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Brood signals dominate social regulation of reproduction and immunity in honey bees

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Abstract

Reproductive division of labour is a defining feature of eusocial insect societies, yet the relative roles of queen and brood signals in shaping the physiology of nestmates remain unresolved. In honey bees (*Apis mellifera*), workers typically remain sterile in the presence of a fertile queen and developing brood. Here we disentangle queen- and brood-derived effects by assessing physiology of workers from colonies with all combinations of queen and brood presence and absence. Queenlessness and broodlessness promoted worker ovary activation additively, with broodlessness exerting a significantly stronger effect. Workers with activated ovaries produced more queen-like pheromone profiles with elevated levels of 9-oxo-(*E*)-2-decenoic acid and 9-hydroxydec-(*E*)-2-enoic acid. Proteomic and transcriptomic analyses revealed a profound impact of broodlessness that was partially reversed by concordant queenlessness, consistent with a return to a more typical physiological status as worker reproduction initiates and pheromonal signalling is partially restored. Broodlessness, both alone and in combination with queenlessness, enhanced worker immunocompetence and led to a > 10-fold reduction in adult viral load. These findings collectively identify brood as a central regulator of worker physiology that coordinates reproduction, pheromone signaling, and immunity, revealing a previously underappreciated link between social environment and immunological defense.

Introduction

Eusociality—characterised by multigenerational colonies, reproductive division of labour, and cooperative brood care—represents the highest level of social organization and is among the most ecologically successful life history strategies[1]. All ant species are eusocial, as well as some bees, wasps, termites, and select other non-hymenopterans (e.g., the ambrosia beetle *Austroplatypus incompertus* and the naked mole-rat *Heterocephalus glaber*). The first eusocial insect genome to be sequenced was from the western honey bee[2], *Apis mellifera*, which has since become a pillar for molecular sociobiological and sociogenomic research, but a central challenge has been understanding how colony-level signals are translated into coordinated physiological states of workers. While queen pheromones were long regarded as the dominant regulators of worker sterility, brood also acts as an important, yet comparatively understudied, organiser of colony physiology[3–7].

Honey bee colonies comprise two female castes (a single reproductive queen and tens of thousands of largely sterile workers) and seasonally produced reproductive males (drones). The long-lived queen is solely responsible for laying fertilized eggs, yet workers retain the fundamental anatomy and physiology required for oogenesis and oviposition (though not fertilization). In queenright colonies, ovary activation among workers is rare, with only approximately 1 in 10,000 individuals exhibiting fully developed ovaries[8,9], though much higher frequencies are observed in *A. mellifera capensis* (a subspecies with notoriously common worker reproduction)[10].

Honey bee worker reproduction is tightly regulated by social cues, with ovary activation being inhibited by the presence of a queen[11–14], young brood[3,4,7], and the eventual development of other laying

workers[3,15,16]. In the bumble bee *Bombus impatiens*, while both the queen and brood are important, queen signals exhibit a greater, and sometimes synergistic, effect than brood signals on worker brain transcript profiles and worker reproduction[17,18]. Similar patterns have been described in some ants[19]; however, in honey bees, the presence of brood — particularly larvae — appears to exert a stronger inhibitory effect than the queen[3,7]. Following queen loss, honey bee workers (sometimes up to 50% of those in *A. m. ligustica* colonies[20]) typically begin laying eggs after three to five weeks[8,20], but this timeline is also influenced by their genetics[21] and likely by the quantity and developmental stage of residual brood.

Queen and brood signals are mediated through pheromones that function as both primers and releasers, inducing long-term physiological changes and immediate behavioral responses, respectively[22]. The multicomponent queen mandibular pheromone (QMP; see Table 1 for composition) and the expanded queen retinue pheromone (QRP) are among the best characterised pheromones in all the social insects[23,24]. Among many other functions, these pheromones elicit worker retinue formation, suppress queen cell rearing, and inhibit worker ovary development[25–31]. Brood-derived signals, including *E*-β-ocimene and the 10-component brood ester pheromone (BEP), are primarily produced by larvae and control larval food provisioning, behavioural maturation, longevity, and, like QRP, also inhibit worker ovary activation[5–7,32–36]. One component, MO, is shared between QRP and BEP; in queens, it is positively correlated with ovary mass and contributes to queen cell rearing inhibition[31], but its specific role in brood signalling remains unclear. Despite this work identifying individual queen and brood pheromones, most studies have examined isolated behavioural or reproductive endpoints. Whether these social signals orchestrate broader physiological states spanning reproduction, chemical communication, immunity, and genome-wide expression patterns remain largely unknown.

Table 1
Queen pheromone compounds and abbreviations

Compound	**Abbreviation**	**Blend**
9-oxo-(*E*)-2-decenoic acid	9-ODA	QMP, QRP
9-hydroxydec-(*E*)-2-enoic acid (R and S enantiomers)	9-HDA	QMP, QRP
4-hydroxy-3-methoxyphenylethanol	HVA	QMP, QRP
Methyl p-hydroxybenzoate	HOB	QMP, QRP
Methyl oleate	MO	QRP
(*E*)-3-(4-hydroxy-3-methoxyphenyl)-prop-2-en-1-ol	CA	QRP
Hexadecan-1-ol	PA	QRP
(*Z*9,*Z*12,*Z*15)-octadeca-9,12,15-trienoic acid	LEA	QRP

Egg-laying worker honey bees can produce queen-like pheromone profiles, although the strength of this phenomenon appears to differ among subspecies. The strongest evidence comes from *A. m. capensis*, in which laying workers produce queen-like mandibular gland pheromones[37–42], and their extracts are sufficient to inhibit ovary activation in other workers[16], thus contributing to the colony's pheromonal environment. Although the amount of combined 9-ODA and 9-HDA (the most abundant QMP components) produced by each laying worker is approximately 80- to 290-fold lower than a laying queen[23,39,40,43], the large number of reproductively active workers could generate a biologically meaningful collective inhibitory signal. This provides a possible mechanism by which the development of laying workers could inhibit ovary activation of nestmates, as previously observed[3,15,16]. Whether laying workers of other *A. mellifera* subspecies similarly produce queen-like pheromones is less clear. Plettner *et al.* reported that laying workers of *A. m. carnica* and *A. m. ligustica* did not significantly upregulate QMP production, in contrast to *A. m. capensis*[44]. This differs from more recent analyses[39] and functional assays that indicate production of queen-like compounds[16]. Notably, Plettner *et al*. did find that egg-laying workers produced approximately four-fold more 9-HDA than controls, although the variance was substantial[44], suggesting the presence of unresolved explanatory factors.

Egg-laying worker honey bees (and ants[19,45]) have been described as exhibiting a more "queen-like" transcript profile compared to non-reproductive workers[46]; however, this is difficult to distinguish from a "nurse-like" profile because there are shared pathways that enable both reproduction and brood care tasks[7]. This overlap is in accordance with the reproductive ground-plan hypothesis[47], which proposes that eusocial reproductive division of labour (i.e., caste-separation of egg laying and brood care tasks) evolved through modification of ancestral reproductive and brood provisioning pathways. Two key regulators of reproduction are vitellogenin and juvenile hormone, which act antagonistically in honey bees, with vitellogenin promoting reproduction while juvenile hormone suppresses vitellogenin expression (and vice versa)[41–46]. Indeed, both reproduction[48,49] and nursing behaviour[50–52] are associated with higher levels of vitellogenin and lower levels of juvenile hormone. Laying queens express elevated levels of vitellogenin relative to virgin queens and workers, and nurse honey bees (who directly care for young) express higher vitellogenin levels than foragers[51,53–55], with juvenile hormone and the associated synthesis enzymes showing the opposite pattern (as is typical in honey bees)[52,55–57]. This demonstrates physiological overlap between egg laying and nursing states, which contrasts with the foraging state.

Extended periods of broodlessness, despite the presence of a mated queen, can occur in two distinct scenarios: 1) during winter in temperate climates, when brood rearing naturally subsides, and 2) when queens are temporarily caged during the spring or summer as part of an integrated pest management technique for controlling the parasitic mite, *Varroa destructor*[58]. The latter technique is employed because the resulting lack of brood slows *Varroa* reproduction and makes the mites more vulnerable to beekeeper-applied acaricides and natural worker grooming behaviour. Winter broodlessness is impossible to mimic during the summer without the necessary environmental cues, but investigating the

impact of broodlessness on spring and summer bee physiology has practical implications for this *Varroa* management technique, since there may be a risk of worker ovary activation and underappreciated impacts on worker physiology (positive or negative). More broadly, queenright, broodless colonies provide a rare opportunity to experimentally uncouple these two social signals that are normally inseparable in eusocial colonies. This enables the independent contributions of queens and brood to be resolved across multiple levels of biological organization, from reproductive physiology to gene and protein regulation.

Here, we combine factorial manipulation of queen and brood presence with worker ovary assessments as well as pheromonal, proteomic, transcriptomic, and virus analyses to determine how these social environments affect worker phenotypes. We first quantify their independent and combined effects on worker ovary activation, then test whether these reproductive changes are accompanied by coordinated shifts in pheromone production, protein and gene expression profiles, and immunity. We revisit the relationship between queen pheromone production and worker ovary activation in European-derived honey bees using a recently described liquid chromatography-tandem mass spectrometry (LC-MS/MS) analytical method that offers improved sensitivity[31,59], and test whether the established inhibition of worker ovary activation by brood reflects a broader role of brood in coordinating worker physiological state. Our findings identify brood as a core regulator of worker physiology, with implications for both the evolution of eusociality and the management of honey bee health.

Methods

Colony establishment

Five queenright double brood chamber colonies (made up of two 10-frame deep Langstroth boxes) that were previously treated with Apivar (to control *Varroa*) were split into 20 nucleus colonies on May 27, 2022, at an apiary located at the University of Lethbridge, Alberta. Each parent colony (replicate) was de-queened and split into four small nucleus colonies (nucs). The nucs from each parent colony were randomly assigned to each of four treatments: queenright with brood (QRBR), queenless with brood (QLBR), queenright with no brood (QRBL), or queenless with no brood (QLBL). Newly mated, unrelated queens imported from California were introduced to the experimental nucs in the two queenright treatments. Nucs containing brood (QRBR and QLBR groups) were supplied with two frames of open brood (larvae), one frame of purple-eyed capped brood, and two frames of honey/nectar from parent colonies at the time of initial setup. Frames containing at least one frame side of open brood from unrelated non-experimental colonies were added to the QLBR colonies every four days to ensure the presence of all brood stages throughout the experiment, despite the absence of a laying queen. At the same time, one frame of emerged brood or capped brood was removed to create space. To mimic the swapping of one frame experienced by all QLBR colonies and to equalize levels of disturbance across groups, all other experimental colonies underwent “sham manipulations,” where a single frame was removed from the colony, the bees shaken into the hive, then the same frame was replaced. This means that QLBR colonies received unrelated brood whereas other colonies did not. While this represents a

potentially confounding factor, any resulting effect is expected to be minimal given that workers exhibit zero to very low levels of genetically biased larval care[60–62]. Broodless nucs (QRBL and QLBL) received two capped brood frames at initial setup to help prevent absconding and to minimize eventual age stratification across groups, as well as a frame of foundation and two frames of honey/nectar at initial setup. Brood remains capped for 12 days prior to adult emergence; therefore, all capped brood emerged well before the time of colony sampling (see below), rendering all BL colonies completely broodless for a minimum of one week. Queens in the QRBL experimental colonies were confined to a large cage (16.8 cm x 12.5 cm x 3.2 cm), made from queen excluder material on one side and steel mesh on the other, that enabled queens to mingle with workers and distribute pheromones but prevented their access to comb to lay eggs.

Queen and brood status were monitored every four days for the presence/absence of a queen and open brood. At each inspection, whether the colonies contained active queen cells (cups containing either an egg or a larva) was noted as well as the number of queen cells observed. Queen cells were destroyed upon each inspection. On June 15, 2022 (19 days after initial setup of the experimental nucs), approximately 100 bees were sampled from the center frame of each experimental colony using 50 mL conical tubes. Tubes were placed on dry ice until storage in a -80°C freezer. These bees were then split into subsamples that were used for ovary activation scoring, pheromone analysis, proteomics, and transcriptomics, described below.

Ovary scoring

Ten bees per colony were dissected and analyzed (N = 200 in total). Although this sample size may appear limited, our preliminary analysis showed that effects were sufficiently strong for statistical significance to be achieved with as few as five bees per colony. Sampling was doubled to further improve our estimates. Two operators dissected the samples, with each dissecting half the bees (n = 5) per colony. Operator was included in the statistical analysis to account for minor, but systematic differences in operator scoring (see Statistical Analysis section). Dissections were conducted with the operators blind to the experimental group. For each dissection, a single bee from a frozen sample was mounted to a dissection plate and partially submerged with 95% ethanol. One or both ovaries were isolated and removed from the worker by creating two incisions at the base of the sting apparatus, producing a ventral flap through which the ovaries could be accessed. Ovaries were photographed and assigned a single ovary development score based on the level of development seen in the most-developed ovary. Ovary development scores were assigned on a 4-point ordinal scale ranging from 0–3 (Fig. 1A) following a modified 5-point Velthuis scale used previously[26,63]. For the purposes of this study, the scale was reduced to 4 ranks by combining Velthuis scores 2 and 3 into a single rank to remove the need to observe egg size compared to nutritive follicle size.

Proteomics LC-MS/MS analysis

Worker samples were shipped on dry ice from the University of Lethbridge to the University of British Columbia, where the bees were subsequently dissected and processed for proteomics analysis in randomized order, essentially as previously described[64]. Protein was extracted from 10 pooled heads (antennae removed) and, separately, 10 pooled abdomens (digestive tract removed) per nucleus colony (20 colonies total, n = 5 per experimental group) by mechanical homogenization in lysis buffer (4% SDS, 100 mM Tris pH 6.8) containing protease inhibitors (Thermo Scientific Halt Protease Inhibitor Cocktail & cOmplete Protease Inhibitor Cocktail). Homogenization was conducted in a Precellys 24 bead mill using 2.8 mm ceramic beads (three cycles of 30 s at 6.5 m/s). Protein concentration was determined using a bicinchoninic acid (BCA) assay. For each sample, 40 µg of protein was reduced with DTT (dithiothreitol, final concentration of 30 mM) at 37°C for 30 min, followed by alkylation with CAA (chloroacetamide, final concentration of 50 mM) at 37°C for 20 min in the dark.

Samples were purified using SDS-polyacrylamide gel electrophoresis on Bio-Rad Mini-PROTEAN® TGX™ Precast gels, followed by in-gel digestion as previously described[65]. Protein bands were excised from the gel, diced into small cubes, and sequentially destained with increasing concentrations of acetonitrile. Each sample was digested using 0.45 µg of trypsin (NEB MS grade) in a pre-chilled digestion buffer (40 mM ammonium bicarbonate, 1 mM $CaCl_2$, 10% v/v acetonitrile, pH 8.0) and incubated at 37°C overnight. Dried peptides were reconstituted in 0.2% trifluoroacetic acid, and desalted using STAGE-tip purification with homemade single C18 membrane stage tips[66]. Desalted samples were resuspended in solvent A (0.5% acetonitrile, 0.1% formic acid), and the peptide concentration was determined by absorbance at 205 nm using a NanoDrop One (Thermo Fisher Scientific)

A total of 150 ng of peptides per sample were injected onto a NanoElute UHPLC system (Bruker Daltonics) with an Aurora Series Gen2 (CSI) analytical column (25 cm × 75 µm, 1.6 µm FSC C18, Gen2 nanoZero and CSI fitting; Ion Opticks, Parkville, Victoria, Australia). The LC system was coupled to a timsTOF Pro 2 mass spectrometer (Bruker Daltonics). The column was maintained at 50°C. A standard 30-minute chromatography gradient at a flow rate of 0.3 µL/min was used: 2% to 12% solvent B (0.1% formic acid in 99.4% acetonitrile) over 15 minutes, then increased to 33% B from 15 to 30 minutes, then ramped to 95% B over 0.5 min and held at 95% B for 7.72 minutes. The analytical column was conditioned with 4 column volumes of solvent A before each run. The NanoElute thermostat temperature was set to 7°C.

Mass spectrometer acquisition settings have been previously described[53]. The timsTOF Pro 2 operated in DIA-PASEF (data-independent acquisition, parallel accumulation-serial fragmentation) mode with timsControl v. 4.1.12 (Bruker). The LC and MS systems were controlled with HyStar 6.0 (6.2.1.13, Bruker). The instrument was set to scan from 100 to 1700 m/z, with a capillary voltage of 1800 V, a drying gas flow of 3 L/min, and a drying temperature of 180°C. The MS1 scan was followed by 17 consecutive PASEF ramps, each containing 22 non-overlapping 35 m/z isolation windows, covering 319.5–1089.5 m/z. The ion mobility range ($1/k_0$) was set to 0.70–1.35 V·s/cm^2, with a 100-ms ramp and accumulation time (100% duty cycle), and a ramp rate of 9.42 Hz (1.91 sec total cycle time). Collision

energy was ramped linearly as a function of mobility from 27 eV at $1/k_0$ = 0.7 V·s/cm^2 to 55 eV at $1/k_0$ = 1.35 V·s/cm^2. The absolute thresholds for mass spectra peak detection were set to 10, and for mobility peak detection to 5,000. Mass accuracy was typically within 3 ppm and not allowed to exceed 7 ppm. For ion mobility calibration, the ions of Agilent ESI-Low Tuning Mix ions were used (m/z [Th], $1/k_0$ [Th]: 622.0290, 0.9915; 922.0098, 1.1986; 1221.9906, 1.3934).

Raw data were searched together using DIA-NN in FragPipe (v1.8)[67] against a previously generated spectral library that was produced from the same tissues analyzed under identical conditions, except with the MS instrument operating in DDA mode[64]. Additional details on spectral library data processing can be found in our previous publication[64]. Within DIA-NN, default settings were used except that DIA-quantification mode was set to “Any LC (high precision).” Data from worker gut samples derived from the same bees described here were also acquired and searched together with the head and abdomen samples, but the results are not analyzed as part of this manuscript.

Transcriptomics analysis

A second subset of workers sampled from each colony were processed for transcriptomic analysis at York University, Ontario. The samples were produced using the same dissection and pooling strategy as for proteomics; that is, 10 pooled heads (antennae removed) and a separate sample of 10 pooled abdomens (digestive tract removed) were analyzed per colony (20 colonies total, n = 5 per experimental group). These pools were homogenized with ceramic beads in a Fisherbrand Bead Mill 24 (Thermo Fisher) according to the RNAeasy (Qiagen) kit manual, then RNA was extracted with the KingFisher Flex system according to the NucleoMag RNA kit manual (Thermo Fisher). Extracted RNA samples were assessed for quality and integrity using an Agilent 4200 TapeStation System with RNA ScreenTape. Purified RNA samples were then sent to Genome Quebec (Montreal, Quebec) for library preparation and paired-end sequencing on a NovaSeq 6000 (Illumina) with an average depth of 50 million reads. The reads were aligned to the current honey bee genome (Amel HAv3.1[68]) using STAR v2.9.7a[69] with default parameters. The resulting BAM files were used to generate count matrices via HTSeq-Count v0.13.5[70] with parameters: non-strandedness and the feature counting mode was intersection-nonempty.

Pheromone analysis by LC-MS/MS

Pheromone analysis was conducted on individual worker head extracts (one bee per sample, three samples per colony, 60 samples in total) exactly as previously described[31]. This sampling strategy differs from the proteomics and transcriptomics section because the pheromone extraction procedure is not well-suited for larger initial sample amounts. Therefore, instead of pooling 10 bees per colony, we analyzed multiple individual bees per colony, allowing us to associate pheromone abundance with ovary scores of specific workers, while incorporating colony as a factor in our statistical model. Whole heads

were analyzed as opposed to mandibular glands because the exact site of synthesis is not known for all pheromone components, but all are readily detectable in the head[23].

We followed a two-phase extraction procedure[71] in which the tissue was homogenized in methanol extraction solvent (400 µl 75% methanol, 0.1% butylated hydroxytoluene, in ddH_2O) that contained 1 ppm of CUDA (1-cyclohexylureido-3-dodecanoic acid) as an internal standard, then methylated tert-butyl ether (1 mL) was added and the samples were incubated for 1 h (1000 rpm shaking, room temperature). Next, clarified extract was mixed with water to induce phase separation. After a brief incubation at room temperature (10 min), samples were centrifuged (14,000 g, 15 min, 4°C) and 250 µl of the upper phase (lipids) was retained for pheromone analysis. Blank samples (n = 6) were processed in parallel with real samples and lacked only the bee tissue. The samples were stored at -70°C in solution until LC-MS/MS analysis.

Pheromone analysis was conducted using the previously described LC gradient and instrument settings[59]. Extracts were dried in a SpeedVac and resuspended in 250 µl of 70% acetonitrile and 30% isopropanol, then lipids were separated using a Vanquish UHPLC system (Thermo) equipped with an ACQUITY UPLC CSH C18 analytical column (130 Å, 1.7 µm, 2.1 mm × 100 mm, Waters). A multi-step gradient was employed, increasing from 20% to 99% mobile phase B over 18 minutes. Mobile phase A consisted of water, while mobile phase B was a mixture of acetonitrile and isopropanol, both containing 10 mM ammonium formate and 0.1% formic acid. The column was maintained at 65°C with a flow rate of 0.4 mL/min, and the samples were kept at 4°C. Mass spectrometry (MS) analysis was performed on a Bruker Impact II QTOF in positive (ESI+) electrospray ionization mode only. The capillary voltages were set to + 4,500 V with a dry gas temperature of 220°C and a scan range of 100–1,700 m/z. The spectral acquisition rate was set to 2 Hz, and a stepped MS/MS collision energy varying from 100% to 250% was applied, with 50% timing at each energy level. Dynamic MS/MS acquisition was utilized, targeting an intensity of 1125 counts, with an acquisition frequency ranging from 20 to 40 Hz. Structural information was improved by implementing a 15-second active exclusion window, minimizing redundant fragmentation, and improving isomer coverage.

Samples were injected in randomized order, except for blanks and pooled QC injections, which were injected every 14 samples. At the end of the sequence, blended pheromone samples (with each component at either 5 ppm or 10 ppm) were analyzed to facilitate confident pheromone peak identification. This blend included the QRP components 9-ODA, 9-HDA, HVA, HOB, MO, CA, and the pheromone and worker mandibular gland component 10-hydroxydec-(*E*)-2-enoic acid (10-HDA). Hexadecan-1-ol (palmityl alcohol; PA) is also a component QRP, but we previously determined it to be unsuitable for quantification by LC-MS/MS[59].

Pheromone data processing

Pheromone relative quantitation data was extracted using Progenesis QI™ software (V3.0.7600.27622) with the METLIN™ plugin V1.0.7642.33805 (NonLinear Dynamics). The data processing steps

encompassed peak picking, alignment, deconvolution, normalization, and database querying, as described previously[59,72,73]. Assignments were confirmed by comparison to spectra and retention times of external pheromone standards run on the same LC-MS/MS system.

Virus analysis

Composite samples of approximately 55 worker bees per colony (20 colonies total, n = 5 colonies per group) were screened, essentially as previously described[74], for nine viruses commonly found in honey bees: Israeli acute paralysis virus (IAPV), deformed wing virus A (DWV-A), *Varroa destructor* virus (VDV; also known as deformed wing virus B or DWV-B), acute bee paralysis virus (ABPV), Kashmir bee virus (KBV), chronic bee paralysis virus (CBPV), black queen cell virus (BQCV), Lake Sinai virus (LSV), and sacbrood virus (SBV). Samples were homogenized in 18 mL of GITC buffer (4.2 M guanidinium thiocyanate, 50 mM Tris, 20 mM EDTA, 1.3% Triton-X 100). An aliquot of 200 µl was used to isolate total RNA using the NucleoSpin®RNA kit following manufacturer instructions (Macherey-Nagel Gmbh & Co. KG, Düren, Germany). RNA was quantified using IMPLEN Nanophotometer NP80 (Munich, Germany). cDNA was synthesized from 800 ng of total RNA for 20 min at 46°C in a final volume of 20 µl using the iScript cDNA synthesis kit (Bio-Rad laboratories, Hercules, USA). cDNA was diluted with 60 µl of molecular grade water to a total of 80 µl from which 3 µl (30 ng) were used for qPCR quantification.

Quantification of virus infection levels was determined by real-time PCR (see Table S1 for primer sequences)[75–78] using SSoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, USA). Amplification assays were performed by triplicate employing ~ 30 ng of cDNA in a CFX384 Touch™ Real-Time Detection System (Bio-Rad Laboratories, Hercules, USA). RP49 was used as the reference gene. Standard curves were prepared from plasmids harbouring the target amplicons with copy numbers serially diluted 10^7-fold to 10^2-fold. PCR conditions were 3 min at 95°C for initial denaturation/enzyme activation followed by 40 cycles of 10 s at 95°C and 30 s at 60°C (except IAPV, where annealing/extension was 45 s at 60°C). Specificity was checked by performing a melt-curve analysis 65–95°C with increments of 0.5°C 2 s/step. Results were analyzed with the CFX Manager™ Software and exported to an Excel spreadsheet to calculate virus copy numbers per sample.

Statistical analysis

All statistical analyses were conducted in R (version 4.3.0)[79] using R Studio (version 2023.09.1 + 494).

Ovary scores were analyzed using a cumulative link mixed model (ordinal package[80,81]) with group (QRBR, QLBR, QRBL, QLBL) as a fixed effect and colony nested within replicate as a random intercept term (i.e., formula: Ovary score ~ Group + Operator + (1|Replicate/Colony)). Post-hoc tests among groups were conducted using emmeans[82] with mode set to “latent”, followed by Tukey multiple hypothesis testing correction.

For pheromone analysis, abundances of each pheromone were first normalized to CUDA, then $\log_2$ transformed and compared among groups using the same model structure as above to model the effect of group. A Bonferroni correction ($\alpha = 0.01$) was used to control false positives. Next, pheromone abundances in the QLBL group were analyzed with respect to ovary score as a continuous predictor and colony as a random effect. The QLBL group was employed in this analysis because it was the only group with meaningful representation across the full spectrum of ovary scores. Finally, to assess relationships with ovary development across the complete dataset, pheromone abundances across all groups were modelled with both group and ovary score as fixed effects and colony nested within replicate as a random effect.

Principal component analysis (PCA) of proteomics and transcriptomics data was conducted via the prcomp() function using scaled and centered complete observations.

Proteomics label-free quantification data were analyzed by first removing reverse hits, contaminant proteins, and proteins identified in < 50% of samples. The data were then $\log_2$ transformed, and the overall data distribution was visually inspected for normality. Heatmaps were plotted to screen for obvious outlier samples, but none were identified. Then, statistical comparisons were assessed using tools within the limma R package (v3.56.2)[83]. We used a limma model with empirical Bayes variance estimation to compare expression of experimental groups (QLBR, QRBL, QLBL) to the reference (QRBR). Replicate (parent colony) was included as a blocking factor using the duplicateCorrelation() function[84]. The false discovery rate was controlled to 5% using the Benjamini-Hochberg correction.

Transcriptomics data were analyzed using tools within the edgeR package (v3.42.4)[85] to convert count matrices to DGEList objects, then sample counts were converted to normalized (trimmed mean of M-values method) counts per million (cpm). A cutoff of 5 cpm was used to filter out lowly expressed genes, which was necessary to obtain an appropriate data distribution. We then $\log_2$ transformed the data and used the same statistical analysis framework as described for proteomics.

Queen cell abundances were compared among the QRBR reference group and treatment groups using a linear mixed model with replicate as a random effect.

Total viral load ($\log_{10}$ transformed) was evaluated among groups using a linear mixed model with group as a fixed effect and replicate as a random effect. Significant differences among all groups were then determined via pairwise contrasts followed by Tukey correction.

GO term enrichment

For proteomics data, GO terms corresponding to refSeq protein accessions were retrieved by first converting refSeq IDs to Uniprot IDs using the ID mapping tool available at https://www.uniprot.org/id-mapping, which were then used to link refSeq IDs to Uniprot gene ontology (GO) annotations of the *A. mellifera* UniprotKB proteome (downloaded September 19, 2025).

For transcriptomics data, functional annotation of differentially expressed genes was performed using eggnog, version emapper-2.1.12[86,87] and the RefSeq annotation database from NCBI (release date: November 4th, 2023; accession: GCF_003254395.2_Amel_HAv3.1_gene_ontology). *A. mellifera* protein sequences (version Amel_HAv3.1) were used as inputs to identify one to one orthologs and their associated GO identifiers. Default parameters were used, with a minimum homology identity of 75%, seed_ortholog_evalue and an e-value threshold of 5^{-3} and 5^{-6} respectively.

To perform enrichment tests, we used the gene score resampling (GSR) approach within ErmineJ (v3.1.2)[88] to identify significantly enriched GO terms. This approach does not depend on a p value threshold to parse the data into a hit-list vs. background; rather, it considers the p value continuum of all evaluated proteins or genes to test for functional bias among those with low p values. Up- and down-regulated proteins and genes were evaluated separately to easily assess the direction of change associated with proteins/genes underlying enriched GO terms. Enrichment FDR was controlled to 5% using the Benjamini-Hochberg procedure.

Data availability

All data underlying the results and figures presented in this manuscript are available within the supplementary material. Supplementary Data 1 includes primers used in qPCR assays (Table S1), worker ovary score data (Table S2), proteomics and transcriptomics sample metadata (Table S3), processed proteomics data (Table S4), results of statistical contrasts of proteomics data (heads: Table S5-S7; abdomens: Table S8-S9), significantly enriched GO terms resulting from differential protein expression (Table S10), processed transcriptomics data (Table S11), results of statistical contrasts of transcriptomics data (heads: Table S12-S14; abdomens: Table S15-S16), significantly enriched GO terms resulting from differential transcript expression (Table S17), pheromone data (Table S18), virus RT-qPCR analysis (Table S19), and queen cell abundances (Table S20).

Raw proteomics data (.d files) are publicly available on the PRIDE proteomics data archive (http://www.ebi.ac.uk/pride; accession/login: PXD077007; reviewer token: 1YJ5vqun6J2V). Raw transcriptomics data are available on the Short Read Archive (https://www.ncbi.nlm.nih.gov/sra; PRJNA1466999: BeeCSI QxB Transcriptomics).

Results

Ovary activation is additively controlled by the presence of larvae and a queen

Average ovary scores in all four groups (QRBR, QLBR, QRBL, and QLBL) sequentially increased (in that order) and were significantly different from one another, except the QRBL vs QLBL comparison, which was marginally not significant ($p = 0.052$) (cumulative link mixed model followed by post hoc Tukey contrasts; Fig. 1B, Table 2). All groups had significantly higher ovary scores than the reference group

(QRBR). No workers in the QRBR group possessed ovaries containing partially or fully developed eggs (ovary scores 2 and 3, respectively), whereas 40% of workers in the QLBL group displayed this level of activation (Fig. 1C).

Table 2
Post hoc testing results of ovary activation analysis

Contrast	Estimate	z ratio	P value
QRBR - QLBR	-2.6	-4.5	0.00010 *
QRBR - QRBL	-3.8	-6.2	< 0.00010 *
QRBR - QLBL	-4.8	-7.6	< 0.00010 *
QLBR - QRBL	-1.2	-3.0	0.017 *
QLBR - QLBL	-2.2	-5.2	< 0.00010 *
QRBL - QLBL	-1.0	-2.6	0.052
* Indicates statistical significance (Tukey adjusted p values)			

Ovary activation increases production of queen-like pheromones

We performed relative quantification of queen mandibular pheromone components (9-ODA, 9-HDA, HVA, and HOB) in addition to two queen retinue pheromone components (MO and CA) and 10-HDA. 9-ODA, 9-HDA, HOB, MO, and 10-HDA were detected in worker head lipid extracts (Fig. 2A), but none were significantly different among groups after Bonferroni correction ($p > \alpha = 0.01$). However, among workers from the QLBL group (which included the best representation of individuals with both developed and undeveloped ovaries), the major QMP components 9-HDA and 9-ODA significantly positively correlated with ovary score (Fig. 2B, Table 3). When the data from all groups are modelled together, the relationship between 9-HDA and ovary score remains significant (estimate = 0.49, df = 52.1, t = 3.7, p = 0.00054), while 9-ODA becomes marginally non-significant (estimate = 0.31, df = 55.0, t = 1.9, p = 0.066). Interestingly, 10-HDA (which is typically associated with nursing[89–91]) was also significantly linked to ovary score across the full dataset (estimate = 0.38, df = 52.5, t = 2.9, p = 0.0062). Worker ovary development therefore coincides with more queen-like pheromone profiles, though apparently not the full QMP suite, as well as the nursing-associated component 10-HDA.

Table 3
Statistical parameters of relative compound abundance with respect to ovary score among QLBL colonies

Compound	Estimate	df	T ratio	P value
10-HDA	0.52	11.5	2.7	0.019
9-HDA	0.033	12.7	3.3	0.0065*
9-ODA	0.010	12.6	3.8	0.0022*
HOB	0.0011	12.4	0.23	0.82
MO	0.012	12.8	1.32	0.21
* Indicates statistical significance (Bonferroni adjusted at $p < \alpha = 0.01$)				

Because we previously determined that MO is positively linked to ovary activation in queens, we hypothesized that the same would be true in workers. Our data do not support this idea; we identified no relationship between MO levels and worker ovary score within the QLBL group (estimate = 0.012, df = 12.8, t = 1.3, p = 0.21) nor the full dataset modelled as described above (estimate = -0.020, df = 53.0, t = -0.13, p = 0.90).

The impact of broodlessness on the proteome and transcriptome is partially masked by queenlessness

We quantified 4,857 proteins and 9,604 transcripts overall. Principal component analyses of the head (Fig. 3A) and abdomen (Fig. 3B) proteome and transcriptome show that expression profiles in the QRBR (reference) and QLBR groups were similar, occupying comparable dimensions in the PC space and diverging from the broodless groups. However, some separation of the QRBR and QLBR groups was observed among head proteins. Similarly, no differentially expressed proteins or transcripts were observed comparing the QRBR and QLBR groups except for 56 significantly different proteins in the head (5% FDR, Benjamini-Hochberg correction; Fig. 3C **& D**). Strong differential expression was observed in the QRBL group, though, most notably among head transcripts, where ~ 40% of the transcriptome was significantly altered.

Queenlessness partially reversed the broodlessness effect, reducing the fraction of differentially expressed features by 12–32% in the head and 75–78% in the abdomen. There were no differentially expressed features in the QRBR vs QLBL contrast that were not also differentially expressed in the QRBR vs QRBL or QRBR vs QLBR contrasts.

We performed GO term enrichment analysis to identify biological functions that were disproportionately represented among differentially expressed proteins and transcripts (Fig. 4). In the head, ribosomal proteins were consistently enriched among downregulated proteins in all contrasts with the QRBR reference group, whereas proteins associated with tRNA aminoacylation were enriched among downregulated proteins in the queenless (QLBR and QLBL) groups. Queenlessness and broodlessness

thus appear to regulate different aspects of protein translation in the head. Relatedly, “protein metabolic process” was significantly enriched among downregulated head transcripts in broodless groups, in addition to transcripts encoding proteins destined for the endoplasmic reticulum (the surface of which is a major site of translation). Broodlessness specifically upregulated proteins involved in defense response (immunity) and extracellular proteins (secretions), while proteins related to vesicular transport were downregulated. Only two GO terms, “oxidoreductase activity” and “positive regulation of establishment of protein localization” were significantly enriched in the QRBL-QRBR contrast but absent from the QLBL-QRBR contrast, representing two functional categories of expression changes that were induced by broodlessness and mitigated by queenlessness.

Expression patterns of MRJPs and vitellogenin suggest that broodless groups retain nursing potential

Because we observed enrichment of extracellular localization among upregulated head proteins in broodless groups (Fig. 4) in addition to an association between ovary development and the fatty acid royal jelly component 10-HDA (Fig. 2), we inspected expression patterns of major royal jelly proteins (MRJPs), which are the primary protein component in the jelly secretion that nurse bees feed to developing larvae. MRJPs were significantly more abundant in both broodless groups relative to the QRBR group (Fig. 5A), suggesting that the proteins accumulate in the workers’ heads as a result of being unable to dispense them to larvae, and that they maintain nursing potential. Accordingly, there were no changes in MRJP abundances in the abdomens, where they are expressed but do not contribute to larval secretions. Transcripts of the same MRJPs (MRJP1-4, MRJP5, and MRJP7) were downregulated in both broodless groups (**Table S12 and S13**), indicating that the high protein abundance was due to failure to dispense and not continued biosynthesis; therefore, the protein abundance does not necessarily indicate nursing potential. Rather, there appears to be a negative feedback mechanism that prevents MRJP synthesis when undispensed loads are already high.

We further hypothesized that vitellogenin would be more abundant in broodless groups owing to its relationship with reproduction, ovary development, and nursing behaviour that has been shown previously[51,53,55,56,92–94]. Vitellogenin and the lipid transport proteins apolipophorins I/II, apolipophorin III, very high-density lipoprotein (VHDL), and apolipoprotein D were all among the upregulated proteins in abdomens of broodless groups. The pattern was weaker in the heads, with only vitellogenin and apoliphophorins I/II being significantly upregulated. This is consistent both with lipid mobilization being important for worker ovary development as well as a reversion to nursing capability, both of which are associated with higher vitellogenin expression. Vitellogenin was the most significantly differentially expressed transcript in the head of both broodless groups, and was in the top 5 most significant transcripts in the abdomens from the same groups.

Table 4
Statistical analysis of JH-related transcripts

Tissue	Transcript	Contrast	Estimate	df	T ratio	P value
Head	JHBP-1	QRBR-QLBR	-0.48	12	-1.4	0.54
		QRBR-QRBL	-1.7	12	-5.0	0.0016 *
		QRBR-QLBL	-1.6	12	-4.7	0.0027 *
		QLBR-QRBL	-1.3	12	-3.6	0.017 *
		QLBR-QLBL	-1.1	12	-3.3	0.029
		QRBL-QLBL	0.11	12	0.31	0.99
	JHE	QRBR-QLBR	-0.47	12	-2.1	0.22
		QRBR-QRBL	-2.1	12	-9.2	< 0.00010 *
		QRBR-QLBL	-1.9	12	-8.3	< 0.00010 *
		QLBR-QRBL	-1.7	12	-7.2	0.00010 *
		QLBR-QLBL	-1.4	12	-6.2	0.00020 *
		QRBL-QLBL	0.22	12	0.94	0.78
	JHMT	QRBR-QLBR	-0.51	12	-1.35	0.55
		QRBR-QRBL	-1.12	12	-3.0	0.050
		QRBR-QLBL	-1.4	12	-3.7	0.013 *
		QLBR-QRBL	-0.61	12	-1.6	0.40
		QLBR-QLBL	-0.90	12	-2.4	0.13
		QRBL-QLBL	-0.29	12	-0.76	0.87
Abdomen	JHBP-1	QRBR-QLBR	0.17	12	0.28	0.99
		QRBR-QRBL	1.0	12	1.7	0.37
		QRBR-QLBL	-0.16	12	-0.27	0.99
		QLBR-QRBL	0.84	12	1.4	0.52
		QLBR-QLBL	-0.33	12	-0.55	0.94
		QRBL-QLBL	-1.17	12	-2.0	0.26
	JHE	QRBR-QLBR	-0.12	12	-0.40	0.98

* Indicates statistical significance (Tukey adjusted p values, further Bonferroni corrected at Tukey $p < \alpha = 0.017$)

Tissue	Transcript	Contrast	Estimate	df	T ratio	P value
		QRBR-QRBL	-1.5	12	-5.0	0.0016 *
		QRBR-QLBL	-2.0	12	-6.4	0.00020 *
		QLBR-QRBL	-1.4	12	-4.6	0.0032 *
		QLBR-QLBL	-1.8	12	-6.0	0.00030 *
		QRBL-QLBL	-0.43	12	-1.4	0.52
	JHMT	QRBR-QLBR	0.044	12	0.11	1.0
		QRBR-QRBL	0.57	12	1.5	0.48
		QRBR-QLBL	-0.87	12	-2.3	0.17
		QLBR-QRBL	0.53	12	1.4	0.54
		QLBR-QLBL	-0.91	12	-2.4	0.14
		QRBL-QLBL	-1.4	12	-3.7	0.013 *

* Indicates statistical significance (Tukey adjusted p values, further Bonferroni corrected at Tukey $p < \alpha = 0.017$)

If workers in the QLBL groups are becoming more queen-like or nurse-like on average, we hypothesized that their elevated vitellogenin levels would be associated with a concomitant decrease in juvenile hormone. We did not acquire direct juvenile hormone measurements, but we reasoned that levels could be inferred based on juvenile hormone methyl transferase (JHMT) and juvenile hormone esterase (JHE) transcript abundances, which positively and negatively influence JH levels, respectively. We therefore expected JHMT levels to be low (indicating reduced biosynthesis) and JHE levels to be high (indicating degradation) in the QLBL group, especially in the head, which is the site of JH production. Surprisingly, both JHMT and JHE were more abundant in QLBL head extracts, with similar patterns in the abdomen (Fig. 5B **& C**). JHE was also elevated in the QLBR group in both tissues. Juvenile hormone binding protein (JHBP), which both transports JH in the hemolymph and protects it from JHE degradation, was also elevated in head extracts from broodless groups. With JHMT, JHBP, and JHE transcripts all changing simultaneously, the genes governing JH availability are undergoing conflicting shifts and may not be used to predict the impact on JH levels.

Queen cell initiation in QLBL colonies indicates worker egg laying and nursing capacity

Queen cell initiation occurred predominantly in the QLBR and QLBL groups (**Figure S1**, Table 5). The QLBR group initiated significantly more total queen cells than the QRBR group (estimate = 4.2, df = 12, t = 3.2, p = 0.0071), as expected, whereas the increase in the QLBL group was marginally non-significant (estimate = 2.6, df = 12, t = 2.0, p = 0.068). This is consistent with the possibility that some workers in the

QLBL group may have become more queen-like (laying occasional eggs that were not removed by policing workers) while others were nurse-like (attempting to rear haploid eggs into queens), despite being > 19 d old (beyond the typical nursing window of 2–14 d[95]). Little cell rearing activity was observed in the QRBL group compared to QRBR (estimate = 0.80, df = 12, t = 0.62, p = 0.55), which is consistent with their lesser degree of ovary activation compared to QLBL, likely corresponding to an absence of available eggs (Fig. 1B).

Table 5
Average total queen cell count per colony across the observational period*

Group	Queen cell count	sd	Estimate	df	T ratio	P value
QRBR	0.4	0.80	-	-	-	-
QLBR	4.6	3.1	4.2	12	3.2	0.0071
QRBL	1.2	1.2	0.80	12	0.62	0.55
QLBL	3.0	2.1	2.6	12	2.0	0.068
*Statistical comparisons were made with respect to the QRBR reference group						

Broodlessness reduces virus infections

Owing to the observed upregulation of immune defense response proteins in the broodless groups (Fig. 4), we conducted pathogen testing of six honey bee viruses to assess the potential association with infection levels. We hypothesized that the higher abundance of immune proteins would occur due to more intense infections (i.e., infection levels would dictate the immune response, rather than the immune response dictating infection levels), possibly as a result of differential *Varroa* dynamics among groups. However, we observed the opposite; the broodless groups with higher immune protein abundances actually had significantly lower total virus burdens (Fig. 6, Table 6), indicating that the causal relationship likely stems from immune protein expression rather than the presence of pathogens. The overall reduction in viral burden appears to be driven by negative impacts of broodlessness on SBV, BQCV, and LSV. Honey bee queens have been previously shown to have elevated immune protein expression than workers[54], thus, the shift for workers to gain improved immunity is consistent with a shift toward queenliness.

Table 6
Statistical analysis of total virus burden

Contrast	Estimate	df	T ratio	P value
QRBR-QLBR	-0.16	12	-0.45	0.97
QRBR-QRBL	1.7	12	4.8	0.0022 *
QRBR-QLBL	1.3	12	3.6	0.017 *
QLBR-QRBL	1.8	12	5.2	0.0010 *
QLBR-QLBL	1.4	12	4.0	0.0077 *
QRBL-QLBL	-0.42	12	-1.2	0.64
* Indicates statistical significance (Tukey corrected p values)				

Discussion

Our results demonstrate that brood functions as a central regulator of worker physiology, extending its influence well beyond the inhibition of worker reproduction. By experimentally disentangling queen and brood signals, we show that brood coordinates not only reproductive physiology, but also pheromone production, molecular expression, and antiviral immunity, while queen signals contribute to modulating these effects in a context-dependent manner. Consistent with previous reports, both queenlessness and broodlessness promoted worker ovary activation, with the absence of brood having the stronger effect[3,4,11–14]. Surprisingly, while the two factors acted additively to promote ovary activation, queenlessness partially reversed the proteomic and transcriptomic changes associated with broodlessness.

We speculate that this attenuation could occur via three non-mutually exclusive mechanisms. First, workers with fully developed ovaries (which was most common in the QLBL group; Fig. 1) may create a somewhat ‘queenright’ environment by producing queen-like pheromone. Indeed, we observed that ovary activation correlated with elevated levels of the major queen pheromone components 9-ODA and 9-HDA (Fig. 2), which could have knock-on physiological effects on other workers, as has been previously observed[16]. Second, these reproductively activated workers may themselves exhibit expression profiles more similar to active nurses than they do to bees that are not nursing, which is consistent with the reproductive ground plan hypothesis[7,47]. Third, workers with developed ovaries may have caused the QLBL group to not be been entirely broodless due to potential initiation of worker egg laying. Classic laying worker syndrome (in which multiple eggs are deposited within cells) was absent from all colonies, but there could have been very low frequencies of worker egg laying in the QLBL colonies that was not readily apparent during inspections. Indeed, queen cell rearing frequencies (in which queen cups were active, containing either an egg or a larva) were highest two weeks after experiment initiation (**Figure S1**), long after any queen-originated eggs or larvae would be viable for cell rearing (all would be at or beyond the pupal stage). Given this timeline, the active queen cells could only have originated from

worker-laid, haploid (drone) eggs, as others have previously observed[96]. These are clearly not viable as queens, but could nonetheless contribute to brood pheromonal signalling to the colony, especially since drones produce larger amounts of some brood pheromones[97]. Therefore, workers in QLBL colonies may have been beginning to re-experience low levels of queen-like pheromone (from the workers with developed ovaries) *and* low levels of brood pheromone (from occasionally hatching worker-laid eggs), thereby returning the non-reproductive workers to a more normal (QRBR) pheromonal and physiological state.

We speculate that these three mechanisms are operating in concert, but their relative contributions cannot be resolved with the current dataset due to pooled sampling and age-related biases. Because each 'omics sample represents ten pooled individuals, our results reflect average worker states (though accuracy is somewhat limited by small samples). Disentangling which expression patterns are contributed by nurse bees versus reproductive bees and the degree to which these processes overlap would require individual-level data paired with ovary and hypopharyngeal gland assessments. Age-matching the samples or including only eggs in the broodright colonies (adult emergence of which would not occur within the experimental timeline) would also be necessary to eliminate the possibility of age biases among groups. In the current experimental design, the broodright groups inherently include a larger proportion of younger workers compared to broodless groups at the end of the experiment, when bees were sampled. This limits our ability to separate the effect of aging from the effect of broodlessness; however, because the QRBL and QLBL groups shared same age distributions, the consistent partial reversal of the broodless effect by queenlessness is particularly robust and intriguing.

Another unanswered question is that it is it not clear how worker ovary development remains so high in QLBL colonies despite the partial restoration of pheromones. An intriguing hypothesis is that, once a worker's ovaries become activated, the bee becomes somewhat immune to inhibitory signals. This may also explain how laying workers (and queens) circumvent self-inhibition, but to our knowledge, the idea is yet to be tested.

Based on previous findings linking one QRP component (methyl oleate) to ovary mass in queens[31], we expected similar relationships in workers; however, our data do not support this hypothesis. We found that only 9-ODA and 9-HDA, the major QMP components, were linked to ovary activation, not methyl oleate (Fig. 2). The lack of correlation between methyl oleate and worker ovary activation suggests that this pheromone serves a queen-adapted function, such as alerting workers to the queen's health and fertility status to help regulate supersedure[31], which is irrelevant in the case of laying workers.

Previous research has identified relationships between other pheromone components (10-HDA, HVA, and HOB) and worker ovary activation[39]; among these, we could not quantify HVA, which fell below our limit of detection (30.3 nM), and the others yielded no significant association after multiple hypothesis testing correction. Indeed, there is little agreement in the literature about which pheromone components, if any, are upregulated in workers that are laying or have activated ovaries, regardless of subspecies[37,39,40,44]. Two studies found that 9-HDA was the only associated compound[37,40], while

another found that only 9-ODA was linked to worker reproduction[39], and another found no relationship[44]. Since 9-HDA is the precursor for 9-ODA biosynthesis[98], it is sensible for them to covary, as we observed here. Despite variation in exact outcomes, our data and existing studies[16,39] do suggest that the link between pheromone production and ovary activation in workers with European-derived genetics warrants reconsideration.

Perhaps the most unexpected result in our data was the negative impact of broodlessness on natural virus infections, which may offer a management strategy to help combat these pervasive pathogens. This initially surprised us, but others have independently established a similar link between worker ovary activation, immune protein abundance, and virus resistance[99–101], suggesting that this is a reproducible biological phenomenon. Our data show that 19 days of broodlessness, even in the presence of a caged queen, significantly reduced overall virus infection levels by ~ 10-fold (Fig. 6). While the mechanism remains unclear, the abundance of hemocytes (important elicitors of immunity) is thought to be controlled by vitellogenin[92], which was within the top 5 and top 20 differentially expressed transcripts and proteins, respectively, in abdomens sampled from both broodless groups. Perhaps not coincidentally, vitellogenin expression in winter bees is also high[102–104], which corresponds with a natural period of broodlessness, and sometimes, but not always, lower virus titres[105–109].

Induced broodlessness is already employed as a means of controlling the parasitic mite, *Varroa destructor*, and our data suggest that it is also a promising strategy for directly mitigating virus infections. Broodless periods may thus offer a two-pronged approach to virus management by both preventing *Varroa* reproduction (thereby suppressing its capability as a virus vector) and reducing existing virus infections by making workers more physiologically resistant. We speculate that the former is less important than the latter, given that in our data, the three viruses most affected by broodlessness were BQCV, SBV, and LSV, none of which are consistently vectored by *Varroa*[58]. While *Varroa* infestation levels were not quantified in our experimental colonies, the parent colonies received a miticide treatment before the experiment began, and regardless, this pattern suggests that any indirect effect of broodlessness on virus infections via *Varroa* control is minor compared to the direct effect on worker physiology. Efficacy trials are warranted to better document the phenomenon, potential trade-offs, and to determine the optimal duration of broodlessness.

Our findings suggest that brood plays a broader role in regulating worker honey bee physiology than previously recognized. We identify context-dependent effects of queen and brood social cues that extends beyond worker reproduction to include pheromone production, molecular expression, and immunological defense. Together, these findings provide a more integrated view of how social cues shape worker phenotype and highlight induced broodlessness as a promising avenue for improving colony health. Future work should aim to determine the degree to which this interactive pattern of queen- and brood-based social regulation is evolutionarily conserved and explore induced broodlessness as an antiviral treatment.

Declarations

Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

AM: statistical analysis, figure generation, writing – original draft. LH: Methodology, investigation, supervision, writing – reviewing and editing. KG: Investigation, statistical analysis, writing – original draft. JK: Methodology, investigation, supervision. AAM: Methodology, investigation, data curation, writing – reviewing and editing. RM: Methodology, investigation, supervision, writing – reviewing and editing. WWTF: Investigation, writing – reviewing and editing. XY: Investigation. YS: Data curation, writing – reviewing and editing. HZ: Data curation, writing – reviewing and editing. PWV: Investigation, writing – reviewing and editing. IMC: Supervision. MP: Methodology, investigation, writing – reviewing and editing. AJ: Methodology, investigation, data analysis, and writing – reviewing and editing. DRT: Supervision, writing – reviewing and editing. SFP: Funding acquisition, writing – reviewing and editing. PG: Funding acquisition, writing – reviewing and editing. EG: Funding acquisition, writing – reviewing and editing. RWC: Funding acquisition, writing – reviewing and editing. MMG: Funding acquisition, writing – reviewing and editing. AZ: Supervision, funding acquisition, writing – reviewing and editing. LJF: Supervision, funding acquisition, investigation, and writing – reviewing and editing. SEH: conceptualization, methodology, investigation, supervision, funding acquisition, writing – reviewing and editing.

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References

1. Nowak, M.A., Tarnita, C.E., Wilson, E.: O. The evolution of eusociality. Nature. **466**, 1057–1062 (2010)
2. Honeybee Genome Sequencing Consortium: Insights into social insects from the genome of the honeybee *Apis mellifera*. Nature. **443**, 931–949 (2006)
3. Jay, S., Nelson, E.: The effects of laying worker honeybees (*Apis mellifera* L.) and their brood on the ovary development of other worker honeybees. Can. J. Zool. **51**, 629–632 (1973)
4. Mohammedi, A., Paris, A., Crauser, D., Le Conte, Y.: Effect of aliphatic esters on ovary development of queenless bees (*Apis mellifera* L). Naturwissenschaften. **85**, 455–458 (1998)
5. Smedal, B., Brynem, M., Kreibich, C., Amdam, G.: Brood pheromone suppresses physiology of extreme longevity in honeybees (*Apis mellifera*). J. Exp. Biol. **212**, 3795–3801 (2009)
6. Le Conte, Y., Mohammedi, A., Robinson, G.E.: Primer effects of a brood pheromone on honeybee behavioural development. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 268, 163–168 (2001)
7. Traynor, K.S., Le Conte, Y., Page, R.E. Jr.: Queen and young larval pheromones impact nursing and reproductive physiology of honey bee (*Apis mellifera*) workers. Behav. Ecol. Sociobiol. **68**, 2059–2073 (2014)
8. Ratnieks, F.L.: Egg-laying, egg-removal, and ovary development by workers in queenright honey bee colonies. Behav. Ecol. Sociobiol. **32**, 191–198 (1993)
9. Visscher, P.K.: Reproductive conflict in honey bees: A stalemate of worker egg-laying and policing. Behav. Ecol. Sociobiol. **39**, 237–244 (1996)
10. Beekman, M., Allsopp, M.H., Jordan, L.A., Lim, J., Oldroyd, B.: P. A quantitative study of worker reproduction in queenright colonies of the Cape honey bee, *Apis mellifera capensis*. Mol. Ecol. **18**, 2722–2727 (2009)
11. De Groot, A., Voogd, S.: On the ovary development in queenless worker bees (*Apis mellifica* L). Experientia. **10**, 384–385 (1954)
12. Butler, C., Fairey, E.M.: The role of the queen in preventing oogenesis in worker honeybees. J. Apic. Res. **2**, 14–18 (1963)
13. Butler, C.: The control of ovary development in worker honeybees (*Apis mellifera*). Experientia. **13**, 256–257 (1957)
14. Voogd, S.: Inhibition of ovary development in worker bees by extraction fluid of the queen. Experientia. **11**, 181–182 (1955)
15. Velthuis, H.: Ovarian development in *Apis mellifera* worker bees. Entomol. Exp. Appl. **13**, 377–394 (1970)
16. Velthuis, H., Verheijen, F., Gottenbos, A.: Laying worker honey bee: Similarities to the queen. Nature. **207**, 1314–1314 (1965)
17. Santos, P.K., Galbraith, D.A., Starkey, J., Amsalem, E.: The effect of the brood and the queen on early gene expression in bumble bee workers' brains. Sci. Rep. **12**, 3018 (2022)

18. Orlova, M., Starkey, J., Amsalem, E.: A small family business: Synergistic and additive effects of the queen and the brood on worker reproduction in a primitively eusocial bee. J. Exp. Biol. **223**, jeb217547 (2020)
19. Carmona-Aldana, F., Yong, L.W., Reinberg, D., Desplan, C.: Phenomenon of reproductive plasticity in ants. Curr. Opin. Insect Sci. **63**, 101197 (2024)
20. Page, R. Jr., Erickson, E. Jr.: Reproduction by worker honey bees (*Apis mellifera* L). Behav. Ecol. Sociobiol. **23**, 117–126 (1988)
21. Ruttner, F., Hesse, B.: Rassenspezifische unterschiede in ovarentwicklung und eiablage von weisellosen arbeiterinnen der honigbiene *Apis mellifera* L. *Apidologie* 12, 159–183 (1981)
22. Slessor, K.N., Winston, M.L., Le Conte, Y.: Pheromone communication in the honeybee (*Apis mellifera* L). J. Chem. Ecol. **31**, 2731–2745 (2005)
23. Keeling, C.I., Slessor, K.N., Higo, H.A., Winston, M.L.: New components of the honey bee (*Apis mellifera* L.) queen retinue pheromone. *Proceedings of the National Academy of Sciences* 100, 4486–4491 (2003)
24. Oi, C.A., et al.: The origin and evolution of social insect queen pheromones: Novel hypotheses and outstanding problems. BioEssays. **37**, 808–821 (2015)
25. Butler, C., Fairey, E.M.: Pheromones of the honeybee: biological studies of the mandibular gland secretion of the queen. J. Apic. Res. **3**, 65–76 (1964)
26. Hoover, S.E., Keeling, C.I., Winston, M.L., Slessor, K.: N. The effect of queen pheromones on worker honey bee ovary development. Naturwissenschaften. **90**, 477–480 (2003)
27. Hoover, S.E., Winston, M.L., Oldroyd, B.P.: Retinue attraction and ovary activation: responses of wild type and anarchistic honey bees (*Apis mellifera*) to queen and brood pheromones. Behav. Ecol. Sociobiol. **59**, 278–284 (2005)
28. Pankiw, T., Winston, M., Robinson, G.: Queen mandibular gland pheromone influences worker honey bee (*Apis mellifera* L.) foraging ontogeny and juvenile hormone titers. J. Insect. Physiol. **44**, 685–692 (1998)
29. Pankiw, T., Winston, M., Slessor, K.: Queen attendance behavior of worker honey bees (*Apis mellifera* L.) that are high and low responding to queen mandibular pheromone. Insectes Soc. **42**, 371–378 (1995)
30. Winston, M.L., Higo, H.A., Slessor, K.N.: Effect of various dosages of queen mandibular gland pheromone on the inhibition of queen rearing in the honey bee (Hymenoptera: Apidae). Ann. Entomol. Soc. Am. **83**, 234–238 (1990)
31. McAfee, A., et al.: Elevated virus infection of honey bee queens reduces methyl oleate production and destabilizes colony-level social structure. *Proceedings of the National Academy of Sciences* 122, e2518975122 (2025)
32. Maisonnasse, A., Lenoir, J.C., Beslay, D., Crauser, D., Le Conte, Y.: E-beta-ocimene, a volatile brood pheromone involved in social regulation in the honey bee colony (*Apis mellifera*). PLoS One. **5**, e13531 (2010). https://doi.org/10.1371/journal.pone.0013531

33. Traynor, K.S., Le Conte, Y., Page, R.E.: Age matters: pheromone profiles of larvae differentially influence foraging behaviour in the honeybee, *Apis mellifera*. Anim. Behav. **99**, 1–8 (2015)
34. Le Conte, Y., Arnold, G., Trouiller, J., Masson, C., Chappe, B.: Identification of a brood pheromone in honeybees. Naturwissenschaften. **77**, 334–336 (1990)
35. Maisonnasse, A., et al.: A scientific note on E-beta-ocimene, a new volatile primer pheromone that inhibits worker ovary development in honey bees. Apidologie. **40**, 562–564 (2009)
36. Peso, M., Barron, A.B.: The effects of brood ester pheromone on foraging behaviour and colony growth in apicultural settings. Apidologie. **45**, 529–536 (2014)
37. Mumoki, F.N., Pirk, C.W., Yusuf, A.A., Crewe, R.M.: Reproductive parasitism by worker honey bees suppressed by queens through regulation of worker mandibular secretions. Sci. Rep. **8**, 7701 (2018)
38. Yusuf, A.A., Crewe, R.M., Pirk, C.W.: Turning workers into false queens: the role of exogenous pheromones in regulating reproduction in worker honey bees. J. Exp. Biol. **221**, jeb175505 (2018)
39. Tan, K., Yang, M., Wang, Z., Radloff, S.E., Pirk, C.W.: The pheromones of laying workers in two honeybee sister species: *Apis cerana* and *Apis mellifera*. J. Comp. Physiol. A. **198**, 319–323 (2012)
40. Niu, D.-F., et al.: Reproductive traits and mandibular gland pheromone of anarchistic honey bee workers *Apis mellifera* occurring in China. Apidologie. **47**, 515–526 (2016)
41. Crewe, R., Velthuis, H.: False queens: A consequence of mandibular gland signals in worker honeybees. Naturwissenschaften. **67**, 467–469 (1980)
42. Simon, U.E., Moritz, R.F., Crewe, R.M.: The ontogenetic pattern of mandibular gland components in queenless worker bees (*Apis mellifera capensis* Esch). J. Insect. Physiol. **47**, 735–738 (2001)
43. Plettner, E., et al.: Species-and caste-determined mandibular gland signals in honeybees (*Apis*). J. Chem. Ecol. **23**, 363–377 (1997)
44. Plettner, E., Slessor, K., Winston, M., Robinson, G., Page, R.: Mandibular gland components and ovarian development as measures of caste differentiation in the honey bee (*Apis mellifera* L). J. Insect. Physiol. **39**, 235–240 (1993)
45. Nagel, M., et al.: The gene expression network regulating queen brain remodeling after insemination and its parallel use in ants with reproductive workers. Sci. Adv. **6**, eaaz5772 (2020)
46. Grozinger, C.M., Fan, Y., Hoover, S.E., Winston, M.L.: Genome-wide analysis reveals differences in brain gene expression patterns associated with caste and reproductive status in honey bees (*Apis mellifera*). Mol. Ecol. **16**, 4837–4848 (2007)
47. Amdam, G.V., Norberg, K., Fondrk, M.K., Page, R.E. Jr.: Reproductive ground plan may mediate colony-level selection effects on individual foraging behavior in honey bees. *Proceedings of the National Academy of Sciences* 101, 11350–11355 (2004)
48. Engels, W.: Occurrence and significance of vitellogenins in female castes of social Hymenoptera. Am. Zool., 1229–1237 (1974)
49. Engels, W., Imperatriz-Fonseca, V.L.: Caste development, reproductive strategies, and control of fertility in honey bees and stingless bees. In: Social insects: An evolutionary approach to castes and

reproduction. Springer (1990)

50. Rodriguez Messan, M., Page, R.E., Kang, Y.: Effects of vitellogenin in age polyethism and population dynamics of honeybees. Ecol. Model. **388** (2018)
51. Amdam, G.V., Norberg, K., Hagen, A., Omholt, S.W.: Social exploitation of vitellogenin. *Proceedings of the National Academy of Sciences* 100, 1799–1802 (2003)
52. Amdam, G.V., Fennern, E., Havukainen, H.: Vitellogenin in honey bee behavior and lifespan. In: Honeybee neurobiology and behavior: a tribute to Randolf Menzel. Springer- (2011)
53. McAfee, A., et al.: Conserved and unique protein expression patterns across reproductive stage transitions in social hymenopteran queens. Mol. Ecol., e17568 (2024)
54. Chan, Q.W., et al.: Honey bee protein atlas at organ-level resolution. Genome Res. **23**, 1951–1960 (2013). https://doi.org/10.1101/gr.155994.113
55. Hartfelder, K., Engels, W.: Social insect polymorphism: Hormonal regulation of plasticity in development and reproduction in the honeybee. Curr. Top. Dev. Biol. **40**, 45–77 (1998)
56. Corona, M., et al.: Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. *Proceedings of the National Academy of Sciences* 104, 7128–7133 (2007). https://doi.org/10.1073/pnas.0701909104
57. Barchuk, A.R., et al.: Molecular determinants of caste differentiation in the highly eusocial honeybee *Apis mellifera*. BMC Dev. Biol. **7**, 70 (2007). https://doi.org/10.1186/1471-213X-7-70
58. Traynor, K.S., et al.: *Varroa destructor*: A complex parasite, crippling honey bees worldwide. Trends Parasitol. **36**, 592–606 (2020). https://doi.org/10.1016/j.pt.2020.04.004
59. McAfee, A., Magaña, A.A., Foster, L.J., Hoover, S.E.: Differences in honey bee queen pheromones revealed by LC-MS/MS: Reassessing the honest signal hypothesis. iScience (2024)
60. Visscher, P.: Colony integration and reproductive conflict in honey bees. Apidologie. **29**, 23–45 (1998)
61. Breed, M.D., Velthuis, H.H., Robinson, G.E.: Do worker honey bees discriminate among unrelated and related larval phenotypes? Ann. Entomol. Soc. Am. **77**, 737–739 (1984)
62. Page, R.E. Jr., Erickson, E.H. Jr.: Kin recognition during emergency queen rearing by worker honey bees (Hymenoptera: Apidae). Ann. Entomol. Soc. Am. **79**, 460–467 (1986)
63. Pernal, S.F., Currie, R.W.: Pollen quality of fresh and 1-year-old single pollen diets for worker honey bees (*Apis mellifera* L). Apidologie. **31**, 387–409 (2000)
64. Zhong, H., et al.: Omics insights into the effects of highbush blueberry and cranberry crop agroecosystems on honey bee health and physiology. *Proteomics*, e70033 (2025)
65. Shevchenko, A., Tomas, H., Havli, J., Olsen, J.V., Mann, M.: In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat. Protoc. **1**, 2856–2860 (2006)
66. Rappsilber, J., Ishihama, Y., Mann, M.: Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. Anal. Chem. **75**, 663–670 (2003)

67. Demichev, V., Messner, C.B., Vernardis, S.I., Lilley, K.S., Ralser, M.: DIA-NN: neural networks and interference correction enable deep proteome coverage in high throughput. Nat. Methods. **17**, 41–44 (2020)
68. Wallberg, A., et al.: A hybrid de novo genome assembly of the honeybee, *Apis mellifera*, with chromosome-length scaffolds. BMC Genom. **20**, 1–19 (2019)
69. Dobin, A., et al.: STAR: ultrafast universal RNA-seq aligner. Bioinformatics. **29**, 15–21 (2013). https://doi.org/10.1093/bioinformatics/bts635
70. Anders, S., Pyl, P.T., Huber, W.: HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics. **31**, 166–169 (2015)
71. Chen, S., et al.: Simultaneous extraction of metabolome and lipidome with methyl tert-butyl ether from a single small tissue sample for ultra-high performance liquid chromatography/mass spectrometry. J. Chromatogr. A. **1298**, 9–16 (2013)
72. Alcazar Magaña, A., et al.: Vitamin C activates the folate-mediated one-carbon cycle in C2C12 myoblasts. Antioxidants. **9**, 217 (2020)
73. Alcazar Magaña, A., et al.: Integration of mass spectral fingerprinting analysis with precursor ion (MS1) quantification for the characterisation of botanical extracts: Application to extracts of *Centella asiatica* (L). Phytochem. Anal. **31**, 722–738 (2020)
74. French, S.K., et al.: Honey bee stressor networks are complex and dependent on crop and region. Curr. Biol. **34**, 1893–1903 (2024). https://doi.org/https://doi.org/ 10.1016/j.cub.2024.03.039
75. Locke, B., Forsgren, E., Fries, I., de Miranda, J.R.: Acaricide treatment affects viral dynamics in *Varroa destructor*-infested honey bee colonies via both host physiology and mite control. Appl. Environ. Microbiol. **78**, 227–235 (2012)
76. McMahon, D.P., et al.: A sting in the spit: widespread cross-infection of multiple RNA viruses across wild and managed bees. J. Anim. Ecol. **84**, 615–624 (2015). https://doi.org/10.1111/1365-2656.12345
77. Vanengelsdorp, D., et al.: Colony collapse disorder: a descriptive study. PLoS One. **4**, e6481 (2009). https://doi.org/10.1371/journal.pone.0006481
78. Daughenbaugh, K.F., et al.: Honey bee infecting Lake Sinai viruses. Viruses. **7**, 3285–3309 (2015)
79. R Core Team: R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria (2023)
80. Christensen, R.H.B.: ordinal—Regression Models for Ordinal Data. R package version 2025.12-29. (2025). https://CRAN.R-project.org/package=ordinal
81. Bates, D., Maechler, M., Bolker, B., Walker, S.: Fitting linear mixed-effects models esing {lme4}. J. Stat. Softw. **67**, 1–48 (2015). https://doi.org/10.18637/jss.v067.i01
82. Lenth, R.V.: (R package version 1.10.1; (2023). https://cran.r-project.org/web/packages/emmeans/emmeans.pdf

83. Ritchie, M.E., et al.: limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. **43**, e47–e47 (2015)
84. Smyth, G.K., Michaud, J., Scott, H.S.: Use of within-array replicate spots for assessing differential expression in microarray experiments. Bioinformatics. **21**, 2067–2075 (2005)
85. Robinson, M.D., McCarthy, D.J., Smyth, G.K.: edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. **26**, 139–140 (2010)
86. Cantalapiedra, C.P., Hernández-Plaza, A., Letunic, I., Bork, P., Huerta-Cepas: J. eggNOG-mapper v2: Functional annotation, orthology assignments, and domain prediction at the metagenomic scale. Mol. Biol. Evol. **38**, 5825–5829 (2021)
87. Huerta-Cepas, J., et al.: eggNOG 5.0: A hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. Nucleic Acids Res. **47**, D309–D314 (2019)
88. Lee, H.K., Braynen, W., Keshav, K., Pavlidis, P.: ErmineJ: tool for functional analysis of gene expression data sets. BMC Bioinform. **6**, 269 (2005). https://doi.org/10.1186/1471-2105-6-269
89. Wang, Y., et al.: Comparison of the nutrient composition of royal jelly and worker jelly of honey bees (*Apis mellifera*). Apidologie. **47**, 48–56 (2016)
90. Li, Y., Zhang, X., Xia, Z., Hao, Y.: Dissecting the molecular mechanism of 10-HDA biosynthesis: Role of acyl-CoA delta (11) desaturase and transcriptional regulators in honeybee mandibular glands. Insects. **16**, 563 (2025)
91. Zhang, X., et al.: Division of labor among worker bees is associated with the lipidomic plasticity in their brains. Agriculture. **12**, 952 (2022)
92. Amdam, G.V., et al.: Hormonal control of the yolk precursor vitellogenin regulates immune function and longevity in honeybees. Exp. Gerontol. **39**, 767–773 (2004)
93. Nelson, C.M., Ihle, K.E., Fondrk, M.K., Page, R.E., Amdam, G.V.: The gene vitellogenin has multiple coordinating effects on social organization. *PLoS Biology* 5, e62 (2007). https://doi.org/10.1371/journal.pbio.0050062
94. Liu, F., et al.: High-abundance mRNAs in *Apis mellifera*: comparison between nurses and foragers. J. Insect. Physiol. **57**, 274–279 (2011)
95. Winston, M.L.: The biology of the honey bee. Harvard University Press, Cambridge, Massachusetts, USA (1991)
96. Uzunov, A., Chen, C., Gabel, M., Kovačić, M.: Queen ringing vs. queen caging for summer brood interruption. J. Apic. Res. **63**, 660–663 (2024)
97. Trouiller, J., Arnold, G., Chappe, B., Le Conte, Y., Masson, C.: Semiochemical basis of infestation of honey bee brood by *Varroa jacobsoni*. J. Chem. Ecol. **18**, 2041–2053 (1992)
98. Plettner, E., Slessor, K.N., Winston, M.L., Oliver, J.E.: Caste-selective pheromone biosynthesis in honeybees. Science. **271**, 1851–1853 (1996)

99. Cardoen, D., et al.: Differential proteomics in dequeened honeybee colonies reveals lower viral load in hemolymph of fertile worker bees. PLoS One. **6**, e20043 (2011)
100. Cardoen, D., et al.: Worker honeybee sterility: A proteomic analysis of suppressed ovary activation. J. Proteome Res. **11**, 2838–2850 (2012)
101. Kennedy, A., Herman, J., Rueppell, O.: Reproductive activation in honeybee (Apis mellifera) workers protects against abiotic and biotic stress. Philosophical Trans. Royal Soc. B. **376**, 20190737 (2021)
102. Ward, R., Coffey, M., Kavanagh, K.: Proteomic analysis of summer and winter *Apis mellifera* workers shows reduced protein abundance in winter samples. J. Insect. Physiol. **139**, 104397 (2022)
103. Doeke, M.A., Frazier, M., Grozinger, C.M.: Overwintering honey bees: Biology and management. Curr. Opin. Insect Sci. **10**, 185–193 (2015)
104. Fluri, P., Wille, H., Gerig, L., Lüscher, M.: Juvenile hormone, vitellogenin and haemocyte composition in winter worker honeybees (*Apis mellifera*). Experientia. **33**, 1240–1241 (1977)
105. Van Herzele, C., et al.: New insights into honey bee viral and bacterial seasonal infection patterns using third-generation nanopore sequencing on honey bee haemolymph. Vet. Res. **55**, 118 (2024)
106. Wham, B.E., et al.: Comparison of seasonal viral prevalence supports honey bees as potential spring pathogen reservoirs for bumble bees. Ecosphere. **15**, e4883 (2024)
107. Prado, A., et al.: Warmer winters are associated with lower levels of the cryoprotectant glycerol, a slower decrease in vitellogenin expression and reduced virus infections in winter honeybees. J. Insect. Physiol. **136**, 104348 (2022)
108. Desai, S.D., Currie, R.W.: Effects of wintering environment and parasite–pathogen interactions on honey bee colony loss in north temperate regions. PLoS One. **11**, e0159615 (2016)
109. Steinmann, N., Corona, M., Neumann, P., Dainat, B.: Overwintering is associated with reduced expression of immune genes and higher susceptibility to virus infection in honey bees. PloS one. **10**, e0129956 (2015)

Figures

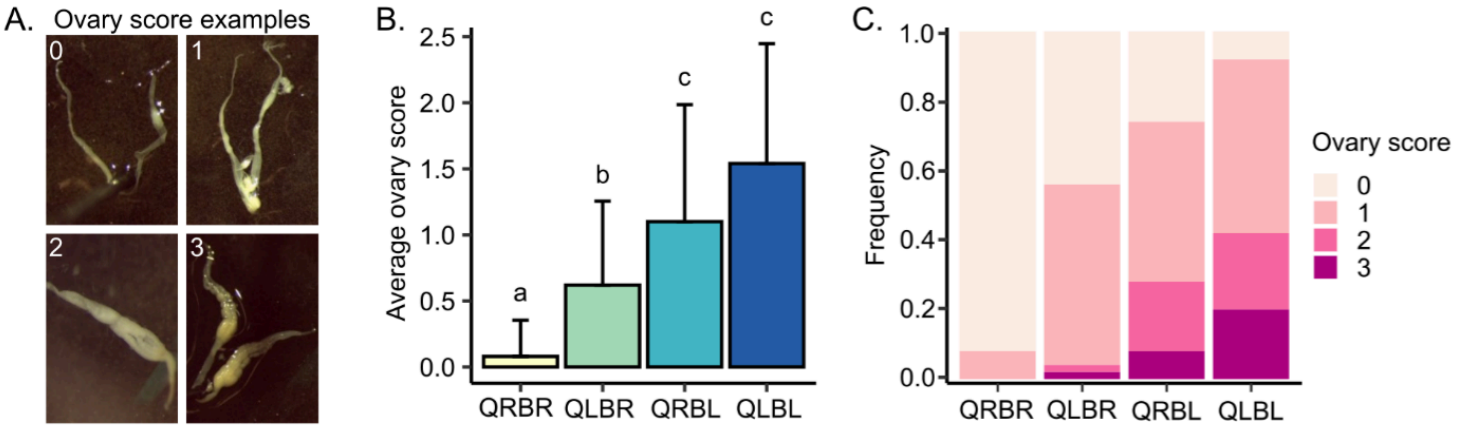


Figure 1

Ovary activation depends on queen and brood status additively, but brood status has the largest effect. Five progenitor colonies (replicates) were each split into four experimental units, one each of QRBR, QLBR, QRBL, and QLBL, where QR = queenright, QL = queenless, BR = broodright, and BL = broodless. A) Ovaries from n = 10 bees per colony (n = 5 colonies) were blindly scored according to a predefined rubric. 0 = undeveloped; 1 = partially developed, no eggs; 2 = partially developed, eggs developing; 3 = developed ovaries, containing at least one fully developed egg. Photos not to scale. B) Average ovary scores in each group, plotted with standard deviations. Shared lowercase letters indicate that two groups were not significantly different (based on Tukey contrasts), while different letters indicate that groups were significantly different. C) Frequencies of different ovary scores among groups.

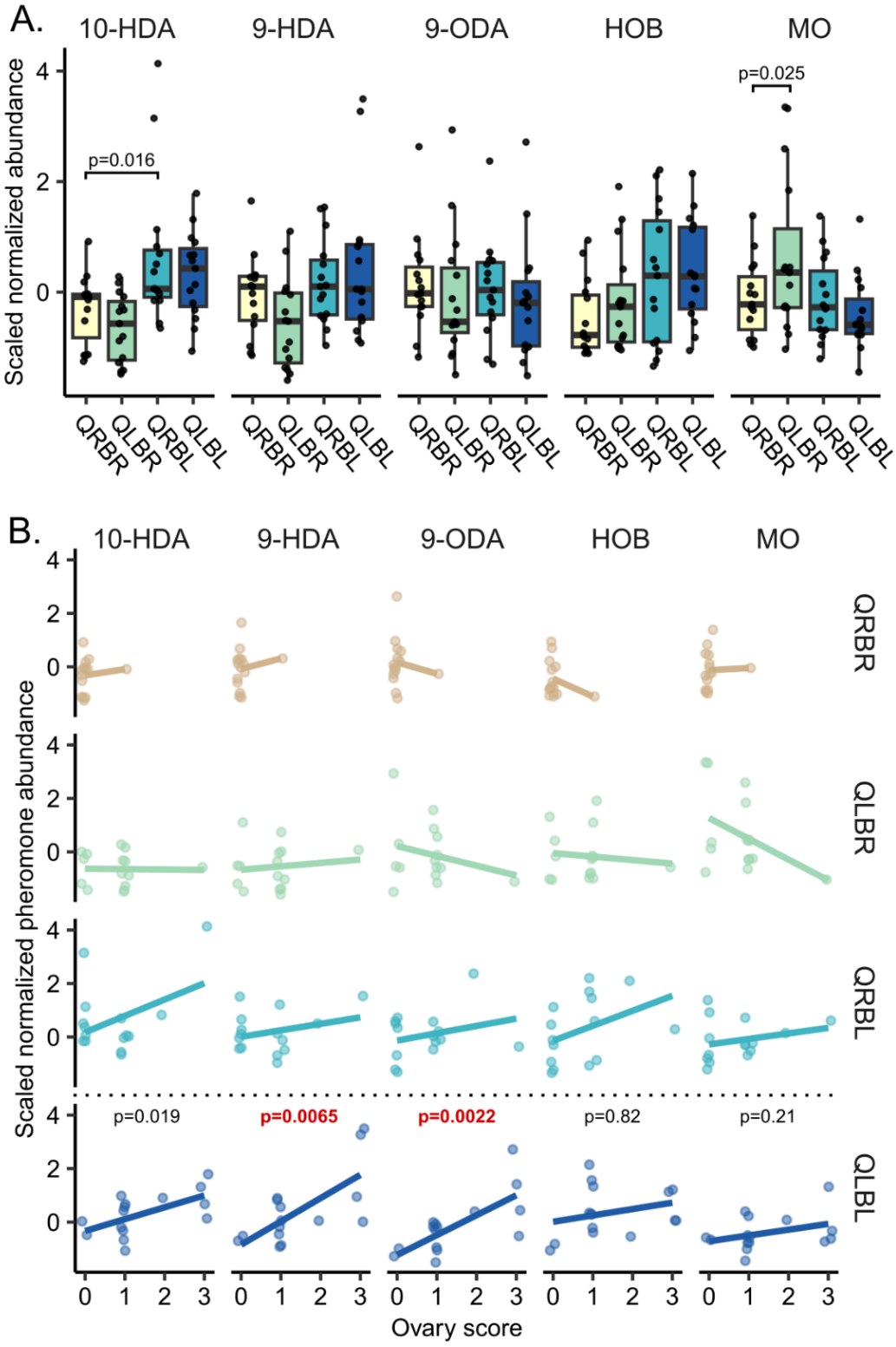


Figure 2

The queen retinue pheromone components 9-HDA and 9-ODA, not methyl oleate (MO), correlate with ovary development in workers. Pheromone components were measured in worker heads (n = 3 bees per colony, n = 5 colonies) using LC-MS/MS. A) No pheromone components were significantly different among groups. P values < 0.05 are shown, but α = 0.01 with Bonferroni correction. B) Within the QLBL group, 9-HDA and 9-ODA significantly positively correlated with ovary development score. The analysis is

limited to the QLBL group because this is the only group with good representation across the ovary score range. Red text indicates $p < \alpha = 0.01$ (Bonferroni correction).

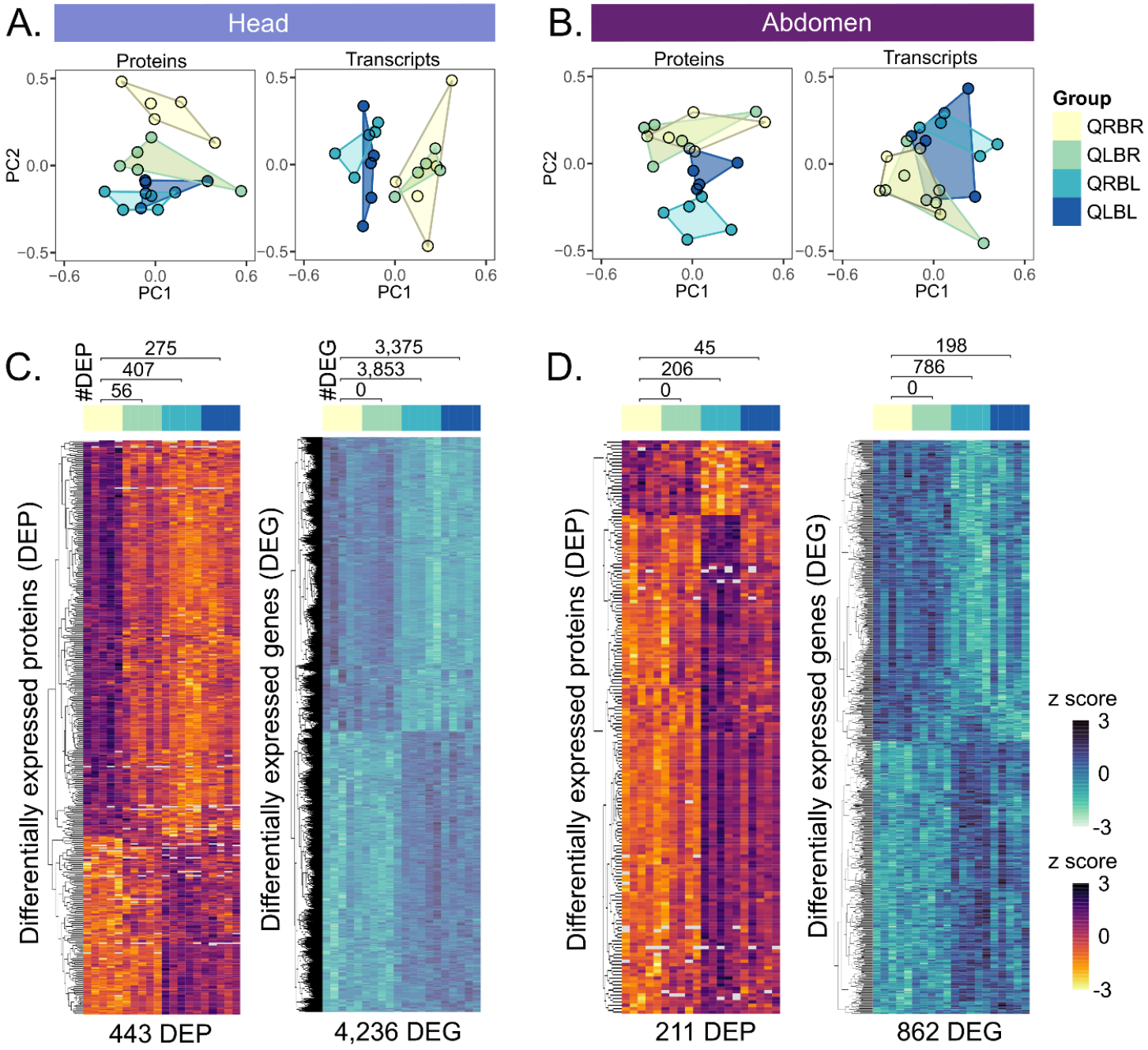


Figure 3

Broodlessness effects are partially masked by queenlessness. Proteomics and transcriptomics were performed on independent samples of 10 pooled bees per colony (n = 5 colonies per group). The data were analyzed using a mean-reference model (limma-trend method). A-B) Principal component analysis shows distinctive sample clustering. Queenlessness has little impact on expression profiles, except in heads, whereas broodlessness induces distinct clusters. C-D) Differentially expressed proteins (DEPs) and genes (DEGs). Numbers above heatmaps indicate the quantity of DEPs or DEGs in each contrast, and numbers below heatmaps indicate the total number of DEPs or DEGs across groups. Grey tiles

indicate missing values. C) 443 proteins were differentially expressed in heads, the largest fraction of which were observed in the QRBR vs. QRBL comparison. 4,236 transcripts were differentially expressed, again with the majority derived from the QRBR vs. QRBL comparison. D) 211 proteins were differentially expressed in abdomens, again with the largest difference observed among QRBR vs. QRBL groups; a pattern that was reflected in the transcriptomics data, in which 862 DEGs were identified. In all cases, the QRBR vs QLBL contrast yielded fewer differentially expressed features than the QRBR vs QRBL contrast (by 12-78%, depending on the comparison).

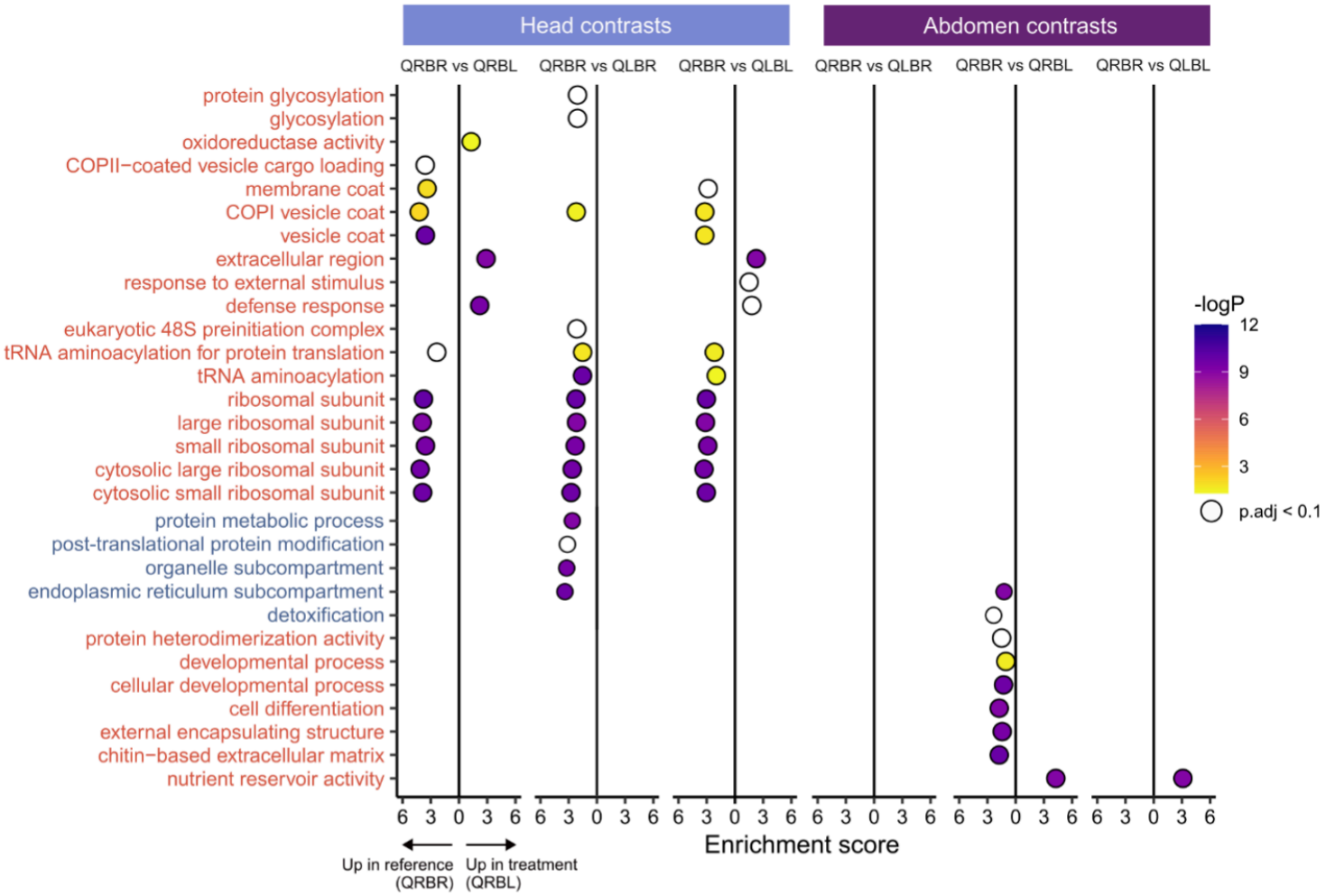


Figure 4

Significantly enriched GO terms across contrasts and tissues. Significantly enriched GO terms (Benjamini-Hochberg correction) are shown as colored dots, while GO terms with marginally non-significant enrichments (adjusted $p < 0.1$) are shown as colorless circles (only plotted in cases where statistical significance is achieved in at least one other contrast). Dots appearing to the right and left of the y axis are derived from upregulated and downregulated features, respectively, in the treatment group compared to the reference (QRBR). Orange text indicates GO terms derived from proteomics data, whereas blue text indicates GO terms derived from transcriptomics data.

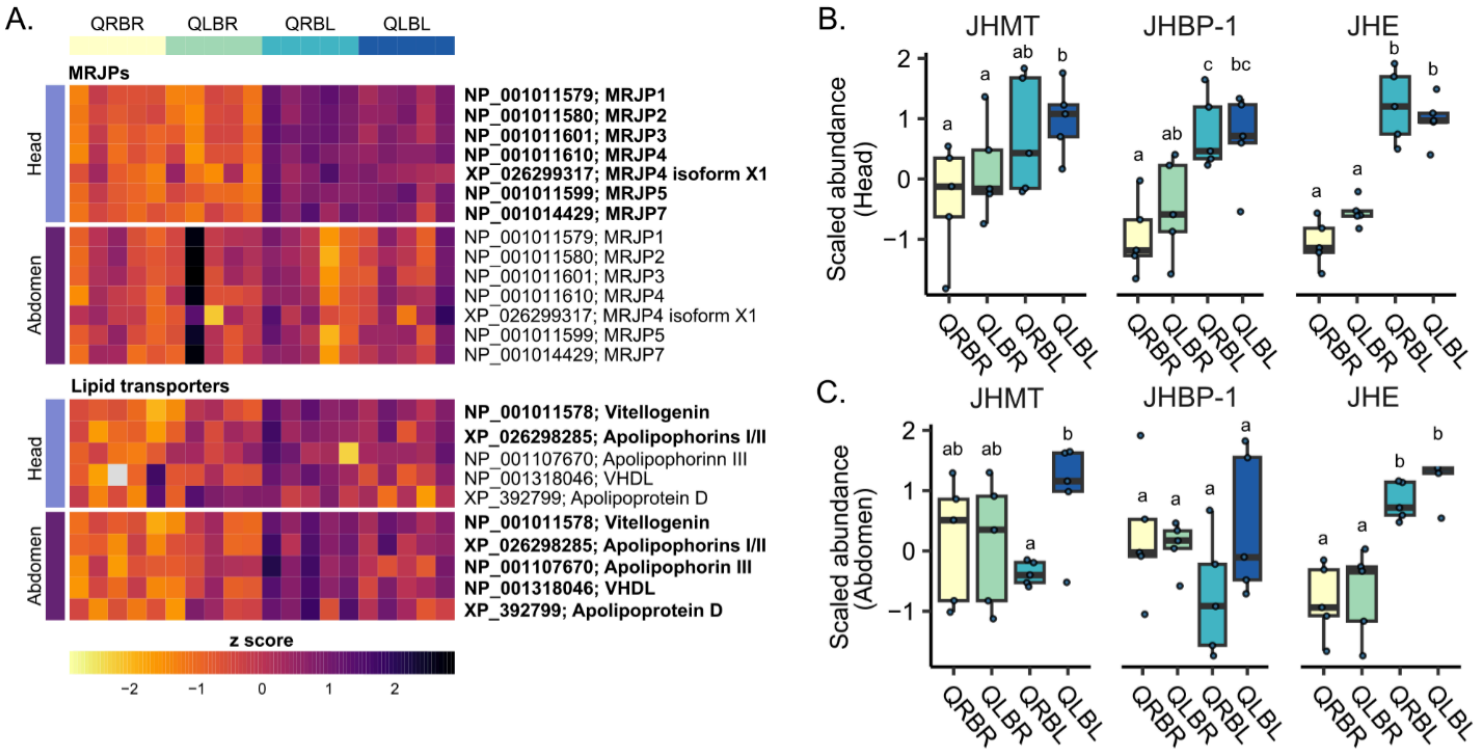


Figure 5

Differential expression patterns of lipid transporter proteins, major royal jelly proteins, and juvenile hormone transcripts. A) Broodlessness affects lipid transporters and major royal jelly proteins (MRJPs). Bold names indicate proteins that were differentially expressed at 5% FDR (Benjamini-Hochberg correction) in at least one contrast. B & C) Juvenile hormone methyl transferase (JHMT), juvenile hormone binding protein 1 (JHBP-1), and juvenile hormone esterase (JHE) gene expression data. Different lower case letters indicate significantly different groups based on Tukey post hoc testing that was further Bonferroni corrected for three hypotheses (transcripts) per tissue (Tukey α = 0.017; **Table 4**; n = 5 per group). Shared lowercase letters indicate no significant difference.

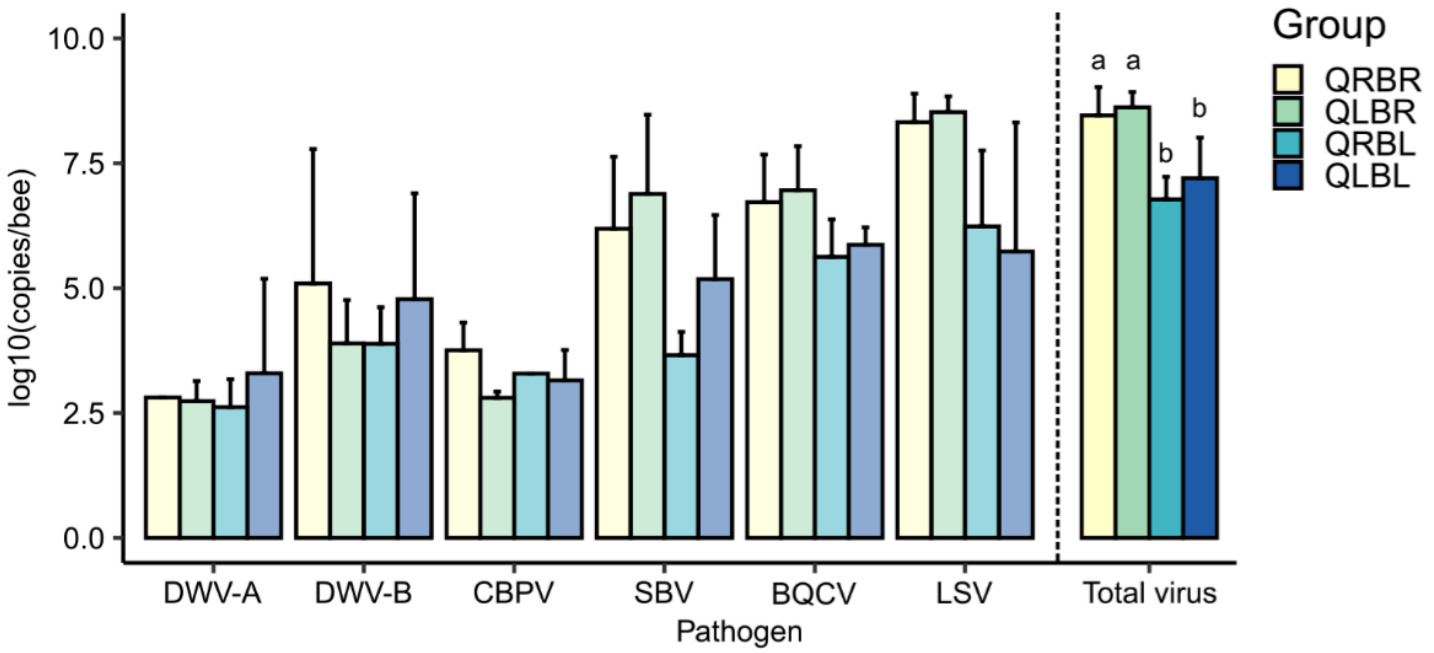


Figure 6

Broodlessness affects colony virus infection severity. Raw copy numbers per bee, determined by RT-qPCR, were summed across viruses to generate the “Total virus” variable. Total virus levels were significantly lower in both broodless groups compared to the QRBR reference (n = 5 per group). Error bars depict standard deviations. Different lowercase letters indicate significantly different groups (Tukey method). Data for individual viruses are displayed to visualize contributions to total viral load, but were not statistically analyzed.

Supplementary Files

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