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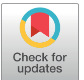

Substances in the mandibular glands mediate queen effects on larval development and colony organization in an annual bumble bee

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Social organization is commonly dynamic, with extreme examples in annual social insects, but little is known about the underlying signals and mechanisms. Bumble bee larvae with close contact to a queen do not differentiate into gynes, pupate at an earlier age, and are commonly smaller than siblings that do not contact a queen. We combined detailed observations, proteomics, microRNA transcriptomics, and gland removal surgery to study the regulation of brood development and division of labor in the annual social bumble bee *Bombus terrestris*. We found that regurgitates fed to larvae by queens and workers differ in their protein and microRNA composition. The proteome of the regurgitate overlaps significantly with that of the mandibular (MG) and hypopharyngeal glands (HPG), suggesting that these exocrine glands are sources of regurgitate proteins. The proteome of the MG and HPG, but not the salivary glands, differs between queens and workers, with caste-specificity preserved for the MG and regurgitate proteomes. Queens subjected to surgical removal of the MG showed normal behavior, brood care, and weight gain, but failed to shorten larval development. These findings suggest that substances in the queen MG are fed to larvae and influence their developmental program. We suggest that when workers emerge and contribute to larval feeding, they dilute the effects of the queen substances, until she can no longer manipulate the development of all larvae. Longer developmental duration may allow female larvae to differentiate into gynes rather than to workers, mediating the colony transition from the ergonomic to the reproductive phase.

social evolution | caste differentiation | mandibular glands | queen substances | bumble bee

Significance

Social insects tightly coordinate almost all aspects of their lives to produce colony-level behavior. We combined detailed observations, proteomics, microRNA transcriptomics, and gland removal surgery to study signals and mechanisms involved in the social regulation of annual bumble bee colonies. We show that workers and queens differ in the protein and microRNA composition of glands in their head and in the food they deliver to larvae. Queens with no mandibular glands lost the capacity to shorten larval development. Based on these findings, we suggest a model stating that the emergence of workers attenuates the effects of the queen substances, enabling larvae to grow over a longer period and differentiate into gynes, and the colony switches from growth to reproduction.

Insect societies represent a prime example of complex social organization in the animal kingdom and are considered to represent a major transition in the evolution of complexity (1). Individuals in these societies coordinate almost any aspect of their life to produce tightly regulated group (colony)-level phenotypes and are therefore commonly referred to as "superorganisms" (2–4). The functional significance of their colony-level properties attracts special attention to the communication signals and social mechanisms enabling up to millions of individuals to tightly integrate their behavior, physiology, and development. Two of the most important organization principles of insect societies are a division of labor in reproduction, which is characterized by a strong reproductive bias toward a single or a few fertile individuals ("queens" and "kings"), and a division of labor among individuals ("workers") performing various nonreproductive tasks (5).

In small colonies, physical contact and agonistic interactions determine and maintain dominance hierarchies underlying division of labor in reproduction (5, 6). In large colonies, in which the queen cannot physically inhibit reproduction of all workers, there is evidence that reproductive dominance is typically maintained by queen substances such as "queen pheromones" (6, 7). The best known of these is the mandibular glands pheromonal blend of the queen honey bee, which regulates developmental processes influencing female reproductive potential as well as ovarian activation in adult workers (8–11). The complexity of the queen mandibular blend, as well as evidence for queen pheromones in additional exocrine glands, suggest that it was shaped by an evolutionary arms race (6, 7, 12).

Social organization is dynamic, with extreme examples in annual colonies (sometimes termed "annual superorganisms") that are founded each year by a single or few foundresses. The emergence of the first workers marks the beginning of an ergonomic social phase which is followed by a reproductive phase, before the colony collapses at the end of the season. The signals and mechanisms mediating these dynamic changes in social organization are still largely elusive. Here, we studied the model bumble bee *Bombus terrestris*. As in most bumble bees, their annual colonies are founded by an overwintered queen who rears a first batch of worker-producing brood (13). Incipient colonies are socially cohesive, with reproduction monopolized by the queen, and foraging and nest maintenance carried

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out mostly by her daughter worker bees. This apparent harmony later breaks down when the colony enters a Competition Phase that is characterized by aggression, worker reproduction, oophagy, and gyne (virgin queen) rearing (14, 15). The higher reproductive potential of the queen is determined by caste differentiation processes that are not well understood in bumble bees (16, 17). Workers have a similar number (eight) of ovarioles, but their ovaries are smaller and typically less developed, and they lay only a few eggs in each egg-laying event, compared to up to few dozen by a queen (14, 15). Workers do not attract males, mate, or enter diapause during the winter season (13, 18). Adult worker reproduction is inhibited by the queen and dominant workers in the colony (19, 20). It is still debated whether these queen effects are mediated by pheromones or whether the queen's intimidating size and overt aggression are sufficient to maintain her reproductive dominance (12, 21–24). The division of labor among bumble bee workers relates to body size with the larger individuals more likely to forage and the smaller ones more likely to tend brood (18, 25). Large workers are apparently better suited for foraging activities because they have better sensory acuity, stronger positive phototaxis, earlier and stronger circadian rhythms, and improved performance at some learning and memory tasks (16). Thus, in bumble bees, variation in body size is important for the regulation of division of labor in both reproduction and among workers performing different tasks.

Colony age is one of the factors influencing body size in bumble bees. The first workers develop over a shorter period and are typically small. Average body size then increases as the colony develops and grows until reaching its peak size with the production of new gynes (26–28). Given that in insects, growth is limited to the juvenile stages, the final size of the larva determines the body size of the emerging adult (29). Brood tending also changes along with colony growth. In incipient colonies, the founding queen tends and cares alone for all the brood, but later, workers start to emerge and take an increasing role in brood care and feeding. Moreover, the increase in worker number is associated with the queen reducing brood care and increasing fecundity (16, 27, 30, 31). A set of cross-fostering and manipulation experiments showed that *B. terrestris* larvae that are tended by the queen develop over a shorter period, are unlikely to develop into gynes, and are commonly (but not always) smaller than bees developing without a queen (27, 32). There is also evidence that the queen influences larval development and ultimate body size in *B. impatiens* (28). Brood developing in a mesh-separated queenless compartment of a colony develop similarly to queenless brood, indicating that the queen influence requires close contact with the brood. The larvae are particularly sensitive to the queen influence during the first and second instars (32).

Here, we combined detailed behavioral observations, proteomics, microRNA transcriptomics, and gland removal surgery to test the hypothesis that substances in the cephalic exocrine glands of the queen are fed to larvae and modulate their developmental program and caste fate. Our findings, which show caste-related differences in the composition of the regurgitate and exocrine glands, lend credence to this hypothesis. We also show that queens subjected to surgical removal of the mandibular glands (MG) failed to manipulate the developmental program of the brood they reared. This experiment identifies the MG as pivotal to the social organization of bumble bee colonies.

Results

The Regurgitates of Queens and Workers Differ in Their Protein and miRNA Content. We tested the hypothesis that the regurgitate of queens and workers differ. To address this hypothesis, we collected regurgitate samples shortly after deposition and subjected them to either microRNA transcriptomic or proteomics analysis. Altogether, we identified 148 and 63 known and predicted insect miRs in the regurgitates using the MD and the WEIZ pipelines (*Materials and Methods*), respectively; 30 miRs were identified with both pipelines (*SI Appendix*, Tables S1 and Dataset S1). Additional analyses of the miRs identified in the regurgitates and their comparison to published lists of bumble bee miRs are reported in the *SI Appendix*. The regurgitate samples can be divided into two types, consistent with earlier *B. terrestris* regurgitate analyses (33). The first type of samples is typically yellowish and includes low levels of arthropod (mainly insect) miRs and is relatively enriched in plant miRs (*SI Appendix*, Fig. S1). Samples of the second type are typically whitish, rich in arthropod miRs, and have low levels of plant miRs. Principal component analysis (PCA) for the three queen and three worker samples with high insect miR content (whitish) is summarized in Fig. 1 *A* and *B*. Although the sample size is small, the PCA with the insect miRs curated either with the WEIZ (Fig. 1*A*) or MD (Fig. 1*B*) pipelines suggests that the queen and worker regurgitates can be separated based on their miR content. This premise is further supported by the finding that six miRs are differentially abundant in queen and worker regurgitate samples, some of which were reported to be associated with developmental processes in *Drosophila melanogaster* and *A. mellifera*, and with caste differentiation in *A. mellifera* (Dataset S1). Given that the regurgitate samples were collected from larvae cells, we explored the possibility that they originated from the larvae themselves, rather than from the nursing adults. We did so by comparing the full list of identified miRs, or only a list of the most abundant 16 miRs in our samples, to similar lists for the larval body analyses of Collins et al. (34). The low agreement between the larvae and regurgitate lists does not support (but does not reject) the premise that the larvae are the source of the miRs in our samples (Dataset S1).

To further test the hypothesis that the queen and worker regurgitate differ, we conducted an intensity-based absolute quantification (iBAQ) proteomic analysis of four queen and three worker regurgitate samples. Proteins were extracted, digested, and analyzed using LC–MS/MS as described in *Materials and Methods*. Although a variation in the color of the regurgitate samples was observed, with some displaying a more yellowish hue and others appearing more whitish, downstream analysis did not differentiate the proteomes of the samples based on their color. Across all samples, we identified 1,203 *B. terrestris* proteins, of which ~900 proteins were detected in both queen and worker samples, and ~300 only in the queen samples (Dataset S2). Hierarchical clustering analysis of the relative abundances (of iBAQ intensities) of identified proteins differentiated between the worker and queen samples. The outcomes of this analysis are visualized in a heatmap (*SI Appendix*, Fig. S2*A*). The separation of the samples was further confirmed by complementary PCA (Fig. 1*D*). Student *t*-test analyses (with FDR < 0.05) identified 325 proteins differing in abundance between the queen and worker samples, 324 of which were more abundant in the queen samples or were absent in the worker samples (Dataset S3; see also Fig. 1*C* and *SI Appendix*, Fig. S2*A*). The list contains diverse types of proteins including enzymes, ribosomal proteins, splicing factors, and tRNA ligases. The queen regurgitate sample consisted of 123 proteins harboring a computationally predicted secretion signal. Out of these proteins, three are significantly enriched in the queen's samples compared to the worker's samples. These proteins are CD109 antigen isoforms X1 and X2 and collagen alpha-1(IV) chain (XP_012163132.1, XP_003394802.1, and XP_003399665.1). In an additional analysis, we found that 23 and 21 of the proteins in the queen and worker regurgitates, respectively, were also found in a published proteomic analysis of *B. terrestris* honey (35). Two of the honey

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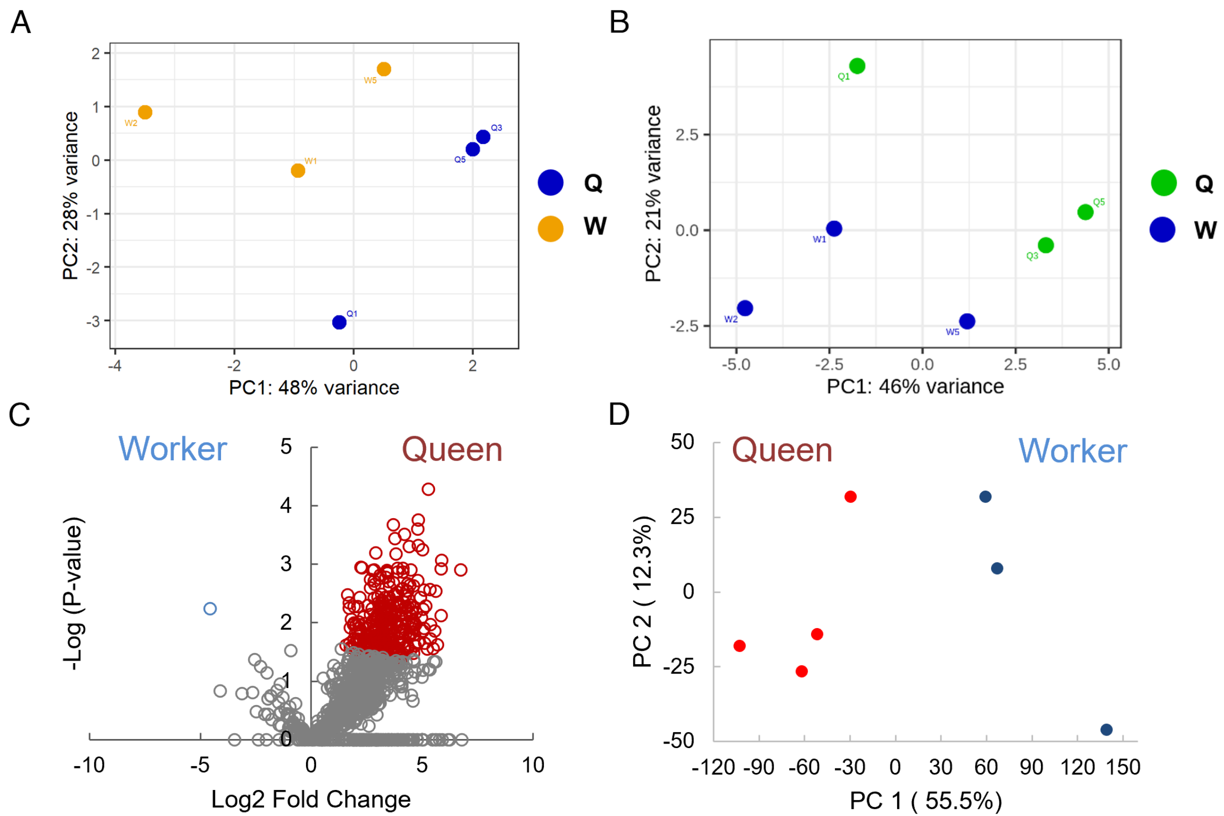

Fig. 1. Regurgitates of queen and worker differ in their protein and miRNA content. (*A* and *B*) Principal component analysis (PCA) of predicted insect miRs in regurgitate samples fed to larvae using two complementary pipelines: (*A*) miR identification was performed with the WEIZ pipeline (queen marked in blue, workers marked in yellow). The plots show the first two principal components (PC1 and PC2). (*B*) miR identification was performed with the MD pipeline (queens marked in green, workers in blue). (*C*) A volcano plot of differentially expressed proteins between queen and worker regurgitate samples. Proteins that are significantly more abundant in queen and worker samples are marked in red and blue, respectively. We used a threshold of *P*-value < 0.05 FDR. (*D*) PCA of all the proteins identified in queen (red) and worker (blue) regurgitate samples.

proteins were found in the queen but not in the worker regurgitate (XP_003399358.1, adenosylhomocysteinase; XP_003398424.1, serine protease inhibitor 88Ea). Enrichment analysis on all proteins in the queen regurgitate, including those also found in worker regurgitate, showed many terms relating to cellular processes including mitochondrial activity, translation, and protein folding. A complementary enrichment analysis, limited to only the proteins more abundant in the queen regurgitate, identified a single statistically significant GO term—Golgi organization (GO:0007030; *P*-adjusted value = 0.03). To test the possibility that the difference in the queen and worker protein profiles can be attributed to a variability in the identification rate caused by the presence of non–*B. terrestris* proteins (e.g., different proportion of pollen proteins), we calculated the ratio of identified *B. terrestris* peptides relative to all peptide-like ions sequenced by MS/MS. The percentage of peak masses that correspond to peptide isotopic profile matching *B. terrestris* proteins was similarly ~30 % for both the queen and worker samples (*SI Appendix*, Fig. S2*C*) indicating that non–*B. terrestris* proteins do not account for the observed caste-related differences. Statistical analysis revealed that identification efficiency of the *B. terrestris* peptides was similar for the queen and worker regurgitate samples (*Wilcoxon test*: $P = 0.7$). This suggests that the observed differences in the regurgitate protein profiles likely reflect genuine biological variability. Taken together, these analyses suggest that regurgitates fed to larvae by queens and workers differ in their miR and protein composition. We aimed to test the hypothesis that this caste-related variation in the regurgitate composition is functionally significant by feeding larvae with worker or queen regurgitate but failed to collect the amounts of regurgitate required to support the full development of a sufficient number of larvae. We therefore took an alternative approach testing whether the major cephalic exocrine glands are the source of regurgitate proteins and differ in expression between queens and workers.

Proteomic Analysis of Cephalic Exocrine Glands. Given that three major cephalic exocrine glands, the hypopharyngeal glands (HPG), the mandibular glands (MG), and the salivary glands (SG), are connected to the mouth cavity and may serve as an important source of proteins in regurgitates and trophallaxis fluids, we next analyzed their proteomes in queen and worker samples. We identified 2,312, 2,119, and 1,442 proteins in the HPG, MG, and SG proteomes, respectively (Dataset S2). The proteomes of the three glands were clearly separated in PCA, with the SG proteins showing a broader distribution compared to the HPG and MG proteomes (Fig. 2*A*). The queen and worker glands were further separated for both the HPG and the MG, but not for the SG samples, revealing caste-specific protein signature in these two glands (Fig. 2 *A* and *B* and *SI Appendix*, Fig. S2*B*).

Both a complementary one-way ANOVA test conducted for clustering all the gland proteomes (*SI Appendix*, Fig. S2*B*) and a pairwise *t*-test analysis performed to compare the gland-specific queen and worker proteins (presented in the volcano plot in Fig. 2*C*) revealed significant differences in protein abundance between queen and worker samples in both the HPG and the MG. The queen HPG showed higher abundance of 82 proteins, and lower abundance in 9 proteins (Fig. 2*C* and Dataset S4). The MG proteome included 187 proteins that were more abundant

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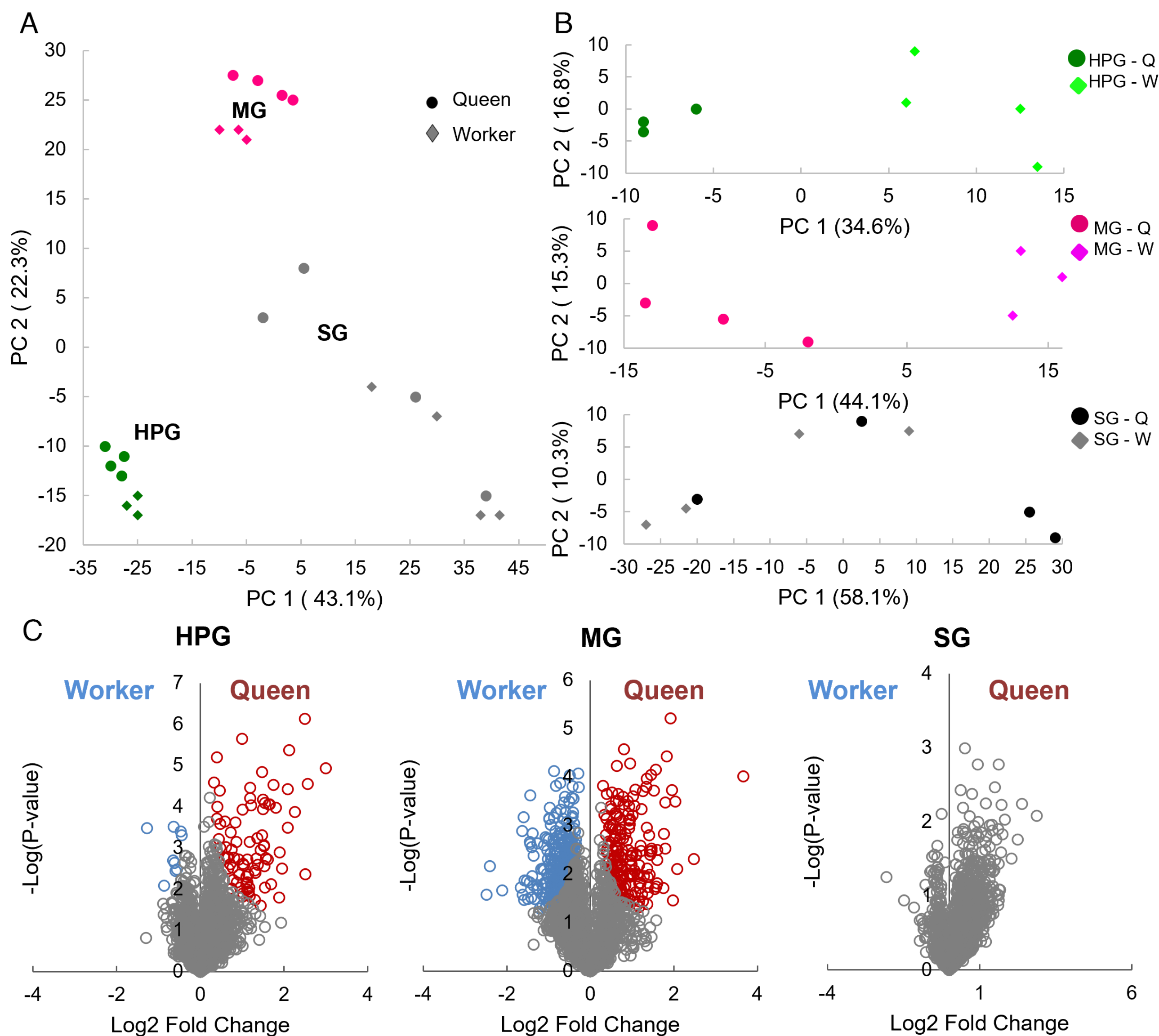

Fig. 2. Protein composition differs between the cephalic exocrine glands of queens and workers. (*A*) Global PCA for queen (circle) and worker (diamond) glands (HPG in green, MG in pink, and SG in gray). (*B*) Gland-specific PCAs: HPG, *Top* panel in green; MG, *Middle* panel in pink; SG, *Bottom* panel in gray highlighting the differences between queen (circle) and worker (diamond) samples. (*C*) Volcano plots of differentially expressed proteins between queen and worker glands: HPG (*Left*), MG (*Center*), and SG (*Right*). Proteins with higher abundance in queen or worker samples are colored red or blue, respectively, using a threshold of $P < 0.05$ FDR.

in queens and 298 that were more abundant in workers (Fig. 2*C* and Dataset S5). The proteins that were more abundant in queen MG were enriched for processes associated with the mitochondria and the Golgi apparatus (*SI Appendix*, Fig. S3). None of the SG proteins differed in abundance between the queen and worker samples (Fig. 3*C*). Taken together, the PCA, hierarchical clustering analysis, and volcano plots, all point toward the MG and HPG as the cephalic exocrine glands in which the protein composition is most strongly associated with caste. Caste-specific protein expression is consistent with the synthesis of queen pheromones or substances that are fed to the larva.

Proteins Common to the Regurgitate and Exocrine Glands. An analysis of the overlap between the regurgitate and exocrine proteomes revealed that the HPG proteins are more prevalent in the regurgitate proteome compared to the MG and SG proteins. Specifically, ~12% and ~9% of the regurgitate proteins were also found in the queen and worker HPG proteomes, respectively, while 4.2% and 5.4% were identified in the queen and worker MG, respectively. Less than 2% of the regurgitate proteins were found in the SG (1.5% in queen SG, 0.5% in worker SG) (Fig. 3 *A* and *B* and Dataset S6). Notably, the fact that only 20% of the regurgitate proteome overlaps with at least one of the analyzed exocrine glands might indicate that the protein composition of the regurgitate is influenced by other tissues such as the digestive tract, crop, esophagus, or other exocrine glands. Moreover, we cannot exclude the possibility that some proteins in the regurgitate samples originated from larvae, but properly testing this possibility is beyond the scope of the current manuscript.

Next, we compared the lists of proteins that differ in abundance between queens and workers in the regurgitate samples and also differ in each one of the glands. Notably, all of the MG proteins that are more abundant in the queen compared to the worker regurgitate are also more abundant in the queen compared to the worker MG. (Fig. 3*C*). A similar comparison for the HPG revealed that 40% of the proteins that are significantly enriched in the queen

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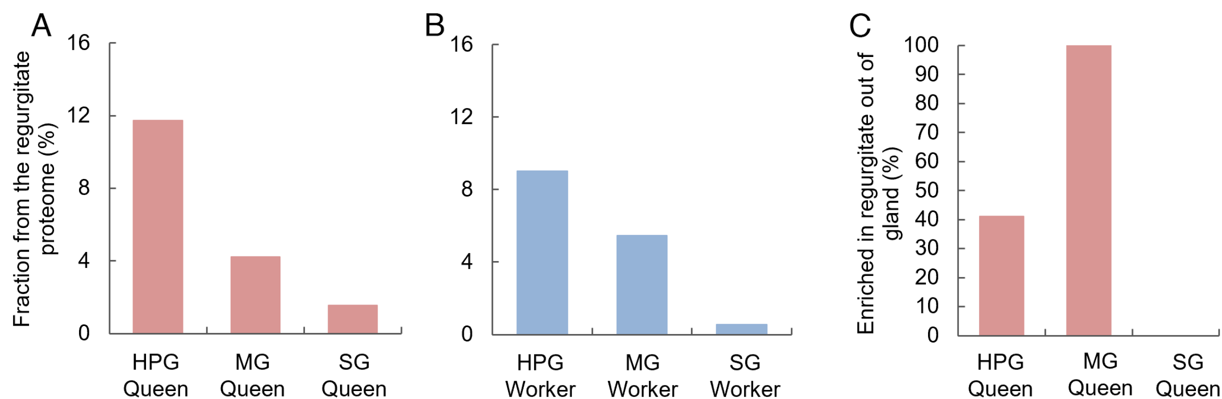

Fig. 3. Many regurgitate proteins are also found in the major cephalic exocrine glands, and some show caste-specific expression in both the regurgitate and glands. (*A*) The fraction of proteins from the queen regurgitate that are also found in each of the queen glands. (*B*) The fraction of proteins from the worker regurgitate that are also found in each of the worker glands. (*C*) Percentage of proteins that are significantly more abundant in the queen regurgitate and are also more abundant in queens compared to workers in each of the gland proteomes (i.e., 40% of HPG proteins more abundant in queen regurgitate, were also significantly more abundant in the queen HPG as compared to the worker HPG).

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HPG (30 proteins) were also significantly more abundant in the queen regurgitate. These analyses highlight the HPG and MG as potentially important sources of proteins in the regurgitate, which also contribute to the observed variation between the queen and worker regurgitate (Fig. 3*C* and Datasets S6 and S7). Given that more queen MG proteins are abundantly expressed in both the regurgitate and glands, we focused our attention on these glands.

The Influence of the Queen MG on Brood Development and on Worker Physiology and Behavior. The proteomic analyses point to the queen MG as a possible source of substances that may be fed to or detected by the larvae, which modulate their developmental program. To further test this hypothesis, we studied the influence of queens, with or without MG, on larvae development.

The influence of MG removal on queen well-being, behavior, and brood care. Our first set of detailed observations suggested that queens with no MG (MG^-) behaved overall similarly to the Sham and Control queens. Queens subjected to the three treatments did not differ in the time they stood still (*Kruskal–Wallis test*: H = 1.94, P = 0.379), groomed (H = 0.584, P = 0.747), handled or touched wax (H = 4.419, P = 0.11), or touched the incision area (H = 1.725, P = 0.422). The MG^- queens also did not differ in the number of times they were observed drinking sugar syrup (H = 2.424, P = 0.298) but were recorded eating pollen more often (H = 9.076, P = 0.011; Dunn–Bonferroni post hoc tests: Sham vs. MG^-, P = 0.016; Control vs. MG^-, P = 0.047; *SI Appendix*, Fig. S4). The treatment did not affect the number of "buzzing" threatening displays performed by the queen toward the worker (H = 1.486, P = 0.476; *SI Appendix*, Fig. S4*G*). We observed only one overt attack by a queen (from the Sham group) during the entire set of observations. These observations suggest that the MG^- queens did not need to be more aggressive in order to dominate the worker in their cage. Overall, queens in all three groups gained weight during this period (*Sign pair test:* Control, P = 0.012; Sham, P = 0.028; -MG, P = 0.021; *SI Appendix*, Fig. S5), with no effect of treatment on queen mass at the beginning (H = 1.879, P = 0.391) or the end (H = 0.599, P = 0.741) of the experiment. These results suggest that food intake was not compromised in the dissected queens.

We observed each queen again when she already had brood. Here too, the treatment did not affect the queen behavior or well-being (eating pollen, H = 2.831, P = 0.243; grooming, H = 1.699, P = 0.428; touching the incision area, H = 3.017, P = 0.221; drinking sugar syrup, H = 4.162, P = 0.125; handling or touching the wax, H = 0.722, P = 0.697; *SI Appendix*, Fig. S6 *A–E*), with the exception that Control queens stood still less time than the Sham queens (H = 6.692, P = 0.035; Dunn–Bonferroni post hoc tests, P = 0.033; *SI Appendix*, Fig. S6*F*). We recorded only a few threatening displays and no overt aggression; buzzing events were recorded four times, twice each by a Control queen and a MG^- queen. Queens from the three treatment groups did not differ in the time they incubated brood cells (H = 1.663, P = 0.435; *SI Appendix*, Fig. S6*G*), in the number of times they were observed feeding larvae (H = 1.194, P = 0.551; *SI Appendix*, Fig. S6*H*), or inspecting brood cells (H = 3.343, P = 0.188; *SI Appendix*, Fig. S6*I*). We did not observe any brood inspection by a worker in any of the observations. The number of feeding events did not differ between workers in cages with queens subjected to the three treatments (H = 0.235, P = 0.889; *SI Appendix*, Fig. S6*J*). Taken together, our observations suggest that MG^- queens overall behaved and consumed food and sugar syrup normally and provided similar brood care as the Sham and Control queens.

The influence of MG removal on queen capacity to inhibit ovary activation in workers. Ovarian activity was similarly low for both 5-d-old and 6-d-old workers developing in the presence of queens subjected to the different treatments and was lower compared to the queenless workers (5 d of age: H = 22.79, P < 0.001; 6 d: H = 14.835, P = 0.002; Fig. 4 *A* and *B*, respectively). These results show that queens without MG can inhibit ovarian activity in a single worker.

The effect of the queen MG on brood development. Two Sham and three Control queens had a mix of male and worker brood and were excluded from the brood analyses; gynes did not develop in any of the cages. The number (H = 1.777, P = 0.411; Fig. 4*C*) and final body size (H = 1.25, P = 0.535; Fig. 4*D*) of the brood did not differ between cages with queens subjected to the three treatments. Developmental duration was significantly longer for brood reared by MG^- compared to Sham-treated queens (H = 7.977, P = 0.019; Dunn–Bonferroni post hoc tests, P = 0.017; Fig. 4*E*). A similar trend of longer developmental duration was seen for the MG^- compared to Control queens (P = 0.139).

Discussion

Our results suggest that substances in the MG of bumble bee queens enable them to alter the course of offspring development by shortening the developmental duration of their larval offspring. Given that in *B. terrestris*, gynes develop over a longer period than workers, this shortening effect is assumed to prevent larvae from developing into gynes. This form of maternal manipulation may influence the social organization of the colony. As the season

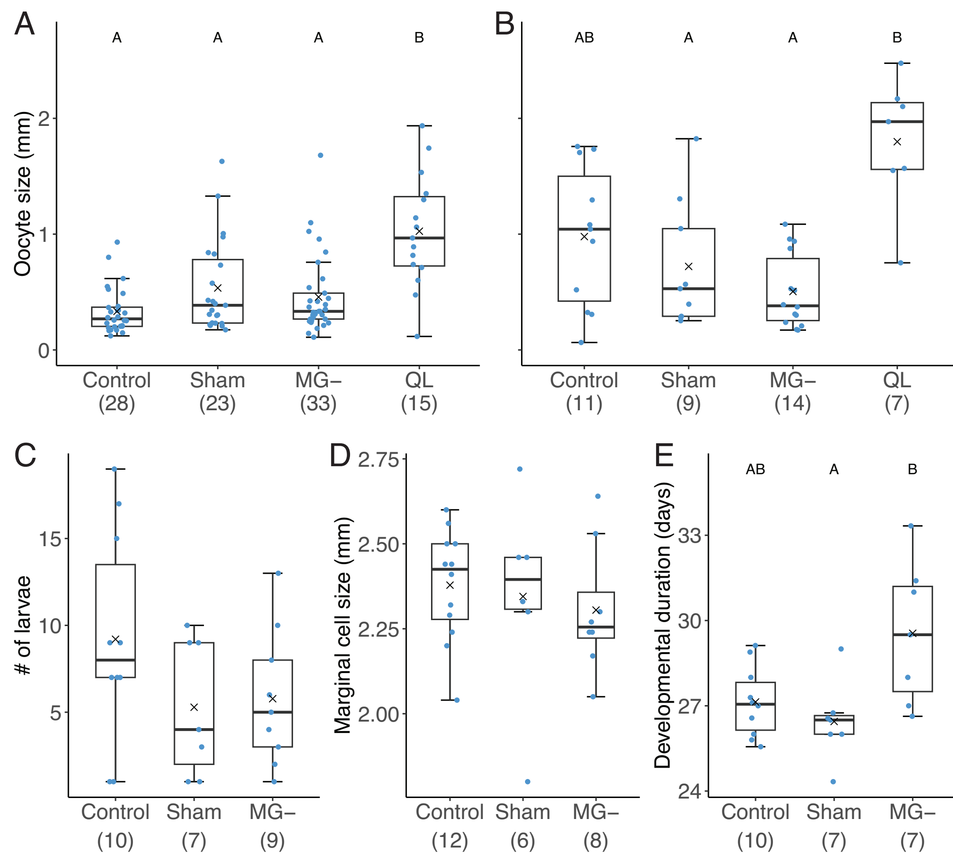

Fig. 4. The influence of the queen MG on brood development and worker ovarian activity. (*A*) Ovarian activity of 5-day-old workers. (*B*) Ovarian activity of 6-day-old workers. QL, queenless (orphan) workers. (*C*) Average number of larvae/cage. (*D*) The body size of emerging adults. (*E*) Developmental duration from the day eggs were laid until adults emerged from the pupae. Numbers in parentheses below the treatment groups depict sample size. The box plots frame spans over the first to the third quartile and include the median (line) and mean (X). The whiskers depict the minimum and maximum values; outliers are depicted with dots but are excluded from the box plots (outliers were defined as values over 1.5 times the interquartile range more and less than the 75th and 25th percentiles, respectively). Boxes with different letters on top represent significant differences in the Kruskal–Wallis test followed by the Dunn–Bonferroni post-hoc test, $P < 0.05$.

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progresses and workers start to emerge, they take an increasing role in brood tending and larvae feeding (27), and the queen effect is predicted to be attenuated. According to this model, the increase in the number of workers enables larvae to extend their developmental duration and differentiate into gynes. This proposed self-organized mechanism may influence two major organization principles of bumble bee sociality: caste differentiation and size-related division of labor among workers performing different tasks in the colony (27, 28, 36). Although the number of caregivers in our experimental design does not support rearing large workers and gynes which is predicted by our model, the differences in developmental duration are consistent with the premise that substances from the queen MG that are fed to larvae modify their development and play crucial roles in the organization of bumble bee societies. In a broader view, these findings are consistent with hypotheses stating that maternal manipulation was important for the evolution of sociality in insects (37–40).

The influence of diet on larval development and caste determination was recognized long ago (41, 42). In honey bees, arguably the best-studied social insect, research on caste determination has emphasized the importance of diet quality with evidence that variations in lipids, proteins, carbohydrates, and water content affect the developmental program of larvae (reviewed in ref. 43). Recent studies further emphasized the importance of diet quantity (44, 45). We first tried to test the influence of nutrition by feeding larvae with queen or worker regurgitates but could not collect sufficient regurgitate samples to obtain a statistically meaningful sample size. We therefore focused on the major cephalic exocrine glands as a likely source of substances in the regurgitate. Previous studies suggest that in *B. terrestris* the queen does not provide less or lower quality food. First, her effect is limited to a "critical period" of about only 5 d during the larval first and second instars during which they consume relatively low amounts of food (32). Detailed video recordings further suggest that queen and worker destined larvae are fed at a similar rate during this period (46, 47), as is also true in *B. impatiens* (28). Second, our new proteomic analyses (Fig. 1) support and extend earlier analyses (33) showing that the queen regurgitate is richer rather than poorer in proteins compared to the worker regurgitate. Moreover, the regurgitate fed to queen- and worker-destined larvae appear to generally have similar pollen, protein, and carbohydrate composition (33). Rather than eating more and growing faster, as in the case of queen-destined honey bee larvae (e.g., a steeper slope), queen-destined bumble bee larvae appear to gain their larger size by extending their developmental duration (37). Third, larvae do not differentiate into gynes in young colonies with several dozen workers that can provide larvae with nutrients deprived from the queen regurgitate (46, 47). Thus, we hypothesized that the MG effects are more likely mediated by specific substances with pheromonal or bioactive functions.

The MG have been suggested to produce a queen pheromone that inhibits JH-mediated reproduction in adult *B. terrestris* workers (21, 48), but later studies could not repeat the inhibitory effect of queen MG extracts on the rate of JH biosynthesis (22). Our findings that MG^{-} queens could effectively inhibit oogenesis in a single worker in her cage (Fig. 4 *A* and *B*) are consistent with this later study suggesting that the MG are not crucial for queen

inhibition of worker reproduction. However, we cannot exclude the possibility that MG⁻ queens are less effective in inhibiting reproduction in a larger number of workers, a possibility that needs to be tested in future studies. In honey bees and some ant species, the queen effects on caste differentiation are mediated by pheromones that modify worker brood tending behavior (49–51). In *B. terrestris*, there is no support for a functionally similar queen pheromone. Workers that interacted with the queen (and her putative pheromones) but were free to move between queenless and queenright compartments did not prevent larvae from differentiating into gynes in the compartment with no queen (52, 53). Queen pheromones may also be perceived directly by premature individuals and prevent them from differentiating into gynes (54). In the bumble bee, the queen needs to closely interact with the larvae in order to exert her affect (27, 52, 54), indicating that if there is a similar queen pheromone, it is not volatile. Transferring wax from a queenright (i.e., exposed to putative queen pheromones) to a queenless compartment failed to prevent gyne production (52, 53). The evidence that close contact is important suggests that if the queen's MG produce contact pheromones influencing larval development, these are detected by the larvae when the queen inspects or feeds them.

Although we cannot exclude effects of the richer protein composition of the queen regurgitate, our findings seem to fit better with the hypothesis that the queen feeds larvae with bioactive substances that modify their developmental program. This premise is consistent with studies with other social insects showing that particular bioactive substances such as proteins [e.g., *Apis mellifera* (55–57)], noncoding RNA [*A. mellifera* (58, 59)], or endocrine signaling molecules [*Camponotus floridanus* ants (60)] in the diet influence larval development by regulating caste-determination or differentiation. The first evidence supporting this premise is the caste-related variability in both the miR and protein composition of the regurgitate (Fig. 1). Second, many of the regurgitate proteins are found in the MG or HPG (Fig. 3), suggesting that these cephalic exocrine glands are the sources of proteins in the regurgitate. Third, the MG and HPG proteomes differ between queens and workers, and all the MG-related proteins that are more abundant in the queen compared to worker regurgitate are also more abundant in the queen MG. These findings suggest that the queen MG produce specific substances that are secreted into her regurgitate. This premise is also consistent with the findings that many MG proteins harbor predicted secretion signals and with the enrichment of processes relating to the Golgi apparatus and mitochondria in the queen MG proteome which is consistent with higher biosynthetic and secretory activity of the queen glands. The finding that queens with no MG appear to behave and tend brood similarly to intact and Sham-treated queens, but fail to shorten brood developmental duration (Fig. 4*C*), is consistent with the hypothesis that the differences between the queen and worker MG are functionally significant.

Our findings for the bumble bee add to the strong evidence that the MG and HPG are pivotal to the social organization of honey bee colonies, suggesting that modifications in these glands were important for the evolution of sociality in bees. Importantly, however, our findings suggest that the effects of the MG in bumble bees are different than in honey bees. In the honey bee, major royal jelly proteins and other substances that are produced by the worker cephalic exocrine glands are differentially fed to worker- and queen-destined larvae to regulate caste differentiation (7, 61). Worker reproduction and task performance are influenced by pheromonal signals from the queen, the brood, and other workers (6, 61). By contrast, our study suggests that in bumble bees, substances in the queen's MG (and perhaps also the HPG) directly act on the larva to shorten its developmental duration, which in turn, may affect both caste differentiation (division of labor in reproduction) and size-related division of labor among workers. There is evidence that chemicals on the queen cuticle inhibit worker reproduction either by functioning as honest signals for her quality or by affecting worker reproductive physiology (21, 22, 26).

Taken together, our results highlight the queen MG as potentially pivotal to the organization of bumble bee societies. Our findings best fit with a maternal manipulation model in which substances secreted from these glands are fed to larvae and shorten their developmental duration. We hypothesize that the queen substances account for her capacity to prevent larvae from differentiating into gynes and also increase the probability that they develop into smaller adults. However, given that our experimental design does not support large workers and gynes, these effects need to be explicitly explored in future studies. A simple self-organized model based on these functions of the queen substances can explain the dynamic changes in the social organization of annual bumble bee colonies (16, 27). According to this model, in incipient colonies, the queen feeds the larvae alone, and substances (and perhaps also contact pheromones) in the regurgitate she feeds to the larvae shorten the developmental duration of the first larvae who develop rapidly into workers. When workers emerge, they contribute to brood feeding. Given that the workers lack these queen substances, they essentially dilute the concentration of the queen substances allowing larvae to grow longer, produce larger workers, and when crossing a certain threshold, enable larvae to differentiate into gynes which develop over a longer period in bumble bees. Gyne production marks the colony transition from the ergonomic to the reproductive phase. Our datasets of queen and worker proteins and miRs in the regurgitate, and proteomics of the cephalic exocrine glands, set the stage for the identification of particular substances modulating larval development, and for further testing this self-organized model.

Materials and Methods

Bumble Bees. Incipient *B. terrestris* colonies containing a queen, brood at various stages of development, and 5–10 workers, were purchased from Polyam Pollination Services, Kibbutz Yad Mordechai. The colonies were transferred into nest boxes and kept in an environmental chamber (dark room; 28 ± 1 °C, 50 ± 10% relative humidity monitored with HOBO UX100 data loggers) and fed ad libitum with sugar syrup and "pollen cakes" (for more details, see ref. 27). During collection, white light was used; at other times, they were kept in the dark. The research was done in the Bee Research Facility at the Edmond J. Safra Campus of the Hebrew University of Jerusalem, Jerusalem, Israel.

Collecting Regurgitate Samples. Queen and worker regurgitate samples were collected from cages with 2–5 workers, and a brood batch, with or without a queen, respectively. A regurgitate sample was collected with a capillary pipette immediately after a queen or a worker was seen feeding the larvae. Most regurgitate samples were collected from large larvae because collecting regurgitate samples from smaller larvae that received smaller amounts of regurgitate (less than 2 µL) proved extremely challenging. The samples were deposited into a 1.5-mL glass vial that was immediately immersed in dry ice. Samples for miR analyses were dissolved into 5 mL of a 50% DDW/50% acetonitrile solvent and frozen in liquid nitrogen. All the samples collected at the same session were transferred to a −75 °C ultra-low freezer in which they were kept until later analysis.

Dissection and Collection of Cephalic Glands. Four healthy queens and four workers were collected from different normally developing colonies before the competition phase and immediately chilled on ice. When the bees stopped moving (i.e., chilled-anesthetized), we decapitated them with surgery scissors and pinned the heads (above the antennae) to a dissection plate filled with melted honey bee wax. We performed the entire dissection under a stereomicroscope (Nikon–SMZ800, magnification: between X20 to X40). First, we immersed the head in honey bee saline (62) to keep the samples from drying. Then, we made four incisions, creating

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a square-shape opening. We then removed the cuticle of the frontal part of the head capsule using a scalpel and forceps. Given that all three gland types were dissected from the same bee sample, we took several measures to assure the integrity of each gland and minimize possible contamination with tissue from the other exocrine glands. We first removed the easily accessible HPG that are located just below the window in the cuticle; second, we removed the MG that are located dorsoventrally to the mandible joint to the head capsule in each side of the mouth. Finally, we turned to dissect the salivary glands (SG) that are positioned superiorly to the brain of the bee. We thoroughly cleaned the dissection tools with EtOH after removing each gland type and moving on to remove the next type of glands. We washed the gland three times in clean bee saline before placing them into a fresh 1.5-mL tube. The samples were stored in a −75 °C ultra-low freezer until proteomic analysis.

Gland Sample Preparation for the Proteomic Analysis. Glands were disturbed using plastic sticks in the Urea-Tris lysis buffer (6 M urea, 200 mM Tris-HCl, 10 mM EDTA, and 0.5% SDS, pH 8.5) supplemented with 10 μL/10 mL protease inhibitor, incubated on ice for 15 min, followed by a 30 min centrifugation at 15,000×*g* at 4 °C. The supernatant was collected, and the proteins were reduced with 10 mM dithiothreitol (DTT) for 30 min at 10 °C under constant shaking at 350 rpm. To remove potential aggregates, the lysates were centrifuged again for 30 min at 15,000×*g* at 4 °C; the supernatant was transferred to a new tube for cysteine alkylation with 100 mM Iodoacetamide for 1 h at room temperature, in the dark.

SDS and urea were removed using the methanol-chloroform precipitation as described in ref. (35). Briefly, 400 μL of HPLC-grade methanol was added at a ratio of 1:4 to each sample and mixed well, followed by the addition of 100 μL of HPLC-grade chloroform. Then, after dilution by double distilled water (DDW) to the 1:3 ratio, and brief centrifugation (14,000×*g* for 2 min at room temp), three layers were obtained, while proteins are in the interphase, covered by methanol. After washing the methanol fraction, the proteins were extracted, dried by SpeedVac (1,300 rpm, 45 °C for 1 h), and resuspended in 6 M urea. The desalted proteins were then digested by trypsin in a diluted digestion buffer (25 mM Tris-HCl pH 8, 10% ACN) overnight at 37 °C, 350 rpm. Then, after quenching using 3 μL of formic acid (to a final concentration of 0.15%), the peptides were desalted using in-house C18 Stage tips as described in ref. 63. Peptides were resuspended in 0.1% formic acid (FA).

Regurgitate Sample Preparation for the Proteomic Analysis. During the collection of regurgitate samples, we noticed some color variation among samples, but this apparent variability was not associated with differences in peptide amount, protein signatures, or protein abundance. The regurgitate samples were dissolved in 250 μL 80% acetonitrile (ACN) and washed twice with 80% acetonitrile. After the acetonitrile removal by SpeedVac (1,300 rpm, 45 °C for 1 h), the samples were resuspended in 8 M urea supplemented by 1 mM DTT and incubated for 1 h at 37 °C, 350 rpm. The aggregates and insoluble fractions were removed by 15 min centrifugation at 15,000×*g* at 4 °C. The thiol alkylation was done by 50 mM Iodoacetamide at room temperature in darkness for 1 h. The digestion by trypsin (Promega) was carried out overnight in the digestion buffer (25 mM Tris-HCl pH 8, 10% ACN, and 1 M urea) at 37 °C, 350 rpm. Then, after quenching using 3 μL of formic acid (to a final concentration of 0.15%), the peptides were desalted using in-house C18 Stage tips as described in ref. 63. Peptides were resuspended in 0.1% formic acid.

nanoLC–MS/MS Analysis. To avoid variability in sample preparation and analyses, all the queen and worker gland and regurgitate samples were treated similarly (e.g., alkylation, tryptic digestion, and desalting). A similar amount (1 μg) of the trypsin-digested peptides, from gland or regurgitate samples, were loaded for the LC–MS/MS analysis. The peptides were desalted using the C18 trap column (Acclaim PepMap 100 C18, 5 μm, 100 Å, Thermo Scientific) and then separated using the 50-cm length C18 EasySpray PepMap RSLC column (2-μm particle size and 100 Å pore size, Thermo Scientific) at a flow of 300 μL/min using the Dionex Nano-HPLC system (Thermo Scientific) coupled online to the Orbitrap Mass spectrometer, Q Exactive Plus (Thermo Scientific). To separate the peptides, the column was applied with a linear gradient of solvent B at 40 °C with a flow rate of 300 μL/min: from 1 to 4% in 45 min, 4 to 28% 90 min, 28 to 50% 22 min, 50 to 80% 10 min, and finally held at 80% for 10 min for washing the column and before equilibrating at 1% for 30 min (solvent A is 0.1% formic acid and solvent B is 0.1% formic acid with 80% ACN).

The Q Exactive Plus orbitrap mass spectrometer was operated with a data-dependent acquisition method, using the following parameters: Full-scan MS spectra were set to 300–1650 m/z; the resolution was 60,000, and the 10 most intense ions with charge states between 2 and 7 were fragmented using a normalized collision energy of 25 and 28. The target value (AGC) for MS was 3×10^6, maximum IT of 20 ms. Fragment ions were then detected in the Orbitrap at a resolution of 15,000, AGC target of 10^5, Maximum IT 25 ms, and isolation window was set to 1.6 m/z. To avoid potential carryover of the peptides, the column was washed with 80% ACN for 55 min between each sample run.

Data Analysis and Statistics for the Proteomic Data. Protein identification and quantification were performed by the MaxQuant software, version 1.6.10.43 (64). Peptide search was done by Andromeda algorithm to search for MS/MS spectra against the *B. terrestris* proteome database derived from NCBI *B. terrestris* genome database 1.0. The proteins were predicted based on the *B. terrestris* genome sequence and RNA expression data from NCBI. The identification parameters allowed for two missed cleavages, and the enzyme specificity was set to trypsin. Peptide length was set between 7 and 30 amino acids. Carbamidomethyl and methionine oxidation were set as variable modifications and the false discovery rate (FDR) was set as $P < 0.05$. Fragment mass deviation was up to 20 ppm and initial precursor mass deviation was up to 4.5 ppm.

Proteins were identified by the appearance of at least two peptides, per protein, and quantification was employed using label-free quantification (LFQ) with default parameters, for the gland samples, and intensity-based absolute qualification (iBAQ) for the regurgitate samples, following the median intensity normalization using the MaxQuant software 1.6.10.43 (64). See *SI Appendix, Supplementary Results* for the rationale behind employing different normalization techniques for regurgitate and gland samples. Specifically, the cross-run normalization between the samples was done using differential peptide features and parameters of the detected MS peaks (e.g., peak intensity, retention time, and others). The sum of the parameters for the grouped peptides associated with the same protein was converted to the normalized intensity, which allows the relative quantification of changes in protein abundance levels, across different biological samples (65). The normalization procedure was done using MaxQuant by applying the default parameters.

Statistical and bioinformatic analyses were performed using the Perseus software, version 1.6.0.7. In the analysis of the gland protein profile, we examined four biological replicates for both the queen and worker samples. Four biological replicates of queen regurgitate and three replicates of worker regurgitate samples were analyzed for the regurgitate protein profile analysis. Only proteins detected in at least three replicates in both gland and queen regurgitate were considered for the subsequent statistical analyses. To maintain the original dataset's integrity, the volcano plots and hierarchical clustering were conducted using the nonmodified dataset, without any value imputation. However, for the principal component analysis (PCA) which required value imputation, nonvalid values were substituted based on the normal distribution of the log2 normalized intensity values. This method was chosen to prevent a significant alteration of the value distribution while ensuring robustness in the analysis.

The hierarchical clustering and PCA of the LFQ/iBAQ values were done using ANOVA tests ($P < 0.05$; Benjamini–Hochberg FDR). For the heatmap visualization, Z-score normalization was applied. Proteomic data were uploaded to the PRIDE database (66) with the dataset identifier: PXD039851. To determine the origin of the regurgitate samples, we utilized a conservative approach, employing the "all or nothing" technique in which proteins were considered "found" if they were identified in at least three out of four replicates, above threshold intensity, while those not meeting this criterion were labeled as "not found."

Analysis of miRNA in Queen and Worker Regurgitate Samples. Regurgitate samples were collected as described above for the proteomic analyses. Seven regurgitate samples of workers and seven of queens were sent on dry ice for miRNA sequencing at the University of Illinois in Urbana Champaign. For quality control, all RNA samples were run on an Agilent Fragment Analyzer with 48 capillaries using the Standard Sensitivity RNA kit (Agilent, Santa Clara, CA). Small RNA libraries were constructed with the TruSeq Small RNA Sample Prep kit (Illumina). The libraries were pooled in equimolar concentration, and the pool was quantitated by qPCR and sequenced on one lane for 51 cycles on a HiSeq2500 using a TruSeq Rapid SBS sequencing kit version 2. FASTQ

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files were generated and demultiplexed with the bcl2fastq v1.8.4 Conversion Software (Illumina).

We analyzed the sequencing results using two complementary microRNA analysis pipelines:

1. miRDeep2 (MD). Analyses were done using the miRDeep2 pipeline [https://www.mdc-berlin.de/n-rajewsky#t-data,software&resources; (67)]. Briefly, preprocessed reads were aligned to the *B. terrestris* genome (NCBI). Sequences of genomic intervals with mappings were then extracted and checked for the possibility that they might be functioning as miR precursors (include a stem-loop structure). The ones which were positively predicted as miRs got a serial id according to their genomic location and their functional role within the miR mechanism (m = mature, s = star). All the sequences which were positively predicted as miRs were submitted to annotation by BLAST search against miRDB set of miRs from *A. mellifera* and *D. melanogaster*. A positive hit results in an additional annotation to the tag which will be the name of the subject hit name from within miRDB. This pipeline is limited to the bee sequence and therefore can predict miRs that were not previously documented, but has more chances to miss miRs from other organisms.
2. A custom-made pipeline of the Weizmann Institute bioinformatics unit (WEIZ). This pipeline analyzes every sample separately and then combines all of them together to a final report. The pipeline includes five key steps: 1) miRNA quality control: This step includes merging FASTQ files, trimming by quality, trimming adapter, and various filtering and QC steps. 2) BLAST merged samples: This subprotocol collects all the filtered reads per sample and combines them to one file which is then converted to FASTA format. 3) Creating Tags Annotation Report: This subprotocol collects all the filtered unique sequences and creates a matrix of sequences vs. samples. 4) Creating miR annotation report. This subprotocol takes the matrix from the previous subprotocol, filters out all the sequences that are not mature miRNA, and merges all the sequences that match the same miRNA id. It then generates a new matrix with all the unique miRNAs containing their miRNA database, the number of sequences that are mapped to this miRNA, their count in each sample, their count in all the samples, and their description. 5) Build report: This subprotocol collects all the information from previous steps and builds a report containing details on every sample and all of them combined. The Tags are subjected to a series of BLAST nucleotide searches against few databases with the aim of detecting similarity to relevant miRs and miR precursors (1. Mature miRs from *A. mellifera* and *D. melanogaster*. 2. mir precursors from *A. mellifera* and *D. melanogaster*. 3. Mature miRs of all other organisms. 4. Precursor miRs of all other organisms. 4. NCBI nucleotide collection – nt). This pipeline can complete the picture regarding miRs of other organisms but cannot predict new miRs that were not previously documented.

The miR counts generated by miRDeep2 analysis pipeline of some of the samples were very low. Further analysis of the phylogenic source of the miRs per sample showed that the samples with significantly lower amounts of miRs were dominated by miRs originating from green plants (i.e., rich in pollen), whereas the others contained a higher fraction of miRs annotated as insects' miR (*SI Appendix*, Fig. S1). Given these differences between the two types of samples, they could not be analyzed together. Therefore, for the downstream analyses, we included only three samples of worker regurgitate and three samples of queen regurgitate with high miRNA content which are likely to represent bumble bee miRs. Most of the samples with high miRs content had a whitish color (five out of the six), whereas the low miRs samples were dominated by miRs originating from green plants, and were more yellowish, thus potentially rich in pollen.

Overrepresentation Analysis and Gene List Comparison for Proteins in the Queen Regurgitate. We used overrepresentation analysis to identify processes and pathways that are enriched in the proteins more abundant in the queen regurgitate. This analysis groups together genes based on similar biological processes, cellular components, or molecular function, and examines which of the terms ("GO terms") are enriched in the samples. We analyzed the proteins that are significantly more abundant in the queen regurgitate produced by a *t*-test with permutation-based FDR correction ($P < 0.1$) using the clusterProfiler package (68) in R version 4.2.2. Gene ontology for *B. terrestris* genes was taken from the Hymenoptera Genome Database (69, 70). GO terms were classified as enriched if their proportion in the highly expressed proteins list was greater than the proportion in the samples' entire set of proteins. Enrichment analysis was also performed using STRING version 11.5 (71) using the *A. mellifera* database. Given that the results overall were similar, we chose to remain with the Hymenoptera Genome Database–based enrichment analysis.

We used the SignalP version 6.0 tool (72) to search for sequences of secretion signals. Finally, we compared our regurgitate protein list against proteins found in the *B. terrestris* honey (35).

The Influence of the Queen MG on Brood Development and Worker Ovarian Activity. Prior to the main experiment (below), we developed and validated a procedure to remove the MG with minimal influence on queen survival and wellbeing. To develop the procedure and reach the required expertise, we altogether dissected 560 queens and gynes (gynes were used during training since they have similar anatomy and morphology, and are easier to obtain). We also performed two preliminary experiments each with about 100 incipient colonies with their founding queens.

For the main experiment, we purchased ninety-two *B. terrestris* foundress queens. We also purchased 12 young colonies as a source of callow (newly emerged) workers to support the experimental colonies (see below). Each of these "donor" colonies contained a queen, brood at various developmental stages, and around 100 workers. All the bees were purchased from Biobee Biological Systems Ltd, Kibbutz Sde Eliyahu, Israel. We housed the colonies in wooden cages (24 × 21 × 12 cm) with a plexiglass cover. The foundress queens were each placed individually in a plastic cage (details below) and kept in a separate environmental chamber at the Bee Research Facility. Colonies were fed ad libitum as described above. After the operation (explained below), we introduced a callow ("helper") worker from a donor colony to each cage. The helper worker was replaced with a newly emerged callow every 5 d, except for a single week in which they were removed after 6 d. This was done in order to assure that helper workers do not activate their ovaries and attempt to lay eggs. We kept the workers and dissected their ovaries in order to assess the influence of queens subjected to the different treatments on their ovarian state (*SI Appendix*, Fig. S7). We collected all the newly emerging workers on a daily basis and stored them at −75 °C until further analyses. The brood developmental duration and final body size were measured as explained below.

We randomly assigned colony-founding queens to one of three treatments: *MG removal (MG^-)* queens were anesthetized on ice, and an incision was cut at the cuticle above the gland on each side of the head. After gently removing the glands with fine forceps, the incision was sealed with glue. *Sham surgery (Sham)* queens were similarly anesthetized, and a similar incision was made above the mandibles, but the glands were only touched and moved gently, but not removed. The incision was sealed as described for the MG^- queens. *Handling control (Control)* queens were handled and anesthetized on ice as described for the two other treatment groups, but were not dissected. A detailed description of our dissection protocol is provided in *SI Appendix, Supplementary Methods*. Dissections were performed during three successive days; on day 1, we dissected 13 MG^- queens, 11 *Sham* queens, and handled 7 *Control* queens; on day 2, 15 MG^- queens, 9 *Sham* queens, and 6 *Control* queens; on day 3, 13 MG^- queens, 11 *Sham* queens, and 7 *Control* queens. Altogether, we dissected 41 MG^- queens, 31 *Sham* queens, and handled 20 *Control* queens. After dissections, each queen was placed in a Petri dish cage supplied with ad libitum food and housed in an incubator (29 ± 0.7 °C, 62 ± 6 % relative humidity). After three days of recovery, we transferred the cages to an environmental room. Twenty-six MG^- queens, 17 *Sham* queens, and eighteen *Control* queens survived the recovery period and were transferred to the experiment room. The operation success was assessed at the end of the experiment; we chilled the focal queens, opened the sealed incision, and visually inspected for the presence of glandular remains and significant wounds. Two queens for which we successfully removed only one gland were not included in all our analyses.

We performed two sets of observations focusing on the queen's health and brood care behavior, respectively. All observations were done in an environmental chamber (27.5 ± 1 °C, 46 ± 8 % relative humidity, recorded using HOBO UX100 data loggers) under dim red light (otherwise, they were kept in a dark chamber). Each queen was observed for a total of 40 min. All observations were done in real-time (not video recorded), single-blinded. The cages were made of clear

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plastic allowing us to observe the queens from all directions. We performed the first set of observations in the second week of the experiment. We observed 12 MG^{-} queens, 13 Sham queens, and 13 Control queens. The duration (in minutes) of the following behaviors was recorded: standing still, grooming, handling, or touching wax. We also recorded the following discrete events: The queen is eating pollen, drinking sugar syrup, touching the incision wound, or buzzing with its wings while facing the worker. We also assessed whether or not the queen could move her mandibles, whether she moved normally, and whether she was clean. The second set of observations was additionally focused on brood care–related behaviors and agonistic behaviors performed by the queen toward workers. These observations were conducted when each of the focal queens had larvae. In this set of observations, we observed 13 MG^{-} queens, 6 Sham queens, and 12 Control queens. The differences between the sample size here and in the first set of observations reflect the fact that not all the queens of the first set of observations laid eggs. In addition to the behaviors recorded in the first set of observations, we also recorded the following behaviors: How long the queen is seen incubating brood clumps and how many times she is seen feeding or inspecting the brood. We also recorded the number of times the worker was seen feeding or inspecting the brood. Additional details on each of these behaviors are provided in *SI Appendix*, Table S2.

Each day during the experiment, we recorded egg laying and the number of newly emerging workers. We followed the development of the brood from the day the egg cup was sealed until bees emerged from pupae developing from this egg cup. Developmental duration was calculated as the interval between these two events. We froze all the newly emerging workers and kept them in a −75 °C ultra-low freezer and later measured the length of their Marginal Cell of the front wing as an index for body size. The Marginal Cell length is strongly correlated with other body size measures (73, 74). We used a stereo microscope (Nikon–SMZ800) to measure the Marginal Cell length with a 100-unit scale on the ocular. The experiment ended once all offspring emerged from the pupae of the first batch of brood (and thus, occurred on different days for different queens). The short period of brood rearing and the fact that only one worker assisted the queen did not allow producing gynes and also limited the size of workers emerging from the brood.

We assessed ovarian activity of the helper workers (at 5–6 d of age) as a proxy for the capacity of queens subjected to the various treatments to inhibit worker reproduction. For the analysis of 5-d-old workers, we dissected workers collected over the first ~14 d of the experiment. Given that the worker in each cage was replaced every 5 d, the number of workers in this analysis is higher than the number of queens. Seven workers died before reaching 5 d of age, and 1 removed from the analysis because her ovaries were damaged during the operation. Four MG^{-} workers were removed because the MG removal from their queen was not successful (see above). Seventeen queens died during the first 2 wk of the experiment.

As an index for ovarian activity, we measured the length of the seven largest terminal oocytes using a Nikon–SMZ645 stereomicroscope equipped with a 100-unit scale ocular ruler. As a positive control, we added an additional group composed of similar workers that were housed without a queen (queenless; QL). The QL group treatment was composed of a pair of workers housed in similar cages to these with the queens. The QL group control enabled us to confirm that the experimental conditions support ovarian activation in workers from the same source colonies and experiencing overall similar conditions. The ovaries of all workers, besides one, were in a good condition, allowing easy dissection and oocyte measurement.

We used the Kruskal–Wallis test for all behavioral, ovarian activity, body size, and developmental duration analyses. In addition to the queens with unsuccessful dissection, we also removed four queens that produced males. A nonparametric test was selected because sample size was small, and data distribution did not meet the assumptions of parametric statistics for all these parameters. We used the Dunn test and a Bonferroni correction for multiple comparisons for post-hoc pair comparisons following tests in which the Kruskal–Wallis tests produced a statistically significant difference (P-value < 0.05). We used the sign test to compare the queens' weight at the beginning and the end of the experiment. We did not use the Wilcoxon signed-rank test because the assumption of symmetry was violated. In a complementary analysis, we used the Kruskal–Wallis test to compare the weight of queens from the three groups at the beginning and then repeated it again, comparing the weight of queens at the end of the experiment. All statistical analyses were performed using SPSS Statistics Version 25 (IBM corporation, USA).

Data, Materials, and Software Availability. Proteomic databases have been deposited in the PRIDE database (https://www.ebi.ac.uk/pride/archive), project number is (PXD039851) (75). The submission was done by Rosi Fassler, on February 4th, 2023. All other data are included in the manuscript and/or supporting information.

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