Title:

Age and QMP exposure affect the nutritional preferences of caged *Apis mellifera* worker honeybees

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Short Title

Age and QMP effect on worker honeybee feeding

Abstract

Eusocial insect queens often use pheromones to prevent reproduction in the worker caste, enforcing the reproductive constraint that is central to eusociality. In *A. mellifera* honeybees, the queen emits several pheromones that affect worker reproduction, the most important being QMP. Although the effects of QMP have been studied in some detail, the mechanisms by which it brings about reproductive constraint in workers are still unclear. Remarkably, QMP is also able to repress reproduction in other insects, including the fruit fly *Drosophila melanogaster*, in which QMP has been shown to induce a starvation-like response. Here we use caged newly-eclosed-workers with an *ad libitum* choice of protein and sugar food sources to investigate whether QMP alters dietary intake in the honeybee. We show that initially, irrespective of QMP exposure, workers only consume protein, before shifting to carbohydrate after four days. We also show that QMP-exposure results in an increased preference and intake of carbohydrates in worker bees, raising the possibility that QMP also induces a starvation-like response in honeybees.

Keywords

honeybee / nutrition / pheromone / QMP / feeding

Introduction

The defining feature of eusociality is the reproductive division of labour (Wilson 1971). In *A. mellifera* honeybees, this is maintained in part by the presence of pheromones produced by the queen (Princen, Oliveira et al. 2019), particularly Queen Mandibular Pheromone (QMP), which suppresses the reproduction of workers by preventing the activation of their ovaries (Hoover, Keeling et al. 2003). QMP is not the only pheromone to mediate reproductive constraint in this species, however, several other compounds (Mohammedi, Paris et al. 1998; Maisonnasse, Lenoir et al. 2010) and queen pheromones (Wossler and Crewe 1999; Princen, Oliveira et al. 2019) are also able to bring about reproductive constraint, indicating a high degree of redundancy in this eusocial regulatory mechanism (Princen, Oliveira et al. 2019).

QMP, produced in the mandibular glands of queens (Slessor, Kaminski et al. 1990) is comprised of five main compounds (Slessor, Kaminski et al. 1990; Plettner, Slessor et al. 1996; Plettner, Otis et al. 1997). In addition to inhibiting reproduction QMP also produces other effects in the honeybee worker including: inducing care behaviours (Fischer and Grozinger 2008), regulation of swarming (Winston, Slessor et al. 1989), inhibiting rearing of queens (Pettis, Higo et al. 1997), and inducing retinue behaviour (Slessor, Kaminski et al. 1988). Despite the large body of research investigating the different functions of this pheromone, the mechanism of action for QMP's repressive effect on worker reproduction is not fully understood at a physiological, or molecular, level.

QMP is also able to bring about the repression of reproduction in other, phylogenetically diverse, species including the bumblebee *B. terrestris* (Princen, Van Oystaeyen et al. 2020), and the fruit fly *D. melanogaster* (Camiletti, Percival-Smith and Thompson 2013). With the latter species being almost 370 million years diverged from *A. mellifera* (Misof, Liu et al. 2014). Work in *D. melanogaster* has shown that QMP induces a starvation-like response, possibly producing reproductive repression as a by-product of starvation-induced diapause (Lovegrove, Dearden and Duncan 2023). This would possibly indicate that QMP may have evolved to inhibit reproduction in honeybee workers via sensory exploitation of highly conserved pathways, as previously suggested (Oi, van Zweden et al. 2015). An example of a target of this this sensory exploitation might be Notch signalling in QMP-mediated reproductive repression in honeybee workers, which has been shown to be activated by the presence of QMP (Duncan, Hyink and Dearden 2016). In this scheme, the highly conserved Notch signalling pathway, may have been coopted to induce reproductive constraint in worker honeybees in a way which also results in reproductive constraint in those phylogenetically diverse species.

Historically, investigations of QMP activity on the various aspects of honeybee behaviour and physiology have been carried out both within a native hive environment (*in alvo*, e.g. (Pankiw, Winston and Slessor 1994)), and in more sterile environments in cages in laboratory settings (*in cavea*, e.g. (Pirk, Boodhoo et al. 2010)). These *in cavea* experiments allow for the strict control of extraneous variables which could impact the phenotype being investigated (for example, the presence of other pheromones produced by the queen, or developing brood), but they may also produce workers that are not entirely biologically equivalent to those reared under normal in-hive (*in alvo*) conditions. These *in cavea* studies also require the artificial supplementation of food. Different studies have used diverse feeding regimens (Williams, Cédric et al. 2013), ranging from a relatively natural sugar fondant/pollen setup (Mohammedi, Paris et al. 1998), to a protein-heavy complete bee food (CBF, used to maximally induce ovary activation) (Duncan, Hyink and Dearden 2016; Duncan, Leask and Dearden 2020).

In colonies, young workers perform nursing and brood-care tasks which require pollen (Crailsheim 1990; Robinson 1992), whereas older foragers consume nectar to fuel flight (Crailsheim 1990). There has been some investigation into the preference of honeybee workers for different food types, such as the preferences for honeybees towards more metabolisable forms of protein (Pernal and Currie 2000; Pirk, Boodhoo et al. 2010). Food preference and nutrient intake therefore vary with worker behavioural role and physiological state. Several pheromones have been shown to affect these feeding dynamics, for example, (E)-β-ocimene produced by brood simulates foraging and brood care (Maisonnasse, Lenoir et al. 2010; He, Zhang et al. 2016), while QMP alters lipid metabolism and fat body composition (Fischer and Grozinger 2008; Corby-Harris, Snyder et al. 2022) as well as protecting against starvation (Fischer and Grozinger 2008). However, the relationship between QMP exposure, feeding preferences and diet consumption has not been directly examined *in cavea* conditions. This study aimed to investigate the effect of QMP exposure on feeding preferences *in cavea* for queenless *A. mellifera* workers; as well as testing the hypothesis that, similarly to *D. melanogaster* fruit flies, QMP induces starvation-like behaviour in worker honeybees, by, for example, increasing the amount of food being eaten.

Methods

Honeybee Husbandry

Polystyrene national-type hives of honeybees were kept at the University of Leeds, with standard beekeeping practice. Colonies were fed sugar fondant (BeeCandee, Beekeeping Supplies UK) during winter and spring, and pollen cake (ApiCandy, Beekeeping Supplies UK) during the early spring.

For experiments, frames of eclosing brood were taken from multiple queen-right hives over the summers (May-September) of 2023 and 2024.

In cavea experiments

Brood frames from the hives were emptied of adult bees and placed into a 35°C incubator for up to 24 h. All the workers which eclosed in this time were mixed, and

100 of these bees were randomly assigned to metal cages (10 cm x 10 cm x 5.5 cm steel with removable glass front and air holes, www.small-life.co.uk). The caged bees were kept in the dark at 35°C, fed *ad libitum* sugar fondant (3:1 ground table sugar to honey by weight), pollen cake (7:3 ground pollen supplied from LiveMoor, to honey by weight), and water, refreshed every 24h, recording consumption of each food type.

Each cage was provided treatment in the form of queen pheromone or solvent control (ethanol) every 24h. QMP was provided as a 20 µl aliquot of 0.1 Queen equivalent per day (Qe; 1 Qe is the amount of pheromone produced in a day by a single queen: 261.8 µg ODA, 104.7 µg HDA of both enantiomers combined, 26.2 µg HOB, and 2.62 µg HVA (Pankiw, Winston et al. 1996), supplied by Intko Supply Ltd. Canada) in ethanol on a microscope slide on the bottom of the cage, with the slide replaced every 24h. Dead bees were also removed, and deaths recorded, every 24h.

After 10 days all remaining bees were dissected to remove their ovaries, which were analysed to confirm QMP-mediated repression of workers. Some cages were taken through to day 20, however high mortality rates made this data unreliable and so it was censored.

Statistics

Graphs were produced in R using the ggplot2 (Wickham 2016) package and finished in Microsoft PowerPoint. For the consumption graph, means of each average consumption for each day were calculated and standard deviation used for error bars. For the Cohen's D graph, Cohen's D values were calculated measuring the effect size between fondant consumption by treatment for each day, with the error bars representing the upper and lower limits.

All analysis was performed in R (R Core Team 2021): The difference of food given to the bees and food removed from the bees 24h later for each cage was calculated into a feeding difference value for each of fondant and pollen. This value was then used to do individual pairwise comparisons between each of the treatments for each day via GLM using a distribution determined via the descdist package from the fitdistrplus package (Delignette-Muller and Dutang 2015) in R. For gaussian fitted models, an ANOVA was performed using an F-test, while for the gamma fitted models, a Log-Rank test was used to generate significance values. When these were significant, post-hoc comparisons were undertaken using a Sidak adjustment for multiple comparisons at a given time point.

Overall significance of treatment effect on food consumption was also calculated using the data aggregated across all days, using a GLM with gaussian distribution. Cage was initially introduced as a covariate, but was found not to significantly predict consumption difference, and so was excluded. The data distribution was determined using the descdist function from the fitdistrplus package (Delignette-Muller and Dutang 2015) in R. Significance was determined using ANOVA with F-test, followed by Sidak post-hoc adjustments as described above

Results

Food Preferences switch from protein-rich food to carbohydrate rich food

Over the course of two summers, a total of 70 ethanol and 62 QMP cages were investigated, and their food intake (either fondant or pollen) was recorded daily.

As seen in Fig. 1a, irrespective of treatment, newly eclosed workers initially prefer protein-rich pollen cake, before a switch of preference to the carbohydrate rich sugar fondant occurring during the fifth day after eclosure. By day 10, the consumption of pollen cake falls to almost zero. This is consistent with previously published research showing the initial importance of protein-rich food in the days immediately after eclosure (Pernal and Currie 2000; Pirk, Boodhoo et al. 2010).

QMP-exposed worker bees consume more fondant than those exposed to solvent control

QMP has no effect on the consumption of protein (in the form of pollen cake) (F=0.0908, df=1316, P=0.7673).

However, when exposed to QMP at a concentration of 0.1 Qe per day, honeybee worker consumption of carbohydrates (in the form of sugar fondant) exceeds that of bees exposed to solvent controls (F=28.745, df=1315, P<0.001). This difference is statistically significant from days two to nine after eclosion, with the greatest effect size occurring from days four to six (Table I and Fig. 1a, b)

When observing total food consumed per cage, the statistically significant difference in overall fondant consumption between treatments, but not pollen consumption, can be clearly seen (Fig. 1c. Fondant: F=28.745, df=1315, P<0.001; Pollen: F=0.0908, df=1315, P=0.7633).

For each biological replicate, bees from QMP-exposed cages and solvent-only control cages were dissected on day 10 to assess ovarian activity. In all cases, QMP exposure resulted in statistically significant repression of ovary activity compared with the ethanol-only solvent (Supplementary Fig. 1).

Interestingly, workers provided with fondant alone did not activate their ovaries, regardless of QMP exposure (Supplementary Fig. 2). Bees fed only pollen exhibited significantly lower survival (Supplementary Fig. 3), whereas those fed either pollen plus fondant or fondant alone showed significantly higher survival (Supplementary Fig. 3).

Discussion

This study aimed to investigate whether QMP alters the nutritional preference of newly eclosed worker honeybees. Building on previous work in *D. melanogaster*, where QMP induces a starvation-like response (Lovegrove et al., 2023), we hypothesised that QMP might similarly influence feeding behaviour in honeybees. Our findings support this hypothesis, but only for carbohydrate consumption. QMP exposed workers showed a significant and sustained increase in carbohydrate rich (fondant) consumption while protein (pollen-cake) intake remained unaffected (Fig. 1).

That QMP exposure results in an increase in sugar consumption is perhaps counterintuitive. Given that QMP-exposed bees are less reproductively active (and therefore devoting fewer metabolic resources to egg production), the energy requirements within these bees should theoretically be lower, all else being equal (Wigglesworth 1960). Similarly, we would expect to see those bees which are more reproductively active to have higher protein needs, due to the role of metabolic protein in vitellogenin synthesis (Izumi, Yano et al. 1994; Wu, Yang et al. 2021). The lack of difference in pollen consumption (the only protein source for honeybees in general, and particularly in the cages, though there are trace amounts of amino acids in the honey used in the sugar fondant), is therefore surprising, and indicates that the effect of QMP on food consumption is likely unrelated to reproduction directly.

The increase in consumption of fondant under QMP exposure suggests that QMP may be triggering a shift in perceived nutritional state or metabolic demand, consistent with a starvation-like response, despite the bees being in a controlled nutrient abundant environment, as was seen for *D. melanogaster*. Interestingly, nutritional state modulates workers' responsiveness to QMP (Walton, Dolezal et al. 2018), further suggesting that diet and pheromonal signalling interact closely in the honeybee, potentially acting through shared or overlapping physiological pathways.

The increase in consumption of carbohydrates might also reflect a QMP-induced increase in metabolic activity, possibly indicating a change in physical activity which necessitates the increase in metabolism and therefore sugar consumption. However, the presence of a queen has been shown to have a calming effect on workers (Grodzicki, Piechowicz and Caputa 2020), and it has been shown that QMP reduces activity in workers (Beggs, Glendining et al. 2007), although this latter study used much higher QMP exposures than in this study, and better techniques for quantifying physical activity have since been developed (Chiara and Kim 2023). It is worth applying these techniques to bees reared under the conditions presented here, in order to confirm the effect that QMP has on activity.

Notably, QMP is known to inhibit "social-aging", whereby the innate age-based polyethism of honeybees is delayed, resulting in less foraging activity (Pankiw, Huang et al. 1998). This would imply that QMP should decrease sugar consumption, due to the lower anticipated metabolic requirements associated with non-foraging activities as foraging requires higher energy expenditure to sustain flight (Casey 1981)). However confirming this would require additional data measuring physiological proxies for social aging must be collected (e.g. changes in haemolymph vitellogenin titres (Nakaoka, Takeuchi and Kubo 2008) or fat body lipid and protein levels (Bertholf 1925)).

It is also important to note that food intake patterns in caged workers are likely to differ from those in colony conditions, where foragers, for example, require more nutrients to sustain flight. In our caged setup, where brood and flight activity are absent, the increased carbohydrate consumption observed in QMP-exposed workers may reflect a shift toward a more nurse-like metabolic state. The increase in fondant consumption we observed is consistent with the increased lipid storage in the fat body that comes about as a result of nursing behaviours in honeybees (for royal jelly production in the hypopharyngeal glands) (Crailsheim, Schneider et al. 1992; Toth and Robinson 2005). This pattern is similar to that reported by Corby-Harris (2022) where exposure of young bees to QMP resulted in altered fat body composition (increased lipid and decreased protein) (Corby-Harris, Snyder et al. 2022), supporting the idea that QMP influences nutritional metabolism as well as reproductive state.

It is possible that QMP is able to bring about repression of worker reproduction and increased sugar consumption via the role of adult diapause mechanisms in honeybees. The role of diapause in QMP-mediated repression of reproduction in *D. melanogaster* has been postulated (Knapp, Norman et al. 2022), whereby QMP has evolved to coopt ancestral diapause mechanisms to bring about reproductive repression in that species. A similar diapause-like dormancy mechanism exists in the honeybee as the winter phenotype, whereby during winter, reproduction is switched off in queens, but also in workers (Seeley and Visscher 1985; Knoll, Pinna et al. 2020), combined with a host of other metabolic, genetic, and behavioural changes (Phillips and Demuth 1914;

Rockstein 1950; Owens 1971; Bresnahan, Döke et al. 2022). Interestingly, recent work has shown that worker exposure to QMP components varies seasonally but does not affect retinue size (Carroll, Brown et al. 2023). This suggests that although QMP levels vary across the year, its behavioural effects may remain stable. The influence of QMP on winter workers is an interesting area for future studies. Notably this adult reproductive diapause is distinct from the larval diapause brought about by nutrient stress that occurs in many insects (Hahn and Denlinger 2011). It is possible that, as is suggested in the fruit fly, in *A. mellifera* QMP acts to induce elements of this adult diapause to prevent worker ovary activation.

It is possible that QMP's ability to bring about repression of reproduction in adult worker honeybees under summer conditions, is a co-option of the seasonal mechanisms which bring about the repression of worker reproduction under winter conditions, and that a secondary effect of this coopted mechanism is the winter phenotype's propensity to consume food as necessary for maintaining temperature homeostasis in the winter cluster (Owens 1971). The increased consumption in carbohydrates (but not pollen) would therefore be a side-effect of QMP-mediated reproductive constraint.

Regardless of the reason for increased consumption of carbohydrates, the fact that the overconsumption of food under QMP-exposed conditions is similar between *A. mellifera* and *D. melanogaster* potentially demonstrates that they are bringing about reproductive constraint via the same mechanism.

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Author Declarations

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Conflicts of Interest /Competing Interests

The authors have no relevant financial or non-financial interests to disclose. The authors declare that they have no potential conflict of interest in relation to the study in this paper

Ethics approval/declarations

Not applicable.

Consent to participate

Not applicable

Availability of data and material/ Data availability

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

Authors' Contribution

AB and EJD conceived and designed the experiments. AB, ZLH, EHP, and KY carried out experiments and collected data. AB analysed the data. AB and EJD wrote the paper and revised it. All authors read and approved final manuscript.

References

Beggs K.T., K.A. Glendining, N.M. Marechal, V. Vergoz, I. Nakamura, et al. (2007) Queen pheromone modulates brain dopamine function in worker honey bees. Proceedings of the National Academy of Sciences **104**(7): 2460-2464.

Bertholf L.M. (1925) The Moults of the Honeybee. Journal of Economic Entomology **18**(2): 380-384.

Bresnahan S.T., M.A. Döke, T. Giray, C.M. Grozinger. (2022) Tissue-specific transcriptional patterns underlie seasonal phenotypes in honey bees (*Apis mellifera*). Molecular Ecology **31**(1): 174-184.

Camiletti A.L., A. Percival-Smith, G.J. Thompson. (2013) Honey bee queen mandibular pheromone inhibits ovary development and fecundity in a fruit fly. Entomologia Experimentalis et Applicata **147**(3): 262-268.

Carroll M.J., N.J. Brown, Z. Ruetz, V.A. Ricigliano, K.E. Anderson. (2023) Honey bee retinue workers respond similarly to queens despite seasonal differences in Queen Mandibular Pheromone (QMP) signaling. PLoS One **18**(9): e0291710.

Casey T.M. (1981) Insect Flight Energetics, in: Herreid C.F. and Fourtner C.R. (Eds.), Locomotion and Energetics in Arthropods, Springer US, Boston, MA, pp. 419-452.

Chiara V., S.-Y. Kim. (2023) AnimalTA: A highly flexible and easy-to-use program for tracking and analysing animal movement in different environments. Methods in Ecology and Evolution **14**(7): 1699-1707.

Corby-Harris V., L. Snyder, C. Meador, E. Watkins-DeJong, B.T. Obernesser, et al. (2022) Diet and pheromones interact to shape honey bee (*Apis mellifera*) worker physiology. Journal of Insect Physiology **143**: 104442.

Crailsheim K. (1990) The protein balance of the honey bee worker. Apidologie **21**(5): 417-429.

Crailsheim K., L.H.W. Schneider, N. Hrassnigg, G. Bühlmann, U. Brosch, et al. (1992) Pollen consumption and utilization in worker honeybees (*Apis mellifera carnica*): Dependence on individual age and function. Journal of Insect Physiology **38**(6): 409-419.

Delignette-Muller M.L., C. Dutang. (2015) fitdistrplus: An R Package for Fitting Distributions. Journal of Statistical Software **64**(4): 1 - 34.

Duncan E.J., O. Hyink, P.K. Dearden. (2016) Notch signalling mediates reproductive constraint in the adult worker honeybee. Nature Communications **7**(1): 12427.

Duncan E.J., M.P. Leask, P.K. Dearden. (2020) Genome architecture facilitates phenotypic plasticity in the honeybee (*Apis mellifera*). Molecular Biology and Evolution DOI: 10.1093/molbev/msaa057.

Fischer P., C.M. Grozinger. (2008) Pheromonal regulation of starvation resistance in honey bee workers (*Apis mellifera*). Naturwissenschaften **95**(8): 723-729.

Grodzicki P., B. Piechowicz, M. Caputa. (2020) The Effect of the Queen's Presence on Thermal Behavior and Locomotor Activity of Small Groups of Worker Honey Bees. Insects **11**(8): 464.

Hahn D.A., D.L. Denlinger. (2011) Energetics of insect diapause. Annual Review of Entomology **56**: 103-121.

He X.J., X.C. Zhang, W.J. Jiang, A.B. Barron, J.H. Zhang, Z.J. Zeng. (2016) Starving honey bee (*Apis mellifera*) larvae signal pheromonally to worker bees. Scientific Reports **6**: 22359.

Hoover S.E.R., C.I. Keeling, M.L. Winston, K.N. Slessor. (2003) The effect of queen pheromones on worker honey bee ovary development. Naturwissenschaften **90**(10): 477-480.

Izumi S., K. Yano, Y. Yamamoto, S.Y. Takahashi. (1994) Yolk proteins from insect eggs: Structure, biosynthesis and programmed degradation during embryogenesis. Journal of Insect Physiology **40**(9): 735-746.

Knapp R.A., V.C. Norman, J.L. Rouse, E.J. Duncan. (2022) Environmentally responsive reproduction: neuroendocrine signalling and the evolution of eusociality. Current Opinion in Insect Science **53**: 100951.

Knoll S., W. Pinna, A. Varcasia, A. Scala, M.G. Cappai. (2020) The honey bee (*Apis mellifera* L., 1758) and the seasonal adaptation of productions. Highlights on summer to winter transition and back to summer metabolic activity. A review. Livestock Science **235**: 104011.

Lovegrove M.R., P.K. Dearden, E.J. Duncan. (2023) Honeybee queen mandibular pheromone induces a starvation response in *Drosophila melanogaster*. Insect Biochemistry and Molecular Biology **154**: 103908.

Maisonnasse A., J.-C. Lenoir, D. Beslay, D. Crauser, Y. Le Conte. (2010) E-β-Ocimene, a Volatile Brood Pheromone Involved in Social Regulation in the Honey Bee Colony (*Apis mellifera*). PLOS ONE **5**(10): e13531.

Misof B., S. Liu, K. Meusemann, R.S. Peters, A. Donath, et al. (2014) Phylogenomics resolves the timing and pattern of insect evolution. Science **346**(6210): 763-767.

Mohammedi A., A. Paris, D. Crauser, Y. Le Conte. (1998) Effect of aliphatic esters on ovary development of queenless bees (*Apis mellifera* L.). Naturwissenschaften **85**(9): 455-458.

Nakaoka T., H. Takeuchi, T. Kubo. (2008) Laying workers in queenless honeybee (*Apis mellifera* L.) colonies have physiological states similar to that of nurse bees but opposite that of foragers. Journal of Insect Physiology **54**(5): 806-812.

Oi C.A., J.S. van Zweden, R.C. Oliveira, A. Van Oystaeyen, F.S. Nascimento, T. Wenseleers. (2015) The origin and evolution of social insect queen pheromones: Novel hypotheses and outstanding problems. BioEssays **37**(7): 808-821.

Owens C.D. (1971) The thermology of wintering honey bee colonies. US Agricultural Research Service.

Pankiw T., Z.Y. Huang, M.L. Winston, G.E. Robinson. (1998) Queen mandibular gland pheromone influences worker honey bee (*Apis mellifera* L.) foraging ontogeny and juvenile hormone titers. Journal of Insect Physiology **44**(7): 685-692.

Pankiw T., M.L. Winston, E. Plettner, K.N. Slessor, J.S. Pettis, O.R. Taylor. (1996) Mandibular gland components of european and africanized honey bee queens (*Apis mellifera* L.). Journal of Chemical Ecology **22**(4): 605-615.

Pankiw T., M.L. Winston, K.N. Slessor. (1994) Variation in worker response to honey bee (*Apis mellifera* L.) queen mandibular pheromone (Hymenoptera: Apidae). Journal of Insect Behavior **7**(1): 1-15.

Pernal S.F., R.W. Currie. (2000) Pollen quality of fresh and 1-year-old single pollen diets for worker honey bees (*Apis mellifera* L.). Apidologie **31**(3): 387-409.

Pettis J., H. Higo, T. Pankiw, M. Winston. (1997) Queen rearing suppression in the honey bee-evidence for a fecundity signal. Insectes Sociaux **44**(4): 311-322.

Phillips E.F., G.S. Demuth. (1914) The temperature of the honeybee cluster in winter. US Department of Agriculture.

Pirk C.W.W., C. Boodhoo, H. Human, S.W. Nicolson. (2010) The importance of protein type and protein to carbohydrate ratio for survival and ovarian activation of caged honeybees (*Apis mellifera scutellata*). Apidologie **41**(1): 62-72.

Plettner E., G. Otis, P. Wimalaratne, M. Winston, K. Slessor, et al. (1997) Species-and caste-determined mandibular gland signals in honeybees (*Apis*). Journal of Chemical Ecology **23**: 363-377.

Plettner E., K.N. Slessor, M.L. Winston, J.E. Oliver. (1996) Caste-selective pheromone biosynthesis in honeybees. Science **271**(5257): 1851-1853.

Princen S.A., R.C. Oliveira, U.R. Ernst, J.G. Millar, J.S. van Zweden, T. Wenseleers. (2019) Honeybees possess a structurally diverse and functionally redundant set of queen pheromones. Proceedings of the Royal Society B: Biological Sciences **286**(1905): 20190517.

Princen S.A., A. Van Oystaeyen, C. Petit, J.S. van Zweden, T. Wenseleers. (2020) Cross-activity of honeybee queen mandibular pheromone in bumblebees provides evidence for sensory exploitation. Behavioral Ecology **31**(2): 303-310.

R Core Team. (2021) R: A language and environment for statistical computing. , R Foundation for Statistical Computing, Vienna, Austria.

Robinson G.E. (1992) Regulation of division of labor in insect societies. Annual Review of Entomology **37**: 637-665.

Rockstein M. (1950) Longevity in the Adult Worker Honeybee. Annals of the Entomological Society of America **43**(1): 152-154.

Seeley T.D., P.K. Visscher. (1985) Survival of honeybees in cold climates: the critical timing of colony growth and reproduction. Ecological Entomology **10**(1): 81-88.

Slessor K.N., L.-A. Kaminski, G. King, M.L. Winston. (1990) Semiochemicals of the honeybee queen mandibular glands. Journal of Chemical Ecology **16**: 851-860.

Slessor K.N., L.-A. Kaminski, G.G.S. King, J.H. Borden, M.L. Winston. (1988) Semiochemical basis of the retinue response to queen honey bees. Nature **332**(6162): 354-356.

Toth A.L., G.E. Robinson. (2005) Worker nutrition and division of labour in honeybees. Animal Behaviour **69**(2): 427-435.

Walton A., A.G. Dolezal, M.A. Bakken, A.L. Toth. (2018) Hungry for the queen: Honeybee nutritional environment affects worker pheromone response in a life stage-dependent manner. Functional Ecology **32**(12): 2699-2706.

Wickham H. (2016) ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Wigglesworth V.B. (1960) Nutrition and reproduction in insects. Proceedings of the Nutrition Society **19**(1): 18-23.

Williams G.R., A. Cédric, C. Cecilia, C. Támas, D. Vincent, et al. (2013) Standard methods for maintaining adult *Apis mellifera* in cages under *in vitro* laboratory conditions. Journal of Apicultural Research **52**(1): 1-36.

Wilson E.O. (1971) The insect societies.

Winston M.L., K.N. Slessor, L.G. Willis, K. Naumann, H.A. Higo, et al. (1989) The influence of queen mandibular pheromones on worker attraction to swarm clusters and inhibition of queen rearing in the honey bee (*Apis mellifera* L.). Insectes Sociaux **36**(1): 15-27.

Wossler T., C., R. Crewe, M. (1999) Honeybee queen tergal gland secretion affects ovarian development in caged workers. Apidologie **30**(4): 311-320.

Wu Z., L. Yang, Q. He, S. Zhou. (2021) Regulatory Mechanisms of Vitellogenesis in Insects. Frontiers in Cell and Developmental Biology **8**.

Table I - Results of the statistical tests investigating the differences in fondant consumption by worker honeybees exposed to QMP and solvent control

Day	Residual degrees of freedom	Residual Deviance	Adjusted P-value	Cohen's D effect size
1	112	2.989	0.2788	0.20
2	112	46.115	0.0028	0.24
3	112	35.011	0.0102	0.51
4	112	11.681	<0.001	1.02
5	112	9.651	<0.001	0.92
6	112	9.663	<0.001	1.03
7	112	9.804	0.001	0.79
8	112	10.997	0.0013	0.62
9	112	9.585	0.0086	0.50
10	112	9.778	0.1959	0.26

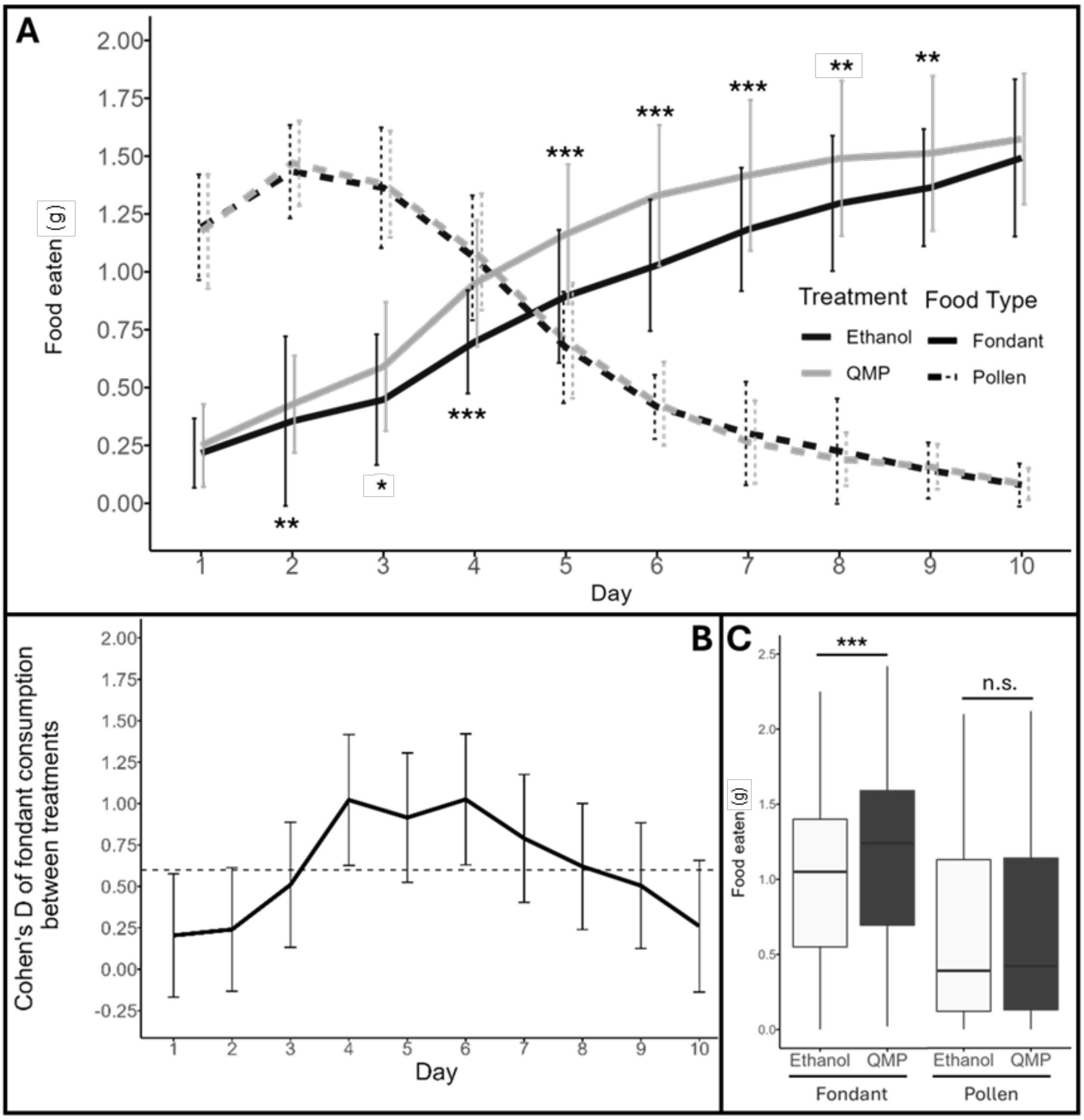

Figure 1 – ***Different food types consumed by queenless worker*** **A. mellifera** ***honeybees reared*** **in cavea** ***in the presence and absence of Queen Mandibular Pheromone .****The consumption of two food sources: sugar fondant (solid lines), and pollen cake (dashed lines); were measured each day for 10 days for each of two treatments: 0.1Qe of QMP per day (grey lines), or ethanol solvent control (black lines). In* ***A****, the mean value is plotted for both treatments and food types with error bars representing one standard deviation, significance is given as * = P<0.05, ** = P<0.01, *** = P<0.001, calculated via glm with post hoc Sidaak adjustment.. In* ***B****, the Cohen's D of effect size between treatments of fondant consumption from panel* ***A*** *is shown, with the dashed line showing a value of 0.6, the threshold between a medium and large effect size. In* ***C*** *the cumulative food consumption is shown for each of treatment and food type, significance is given as n.s. = P>0.05; *** = P<0.001; calculated via glm with post hoc Sidaak adjustment..*

Title:

Age and QMP exposure affect the nutritional preferences of *Apis mellifera* worker honeybees

Supplementary Data

Journal:

Apidologie

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QMP-exposure produces ovary repression in all cages

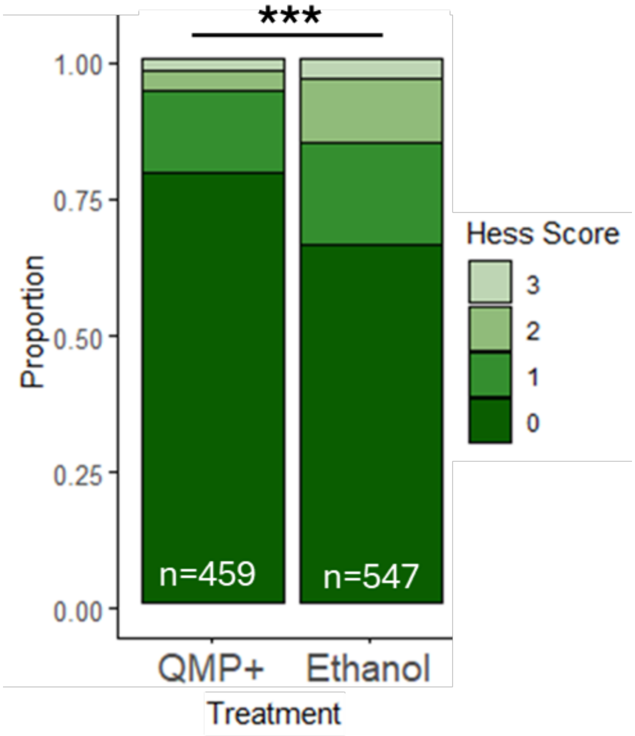

*Figure 1 – Honeybee workers exposed to QMP from eclosure, show repressed ovaries when compared with bees not exposed to QMP, as measured by a modified Hess score (3 = fully developed ovum present; 2 = egg yolk visible; 1 = cell differentiation between ovum and nurse cells visible; 0 = no cell differentiation visible). The x-axis shows treatment (QMP at 0.1 Qe per day provided as liquid on microscope slide, or ethanol solvent control), while the y-axis shows proportion of workers at each Hess score, shown in the legend. Significance was calculated using CLMM with post-hoc tukey-adjusted test; *** = P<0.001.*

In Figure 1, an example of a given experiment, in which six cages of each treatment are analysed for ovary activity via a modified Hess score. Similar analyses were undertaken for all experiments from which the 149 cages were taken to investigate food choice and consumption, in which all experiments indicated a significant difference in ovary activity between QMP and ethanol-only exposed worker honeybees. The overall activation was different between experiments, due to differences in origin of worker honeybees and different times of year, and so each assay was always carried out with both QMP and ethanol-only controls in order to demonstrate the efficacy of the QMP.

Honeybee workers fed only fondant do not activate their ovaries

As can be seen in Figure 2, if fed an *ad libitum* pollen and sugar fondant diet (FandP), workers activate their ovaries (though much less so if also exposed to QMP). If only fed fondant (i.e. without pollen, or any other protein source), workers do not activate their ovaries regardless of pheromone exposure. Fondant or FandP diet has no effect on mortality in the 10-day period of this assay, however bees only fed pollen, with no fondant do not survive past day 9 (Figure 3)

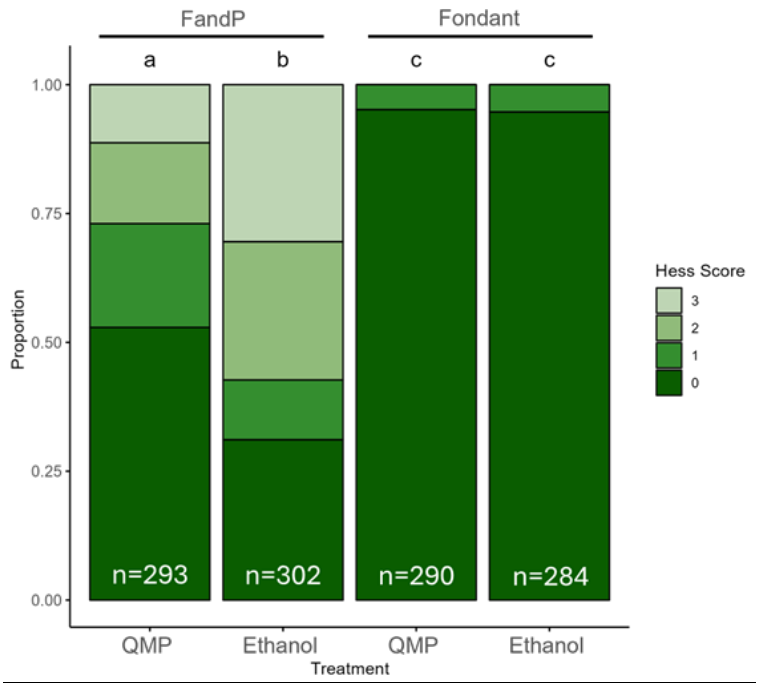

Figure 2 - Showing a proportional stacked bar chart of ovary activity of A. mellifera workers when fed different quality foods. *Ovary activity was measured via modified Hess score (0 = inactive ovaries, 1 = cell differentiation present, 2 = yolk deposition present, 3 = fully developed ovum present). The y-axis shows proportion of ovaries of a given Hess score, while the x axis shows treatment of either 0.1Qe QMP per day or ethanol solvent control, or food types of either FandP diet, or fondant only. Significance given as letters (P<0.05) calculated via CLMM and post-hoc tukey pairwise test. Under the FandP diet, QMP is able to repress the activity seen in the ethanol control, but under fondant diet, no activation of ovaries occurs.*

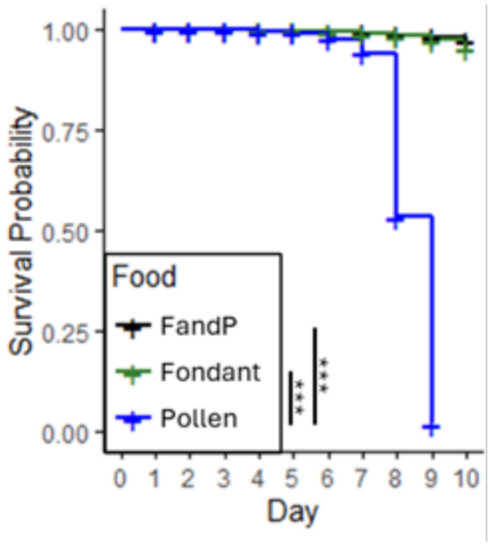

Figure 3 – Showing a Kaplan-Meier survival curve of honeybee workers which have been fed different food sources. *The y-axis shows survival probability and the x-axis day of experiment, food is given as colour, where "fondant" is fondant only, "pollen" is pollen only and "FandP" is a choice of either. Bees fed only pollen could not survive to the end of the experiment, but there is no difference in survival between the other two food types.*