Behavioural, physiological and molecular changes in alloparental caregivers may be responsible for selection response for female reproductive investment in honey bees

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Abstract:

Reproductive investment is a central life history variable that influences all aspects of life. Hormones coordinate reproduction in multicellular organisms, but the mechanisms controlling the collective reproductive investment of social insects are largely unexplored. One important aspect of honey bee (*Apis mellifera*) reproductive investment consists of raising female-destined larvae into new queens by alloparental care of nurse bees in form of royal jelly provisioning. Artificial selection for commercial royal jelly production over 40 years has increased this reproductive investment by an order of magnitude. In a cross-fostering experiment, we establish that this shift in social phenotype is caused by nurse bees. We find no evidence for changes in larval signalling. Instead, the antennae of the nurse bees of the selected stock are more responsive to brood pheromones than control bees. Correspondingly, the selected royal jelly bee nurses are more attracted to brood pheromones than unselected control nurses. Comparative proteomics of the antennae from the selected and unselected stocks indicate putative molecular mechanisms, primarily changes in chemosensation and energy metabolism. We report expression differences of several candidate genes that correlate with the differences in reproductive investment. The functional relevance of these genes is supported by demonstrating that the corresponding proteins can competitively bind one previously described and one newly discovered brood pheromone. Thus, we suggest several chemosensory genes, most prominently OBP16 and CSP4, as candidate mechanisms controlling queen rearing, a key reproductive investment, in honey bees. These findings reveal novel aspects of pheromonal communication in honey bees and explain how sensory changes affect communication and lead to a drastic shift in colony-level resource allocation to sexual reproduction. Thus, pheromonal and hormonal communication may play similar roles for reproductive investment in superorganisms and multicellular organisms, respectively.

Keywords: artificial selection response | brood care | EAG | larval pheromones | life history syndrome | reproduction | social evolution | social phenotype

Article:

1 INTRODUCTION

The cost of reproduction leads to central life-history tradeoffs and the evolutionary optimization of the timing and magnitude of reproductive effort is generally well documented (Stearns, 1989). Systemic hormones control and coordinate the reproductive effort in multicellular organisms, communicating between cells and tissues (Ketterson & Nolan, 1992). However, across other levels of biological organization, the mechanisms that control the investment in reproductive functions are poorly understood (Barban et al., 2016). Colonies of honey bees rely on cooperation between one reproductive queen and her functionally sterile workers that collectively determine reproductive investment (Pratt, 1998). Honey bees and other social insects thus harbour a unique level of complexity that relies on communication and interactions among colony members, which turns reproduction into a complex social phenotype.

To initiate female reproduction, colonies of the Western honey bee (*Apis mellifera*) typically raise 12–24 new queens by differential feeding of female, bipotent eggs that are laid in enlarged, vertical cells (Tarpy, Gilley, & Seeley, 2004). Brood food is provided by young workers (=nurse bees) in the form of proteinaceous royal jelly (RJ), secreted from their hypopharyngeal and mandibular glands (Fujita et al., 2013). Quantity, quality, and timing of RJ feeding determine hormonal signalling and epigenetic patterning that control regulatory gene networks and canalize larval development into either of the widely differing worker or queen phenotypes (Chen et al., 2012; Hartfelder & Emlen, 2005; Kucharski, Maleszka, Foret, & Maleszka, 2008; Wheeler, Buck, & Evans, 2006), but the exact mechanisms remain controversial (Maleszka, 2018). At the phenotypic level, worker-destined larvae are switched from RJ to a poorer diet after 3 days by nurse bees, while queen-destined larvae continue to be fed RJ ad libitum in their larger, vertically-oriented cells (Haydak, 1970). Experimental grafting of young female larvae into artificial queen cells that are transferred into a queenless colony can produce new queens (Laidlaw & Page, 1997). However, little is known about how the reproductive effort of raising a certain number of new queens is regulated.

All brood care in honey bee colonies is performed by nurse bees that provide alloparental care to their sisters, raising female larvae mostly into new workers and sometimes into new queens (Tarpy et al., 2004). Pheromones emitted by the brood are the primary signals of larvae to communicate their needs to the nurses (Slessor, Winston, & Conte, 2005). Brood ester pheromone (BEP) is composed of 10 methyl and ethyl esters of straight-chain fatty acids that serve in workers' recognition of brood and can trigger feeding or cell capping (Le Conte, Arnold, Trouiller, Masson, & Chappe, 1990; Le Conte, Sreng, & Poitout, 1995). Individual esters or ester subsets also have some biological activity (Slessor et al., 2005). Additionally, β-ocimene, a highly volatile component, is emitted by larvae to modulate honey bee behavioural maturation and nursing behaviour (He et al., 2016; Maisonnasse, Lenoir, Beslay, Crauser, & Le Conte, 2010; Traynor, Le Conte, & Page, 2015). These brood signals are mainly perceived by the

antennae (Robertson & Wanner, 2006), the central olfactory organ of all insects, in which odourant transduction occurs within a few milliseconds from the boundary layer of the antenna to olfactory receptor neurons (Leal, 2013; Szyszka, Gerkin, Galizia, & Smith, 2014).

Many proteins are involved in olfaction (Leal, 2013), including odourant binding proteins (OBPs), chemosensory proteins (CSPs), and odourant receptors (ORs). OBPs and CSPs are small soluble proteins that are located in the fluid surrounding the sensory dendrite and contribute to the sensitivity of the olfactory system by transporting odourants through the sensillar lymph to ORs (Leal, 2013; Pelosi, Calvello, & Ban, 2005). CSPs are comparatively poorly understood components in the peripheral chemosensory system (Li et al., 2015). In many cases, the ORs are the primary determinant of the odour response spectrum of the olfactory receptor neurons (Wanner et al., 2007). The annotation of 21 OBPs, 6 CSPs and 170 ORs has contributed some understanding of the peripheral olfactory processing of honey bees (Foret, Wanner, & Maleszka, 2007; Robertson & Wanner, 2006). Some information on central olfactory processing in honey bees exists (Galizia, Sachse, Rappert, & Menzel, 1999) but most studies of honey bee behavioural variation have implicated the peripheral nervous system (Guarna et al., 2015; Mondet et al., 2015; Sánchez-Gracia, Vieira, & Rozas, 2009) or endocrine organs (Page, Rueppell, & Amdam, 2012; Ronai et al., 2015). Affinity and functional assays have established biological function for few CSPs in honey bees, such as OBP1 binding to queen mandibular pheromone (Danty et al., 1999) and OR11 as a specific receptor of 9-oxo-2-decenoic acid (Wanner et al., 2007). However, most OBPs, CSPs, and the numerous ORs have yet undescribed functions and correspondingly the molecular receptors of most honey bee pheromones have not yet been discovered.

The regulation of new queen rearing has drastically changed in a stock of honey bees that has been selected from Italian bees (ITBs, *A. mellifera* ligustica) since the 1980s in China for improved RJ production. These royal jelly bees (RJBs) exhibit strong, heritable differences in RJ production (Li, Chen, Zhong, & Su, 2003a). The RJBs accept more larvae to be raised as new queens than ITBs (Han et al., 2017) and rear artificially grafted larvae into queens even in the presence of a fertile adult queen. RJB nurses have larger acini size in the hypopharyngeal glands accompanied by more abundant proteins related to protein synthesis, cytoskeleton, and carbohydrate metabolism (Li, Feng, Begna, Fang, & Zheng, 2010) and enhanced mandibular glands (Huo et al., 2016). Therefore, some mechanisms that enable increased brood care by RJB nurses have been elucidated. However, the proximate causation of the social phenotype of the RJBs has not yet been sufficiently studied. Specifically, it is unclear whether signallers (brood) or receivers (nurses) of this social communication system changed and which particular individual properties were modified in the RJBs to increase reproductive investment at the colony level.

To explore how queen production, a key component of the reproductive investment of social insects, is regulated in honey bee colonies, we compared behaviour, sensory physiology, and molecular biology of ITBs and RJBs. Multiple, mutually corroborating results suggest that olfactory differences in nurse bee antennae play a key role in the evolution of the commercially important RJB syndrome, a model for the study of the regulation of reproductive investment through alloparental care in social insects.

2 MATERIALS AND METHODS

Two different populations of the Western Honey Bee, *A. mellifera* L., were compared: Unselected Italian Honey Bee (ITB) queens from a commercial breeder in California, USA, and selectively bred High RJB queens from the Pinghu honeybee breeding station, Zhejiang Province, China, were introduced into existing honey bee colonies at the Institute of Apicultural Research, Chinese Academy of Agricultural Science.

2.1 Cross-fostering study

In a reciprocal cross-fostering experiment, larvae were grafted from ITB and RJB sources into ITB and RJB host colonies. One frame with 132 newly-grafted larvae was placed into the upper, queenless box of two-story experimental hives. Approximately 70 hr after larval grafting, the frames were removed from their colonies to count cells that contained a viable larva. The RJ from all cells was then collected and weighed with an electronic balance (AL204-IC; Mettler Toledo). Three ITB and three RJB host colonies were used in six rounds of larval grafting from three ITB and three RJB sources so that each of the source colonies was grafted once into each host colony. Data on individual cell acceptance probability were analyzed with a binary logistic regression, assessing the simultaneous effects of individual host colony and larval graft source, while the influence of host colony and graft source on pooled RJ weight was assessed with GLM in spss v.21 (IBM).

2.2 Measurement of volatile chemicals emitted by larvae and RJ

Volatiles of 1-day-old larvae (between 24 and 36 hr old) were compared between ITB and RJB because this age of larvae is relevant, representing the grafting age in commercial RJ production (Li & Wang, 2005). An auto-sampler GC-MS system (GCMS2010; SHIMADZU) was used in combination with a solid phase micro-extraction (SPME) fibre (DVB/CAR/PDMS) to collect and identify the volatiles. Given that the larvae cannot be maintained without RJ, we analyzed larvae with RJ compared to RJ alone to identify the volatiles of larvae. Four samples of RJ and five of RJ + larvae were analyzed from ITBs and four and six corresponding samples from RJBs. For each sample, the content of 25 cells was collected randomly from three colonies of either ITB or RJB. Pooled larvae were transferred immediately into a 20 ml airtight glass tube and kept at 35°C for 15 min. A 3 µl aliquot of n-heptadecane (25 µg/ml in n-hexane) was added as internal standard (Maisonnasse et al., 2009; Zhao et al., 2016). The remaining RJ was collected from the same cells and analyzed separately. The SPME fibre was conditioned for 7 min in the GC injector port at 250°C, inserted into the headspace of the samples for 30 min at 35°C, and then thermally desorbed at 265°C for 1 min (Zhao et al., 2016). The capillary column DB-5 (30 m × 0.25 mm × 0.25 µm) was initially kept at 35°C for 1 min, heated to 200°C at 5°C/min, and kept at 200°C for 2 min. It was then further heated to 250°C at 15°C/min and kept at 250°C for 2 min. Ultrapure helium was used as a carrier gas with a constant flow rate through the column of 1 ml/min. The temperature of the ion source was kept at 230°C and that of the transmission line was 270°C. Solvent delay was set at 3.5 min. The ionization energy was set to 70 eV with continuous scans from a mass to charge ratio (m/z) of 40–400.

Data were processed using the XCMS metabolomics platform (http://xcmsonline.scripps.edu) with the matched filter algorithm for feature detection (Tautenhahn, Patti, Rinehart, & Siuzdak, 2012). The peak area of each compound was normalized based on the internal standard. Compounds were identified by comparison to the NIST14 and retention index (RI) matching. For RI matching, C8-C30 n-alkanes dissolved in n-hexane were separated under the same conditions as described above. RI was calculated based on retention time of each individual peak (Zhao et al., 2016). Differences between ITB and RJB in the relative quantity of each compound were tested by one-way ANOVA.

2.3 Nurse bee antennal responsiveness to brood signals

The responsiveness of ITBs and RJBs to β-ocimene, allo-ocimene, and BEP, a blend of the 10 fatty aliphatic esters (Le Conte et al., 1989), was quantified in electroantennogram (EAG) assays with a Syntech EAG system equipped with micromanipulator-12 (Syntech). The antennae of nurse bees with clipped tips were connected to chloridized silver wires in glass capillaries filled with 0.1% KCl and 0.5% polyvinylpyrrolidone (Pelletier, Guidolin, Syed, Cornel, & Leal, 2010). Tested compounds (Table S9) were diluted in mineral oil, applied to filter-paper strips, and inserted into Pasteur pipettes. All compounds were tested at four concentrations over four orders of magnitude that were deemed biologically most relevant (Maisonnasse et al., 2009): β-ocimene, allo-ocimene and liquid components of BEP (Le Conte et al., 1990) were used at 100%, 10%, 1%, and 0.1% (*v*/*v*), and solid reagents of BEP were used at 100, 10, 1, and 0.1 µg/µl. The odours were transmitted to the antennae in a high humidity air flow (500 ml/min). Response scores were calculated relative to solvent responses before and after stimulus presentation (Guo & Li, 2009). Approximately 45 antennae sampled randomly from three colonies of each stock (exact sample sizes are given in Figure 3) were measured at 30°C. Results were evaluated separately for each pheromone by a full-factorial general linear model, followed by separate post-hoc tests with Bonferroni correction for multiple testing in spss v.21 (IBM).

Response time of the olfactory system of ITBs and RJBs to the tested compounds was calculated by subtracting the arrival time of the stimulus from the time of the EAG response. The arrival time was determined with a photodiode, a green laser, and $TiCl_4$ smoke as tracer substance. The reflectance of the $TiCl_4$ smoke that was mixed into the odour was recorded with a photodiode (Szyszka et al., 2014). The EAG procedures and analyses were performed as described as above.

2.4 Behavioural attraction tests of larval signals in ITBs and RJBs

The attractiveness of larval signals to ITB and RJB nurses was tested in a three-chambered testing arena, built from airtight acrylic boards with outer dimensions of 40 cm × 10 cm × 10 cm. The central chamber (20 cm × 10 cm × 10 cm) was separated from the two side chambers by removable acrylic dividers that were perforated with dozens of 1 mm holes (Figure S3). For each trial, groups of 300 nurse bees (100 from each of three colonies of either RJBs or ITBs) were put in the central chamber with the test substance and solvent control in each of the side chambers. We tested the chemicals β-ocimene, allo-ocimene, the BEP blend, and a 1:1:1 (*v*/*v*/*v*) mixture of these three pheromones. All compounds were diluted to 10% (*v*/*v*) in mineral oil. These high concentrations were chosen after initial pilot studies had revealed that lower doses were not sufficiently effective in the laboratory cage context. After 3 min, the numbers of bees within

1 cm of the partition on either side of the central chamber were counted. Then, the partitions were removed and bees within 2 cm of the odour sources were counted after an additional 3 min interval. Tests for each substance were repeated in six independent trials involving bees from either three ITB or three RJB colonies. To reduce the potential effect of cage direction, the chemicals and the solvent control were alternatingly put in the left and right side. The experiment was executed in a closed room with artificial overhead lights at 30°C. The combined data from all 48 tests with dividers and afterwards without dividers were analyzed separately with general linear models, followed by Tukey's post-hoc tests when appropriate, in spss v.21 (IBM) to test the effect of bee source (ITB vs. RJB) and compound (β-ocimene, allo-ocimene, the BEP blend, and the mixture of all compounds) on the number of attracted individuals.

2.5 Comparison of antennal proteome of nurse bees

Antennal proteomes of ITB and RJB nurse bees were compared following previously established procedures (Hu et al., 2016). Approximately 180 pairs of antennae of nurse bees were randomly sampled from three ITB and three RJB colonies (each colony was represented with about 60 pairs) and divided into three ITB and RJB pools. Antennae were immediately homogenized in liquid nitrogen. Proteins were extracted using lysis buffer (8 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris-base, 30 mM dithiothreitol, and protease inhibitors [Roche]), and were digested using sequencing grade modified trypsin (Promega) at 37°C overnight. To reduce sample complexity and identify as many proteins as possible, the digested peptides were fractioned using Pierce High pH Reversed-Phase Peptide Fractionation Kit (ThermoFisher Scientific) by hydrophobicity prior to LC/MS analysis following the manufacturer's protocol. Each of the six pooled samples (three ITB and three RJB) was divided into seven fractions. LC-MS/MS was run on a Q-Exactive HF mass spectrometer coupled to a two-column EASY-nLC 1,200 nano-flow system (Thermo-Fisher Scientific).

The RAW data of the LC-MS/MS were retrieved with Xcalibur 3.0 (Thermo-Fisher Scientific), and were deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD011149. Protein identification was performed with the PEAKS search engine (v8.5; Bioinformatics Solutions Inc.) against the protein database of *A. mellifera* (downloaded from NCBI in April 2017) that contained 22,575 entries after incorporation of common contaminants. The search parameters were: parent ion mass tolerance = 15.0 ppm; fragment ion mass tolerance = 0.05 Da; enzyme = trypsin; max missed cleavages = 2; and maximum allowed variable post translational modification per peptide = 3. Statistical evaluation for identification of proteins and petide levels was performed with an FDR threshold of 0.01, using the fusion-decoy database search strategy. Identifications were accepted when at least two unique peptides matched to a protein that was present in all three replicates. The relative abundance levels of proteins in the antennae were quantified by the label-free approach in peaks q module (Meng et al., 2018). Triplicates of RJB or ITB antennal samples were analyzed. Peptide features (defined by *m*/*z*, retention time, and mobility of a peptide) were detected independently in each sample using the expectation-maximization algorithm. The features of the same peptide from three replicates of each sample were reliably aligned, using the high-performance retention time alignment algorithms embedded in the software. Protein abundance was normalized by dividing the total ion current (TIC) multiple by the TIC of the automatically selected reference sample. The abundance levels of proteins were calculated by

summing the three highest peaks of peptides corresponding to the particular protein. A protein was considered significantly different between the ITB and RJB samples only with a fold-change of ≥2 and a significance of multiple-testing corrected <0.05, as implemented in the peaks q module.

clue-go v2.1.7, a Cytoscape plug-in (http://www.ici.upmc.fr/cluego/), was used to test for functional category and pathway enrichment among the different protein sets to interpret the biological implications of the differences in antennal proteins between ITBs and RJBs. The identified proteins were compared to the entire functionally annotated genome of *A. mellifera*. Significantly enriched functional GO categories and pathways in biological processes were determined by right-sided hypergeometric tests, followed by a Bonferroni step-down correction for multiple testing. Nodes in functionally grouped networks were linked based on their kappa score level (0.4). GO tree levels ranged from three to eight, and GO term were restricted to group with a minimum of three genes that covered a minimum of 4% genes.

2.6 Candidate gene sequence and expression analysis

Based on the results of the proteome comparison, candidate genes related to odourant binding (n = 6), chemosensory (n = 2), and hydrogen ion transmembrane transport and oxidative phosphorylation (n = 12) were selected for further analysis. In addition, we chose to also further analyze the remaining 13 OBPs, four CSPs, and two odourant receptor proteins. Pools of nurse bee antennae were collected from three ITB or three RJB colonies (60 pairs of antennae per colony for a total of 180 per replicate). Three biological replicates were analyzed for ITBs and RJBs, each with two technical replicates. Total RNA was extracted with TRIzo (Invitrogen), including a DNA digestion using the Ambion DNA-Free kit, and quantified using a NanoDro 2000 spectrophotometer (Thermo-Fisher Scientific). RNA integrity was verified on a 1% agarose gel. First-strand cDNA was synthesized from 1 µg of total RNA using PrimeScript RT reagent Kit (TakaRa) with oligo dT primer and random hexamers in 20 µl per reaction.

cDNA of all odourant binding and CSPs was amplified to search for sequences differences between ITBs and RJBs. The primers were designed with primer premier 5.0 software based on NCBI reference sequences (Table S10). PCRs were performed with Taq polymerase (TakaRa). The product was purified with the MiniBEST agarose gel DNA extraction Kit Ver 3.0 (TakaRa), cloned into a pMD-T 18 vector, and grown in liquid LB medium of *Escherichia coli*. Five independent clones of each gene of the ITB and RJB libraries were sequenced by standard Sanger sequencing by Sangon Biotech.

Additionally, qRT-PCR was used to compare the expression of candidate genes between ITBs and RJBs. The primers were designed with primer premier 5.0 software for annealing temperatures of 56–65°C, amplicon sizes of 80–300 bp, and a minimum and maximum GC content of 40% and 70% (Table S10). Two pairs of genes, OBP6/8 and OBP19/20, had to be analyzed together due to their sequence similarity. qRT-PCR was performed by using Bio-Rad iQ5 (Bio-Rad) and the SYBR Premix Ex Taq II Kit (TakaRa). Expression levels of analyzed genes were calculated as the average of both technical replicates using $2^{-\Delta\Delta CT}$ of RJBs vs. ITBs (Livak & Schmittgen, 2001), normalized by two references genes, ribosomal protein S8 (Foret & Maleszka, 2006) and ribosomal protein S18 (Scharlaken et al., 2008).

2.7 Binding assays of recombinant olfactory proteins and larval volatiles

To measure the binding force between putative olfactory proteins and brood chemicals, the OBP and CSP genes without signal peptide were subcloned into the prokaryotic expression vector pET-30a (+) and expressed in *E. coli*, BL21 (DE3) strain. Recombinant proteins were induced by 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) in LB medium. The cloned proteins were purified by Ni^{2+}-NTA agarose chromatography (TransGen Biotech) and dialyzed with PBS (pH 7.4). The concentration of the proteins was quantified with a Bradford assay using the Multiskan Go (Thermo-Fisher Scientific). The competitive binding affinity between proteins and tested ligands was measured on a Horiba Scientific Fluoromax-4 spectrofluorometer. The purified proteins were dissolved in 20 mM PBS (pH 7.4) at a final concentration of 1 μM. The fluorescent probe N-phenyl-1-naphthylamine (1-NPN) was excited at 337 nm, and emission was recorded between 380 and 480 nm. The ligands were dissolved at 1 mM in methanol and added to the protein solution. Competitive binding was measured by titrating a solution of protein and 1-NPN with 1 mM ligand in methanol. Dissociation constants for 1-NPN were calculated using Graph Pad Prism (Zhu et al., 2017). Comparative dissociation constants between olfactory proteins and ligands were calculated by the equation $K_d = IC50/(1 + [1\text{-NPN}]/K_{1\text{-NPN}})$, where IC50 is the concentration of ligands halving the initial fluorescence value of 1-NPN, [1-NPN] is the free concentration of 1-NPN, and $K_{1\text{-NPN}}$ is the dissociation constant of the protein/1-NPN complex. Experiments were performed in triplicates, except for ligands showing no significant binding (Figure 7 and Figure S2).

3 RESULTS

3.1 Cross-fostering indicates nurse bee differences

To investigate the effects of brood and nurse bees on the difference in queen rearing behaviour between the ITB and RJB colonies (Figure 1a), we reciprocally cross-fostered larvae grafted into queen cells. In the ITB colonies, queen cells containing ITB larvae (36.45 ± 3.09%) and RJB larvae (34.54 ± 3.88%) were overall less accepted (Figure 1b) than queen cells in RJB colonies containing ITB larvae (91.84 ± 1.35%) or RJB larvae (92.59 ± 1.46%). Grafts in all three RJB colonies were significantly more likely to be accepted than grafts in any of the ITB colonies (all hazard ratios >20, $n = 4{,}752$, all $p < .001$) but ITB colonies relative to each other and RJB colonies relative to each other were not significantly different (all hazard ratios >0.94 and <1.3, $n = 4{,}752$, $p > .33$). The identity of the grafted larvae did not significantly influence the probability of their acceptance (all hazard ratios between and among ITB and RJB source colonies were >0.98 and <1.2, $n = 4{,}752$, all $p > .75$). In the ITB colonies, RJ provisioning of ITB (9.28 ± 2.41 g) and RJB (8.87 ± 2.17 g) larvae was also significantly lower (Figure 1c, $F(1,8) = 2{,}095$, $p < .001$) than of ITB (82.73 ± 5.86 g) and RJB (84.85 ± 5.92 g) larvae in RJB colonies (Table S1).

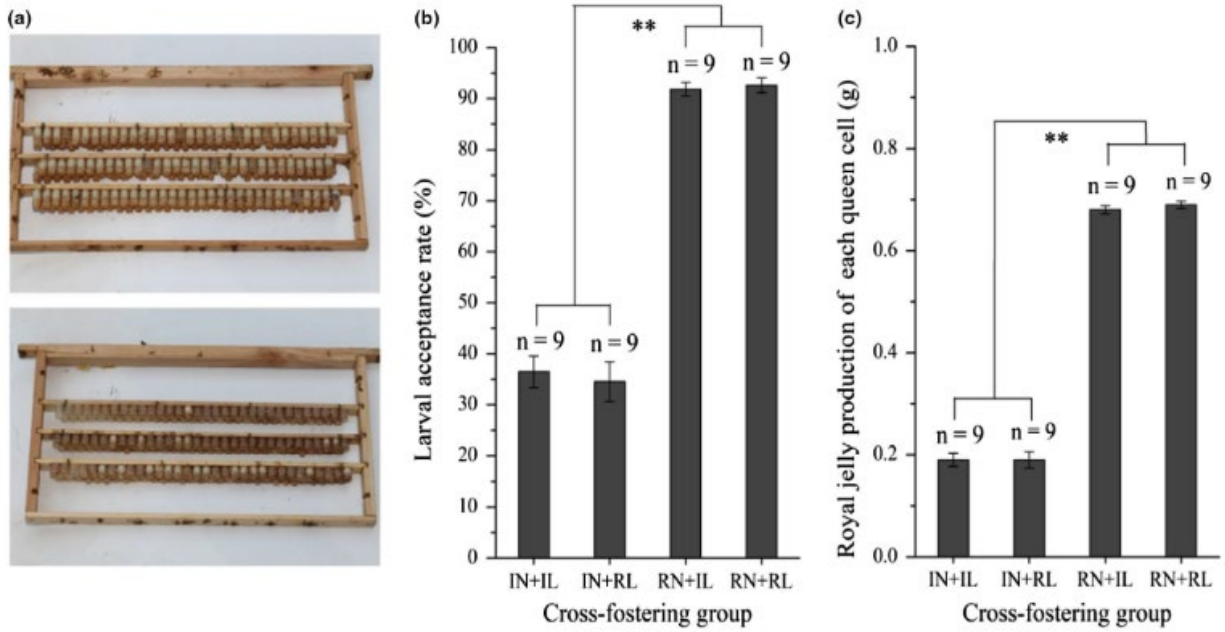

Figure 1. Cross-fostering of queen larvae demonstrates colony effect. (a) The number of queen cells raised from artificially grafted larvae is much higher in royal jelly bee (RJB; top frame) than in Italian honey bee (ITB; bottom frame) colonies, regardless of larval genotype. Accordingly, (b) larval acceptance rate and (c) royal jelly provisioning per cell were significantly higher in colonies of RJBs than in ITBs ("**": $p < .001$), but the identity of the grafted larvae did not affect either variable ($p > .05$) and there were no significant interaction effects. Mean ± *SD* is presented. IL, ITB larva; IN, ITB nurse bee; RL, RJB larva; RN, RJB nurse

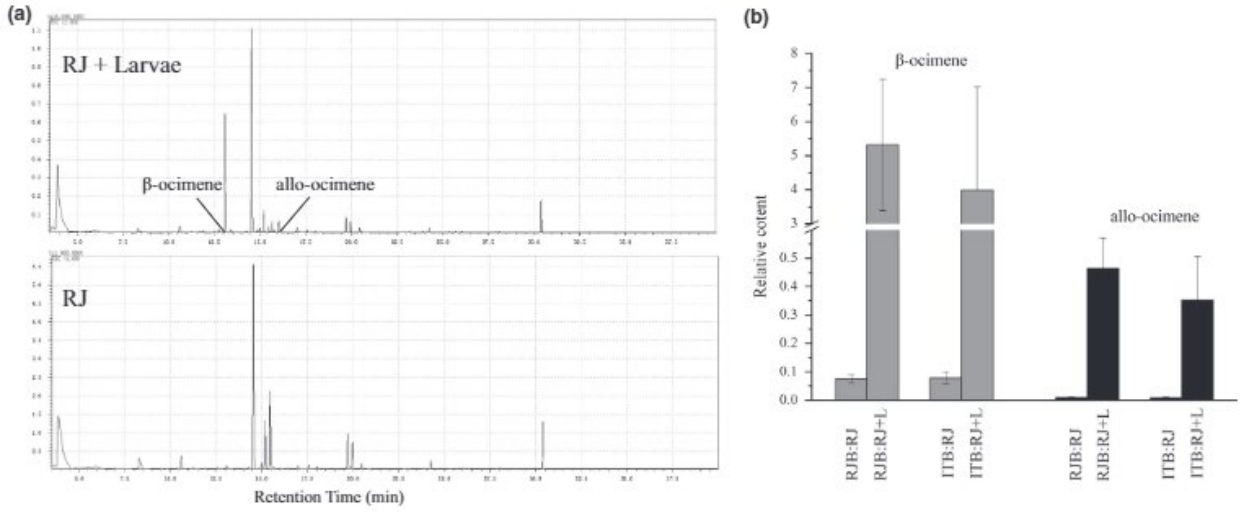

Figure 2. Volatiles of larvae and royal jelly of Italian honey bees (ITBs) and royal jelly bees (RJBs). (a) GC-MS analysis of volatiles from larvae with RJ and from RJ alone identified compounds that were larvae-specific due to large quantitative differences in their relative abundance (arrows). A new putative pheromone, allo-ocimene, was identified. (b) Relative abundance of β-ocimene (grey) and allo-ocimene (black) from larvae with RJ and RJ only of ITBs and RJBs. Samples with larvae contained significantly higher amounts of β-ocimene ($F(1,15) = 26.3$, $p < .001$) and allo-ocimene ($F(1,15) = 72.8$, $p < .001$) than RJ samples but there were no significant differences between ITBs and RJBs or interaction effects between larval presence and stock (all $p > .05$). Mean ± *SD* is presented in each bar. L, larvae; RJ, royal jelly

3.2 Volatiles of ITB and RJB larvae are similar and include novel putative pheromone

Headspace compounds were compared between ITB and RJB samples from 1-day-old larvae in RJ and from RJ alone by solid phase microextraction and GC-MS analysis. Overall, seven compounds were identified: acetoin, 2-heptanone, β-ocimene, 2-nonanone, 2,6-dimethyl-2,4,6-octatriene (allo-ocimene), octanoic acid, and 2-decanone (Table S2). These compounds were present in all samples and no significant differences between ITB and RJB samples were found. However, β-ocimene ($F(1,15) = 26.3$, $p < .001$) and the newly-discovered allo-ocimene ($F(1,15) = 72.8$, $p < .001$) were significantly more abundant in the headspace of larvae with RJ than in the headspace of RJ alone (Figure 2).

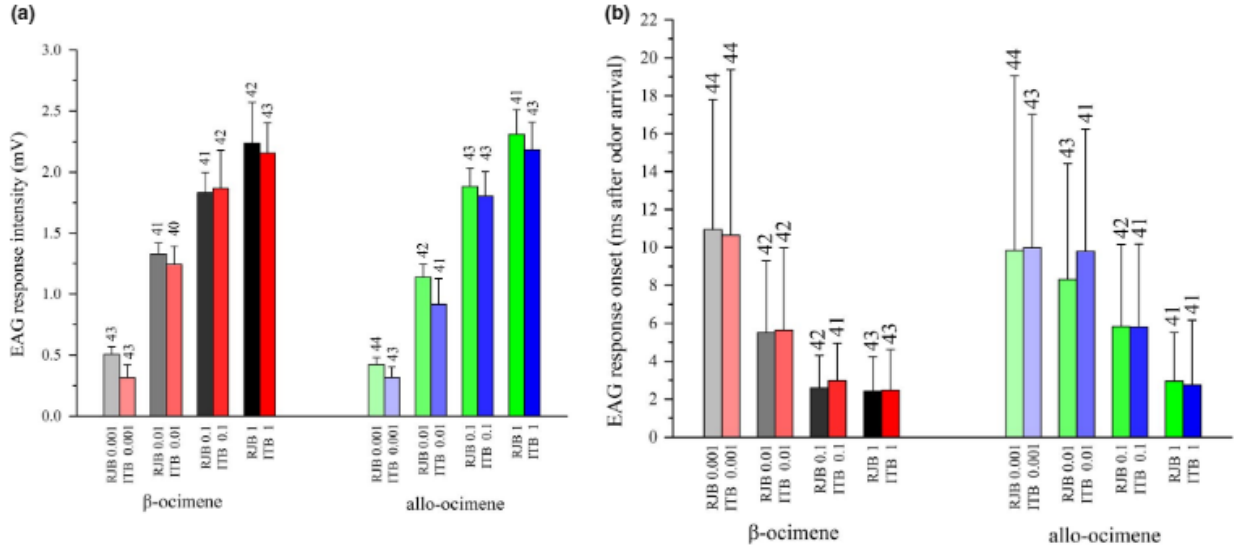

Figure 3. Electroantennograms (EAG) show different Italian honey bee (ITB) and royal jelly bee (RJB) responsiveness to brood pheromones. Particularly at low doses of β-ocimene and allo-ocimene, (a) the electrophysiological response was higher in RJB than in ITB nurse bees. Pairwise comparisons indicated significant differences for β-ocimene at 0.1% ($F(1,84) = 98.0$, $p < .001$) and 1% ($F(1,79) = 10.2$, $p = .008$) and for allo-ocimene at 0.1% ($F(1,85) = 46.9$, $p < .001$), 1% ($F(1,81) = 37.6$, $p < .001$), and 100% ($F(1,82) = 7.6$, $p = .028$). Overall, response intensity increased with increasing doses of both pheromones (see main text). In contrast, (b) EAG response times did not significantly differ between ITBs and RJBs at any dose, although higher doses elicited faster responses in general. For both panels, mean ± *SD* is presented and sample sizes are given above each bar

3.3 RJBs exhibit higher antennal response to brood signals than ITBs

Among the brood pheromones tested in our electroantennogram (EAG) assay, only β-ocimene and allo-ocimene elicited an electrophysiological response in nurse bee antennae. The EAG responses were dose-dependent for β-ocimene ($F(3,327) = 1,746$, $p < .001$) and allo-ocimene ($F(3,332) = 2,187$, $p < .001$; Figure 3a and Table S3). Overall, RJB responses were significantly greater than those of ITBs for β-ocimene ($F(1,327) = 17.9$, $p < .001$) and allo-ocimene ($F(1,332) = 55.3$, $p < .001$). Due to a significant interaction between bee type and concentration on the response to β-ocimene ($F(1,327) = 6.1$, $p < .001$) and allo-ocimene ($F(1,332) = 2.9$, $p = .033$), differences between RJBs and ITBs were evaluated separately for each concentration, confirming higher responsiveness of the RJBs for most but not all concentrations (Figure 3a). The response time of ITB and RJB nurse bees' antennae to β-ocimene and allo-ocimene, measured as the EAG response delay from the time of contact of the stimulus

with the antennae, decreased from lower to higher concentrations (β-ocimene: $F(3,341) = 1,319$, $p < .001$; allo-ocimene: $F(3,328) = 761$, $p < .001$) but was not significantly different between ITB and RJB stocks for either chemical overall or at any specific concentration (Figure 3b and Table S3).

3.4 Brood signals exhibit synergistic effects and attract more RJBs than ITBs from distance

To verify the biological activity of the newly discovered allo-ocimene and compare the attractiveness of brood pheromones to RJB and ITB nurses under controlled conditions, we conducted behavioural attraction assays. When physically separated from the odour source, RJB nurse bees were significantly more attracted than ITBs ($F(1,40) = 4.6$, $p = .038$) and the different compounds exhibited different attractiveness ($F(3,40) = 52.2$, $p < .001$), while no significant interaction effect was found. Post-hoc testing indicated that the BEP blend attracted significantly less bees than any of the other substances and was comparable to the solvent control (Figure 4). Without physical separation, the number of attracted RJBs and ITBs was not significantly different ($F(1,40) = 0.4$, $p = .540$), while significant differences among compounds persisted ($F(3,40) = 7.4$, $p < .001$). Post-hoc testing indicated that the mixture of all compounds attracted significantly more bees than β-ocimene, allo-ocimene, or BEP blend alone, which in turn attracted significantly more bees than the solvent control (Figure 4).

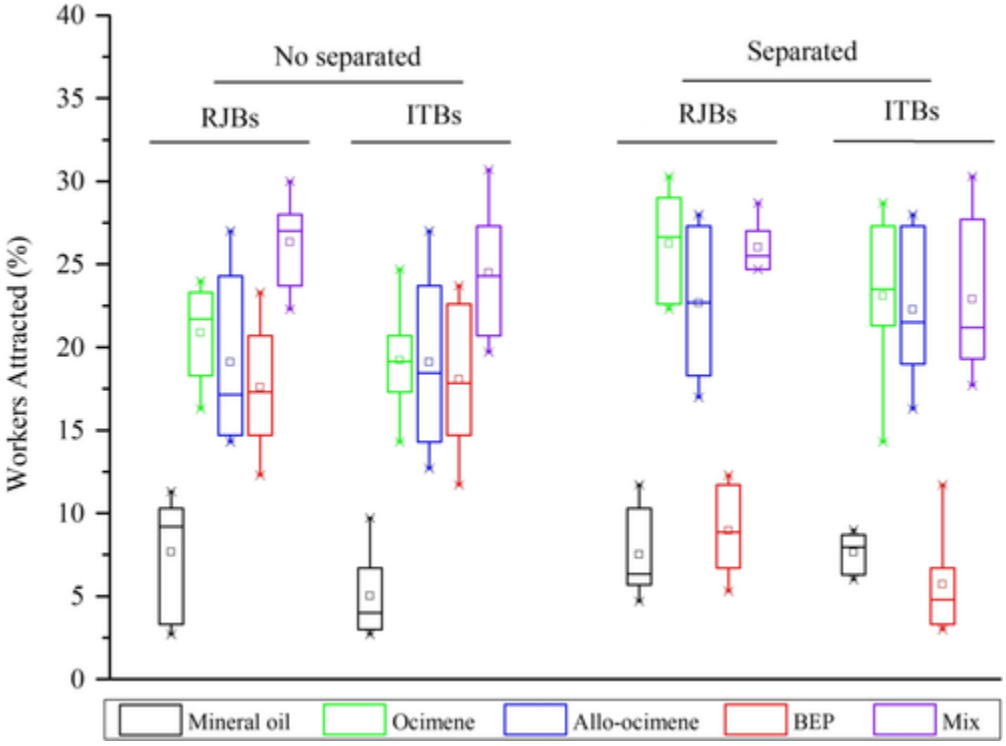

Figure 4, Attraction of Italian honey bee (ITB) and royal jelly bee (RJB) nurses to brood pheromones. Linear olfactometer tests were performed separately on groups of RJBs and ITBs, with or without perforated boards to physically separate bees from odour sources. Bars depict the quartiles of the percentage of workers attracted after 3 min in separate tests, with range indicated by stars and the mean indicated by a small square. RJBs are significantly more attracted by brood pheromones than ITBs (for statistics see main text). While all tested substances were significantly more attractive than the vehicle control (mineral oil) without separation, ocimene, the newly discovered allo-ocimene, and their mixture with BEP proved significantly more attractive than the vehicle control or BEP alone when the bees were separated from the odour source

3.5 Proteomic differences between ITB and RJB antennae

The antennal proteome of ITB and RJB nurses was compared by mass spectrometry to corroborate the behavioural and electrophysiological findings. Overall, 4,728 and 5,154 proteins (corresponding to 2,824 and 3,124 groups) were identified in the nurses' antennae of ITBs and RJBs, respectively. A total of 4,018 (corresponding to 68.5%) protein identifications were shared, and 710 and 1,136 proteins were only identified from antennae of ITBs and RJBs, respectively (Tables S4 and S5). Intracellular protein transport, cellular amide metabolic process, and phagosome were enriched in proteins only identified from ITBs (Figure 5c and Table S6), while oxidative phosphorylation, peptide biosynthesis, organic acid metabolic process, protein-containing complex assembly, and several other GO terms were enriched in the proteins identified only from RJB antennae (Figure 5d and Table S6). Among the investigated OBPs and CSPs, OBP1-5, OBP6, OBP8, OBP11, OBP13-19, OBP21, CSP1, and CSP4 were identified in both stocks, OBP20 and CSP6 were found only in ITBs, CSP3 only in RJB, and OBP7, OBP9, OBP10, OBP12, CSP2, and CSP5 were not found at all. The only ORs identified were OR2 (in both stocks) and OR26 found in RJBs (Tables S4 and S5). Amongst the antennal proteins identified in both stocks, 82 and 327 proteins were upregulated in ITB and RJB antennae, respectively (Table S7). ITB upregulated proteins were enriched in cellular amino acid metabolic process and lysosome, while the RJB upregulated proteins were enriched in hydrogen ion transmembrane transport and oxidative phosphorylation (Figure 5e). For the olfactory proteins, OBP6 and OBP13 were upregulated in ITBs, whereas OBP2, OBP4, OBP16, OBP18 and CSP1, CSP4 were upregulated in RJBs (Figure 5f).

3.6 Candidate gene studies suggest regulatory differences

Sequences of OBP and CSP genes were determined from PCR-amplified cDNA of ITBs and RJBs. Among the 24 sequences analyzed, only OBP15 exhibited one amino acid sequence difference between ITBs and RJBs (Figure 6a), which did not change its binding properties of larval pheromones (see below). To corroborate the quantitative proteomics results, qRT-PCR was used to compare nurse bee antennae of ITBs and RJBs with regard to the expression of top functional candidates: genes related to olfactory proteins, energy metabolism, and signal transduction. Most OBPs and CSPs were highly expressed, including OBP1, OBP2, OBP6/8, OBP21, CSP1 and CSP4. Relative to two reference genes, mRNA levels of OBP1 and OBP17 were higher in ITBs, while OBP2, OBP11, OBP16, OBP18 and CSP4 were significantly higher in RJBs (Figure 6b). Genes related to energy metabolism were consistently more expressed in the RJBs than in the ITBs, although the differences were only significant for Ndufs5 and ATP-F.

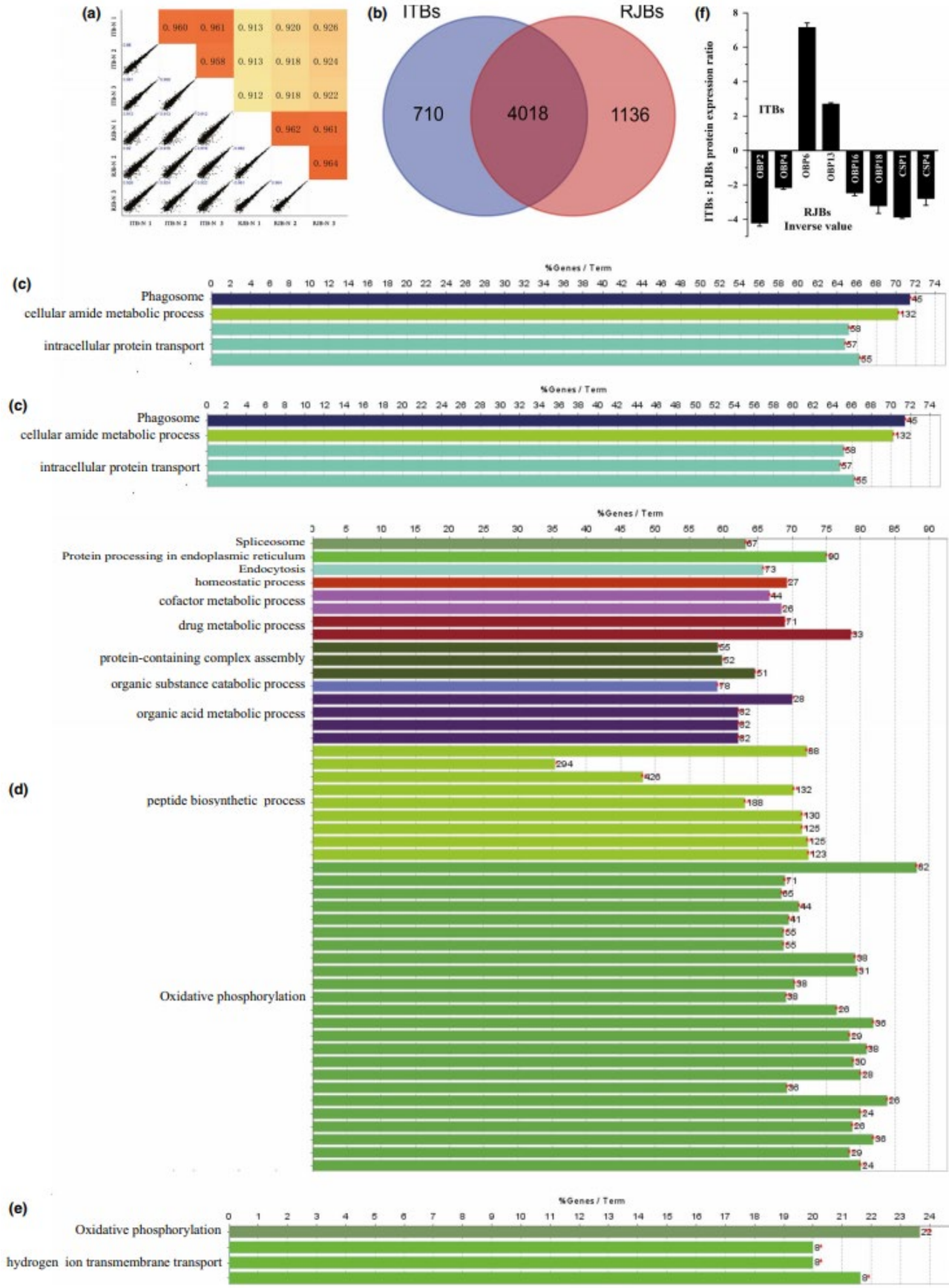

Figure 5. Proteome comparison of antennae of royal jelly bee (RJB) and Italian honey bee (ITB) nurse bees. (a) Overall correlations of protein abundances in antennal samples from ITB and RJB nurses indicate a higher similarity among ITB samples and among RJBs than between ITB and RJB samples. Numbers indicate Pearson correlation coefficients (all $p < .001$). (b) Venn diagram denoting the proteome differences between ITB and RJB antennae. Based on our analysis method, over 30% of proteins were uniquely identified from ITB or RJB antennae. Quantitative differences that lead to nondetection in ITBs or RJBs cannot be excluded. (c) GO enrichment analysis revealed five significantly enriched GO terms in proteins specific to nurse bee antennae of ITBs, (d) Fity-one GO terms in proteins specific to RJB antennae and (e) four GO terms in proteins that were upregulated in RJB relative to ITB antennae. GO terms were functionally combined into categories that are listed and indicated by colour, the *x*-axis represents the percentage of significantly different proteins of all proteins in the respective GO category, and the numbers to the right of each bar indicate the number of genes associated with each category. (f) Specific comparison of olfactory proteins in nurse bee antennae of ITBs and RJBs indicate quantitative changes: Most of these proteins were found more abundant in RJB antennae

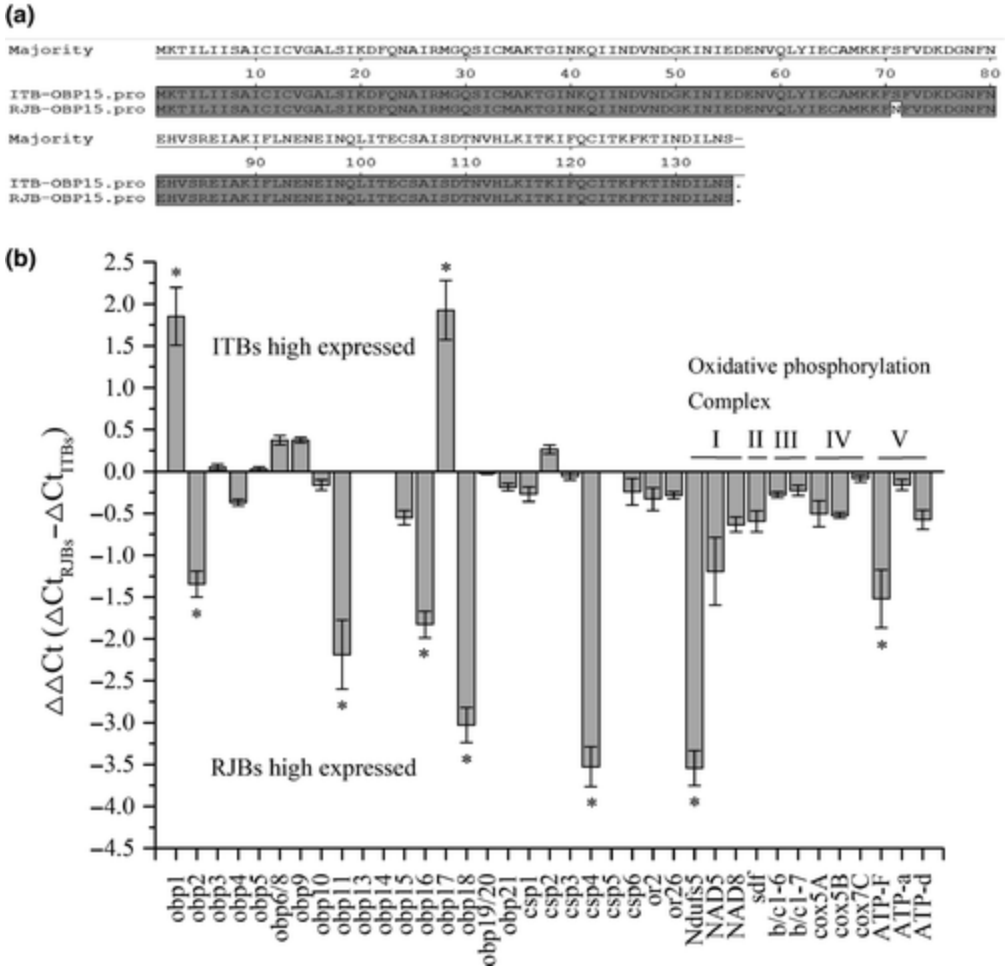

Figure 6. Results of candidate gene analyses. (a) Determination of coding sequences revealed only one nonsynonymous difference between Italian honey bees (ITBs) and royal jelly bees (RJBs), changing a serine of the OBP15 gene of ITBs into an asparagine in the RJB gene. (b) Relative gene expression differences in the nurse bee antennae of ITBs and RJBs indicated a higher expression of olfactory and energy metabolism genes in RJBs in general. Differences were significant for OBP2, OBP11, OBP16, OBP18, and CSP4 (*$p < .05$), while OBP1 and OBP17 exhibited significant higher expression in ITBs. Means ± *SD* are shown. OBP, odourant binding protein

3.7 OBPs and CSPs display high affinities for β-ocimene and llo-ocimene

To test whether OBPs and CSPs are functionally relevant for the perception of brood pheromones, the binding force between these proteins and β-ocimene, allo-ocimene, and the 10 components of BEP were compared in a competitive fluorescence binding assay. All tested OBPs and CSPs had good binding affinities to the fluorescence reporter 1-NPN (e.g., OBP8 and CSP4, Figure 7). Binding affinities of β-ocimene and allo-ocimene varied (Figure 7b and Table S8). OBP8 and CSP4 had the strongest binding affinities to β-ocimene and allo-ocimene, respectively (Figure 7c,d). Overall, the binding affinities of the tested proteins to β-ocimene and allo-ocimene were correlated (Spearman's $R = .75$, $n = 25$, $p < .01$). The 10 components of BEP exhibited very weak or no binding affinity to any OBPs and CSPs (e.g., Figure 7c,d), and dissociation constants of the 10 BEP components could not be determined.

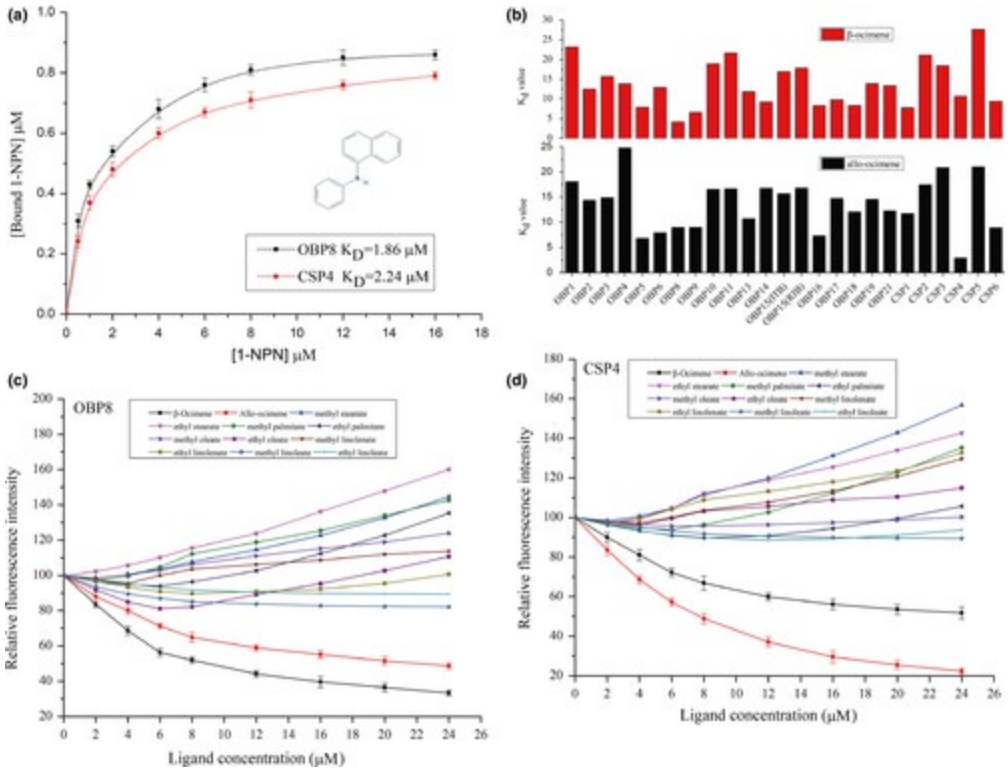

Figure 7. Affinity of odourant binding proteins (OBPs) and chemosensory proteins (CSPs) to brood pheromones. Affinity was assessed by competitive binding experiments with 1-NPN as a fluorescent reporter. (a) Exemplified by OBP8 and CSP4, the purified proteins had good affinities to 1-NPN. Affinity assays for all other proteins are reported in Figure S1. (b) The dissociation constants (K_d) values of OBPs and CSPs for β-ocimene (red) and allo-ocimene (black) revealed strong binding that was variable among proteins and ligands. (c and d) Examples of the competitive binding curves (of OBP8 and CSP4) illustrate the differences between β-ocimene (red) and allo-ocimene (black) and BEP components. Binding curves of all other olfactory proteins are reported in Figure S2

4 DISCUSSION

The long-term breeding programme for high royal jelly producing honey bees in China has resulted in an extraordinary and stable social phenotype, enabling the entire royal jelly production industry and providing a unique opportunity for understanding female reproductive investment in social insects. The study of complex social phenotypes using selected strains has revealed underlying molecular changes in a few cases that involve individual reproductive physiology (Aumer et al., 2019; Page et al., 2012; Ronai et al., 2015), but the breadth and duration of the selection programme for high royal jelly production is unprecedented and has led to a novel social phenotype. The increased production of new queens and royal jelly (RJ) provisioning per queen correspond in >10-fold higher RJ production of RJBs compared to unselected Italian (ITB) honey bees that the RJBs were originally derived from (Li & Wang, 2005). Here, we contribute to the understanding of this social syndrome of increased female reproductive investment through alloparental (worker) care. Our results indicate that the RJB syndrome might be due to increased sensory responsiveness of nurse bees that translates into different attraction of nurse bees to brood pheromones. Furthermore, we provide some support for the hypothesis that the heightened sensitivity to brood signals of RJBs is caused by increased antennal expression of functionally-relevant olfactory proteins and genes involved in energy metabolism. Changes in energy metabolism and communication have emerged in a

number of behavioural and microevolutionary contexts (Clarke, 2003; Li-Byarlay, Rittschof, Massey, Pittendrigh, & Robinson, 2014). The specific genes identified in our study require further study to confirm their role in the RJB phenotype and social evolution more generally.

Our study supports the central role of communication for social organization and phenotypes (Slessor et al., 2005). Our cross-fostering experiment indicates that the RJB selection has affected signal perception, similar to other colony-level selection programmes, such as breeding for pollen hoarding (Page et al., 2012) and hygienic honey bees (Masterman, Ross, Mesce, & Spivak, 2001). However, in contrast to our findings on RJBs, pollen hoarding and hygienic selection has also affected the brood signals (Linksvayer, Fondrk, & Page, 2009; Wagoner, Spivak, Hefetz, Reams, & Rueppell, 2019; Wagoner, Spivak, & Rueppell, 2018). The higher acceptance and better provisioning of grafted larvae in queen cells is central to the royal jelly industry and our results suggest candidates genes that may trigger this highly heritable phenotype (Li et al., 2003a; Li, Chen, Zhong, & Su, 2003b). Complementary previous studies of the RJBs have characterized molecular changes that enable the RJB nurses to produce large quantities of brood food. The nurses of RJBs have expanded acini size and elevated protein synthesis, cytoskeletal functions, and carbohydrate metabolism in their hypopharyngeal glands relative to ITBs (Li et al., 2010). The mandibular glands of RJBs have also enhanced the capacity of lipid synthesis to match the elevated production of RJ (Huo et al., 2016).

The electrophysiological responses of the antenna to β-ocimene and allo-ocimene are higher in RJBs than ITBs, particularly at lower concentrations. The low doses are significant because they are closer to biologically relevant concentrations (Schott, Wehrenfennig, Gasch, & Vilcinskas, 2013), even though higher concentrations were required for a significant response in our laboratory attraction assay. Communication signals and their perception often coevolve and may even involve the same genes (Bousquet et al., 2012). However, the complex phenotype of RJBs seems to involve changes in the sensitivity of the antennae that appear to lower the nurse bees' response threshold to larval pheromones without affecting any larval signalling. Breeding efforts for hygienic honey bees have also increased olfactory sensitivity of the antennae to discriminate diseased from healthy brood (Guarna et al., 2015), lowering their response thresholds (Masterman et al., 2001). Together, studies in honey bees support the central role of the insect antenna for behavioural tuning and rapid evolutionary change (Leal, 2013; Schott et al., 2013) but we cannot rule out additional differences in the central nervous system.

The ability to quickly track different odours is essential to differentiate among odours (Nikonov & Leal, 2002). The speed of the antennal response to β-ocimene and allo-ocimene was sufficiently fast (<3 ms) to distinguish these odours in a complex olfactory environment (Szyszka, Stierle, Biergans, & Galizia, 2012). No significantly differences in response time between ITBs and RJBs were detected, indicating that the tempo of the brood pheromone perception does not contribute to the differences in nursing behaviour between ITBs and RJBs. To our knowledge, no other study has linked intraspecific variability in the speed of peripheral odour processing to behavioural differences, although temporal patterns of olfactory processing can be behaviourally relevant (Saha et al., 2015).

Our indepth proteome comparison identified 4,728 and 5,154 proteins, representing 2,824 and 3,124 protein groups, in ITB and RJB nurse bee antennae, respectively. The amount of proteins

discovered here exceeds previous studies due to technological and procedural advances and significantly improves our understanding of the antennal proteome in general (Guarna et al., 2015; Hu et al., 2016). More than 1,800 proteins were specifically detected in either ITB or RJB antennae and more proteins were discovered in the RJB antennae. However, most unique protein identifications were probably caused by values near the detection limit and thus presumably reflect quantitative rather than qualitative differences. This interpretation is supported by the more sensitive qRT-PCR results that reveal mRNA expression of OBP20, CSP3, and CSP6 in ITB and RJB antennae, even though the corresponding proteins were not detected in one of the stocks. Nevertheless, the identified qualitative and quantitative proteome differences suggest a systematic remodelling of the RJB worker antennae by the artificial selection.

The enrichment of intracellular protein transport, cellular amide metabolic process, and phagosome functions in ITBs, and of oxidative phosphorylation, peptide biosynthetic process, organic acid metabolic process, and nucleotide biosynthetic process in RJBs suggests molecular pathways that have been changed by the selection for RJ production and underlie the heightened olfactory responsiveness to larval signals, increasing the acceptance and provisioning of queen cells. Intracellular protein transport can serve many relevant functions, such as forming olfactory cells and sensilla (Keil & Steiner, 1991). Amides are also involved in a wide variety of biological functions and include insect pheromones that elicit alarm and attack behaviour (Weston, Woolhouse, Spurr, Harris, & Suckling, 1997). Moreover, nucleotide biosynthetic processes and cellular compound metabolism are associated with various neurotransmitter systems (Hill et al., 2002), which could be the basis of increased signal transmissions in RJB antennae to enhance the olfactory functions.

The two most significant differences between ITB and RJB antennae were found in components of the oxidative phosphorylation pathway and in chemoreception. The antennal chemoreception requires a sophisticated, compartmentalized organization (Leal, 2013) and is energetically demanding (Fang et al., 2012). Antennae of RJBs contained higher amounts of proteins that function in oxidative phosphorylation and hydrogen ion transmembrane transport, which presumably enable ATP production. These proteins may be expressed as a consequence of higher antennal activity, but presumably they also increase olfactory responsiveness. Thus, a stronger overall olfactory performance is a likely contributor to the RJB nursing phenotype. Similarly, the upregulation of metabolic functions has presumably led to nurse bees that are more sensitive to disease stimuli (Mondet et al., 2015).

Specifically, several proteins of the electron transport chain were more abundant in RJB than ITB antennae, including dufs5, which was more than 10 times more abundant in RJBs than ITBs. These protein differences were confirmed by higher mRNA levels of Ndufs5, NADH dehydrogenase 1 beta subunit 8 and 5 (complex I), flavoprotein subunit (complex II), cytochrome b-c1 subunit 6 and 7 (complex III), cytochrome c oxidase subunit 5A, 5B and 7C (complex IV), and V-type proton ATPase subunit d and F (complex V). Hydrogen ion trans-membrane transport by V-type ATPase is also involved in the regulation of the cellular membrane potential, mediating depolarization, and synaptic transmission (Paunescu, Jones, Tyszkowski, & Brown, 2008). Thus, the elevated level of V-type ATPase in RJBs may also be linked to increased neuronal transmission (Heming & Bidani, 2003).

The second important group of proteins that differed between the ITB and RJB antennae was CSPs, including OBPs, CSPs, and ORs (Leal, 2013). Presumably due to their low abundance, only two of the many ORs were detected in our proteome studies. Our sequencing analysis showed only one amino acid difference between ITBs and RJBs in one gene, OBP15. This difference did not affect OBP15's affinity to the tested brood pheromones and is therefore unlikely to contribute to the RJB phenotype. However, several OBPs and CSPs exhibited different mRNA levels in RJBs and ITBs that confirmed the protein measurements. Thus, regulatory changes in response to selection for royal jelly production could be causing the increased sensitivity and consequently reproductive behaviour of the RJBs. Regulatory changes have been critical for social evolution (Kapheim et al., 2015) and also seem to underlie other social syndromes, such as the pollen hoarding syndrome (Wang et al., 2009) and the anarchistic worker syndrome (Ronai et al., 2015) in honey bees.

In contrast to the unidirectional changes in energy metabolism, the chemosensory differences between ITBs and RJBs were more variable, suggesting that multiple sensory changes may contribute to the RJB phenotype. With respect to the initial stages of odour perception, OBP1 expression was decreased in RJBs compared to ITBs. OBP1 has a strong affinity to one component of the queen mandibular pheromone (Danty et al., 1999). Thus, RJB selection has reduced queen perception, which may decrease the queen's inhibitory effect on rearing new queens (Keeling, Slessor, Higo, & Winston, 2003) in RJBs. Consequently, RJBs readily rear many new queens in the presence of a reproductively active queen, in contrast to ITBs. RJBs exhibited a stronger mRNA expression of OBP2, OBP11, OBP16, OBP18 and CSP4 and higher protein levels of OBP4 and CSP1. In particular, the strong and consistently upregulated OBP2, OBP16, OBP18, and CSP4 are potential explanations for the RJB syndrome. Most of the numerous odourant receptors of the honey bee (Robertson & Wanner, 2006) were not identified in the antennae, but OR2 and OR26, are also more expressed in the RJB over ITB antennae. We cannot rule out that one or several ORs play a significant role in the RJB phenotype.

Among our tested olfactory proteins, OBP16 and OBP18 exhibit high affinities to β-ocimene and OBP16 and CSP4 to allo-ocimene, making them prime candidate to explain the selective increase in responsiveness to brood pheromone of RJBs. All three are abundant proteins, and OBP16 and OBP18 also play a role in hygienic behaviour (Guarna et al., 2015), while CSP4 seems to be specific to our system. In sum, our results suggest that multiple processing differences may explain the evolution of receiver responsiveness in the complicated colony environment of social insects (Le Conte & Hefetz, 2008; Slessor et al., 2005).

Incidentally, we also report a new honey bee larval pheromone: allo-ocimene. Allo-ocimene has not been reported as a pheromone from any insect but it matches the larva-specific release of β-ocimene, elicits antennal signalling, attracts nurse bees, and demonstrates significant binding affinities to olfactory proteins in our study. Allo-ocimene may thus complement β-ocimene. Its similarity to β-ocimene and its release in similar contexts in lower quantities may have prevented its discovery in previous studies (He et al., 2016; Maisonnasse et al., 2009). The detection of a new volatile compound of honey bee larvae is significant and different binding affinities of β-ocimene and allo-ocimene to olfactory proteins suggest different functions. Together, the two ocimenes may complement the less volatile BEP in modulating nursing behaviour in general

(Maisonnasse et al., 2009) and in the specific context of queen cell acceptance and provisioning (Le Conte et al., 1995). BEP signalling or perception was considered a prime candidate for the observed phenotype of the RJBs but our results failed to support a role for BEP. The selective breeding of RJBs has affected the perception of the volatile β-ocimene and allo-ocimene, which may suggest that pheromonal primer effects also play a role in the high RJB syndrome. The details of how nurse bees distinguish between different larval types and needs are still insufficiently understood and more research is needed (Slessor et al., 2005).

Brood care requires the precise coordination between caregivers and the brood (Le Conte et al., 1995) and a combination of volatile and nonvolatile compounds is likely. The behaviuoral attraction assay demonstrated the biological activity of the nonvolatile BEP and the volatile β-ocimene and allo-ocimene. In addition, our findings also reveal a functional differentiation between them: while the volatile compounds attracted nurse bees from a distance across an experimental divider, BEP attracted nurses only by direct contact. Furthermore, the pheromones are separated by larval age: BEP is emitted principally by old larvae and reaches a maximum concentration during the capping stage (Trouiller, Arnold, Le Conte, & Masson, 1991). In contrast, β-ocimene has been identified from different instars with multiple functions (He et al., 2016; Ma, Mueller, & Rangel, 2016; Maisonnasse et al., 2009; Traynor, Le Conte, & Page, 2014; Traynor et al., 2015). Although we cannot exclude its existence in different developmental ages, allo-ocimene has only been found so far in 1-day old larvae, a critical age for queen development that requires a specific signal for the nurse bees to identify queen-destined larvae. This attractive function of allo-ocimene may have been co-opted by plants, which also use this odour to attract adult insects (Jayanthi, Woodcock, Caulfield, Birkett, & Bruce, 2012; Terry, Walter, Moore, Roemer, & Hull, 2007).

The reproductive investment of honey bees and other social insects has received considerable attention in the context of kin selection theory (Sundström, 1994). However, the long-term artificial selection on RJBs provides an opportunity to elucidate the neglected proximate causes of variation in colony-level reproduction. Our results indicate that the increased reproductive investment of RJB has evolved by altering communication, similar to other colony-level selection experiments. However, it is important to realize that selection experiments entail reproductive isolation and thus entail also many nonspecific genetic differences. The breadth and rigor of the RJB breeding programme may buffer against it, but we cannot exclude the possibility that the reported differences are not causal. Follow-up studies, including genetic mapping is needed. However, we provide multiple corroborating data sets that point to specific molecular changes in the peripheral nervous system of nurse bees that might have changed their alloparental care behaviour to produce the colony-level RJB syndrome. The reproductive phenotype of RJBs is unparalleled among social and solitary organisms and further studies of the RJBs across all levels of organization could more generally determine causes and consequences of the evolution of extreme reproductive investment.

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AUTHOR CONTRIBUTIONS

F.W., and J.-K.L. conceived and designed the experiments. F.W., C.M., Y.F., M.F., and X.-F.Z. performed the experiments. F.W., O.R., B.H., L.-F.M., and H.H. analyzed the data. F.W., O.R., and J.-K.L. drafted and revised the manuscript.

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