stress. In turn, *defensin 1* expression can be used to monitor the health of honey bee colonies (Barroso-Arévalo et al., 2019). The other tested genes (*abaecin, apidaecin, apimisin, relish, hymenoptaecin* and *lysozyme*), which can be activated by abundant pollen, play crucial roles in individual immunity against a broad range of microorganisms, including bacteria, fungi, parasites and viruses (Castelli et al., 2020; Corona et al., 2023; Danihlík et al., 2018). One possible explanation for a lack of a treatment effect on the expression of these genes might be that they are activated in response to pollen availability rather than honey stores.

Previous studies have found that fat stores predict overwintering survival and foraging activity in honey bees (Döke et al., 2015; Toth et al., 2005; Toth & Robinson, 2005). The composition of fatty acids can be affected by diet; for instance, linoleic acid can only be acquired through the diet (Arien et al., 2020; Rosumek et al., 2017). However, the relative proportions of fatty acids and total fatty acid content did not differ between treatments after 6 days of honey depletion (Fig. 4). It is possible that body fat content and composition depend more on the pollen diet of bees, the main source of lipids (Brodschneider & Crailsheim, 2010), rather than on honey. Accordingly, pollen lipid profiles affected the amount of total fatty acids and essential fatty acids in bees (Arien et al., 2020). Alternatively, our experimental period of 6 days may have been too short to cause changes in fat stores. Physiological changes do not

happen as quickly as behavioural changes, which are often the first response to environmental changes (Wong & Candolin, 2015). Our findings suggest that body fat stores are unlikely to change in response to brief periods of low nectar availability, for example those caused by several days of bad weather. More studies of fatty acid content in pollinators are needed to better understand how fat stores relate to landscape level changes, nutritional challenges and health. Nutritional stress is likely to have varied impacts on behaviour, the immune system and the physiology of pollinators, with knock-on effects for their survival. Our study is a step towards a better understanding of how nutritional stress caused by honey depletion influences foraging behaviours, the expression of immune-related genes and physiological changes.

Author Contributions

Yongqiang Wu: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Funding acquisition, Formal analysis, Data curation. **Tianfei Peng:** Writing – review & editing, Methodology, Funding acquisition, Data curation. **Florian Menzel:** Writing – review & editing, Methodology, Formal analysis. **Christoph Grüter:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Formal analysis, Conceptualization.

Data Availability

The data set used for this study is available in the Supplementary Material.

Declaration of Interest

We declare no conflict of interest for this study.

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Supplementary Material

Supplementary material associated with this article is available in the online version at https://doi.org/10.1016/j.anbehav.2024.07.017.

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Appendix

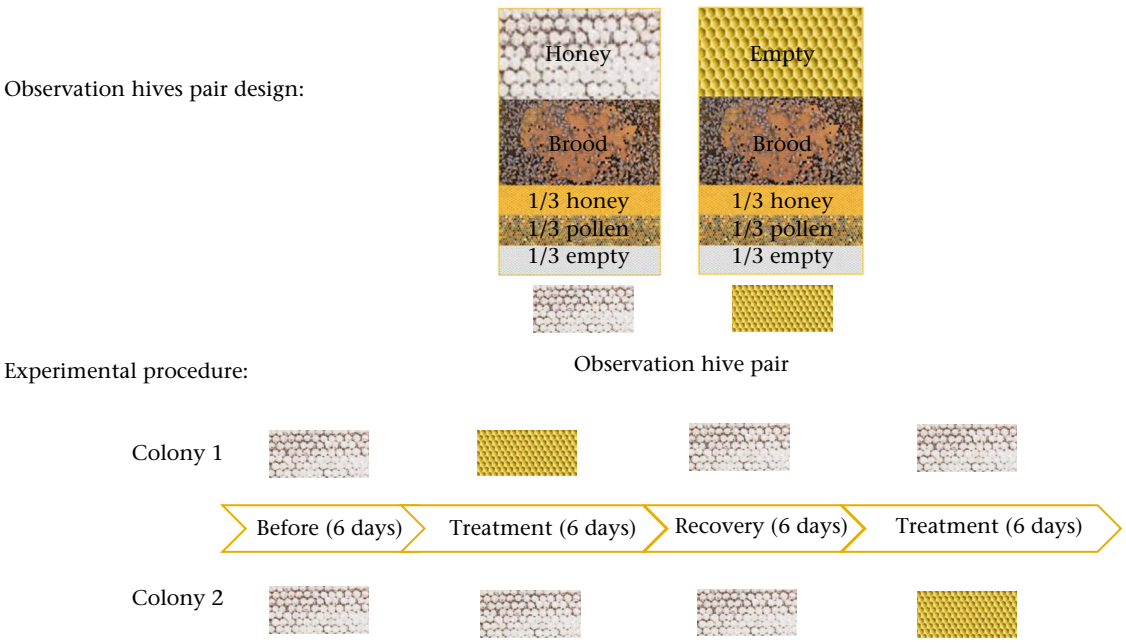

Figure A1. (a) The paired design with two observation hives. Each hive had a honey store frame, a brood frame and a mixed frame with honey, pollen and empty space. (b) Experimental procedure. The hives were first left for 6 days. Then we replaced the top honey frame in one hive with an empty frame (EH treatment) and in the other with a full honey frame from another colony (FH treatment) The treatments lasted 6 days. After another 6-day recovery period with a full honey frame in both hives, we reversed the treatments for another 6 days.