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Original article

Dietary dopamine supplementation has no effect on ovary activity in queen-less or queen-right honeybee (*Apis mellifera*) workers

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Abstract – Eusociality in honeybees (*Apis mellifera*) is characterised by a reproductive division of labour, where the queen monopolises reproduction while worker reproduction is constrained. This constraint is mediated by queen mandibular pheromone (QMP), which inhibits worker ovary development through Notch signalling and possibly oocyte apoptosis. Dopamine has been implicated in regulating reproduction in worker honeybees, with prior studies suggesting that dietary dopamine enhances ovary activity in queen-less workers. This study aimed to test whether dopamine supplementation could overcome QMP-mediated reproductive constraint in worker honeybees. Using caged honeybee experiments, we administered dopamine and its precursor L-dopa at a range of concentrations, both in the presence and absence of QMP. Results showed that neither dopamine nor L-dopa supplementation affected ovary development, survival rates, or food intake, which contrasts with a previous study. These findings suggest that dopamine may not be a major factor in QMP-mediated reproductive inhibition. Instead, we suggest that the multifaceted nature of QMP's components together with the complexity of neuroendocrine signalling makes it likely that multiple redundant mechanisms regulate worker reproduction. Future research should investigate the interplay between nutrition, dopamine and QMP components to fully understand the regulation of ovary activation in honeybee workers.

honeybee / dopamine / ovary activation / QMP / *Apis mellifera* / neuroendocrine

1. INTRODUCTION

Eusociality is defined by the reproductive division of labour, where one female (the queen or dominant) is responsible for most of the reproduction (Wilson 1971). This requires reproduction in the subordinates or workers to be constrained, either during development or in adulthood with a number of mechanisms that allow that to happen (Khila and Abouheif 2008, 2010). In honeybees (*Apis mellifera*), the reproductive potential of workers is reduced, but not entirely eliminated, by developmental mechanisms (Hartfelder et al. 2018). Reproduction in adult workers is constrained, in part, by the presence of queen mandibular pheromone (QMP) (Butler and Fairey 1963; Free 1987; Hoover et al. 2003). In the absence of QMP, worker ovaries

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are completely remodelled, oogenesis is initiated (Duncan et al. 2016; Duncan et al. 2020), and the workers lay haploid eggs destined to become drone (male) bees (Jay 1968).

QMP acts on the ovary to repress reproduction via Notch signalling (Duncan et al. 2016) and possibly mediates apoptosis of oocytes that do develop (Ronai et al. 2015). Repression of reproduction requires direct contact between the worker and QMP (Lovegrove et al. 2020), indicating a role for olfactory and gustatory receptors as QMP is passed via trophallaxis in the hive (Naumann 1991). We do not yet have a comprehensive picture of how all of the components of QMP are detected by worker bees, but research has focussed primarily on antennal detection. In particular, odorant receptors (e.g. Or11 (Wanner et al. 2007)), odorant binding proteins (e.g. OBP11 in *A. cerana*, (Song et al. 2018)) and antenna-specific proteins (e.g. ASP1 in *A. cerana* and *A. mellifera* (Wu et al. 2022)) have all been implicated in binding components of QMP.

Once detected, it also is not clear how the QMP signal is integrated and relayed to the ovary to repress reproduction; however, in all animals, the brain and reproductive system are intricately connected. In insects, the neuroendocrine signalling links the brain and reproductive system and incorporates hormones (e.g. juvenile hormone and ecydsteriods), biogenic amines (e.g. dopamine and octopamine) and nutrient sensing (e.g. insulin signalling) (Knapp et al. 2022). Through altered neuroendocrine signalling, reproduction can be regulated by environmental cues like nutrition and temperature (Knapp et al. 2022), and it is likely that QMP acts by disrupting neuroendocrine signalling pathways to repress reproduction in honeybee workers (Knapp et al. 2022). In the absence of a queen, workers have low levels of juvenile hormone similar to nurse bees, but elevated ecdysteriod levels similar to queens (Robinson et al. 1991, 1992).

In honeybees, evidence also points towards the importance of biogenic amines, in particular dopamine, as being involved in QMP's inhibition of oogenesis in workers. QMP exposure represses brain dopamine levels (Harris and Woodring 1995), possibly through one of the specific components, homovanillyl alcohol (HVA) (Beggs et al. 2007). Dopamine levels also positively correlate with the degree of ovary activity in queen-less workers (Harris and Woodring 1995; Sasaki and Nagao 2001) (reviewed in Amsalem 2020). Additionally, the honeybee ovary expresses two dopamine receptors (*Dop1* and *Dop3*), and the expression of these receptors is altered in queen-less workers (Vergoz et al. 2012), supporting a role for dopamine in regulating reproduction in worker honeybees. Despite decades of research, just one study has causally tested this hypothesis (Dombroski et al. 2003). This study showed that dietary supplementation of dopamine enhances rates of ovary activity in queen-less workers (Dombroski et al. 2003), supporting a role for dopamine as a gonadotropin when QMP is absent. However, it remains unclear whether dopamine supplementation can overcome the repression of reproduction caused by QMP, which would indicate a causative role for dopamine in QMP-mediated reproductive constraint. Here, we aimed to test this hypothesis by expanding upon previous work (Dombroski et al. 2003) through dietary supplementation experiments. Specifically, we tested whether QMP influenced ovarian responses to dopamine supplementation, examined a wider range of dopamine doses and included L-dopa, the precursor to dopamine, to assess its potential effects on ovary activity in honeybees..

2. MATERIALS AND METHODS

2.1. *Caged honeybee experiments*

A. mellifera were kept according to standard beekeeping practices in British National hives at the University of Leeds School of Biology Research Apiary. Experiments were conducted between July and October in 2018 and 2019. Frames of emerging brood were collected from three source hives and incubated overnight at 35 °C. The following day, newly emerged bees were transferred into metal cages with a glass sliding door and holes for the insertion of food caps and a water tube (10 × 10 × 5.5 cm, Small

Life Supplies UK). Eighty to one hundred bees were randomly allocated to each cage, and cages were randomly assigned to treatments. Three independent replicates were carried out for each treatment. Cages were maintained in the dark at 35 °C and 20–40% RH for 10 days. All cages were fed complete bee food (CBF; 20 g pollen, 52 g sucrose, 18.8 g brewer's yeast and 9.2 g lactalbumin) mixed with honey to form a thick paste, a standardised high-protein diet formulated to permit ovary activity (Duncan et al. 2016). Each day, food and water intake were recorded for each cage by weighing the food caps and water tubes and subtracting the weight recorded the previous day. Cages were given fresh CBF daily and dead bees were removed and counted daily. QMP + cages received 0.1 queen equivalents (QE) of synthetic QMP (Intko Supply Ltd, Canada) each day (10 µL droplet of 0.01 QE/µL dissolved in ethanol) on a glass slide. Slides were replaced daily. Synthetic QMP contained a blend of the five major QMP components: 9-oxo-2-decenoic acid (9-ODA), cis- and trans-9-hydroxydec-2-enoic acid (9-HDA), methyl p-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA). QMP- cages received 10 µL ethanol each day as a solvent control.

2.2. Dopamine and L-dopa exposure

Dietary dopamine was administered following the methodology of Dombroski et al. (2003) by adding 0.01 mg dopamine/g food offered to DA + cages, but not DA (control) cages. To ensure an even distribution of the dopamine in the food, the solution was first mixed with the honey, followed by the addition of the CBF powder, and then ground into a homogenous paste using a mortar and pestle. To assess whether dietary dopamine can overcome QMP's repression of ovary activity, dopamine treatments were tested in both the presence and absence of QMP (QMP + or QMP-). In subsequent attempts to reproduce the ovary activation phenotype associated with dopamine in previous studies (Dombroski et al. 2003), dietary dopamine was also tested at a range of concentrations: 0.1, 0.5, 1 and 2 mg/g food in the absence of QMP only.

L-Dopa is the precursor to dopamine and is converted to dopamine by dopa decarboxylase. L-Dopa is generally considered to be more stable than dopamine, and supplementation of L-dopa should lead to localised increases in dopamine specifically where there are high levels of dopa decarboxylase activity, which in the honeybee is the brain (Sasaki et al. 2012), as opposed to systemic supplementation with dopamine. Effects of the dopamine precursor L-dopa on ovary activation were also tested in the absence of QMP. L-Dopa was administered by spiking water solutions offered to cages at concentrations of 0.01, 0.1 and 0.5 mg/mL, while controls received just water. L-Dopa was administered directly in water rather than mixed into the food due to its lower solubility compared to dopamine (0.99 mg/mL versus 18.96 mg/mL). Adding a concentrated L-DOPA solution directly to the food made the mixture too runny and resulted in excess mortality; therefore, it was added directly to the water. L-Dopa solutions were replaced daily, and intake was recorded as detailed above to monitor dosages received.

2.3. Ovary scoring

In our laboratory setup, a 10-day exposure period has been previously shown to be sufficient for observing ovary activation and assessing the effects of experimental manipulations on the ovary activation process (Duncan et al. 2016; Duncan et al. 2020). After 10 days, the ovaries of all surviving bees were dissected in PBS and photographed using a GXM-XTL stereomicroscope with GXCAM-U3 Series 5MP camera and GX Capture Software (GT Vision, UK). As previously described (Duncan et al. 2016), ovary activity was scored blindly using a modified Hess scale (Hess 1942). Ovaries that were thin, lacked defined oocytes and were morphologically indistinguishable from queen-right worker ovaries were scored as 0; ovaries that were slightly thickened, showing signs of differentiated cells but with no deposition of yolk,

were scored as 1; ovaries with clearly defined oocytes and yolk deposition were scored as 2; and ovaries with at least one oval fully mature oocyte were scored as 3.

2.4. Statistics

All data analysis was carried out in R version 4.1.2 (R Core Team 2021). Linear mixed-effects models (LMMs) and generalised linear mixed-effects models (GLMMs) were built using the package *lme4* (Bates et al. 2015). The packages *survival* and *coxme* were used to construct Cox proportional hazards models (CPH) with mixed effects (Therneau 2020, 2021), and cumulative link mixed models (CLMMs) were carried out using the package *ordinal* (Christensen 2019). Post hoc testing was carried out using the package *emmeans* (Lenth 2022). Graphs were prepared using *ggplot2* (Wickham 2016) and *ggpubr* (Kassambara 2022).

2.4.1. Ovary activation

CLMMs were fitted for ovary scores with dopamine, QMP presence and their interaction as fixed effects and replicate as a random effect. For subsequent experiments testing dopamine and L-dopa at a range of doses, dose was considered an ordinal variable and modelled as the sole fixed effect. Statistical significance of fixed effects was determined by comparing the likelihood ratio of the maximal model to that of the null model, or model without the fixed effect of interest. Where fixed effects were statistically significant, post hoc testing was carried out by computing least squares means to determine the significance of pairwise comparisons. *P*-values were Tukey adjusted to control for multiple testing.

2.4.2. Survival

Survival distributions were compared between treatment groups using CPH models with mixed effects. Dopamine, QMP presence and their interaction were included as fixed effects, while in subsequent experiments, dopamine or L-dopa dosage was the sole fixed effect. Replicate was included as a random effect in all models. The assumption of proportional hazards was verified by visual examination of the correlation of scaled Schoenfield residuals with time to test for independence. CPH models were used to predict hazard ratios (HR) and confidence intervals (CI) for fixed effects. HRs are presented as HR (± 95% CI).

2.4.3. Food and water intake

Food intake was compared across treatment groups for each experiment by fitting LMMs with mean food intake per bee per day as the response variable. To account for repeated measures, day was included as a random effect and nested within replicate for all models. Dopamine, QMP presence and their interaction were included as fixed effects, while in subsequent dopamine and L-dopa dosage experiments, dose was included as the fixed effect. Visual examination of model residual plots revealed no obvious deviations from normality or homoscedasticity. Statistical significance of fixed effects was determined by comparing the likelihood ratio of the maximal model to that of the null model, or model without the fixed effect of interest. In L-dopa experiments, where L-dopa was administered via the water solution, water intake was also modelled as described above.

3. RESULTS

3.1. Supplementary dopamine feeding does not influence worker reproduction in the presence or absence of QMP

As expected, QMP exposure significantly reduced reproductive activity in 10-day-old worker honeybees (Figure 1A; CLMM: $x^2 = 64.23$, d.f = 2, 759, $p < 0.001$; detail of the post hoc tests are supplied in Supplementary

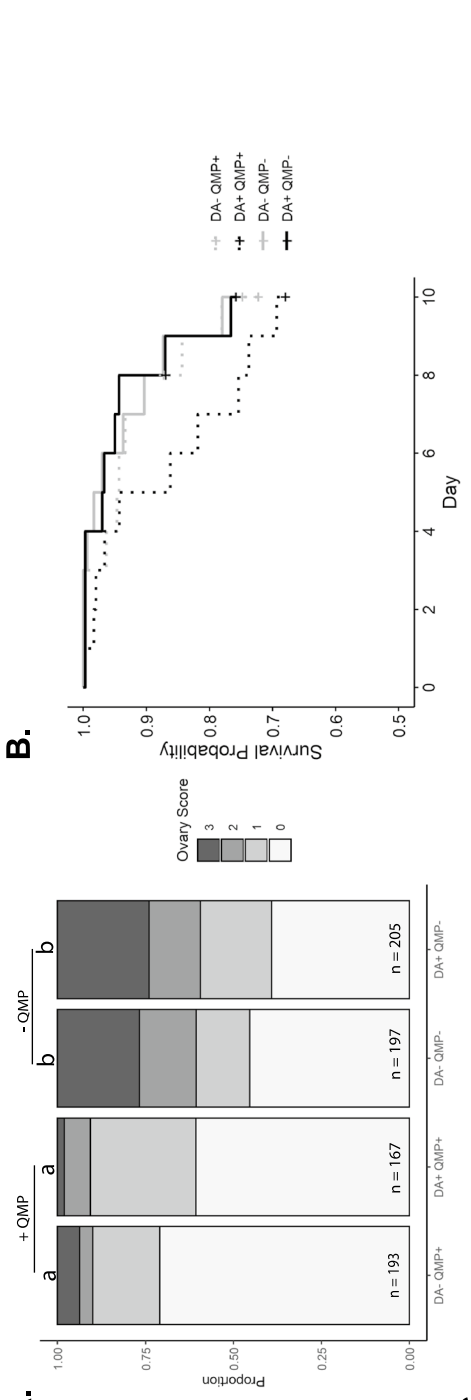

Figure 1. Dopamine supplementation (10 µg/g food) does not affect ovary activity in the presence or absence of QMP. **A** Ovary activity is reduced in bees exposed to QMP (left two bars, +QMP) relative to unexposed bees (right two bars, -QMP), while dopamine supplementation at 10 µg/g food (DA+ vs DA) has no effect. Ovary activity is shown as the proportion of bees within each treatment group with each ovary score (ranging from 0 to 3), where a higher score (darker shade) relates to a higher degree of development. *N* values for each treatment pooled across three replicates are displayed at the base of each bar. Statistical significance of post hoc pairwise comparisons is denoted by bars not sharing a letter (least squares means, $p<0.05$). **B** Survival probability distributions of cages of 100 bees over 10 days. Data shown consists of three replicates of each treatment cage pooled together. The *Y* axis has been truncated to begin at a survival probability of 0.5. **C** Mean food intake per bee per day does not differ between treatment groups. Food intake was recorded daily for each cage ($n=10$ days).

Table A.1). The suppression of ovary activation observed with synthetic QMP is not absolute, as some worker bees had active ovaries even in the presence of QMP (Figure 1A). However, the frequency and extent of this activation are consistent with levels reported in our previous work, suggesting that the experimental conditions replicate established responses to QMP (Duncan et al. 2016; Duncan et al. 2020). However, there was no evidence to support the effect of feeding supplementary dopamine at 10 µg/g on reproductive activity (Figure 1A; CLMM: $\chi^2 = 3.44$, d.f = 2, 759, $p = 0.18$), and no interaction effects between QMP and dietary dopamine were observed (CLMM: $\chi^2 = 0.31$, d.f = 1, 760, $p = 0.58$). This is in contrast with previous studies where it has been shown that dietary dopamine enhanced ovary activity in workers maintained in the absence of QMP (Dombroski et al. 2003).

Dietary dopamine treatment did not significantly compromise survival rates (Figure 1B; CPH: HR = 0.96 ± 0.17 SE, $z = -0.28$, $p = 0.78$), nor did QMP exposure (Figure 1B; CPH: HR = 1.16 ± 0.16, $z = 0.91$, $p = 0.36$) or the combination of the two (Figure 1B; CPH: HR = 1.38 ± 0.22, z = 1.44, $p = 0.15$). Food consumption rates were also consistent across treatments and did not differ significantly with dopamine, QMP exposure or their interaction (Figure 1C; GLMMs: dopamine: $\chi^2 = 1.03$, d.f = 2, 117, $p = 0.60$; QMP: $\chi^2 = 1.82$, d.f = 2, 117, $p = 0.40$; Interaction: $\chi^2 = 0.37$, d.f = 1, 118, $p = 0.54$). Bees from dopamine-treated cages consumed an average of 0.13 µg dopamine per bee per day.

3.2. Supplementary dopamine feeding at concentrations of 0.1–2 mg/g does not influence worker reproduction

Dopamine was administered to bees in the absence of QMP at escalated doses to assess whether the effects on ovary activity were dose-dependent. Despite the increased dosages, dopamine supplementation had no effect on ovary activity rates in the absence of QMP (Fig. 2A; CLMM: $\chi^2 = 1.68$, d.f = 4, 1009, $p = 0.79$).

Dopamine treatment had no significant effect on survival rates at the doses tested (Figure 2B; CPH: 0.1 mg/g HR = 0.94 ± 0.21 SE, $z = -0.29$, $p = 0.78$; 0.5 mg/g HR = 1.09 ± 0.20 SE, $z = 0.42$, $p = 0.68$; 1 mg/g HR = 1.30 ± 0.20 SE, $z = 1.32$, $p = 0.19$; 2 mg/g HR = 1.42 ± 0.18 SE, $z = 1.92$, $p = 0.05$). Food consumption rates were also not significantly affected by dietary dopamine at these doses (Figure 2C; LMM: $\chi^2 = 8.60$, d.f = 4, 145, $p = 0.07$; intake per bee is shown in Supplementary Figure B.1 and the results of the post hoc analysis are shown in Supplementary Table B.1).

3.3. L-Dopa does not influence worker reproduction

Supplementation with the dopamine precursor, L-dopa, did not affect worker reproduction at any dose in the absence of QMP (Figure 3A; CLMM: $\chi^2 = 7.71$, d.f = 3, 834, $p = 0.05$). L-Dopa treatment caused no negative effects on survival rates at the doses administered (Figure 3B; CPH: 0.01 mg/mL HR = 1.76 ± 0.40 SE, $z = 1.41$, $p = 0.16$; 0.1 mg/mL HR = 0.49 ± 0.55 SE, $z = -1.29$, $p = 0.20$; 0.5 mg/mL HR = 0.79 ± 0.47 SE, $z = -0.49$, $p = 0.62$). Additionally, food consumption rates did not differ with L-dopa dose (Figure 3C; LMM: $\chi^2 = 5.08$, d.f = 3, 116, $p = 0.17$; intake per bee is shown in Supplementary Figure C.1 and the results of the post hoc analysis are shown in Supplementary Table C.1). Water intake was also consistent across L-dopa treatment regimes, indicating that received dosages were not compounded by differential rates of consumption (Figure 3D; LMM: $\chi^2 = 2.08$, d.f = 3, 116, $p = 0.56$).

4. DISCUSSION

The inhibition of adult honeybee worker reproduction by pheromones including QMP (Butler and Fairey 1963; Free 1987; Hoover et al. 2003) enforces the reproductive division of labour in this species. In honeybees, dopamine is thought to be a key signalling molecule

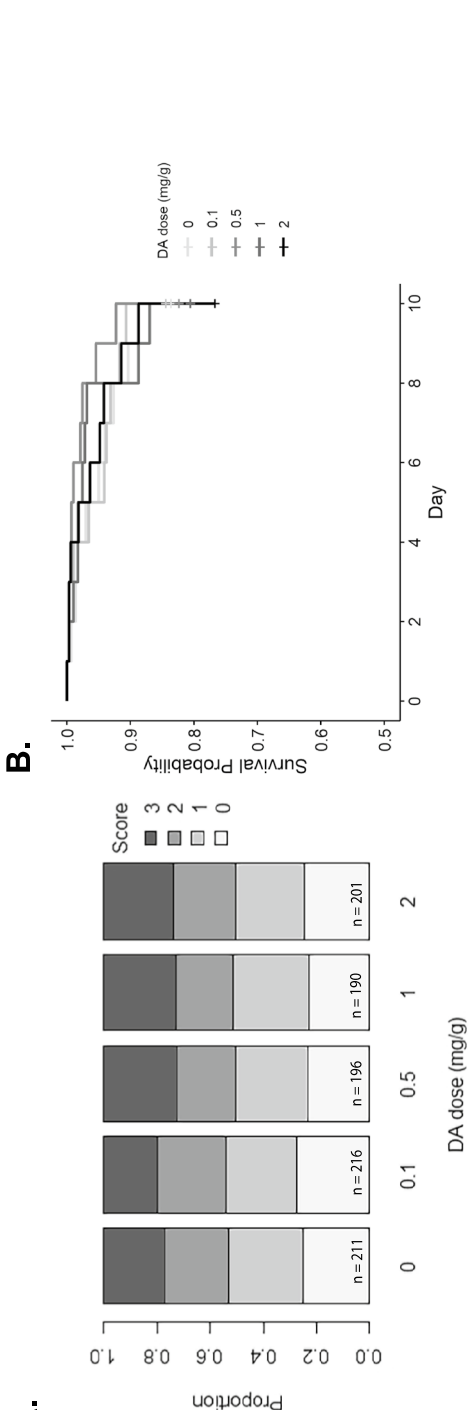

Figure 2. Worker reproduction is insensitive to a wide range of dopamine supplementation doses. **A** Ovary activity unaffected by dopamine supplementation in the absence of QMP at a range of concentrations from 0.1 to 2 mg/g of food. Ovary activity is shown as the proportion of bees within each treatment group with each ovary score (ranging from 0 to 3), where a higher score (darker shade) relates to a higher degree of development. Statistical significance of post hoc pairwise comparisons is denoted by bars not sharing a letter (least squares means, $p < 0.05$). **B** Survival probability distributions of cages of 100 bees over 10 days. Data shown consists of three replicates of each treatment cage pooled together. The Y axis has been truncated to begin at a survival probability of 0.5. **C** Mean food intake per bee per day does not differ between treatment groups. Food intake was recorded daily for each cage ($n = 10$ days).

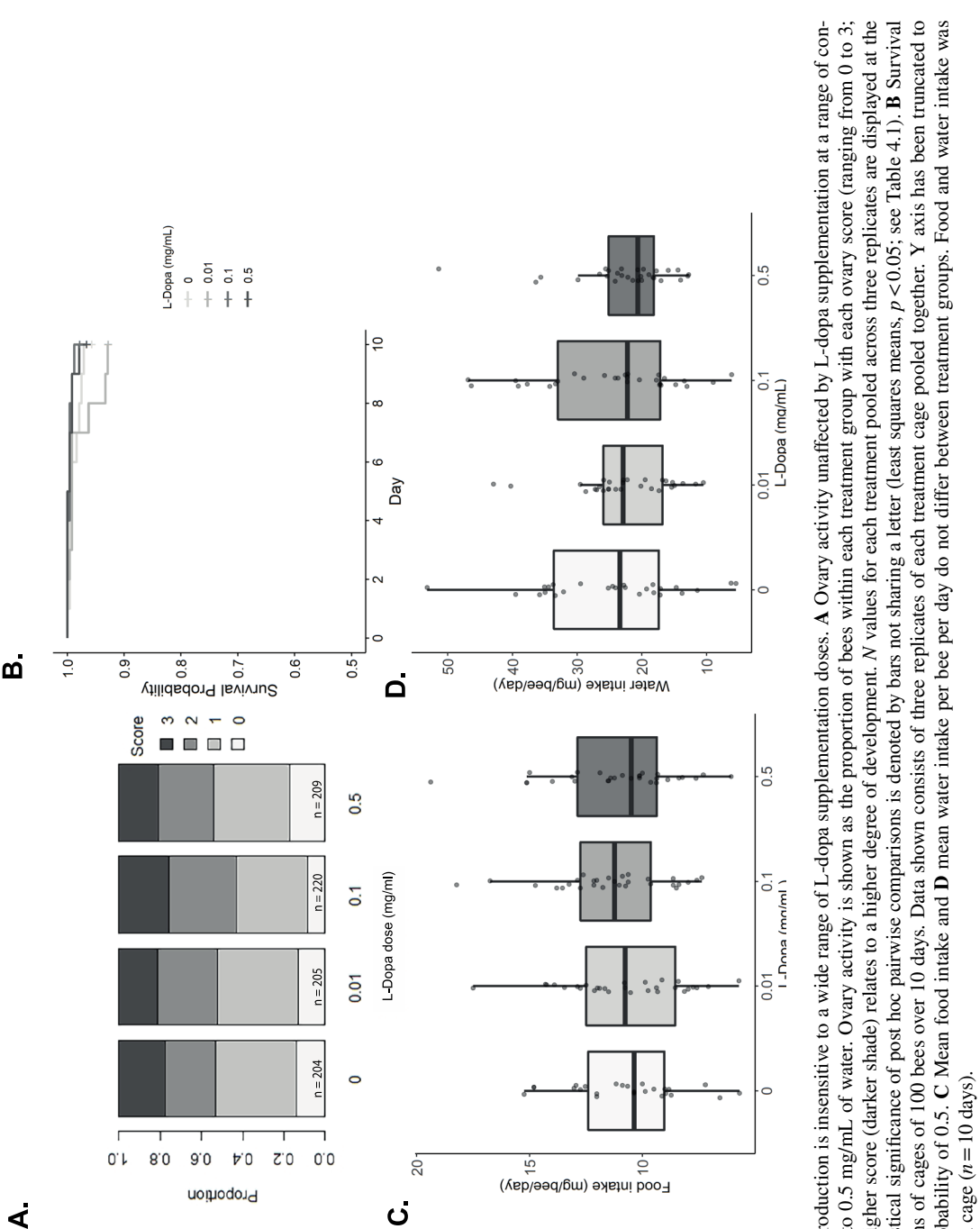

Figure 3. Worker reproduction is insensitive to a wide range of L-dopa supplementation doses. **A** Ovary activity unaffected by L-dopa supplementation at a range of concentrations from 0.01 to 0.5 mg/mL of water. Ovary activity is shown as the proportion of bees within each treatment group with each ovary score (ranging from 0 to 3; Figure 2.2), where a higher score (darker shade) relates to a higher degree of development. *N* values for each treatment pooled across three replicates are displayed at the base of each bar. Statistical significance of post hoc pairwise comparisons is denoted by bars not sharing a letter (least squares means, $p < 0.05$; see Table 4.1). **B** Survival probability distributions of cages of 100 bees over 10 days. Data shown consists of three replicates of each treatment cage pooled together. Y axis has been truncated to begin at a survival probability of 0.5. **C** Mean food intake and **D** mean water intake per bee per day do not differ between treatment groups. Food and water intake was recorded daily for each cage ($n = 10$ days).

mediating QMP's biological effects, including repression of reproduction in adult workers (Harris and Woodring 1995; Sasaki and Nagao 2001; Beggs et al. 2007; reviewed in Amsalem 2020). However, despite decades of research, only one study has causally tested this hypothesis (Dombroski et al. 2003). Dombroski et al. (2003) showed that dietary supplementation of dopamine enhances rates of ovary activity in queen-less workers, supporting a role for dopamine as a gonadotropin in worker honeybees when QMP is absent.

Here, we set out to extend this finding and determine whether dopamine supplementation could overcome the repression of reproduction caused by QMP, which would indicate a causative role for this biogenic amine in QMP-mediated reproductive constraint. However, in contrast to Dombroski et al. we found that neither dopamine nor L-dopa supplementation at a wide range of concentrations affected worker reproduction in our study (Figures 1, 2, 3), either in the presence or in the absence of QMP.

This may be due to differences in the strains used in both studies; the original study (Dombroski et al. 2003) used Africanised honeybees (a cross between *A. mellifera scutellata* and European strains such as *A. mellifera mellifera*), while our study used *A. mellifera ligustica* (Italian honeybee)*/A. mellifera carnica* (Carniolan honeybee). Africanised honeybees have larger ovaries (Linksvayer et al. 2009) and are more likely to be reproductive (Makert et al. 2006). Indeed, Dombroski et al. saw relatively high, but also very variable levels of ovary activity compared to our study after dopamine supplementation (Dombroski et al. 2003). It is possible, therefore, that there may be strain-specific effects of dopamine on ovary activity or in the threshold at which ovaries respond to dopamine levels. Dombroski also reported a significant effect of the number of bees per cage on ovary activation (Dombroski et al. 2003), suggesting that social cues may be important in the response to dietary dopamine, with the effect reduced at larger cage sizes. Here, we used 80–100 bees per cage, thereby keeping the social environment constant across treatments.

It is also possible that there is an interaction between diet and dopamine supplementation. In the original study (Dombroski et al. 2003), the diet was composed of 12% protein derived from a single source, pollen. However, in this study, we used 'complete bee food', a combination of protein sources that have been shown to support ovary activity in *A. mellifera* (Duncan et al. 2016) and that is 26.6% protein. Pollen can be limited in specific amino acids such as methionine, tryptophan, histidine and valine (Cook et al. 2003; Wang and Li-Byarlay 2015; Végh and Csóka 2023). In contrast, our study's diverse protein sources likely provided a balanced and abundant amino acid profile. Since dopamine is synthesised from tyrosine, the higher protein content of our diet may have elevated dopamine levels relative to the levels seen in Dombroski et al. making supplementary dopamine ineffective or redundant for ovary activity. This higher protein content may have not only elevated dopamine levels but also promoted ovary activation more broadly, even in the presence of QMP. This may explain why QMP-mediated suppression of ovary activity was incomplete in our study (Figure 1), suggesting that adequate nutrition can directly enhance ovary development, potentially overriding partial inhibitory effects of QMP.

When comparing diet intake per bee between the two studies, the original study (Dombroski et al. 2003) showed a wide range of 10–45 mg/bee/day (1.2–5.2 mg protein/bee/day). In contrast, our study had a narrower range of 10–20 mg/bee/day (2.7–5.3 mg protein/bee/day). While the upper limits of protein intake were similar (5.2 vs. 5.3 mg protein/bee/day), the lower limits differed, with our study showing 2.7 mg/bee/day compared to 1.2 mg/bee/day in the original study. There is a strong link between dietary protein intake and the level of ovary development in honeybees (Maurizio and Hodges 1950; Lin and Winston 1998; Stephen and Robert 2000). It may be, therefore, that dopamine supplementation only promotes ovary activity in honeybees when they are relatively protein-poor or protein-limited as adults. More work is needed to determine if there are strain-specific effects of dopamine thresholds or if there

is an interaction between diet and dopamine levels that influence ovary activity.

In this study, as in the original study (Dombroski et al. 2003), the absorption of dopamine from the diet was not directly measured, which could influence the interpretation of the results. While dietary supplementation with dopamine or L-dopa is commonly used to manipulate dopamine levels in invertebrates (e.g. Sasaki et al. 2009), the bioavailability and tissue-specific effects of dopamine in invertebrates following dietary supplementation remain poorly understood. Given the susceptibility of dopamine and L-dopa to oxidation (Pendleton et al. 1996) and self-polymerisation (Herlinger et al. 1995; Fichman and Schneider 2021), dietary dopamine supplementation may have limited bioavailability, and any increases in dopamine levels resulting from such supplementation may be transient. However, other studies have demonstrated that dietary supplementation of dopamine can effectively elevate dopamine levels in other insects (e.g. Sasaki et al. 2009). Regardless, future studies should quantify dopamine and its metabolites in specific tissues using high-performance liquid chromatography (HPLC) (Sasaki and Nagao 2001; Sasaki et al. 2012), following dietary supplementation to confirm whether these methods effectively alter endogenous dopamine levels. Such work would provide critical validation for the use of dietary supplementation in manipulative experiments and clarify the potential interactions between diet composition, dopamine biosynthesis and supplementation outcomes.

Our study does, however, indicate that dopamine is likely not the sole factor that controls ovary development in honeybee workers in response to QMP. This is perhaps unsurprising given that reproduction in female insects is tightly regulated by several components, collectively referred to as the neuroendocrine system, which includes neuropeptides, juvenile hormones (JH), ecdysteriods like 20-hydroxyecdsone and, in honeybees, Makisterone A (Feldlaufer et al. 1985), biogenic amines (including dopamine) and insulin signalling (reviewed in Knapp et al. 2022). Manipulation of a single component, like dopamine, may be insufficient to tip the balance between being reproductively repressed and reproductively active in worker honeybees. While a minimum level of dopamine has been shown to be necessary for normal ovarian development in insects like *Drosophila melanogaster* (Neckameyer 1996; Pendleton et al. 1996), additional signals or changes to neuroendocrine signalling may be required to activate reproduction in honeybee workers. Although QMP does repress brain dopamine levels (Harris and Woodring 1995) and dopamine levels in honeybee worker brains positively correlate with the degree of ovary activity in queen-less workers (Harris and Woodring 1995; Sasaki and Nagao 2001) (reviewed in Amsalem 2020), this is likely just one of several cues triggering ovary activation in the absence of the queen.

In some species, dopamine positively regulates reproduction by stimulating the production and release of juvenile hormone in the corpus allatum which (Pastor et al. 1991; Cassier et al. 1993; Granger et al. 1996), in turn, increases levels of vitellogenin and yolk proteins in the fat body (Tufail and Takeda 2008), which are essential for oogenesis. However, in honeybees, juvenile hormone does not promote reproduction and is instead a negative rather than a positive regulator of vitellogenin and yolk proteins (Robinson et al. 1992; Pinto et al. 2000; Corona et al. 2007; Rodrigues and Flatt 2016). Although dopamine seems essential for reproduction in honeybees, its mechanism of action likely differs from that of solitary insects. This underscores the need for a deeper understanding of the complex feedback within the neuroendocrine system that controls reproduction in worker honeybees.

If dopamine levels are a major factor governing ovarian development, this may appear to be at odds with the finding that dopamine supplementation did not overcome QMP's inhibitory effects. Possible limitations of dopamine supplementation methods that may play into this have been discussed above along with the complexities of neuroendocrine signalling. However, it is perhaps unsurprising that QMP's repression cannot be overcome simply via the restoration of dopamine given the multiple levels of functional redundancy within QMP's

components (Princen et al. 2019). QMP contains a blend of five major compounds: (2E)−9-oxo-dec-2-enoic acid (9-ODA), both enantiomers of (2E)−9-hydroxydec-2-enoic acid (9-HDA), methyl-4-hydroxybenzoate (HOB) and the component responsible for lowering dopamine, homovanillyl alcohol (HVA) (Slessor et al. 1988). Exposure of honeybee workers to HVA alone results in partial inhibition of reproduction (Princen et al. 2019), while exposure to 9-ODA or 9-HDA causes equivalent levels of repression to that seen with the five-component blend (Princen et al. 2019). This indicates that the depression of brain dopamine by HVA is likely just one process among several redundant mechanisms acting to constrain worker reproduction in the presence of QMP.

Our study suggests that QMP likely represses ovary activation through multiple mechanisms, making it challenging to overcome this inhibition by simply supplementing with dopamine or L-DOPA. While adequate nutrition and potentially dopamine levels are essential for ovary activation, they are not the sole factors. The complexity of QMP's effects, involving a blend of compounds like homovanillyl alcohol that individually and collectively contribute to reproductive inhibition, highlights the multifaceted nature of this regulatory system. Therefore, future research should explore the interplay between nutrition, dopamine and the various components of QMP to fully understand the regulation of ovary activation in honeybee workers

SUPPLEMENTARY INFORMATION

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AUTHOR CONTRIBUTION

RAK: experimental design, carried out experiments, blind-scored honeybee ovary images, assisted with the preparation of figures and drafted the manuscript.
MRL: assisted with honeybee dietary supplementation experiments, edited the manuscript.
VCN: assisted with honeybee dietary supplementation experiments.
EJD: experimental design, supervised experiments, blind-scored honeybee ovary images, assisted with the preparation of figures and drafted the manuscript.
RAK and EJD conceived the research. RAK conducted experiments, assisted by MRL and VCN, performed the analysis and drafted the figures. RAK and EJD wrote the paper. All authors approved the manuscript.

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DATA AVAILABILITY

The data that support the findings of this study are available from the author upon reasonable request.

DECLARATIONS

Competing interests The authors declare no competing interests.

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