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Drosophila melanogaster and worker honeybees (*Apis mellifera*) do not require olfaction to be susceptible to honeybee queen mandibular pheromone

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Abstract

Eusociality is characterised by the reproductive division of labour; a dominant female (queen) or females are responsible for the majority of reproduction, and subordinate females are reproductively constrained. Reproductive constraint can be due to behavioural aggression and/or chemical cues, so-called queen pheromones, produced by the dominant females. In the honeybee, *Apis mellifera*, this repressive queen pheromone is queen mandibular pheromone (QMP). The mechanism by which honeybee workers are susceptible to QMP is not yet completely understood, however it is thought to be through olfaction via the antennae and/or gustation via trophallaxis.

We have investigated whether olfaction is key to sensing of QMP, using both *Drosophila melanogaster*- a tractable non-eusocial insect which is also reproductively repressed by QMP- and the target species, *A. mellifera* worker honeybees. *D. melanogaster* are still capable of sensing and responding to QMP without their antenna and maxillary palps, and therefore without olfactory receptors. When worker honeybees were exposed to QMP but unable to physically interact with it, therefore required to use olfaction, they were similarly not reproductively repressed. Combined, these findings support either a non-olfactory based mechanism for the repression of reproduction via QMP, or redundancy via non-olfactory mechanisms in both *D. melanogaster* and *A. mellifera*. This study furthers our understanding of how species are susceptible to QMP, and provides insight into the mechanisms governing QMP responsiveness in these diverse species.

Introduction

Eusociality is a successful life-history strategy that relies on the reproductive division of labour- whereby a single, or small number, of dominant females reproduce. The other subordinate female members of the group are reproductively repressed and perform the other tasks in the colony, including foraging and brood-care (Oster and Wilson, 1978). In insects, reproductive dominance is established by behavioural aggression and/or chemical cues (Le Conte and Hefetz, 2008; Padilla et al., 2016). Chemical cues are often in the form of queen pheromones (Matsuura et al., 2010; Van Oystaeyen et al., 2014; Princen et al., 2019; Vargo and Laurel, 1994; Winston and Slessor, 1992). Perhaps the most well-studied queen pheromone is Queen Mandibular Pheromone (QMP), produced by queen honeybees (*Apis mellifera*) (Keeling et al., 2003; Pankiw et al., 1996; Slessor et al., 1988).

QMP can be viewed as both a releaser, and a primer pheromone. Releaser pheromones have a role in inducing rapid behavioural changes upon reception. For instance, the role of QMP in the attraction of drones during mating, inducing caring behaviour and lowering aggressiveness in workers, alarm signalling, orientation and trail marking is characteristic of a releaser pheromone (Kaminski et al., 1990; Slessor et al., 1988, 1990; Winston and Slessor, 1998). In contrast, Primer pheromones induce physiological changes which then result in a downstream behavioural response. For QMP, examples od acting as a primer pheromone include aspects of colony organisation, such as caste structure, and division of labour. Here, we investigate QMPs Primer pheromone role in it's action to supress worker reproduction (Kaatz et al., 1992; Pankiw et al., 1998; Pettis et al., 1995; Winston and Slessor, 1992, 1998).

In general, Queen pheromones activity on the ovary have a mechanism of action that consists of three components; the sensing of the pheromone, the transduction of that signal to the ovary, and lastly the ovarian repression itself. Initial sensing of queen pheromones is hypothesised to be via odorant receptors (ORs) in insect antennae (Brockmann et al., 1998; Pask et al., 2017; Wanner et al., 2007). In honeybees, workers feed, groom and antennate the queen and thus become exposed to QMP (Allan, 1955). QMP is thought to be propagated through the hive via antennation and grooming between workers, and also potentially via trophallaxis (Naumann, 1991) implicating both olfactory machinery and gustatory receptors in mediating the effects of QMP.

QMP is a complex blend of semiochemicals with five major non-volatile components (9-oxo-2-decenoic acid (9ODA), cis- and trans-9-hydroxydec-2-enoic acid (9HDA), methyl p-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA) (Slessor et al., 1988). QMP is derived and distinct from the other queen pheromones found within the social insects (Van Oystaeyen et al., 2014). It is capable of repressing reproduction in a broad range of non-target arthropods, from eusocial insects such as ants (Carlisle and Butler, 1956) and termites (Hrdy et al., 1960) to non-eusocial ones such as the house fly (Nayar, 1963), and *Drosophila melanogaster* (Camiletti et al., 2013; Lovegrove et al., 2019; Sannasi, 1969). Perhaps most surprising is the repression of reproduction in response to QMP in a non-insect species; for example a prawn (Carlisle and Butler, 1956)- which means QMP is capable of repressing reproduction across species up to ~530 million years diverged (Misof et al., 2014). Repression of a broad range of non-target insects is not a feature of ancestral hymenopteran queen pheromones. Instead, it appears to be a derived feature unique to QMP. When *D. melanogaster* are exposed to queen pheromones from other hymenopterans, including the bumblebee *Bombus terrestris*, there is no evidence of reproductive repression (Lovegrove et al., 2019). Hence QMP appears to be able to act upon conserved mechanisms by which insects regulate their reproduction (Lovegrove et al., 2019). Understanding how QMP is acting in non-target species, such as *D. melanogaster* may therefore provide insight into conserved mechanisms of action in *A. mellifera*, as well as aid our understanding of the evolution of queen pheromones and eusociality.

D. melanogaster exposed to QMP have a significant reduction in the number of mature oocytes in their ovaries (Camiletti et al., 2013; Lovegrove et al., 2019). In *D. melanogaster* this maximal repression of reproduction by QMP requires the flies to have direct contact with the QMP. When direct contact is inhibited (by separating flies from QMP by a mesh barrier), flies exhibit an intermediate level of repression (Camiletti et al., 2016). This implies that physically touching QMP may be required for QMP sensing in this species. However, this was contradicted by disruption of *Orco* (the co-receptor required for all ORs to act (Larsson et al., 2004)) which completely abolished the response of *D. melanogaster* to QMP (Camiletti et al., 2016), implying that ORs are the primary mechanism by which *D. melanogaster* detects QMP. However, in this experiment, *Orco* mutants also had fewer mature oocytes than wild-type controls which had been exposed to 20 Qe of QMP (Camiletti et al., 2016). This significant decrease in reproductive capacity in *Orco* mutant is a potentially confounding factor, as it limits the ability to detect additional repression of oogenesis by QMP.

In this study, we use physical manipulation to further investigate whether olfaction is key to sensing of QMP in both the target species (*A. mellifera*) and also in the non-target species *D. melanogaster*.

Methods

QMP dilutions

QMP is measured in Queen equivalents (Qe)- where one Qe is the amount a mated queen will produce in a 24 hour period (Pankiw et al., 1996). QMP is made up of five major components (Slessor et al., 1988). A single Qe for a European mated queen contains; 200 µg 9-keto-(E)-2-decanoic acid (ODA), 80 µg 9-hydroxy-(E)-2-decanoic acid (9-HDA), 20 µg methyl *p*-hydroxybenzoate (HOB) and 2 µg 4-hyroxy-3-methoxyphenylethanol (HVA) (Pankiw et al., 1996). QMP (Intko Supply Ltd, Canada) was dissolved in absolute ethanol to a concentration of 26 Qe/20 µl, and stored at -20 °C until use.

D. melanogaster stocks and maintenance

All *D. melanogaster* used for this study were Oregon-R modENCODE line (Stock #25211) from the Bloomington *Drosophila* stock centre. Stocks were maintained at 25 °C on a 12h:12h light/dark cycle. Flies were reared on a yeast/sugar medium; 3 L dH_2O, 200 g organic cornmeal, 50 g brewer's yeast, 140 g sugar, 20 ml propionic acid and 15 ml 10 % methyl *p*-hydroxybenzoate in absolute ethanol.

D. melanogaster virgin collection

All *D. melanogaster* used for this study were virgin females. These were anaesthetised with CO_2 and observed under a Leica L2 dissection stereomicroscope. Phenotypically virgin females were isolated based on the characteristics of enlarged abdomens, the presence of the meconium and pale colouration. Virgin collection was carried out within one hour of emergence, and these individuals were isolated with other virgin females, and allowed to mature at room temperature for 24 h.

Antennae and/or maxillary palp removal

To test the dependence of QMP sensing on olfaction either the antennae, maxillary palps or both organs were surgically removed (Fig. 1). These are the major sites of OR activity in this

species (Joseph and Carlson, 2015; Su et al., 2009; Vosshall and Stocker, 2007). Virgin female *D. melanogaster* (24 hours post eclosion) were anesthetised with CO_2. Under a Leica L2 dissection microscope, the antennae were removed with fine dissection tweezers. This was carried out by pinching at the most proximal point of the antennae (Fig. 1), which sliced off the antennae at the joint with the head. This was repeated on the second antennae. For individuals which were to have their maxillary palps removed, the same process was carried out on the maxillary palps (Fig. 1). Individuals which had both their maxillary palps and antennae removed underwent both of these procedures. Any individuals which were otherwise damaged, or lost haemolymph during this process were discarded.

Queen pheromone exposure

Vials for pheromone exposure were created by modifying 50 ml centrifuge tubes, as described in Lovegrove et al. (2019). Briefly, tubes were heated, and the collection end removed. Into the lid end of the tube, two layers of Whatman number 1 filter paper were added and screwed into place. A cotton ball was used to plug the open collection end of the tube. Liquid diet (500 µl) was added to the filter paper. This consisted of 4.75 ml dH_2O, 5% absolute ethanol, 0.15 g sugar and 0.1 g brewer's yeast (Camiletti et al., 2013). On top of this liquid diet 20 µl of either 26 Qe QMP solution was added, or 20 µl of an absolute ethanol solvent control- allowing the *D. melanogaster* to have direct contact with the treatment. The 24 h old virgin female *D. melanogaster* that had no sensory organs ablated, antennae removed, maxillary palps removed, or antennae and maxillary palps removed were added to the vial which was laying on its side. These were housed in separate vials based on exposure type and surgical status. This allowed time for recovery from CO_2 narcosis before being incubated upright at 25 °C for 48 h. Each treatment consisted of seven replicates, and each replicate included 10 individuals (n = 70).

D. melanogaster ovary dissection and fixation

Ovary dissections were carried out using fine dissection tweezers and a Leica L2 dissection microscope. After *D. melanogaster* were anesthetised with CO_2, their ovaries were dissected into a petri dish containing ice-cold Phosphate buffered saline (PBS). Any ovaries which were damaged or lost oocytes during dissection were discarded. Ovaries were stored in 400 µl of PBS on ice until all dissections were complete (<30 min).

The PBS in the microcentrifuge tube containing the dissected ovaries was removed until only 50 µl remained. To this tube, 900 µl fresh PBS and 4% formaldehyde were added. Ovaries were fixed by rocking at room temperature for 10 min. The fixative was removed, and the ovaries washed four times with PTx (PB with 0.1 % Triton X100). Fixed ovaries were stored in the dark at 4 °C in 70 % ultrapure glycerol. They were stored in this way for at least 24 h prior to bridge-mounting for microscopy. Manual counting was used to determine the number of mature (vitellogenic) oocytes per ovary, using a Leica L2 dissection microscope. This number was used as a measure of fecundity (King, 1970).

A.mellifera worker rearing and QMP exposure

Apis mellifera mellifera were kept according to standard practices in British National hives at the University of Leeds School of Biology Research Apiary. Frames of capped brood were sourced from queen-right hives and incubated overnight at 35 °C to allow workers to emerge. Glass fronted metal cages (Small Life Supplies, UK) for trials were set up by collecting newly emerged workers throughout the day. Each cage contained 100 age-matched workers collected within 24 hours of emergence. Cages had space for a water tube, provided *ad libitum*, as well as a food cap, and pheromone tube. Complete bee food (CBF) was created by grinding 20 g of pollen, 52 g of sugar and 18.8 g of brewer's yeast into a fine powder. To this, 9.2 g of lactalbumin was added (Duncan et al., 2016). The dry components were stored at -20 °C until use. Each cage received 2 g of CBF per day, which was mixed with honey to form a sticky but crumbly paste. This paste was provided in the food cap, held in the slot within the cage.

Each day dead workers were removed and counted, to record survival rates. Food consumption was measured daily. This data, combined with survival rates allowed for the monitoring of food intake per worker per day (Supplementary Figure 1). QMP was provided in a 15 ml centrifuge tube, which had the collection end removed- leaving the cap and the connecting 4 cm of tube. In the cap, two layers of Whatmann filter paper were screwed into place, onto which 1 Qe QMP or 20 µl of ethanol solvent control was pipetted. In the full access trials workers were able to enter the tube and physically contact the filter paper containing QMP. In the no-touch assay, a layer of mesh was inserted and fixed into place in the tube 2 cm from the filter paper, preventing workers from touching the QMP source. Food

and pheromone/solvent control were provided daily and water was provided as required. Workers were incubated at 35 °C for 10 days prior to dissection

Worker ovary dissection, imaging and scoring

After 10 days, workers were chilled at -20°C to anesthetise them, and their ovaries were dissected in PBS under a GXM-XTL stereomicroscope (GT Vision, UK). Ovaries were removed and transferred to a depression microscope slide containing a drop of PBS. Each pair of ovaries was imaged separately under a Leica M165FC microscope, with Q Capture pro 7 software.

The worker honeybee ovary was classified based on their morphology into three categories of ovary activity (a modified Hess scale (Duncan et al., 2016)). Ovaries which were indistinguishable from queen-right workers were classified as 0. When there were signs of cell differentiation and slight thickening of the ovariole, ovaries were classified as 1. After yolk is deposited, and oocytes are clearly defined, they were scored as 2. Once a mature ovum is present, the ovary as classified as a 3. Ovary images were blind scored independently by two experienced scorers.

Statistical analysis

The number of mature oocytes per ovary in *Drosophila* was analysed using R Studio version 3. 5. 2. Assessment of whether the data fit a normal distribution was carried out using a Shapiro-Wilk test, all data showed a non-normal distribution and so Generalised Linear Mixed Models (GLMMs) with a negative binomial distribution were calculated using lme4. Surgical manipulation and pheromone exposure were treated as fixed effects and the slide number as a random factor. The maximal model was simplified using Analysis of Deviance (AOD) to assess the effect of removing terms. Where an effect of treatment was found, pairwise comparisons between treatments were carried out using emmeans using a Tukey post-hoc test, to correct for multiple testing. Effect sizes (Log odds) and 95% confidence intervals were calculated from the GLMMs using R Studio version 3. 5. 2. (Supplementary Fig. 2). In order to determine whether the levels of ovary activation differed based on whether honeybee workers were allowed full or restricted access to QMP, or no QMP, a Fisher's exact test with a Bonferroni correction for multiple testing was used. This was carried out using R Studio (version 3.5.2). A log rank test was used to determine if there was any difference in survival between honeybee treatments and a Kruskal-wallis test was used to

determine if treatments resulted in differences in food intake (following a Shapiro-Wilk test to assess whether the data fit a normal distribution).

Results

Antennae are not required for D. melanogaster to be susceptible to QMP

To determine whether antennae are required for *D. melanogaster* to be susceptible to QMP, antennae were removed, and virgin females were exposed to 26 Qe QMP for 48 h, or an ethanol solvent control. Intact females were used as a control. The number of mature oocytes present in the ovary was used as a measure of fecundity (Fig. 2) and statistically significant differences were assessed using GLMM with surgical manipulation (either removal of antenna, maxillary palps or both) and pheromone exposure treated as fixed effects and slide as a random factor. Removal of either surgical manipulation, pheromone exposure or the interaction between these effects from the model indicated that data was best described by the full model (AOD $\chi^2 = 14.941$, df = 3 p = 1.868×10^{-3}). We then examined pairwise differences between treatments using a Tukey post-hoc test.

As previously reported (Camiletti et al., 2013; Lovegrove et al., 2019) exposing intact virgin *D. melanogaster* to 26 Qe of QMP resulted in a significant reduction (the number of mature oocytes reduced by 71 %) in the number of mature oocytes after 48 hours of exposure (Fig. 2A, Ethanol mean = 17.47, 26 Qe mean = 5.01, GLMM with Tukey post-hoc test $p < 0.001$).

In the absence of QMP the removal of the antennae did not disrupt the reproductive capacity of these individuals when compared to the intact controls (Intact control ethanol mean = 17.47, Antennae removed ethanol mean = 14.73, GLMM with Tukey post-hoc test $p = 0.849$) (Fig. 2A,B). However, the antennae removed group still showed a reduction in their number of mature oocytes in response to QMP, with the number of mature oocytes being reduced by 63 % (Fig. 2B, Antennae removed ethanol mean = 14.73, Antennae removed 26 Qe mean = 5.50, GLMM with Tukey post-hoc test $p < 0.001$) (Fig. 2B). Whether the *D. melanogaster* were intact, or had their antenna removed, they were repressed by QMP to the same extent (Intact 26 Qe QMP mean = 5.01, Antenna removed 26 Qe QMP mean = 5.50, GLMM with Tukey post-hoc test $p = 0.963$) (Fig. 2A,B). The removal of both antennae therefore did not abolish the ability of *D. melanogaster* to be susceptible to QMP.

Maxillary palps are not required for D. melanogaster to be susceptible to QMP

To test whether the maxillary palps are required for the sensing of QMP in *D. melanogaster*, these sensory organs were ablated before virgin females were exposed to 26 Qe QMP, or an ethanol solvent control for 48h. The number of mature oocytes per ovary was used as a measure of fecundity. The removal of the maxillary palps did not disrupt their reproduction when compared to the intact controls (Intact control ethanol mean = 17.47, Maxillary palps removed ethanol mean = 16.00, GLMM with Tukey post-hoc test $p = 0.999$ (Fig. 2A,2C). After the maxillary palps were removed, repressed reproduction in response to QMP was observed. The number of mature oocytes produced by MPR (maxillary palps removed) individuals exposed to QMP was reduced by 47 % when compared to their controls (Fig. 2C, Maxillary palps removed ethanol mean = 16.00, Maxillary palps removed 26 Qe mean = 8.58, GLMM with Tukey post-hoc test $p < 0.001$). When the intact QMP exposed were compared to the maxillary palps removed QMP exposed, there was significantly more repression in the intact controls (Intact 26 Qe QMP mean = 5.01, Maxillary palps removed 26 Qe QMP mean = 8.58, GLMM with Tukey post-hoc test $p < 0.001$ (Fig. 2 A,C). Regardless, the removal of the maxillary palps did not abolish the ability of *D. melanogaster* to be susceptible to QMP.

Antennae and maxillary palps do not have a compensatory action in the sensing of QMP

To investigate whether the antennae or maxillary palps were compensating for each other in sensing QMP, both of these sensory organs were removed from individuals and the response to QMP was subsequently tested. Ablation of both of these sensory organs did not significantly disrupt the reproductive capacity of these individuals (Intact control ethanol mean = 17.47, Antennae and maxillary palps removed ethanol mean = 12.73, GLMM with Tukey post-hoc test $p = 0.250$) (Fig. 2A,2D). There was also no significant difference in reproductive capacity observed when the controls from all surgery types were compared (Antenna removed control vs antenna and maxillary palps removed control, GLMM with Tukey post-hoc test $p = 0.973$, maxillary palps control vs antenna and maxillary palps removed control, GLMM with Tukey post-hoc test $p = 0.597$). After the removal of both sensory organs was carried out, females were exposed to 26 Qe QMP or an ethanol solvent control for 48 h. Individuals with their antennae and maxillary palps removed still showed a reduction in the number of mature oocytes they produced in response to QMP. Compared to their controls, these individuals had 70 % fewer mature oocytes (Fig. 2D, Antennae and

maxillary palps removed ethanol mean = 12.73, Antennae and maxillary palps removed 26 Qe mean = 3.96, GLMM with Tukey post-hoc test $p < 0.001$). The intact controls exposed to QMP, and the antennae and maxillary palps removed individuals exposed to QMP were repressed to the same extent (GLMM with Tukey post-hoc test $p = 0.535$) (Fig. 2, Supplementary Fig. 2). Therefore *D. melanogaster* are capable of sensing QMP without the presence of their antennae and maxillary palps.

A. mellifera workers are not repressed by QMP without physical access

As our work in *D. melanogaster* had shown that they do not require olfaction to be susceptible to QMP (Fig. 2), we asked whether worker honeybees are susceptible to QMP without olfaction also. Newly emerged workers bees were either exposed to QMP they could physically interact with, or QMP that was separated from the main cage by a mesh barrier ("no touch"). Control cages were provided with a solvent control as well as a mesh barrier. Worker ovaries were scored on a scale from 0 – 3 (Fig. 3), with 0 being inactive, and indistinguishable from queen-right ovaries, 1 showing a slight thickening of the ovariole, and signs of cell differentiation, 2 having clearly defined oocytes with yolk deposited in them, and the most active being 3- having at least one mature oocyte present (Duncan et al., 2016). Those workers which could physically interact with QMP without a barrier showed reduced reproduction compared to those without QMP, having a greater proportion of ovaries scored as showing no signs of activation (0), or only the initial stages of activation (1) (Fishers Exact Test with Bonferroni correction $p = 2.368 \times 10^{-4}$). Those able to physically interact with QMP also showed reduced reproduction compared to workers exposed to QMP, but were unable to touch it (Fisher Exact Test with Bonferroni correction $p = 1.431 \times 10^{-2}$) (Fig. 3). However, when worker bees were prevented from physically interacting with the QMP the reproductive repression was lost and workers activated their ovaries to the same extent as workers which had no QMP present (Fishers Exact Test with Bonferroni correction $p = 0.4209$) (Fig. 3). This was seen as a greater proportion of ovaries scoring at the higher levels of activation- with yolk being deposited (2), or having mature oocytes present (3). This indicates that worker honeybees are not able to be reproductively repressed by QMP with olfaction alone and that direct physical contact between the bees and QMP is required in order for reproduction to be repressed.

Discussion

In this study we have shown that *D. melanogaster* are able to be susceptible to QMP and are reproductively repressed without the presence of their antennae or maxillary palps (Fig. 2). We have also shown that these tissues are not acting in a redundant manner- whereby the loss of one sensory tissue is compensated for by the presence of the other. Removing both of these tissues did not reduce the reproductive repression observed in response to QMP. Without these tissues - thought to be the only sources of ORs (Joseph and Carlson, 2015; Su et al., 2009; Vosshall and Stocker, 2007) - QMP is still able to repress reproduction. This indicates that olfaction and olfactory receptors are not essential for QMP sensing in *D. melanogaster.* This is in contrast to previous findings in *D. melanogaster*, where the loss of function of all ORs abolished the sensing of QMP, and subsequent reproductive repression (Camiletti et al., 2016). In this previous study, it was determined that individuals without a functional *Orco* were not susceptible to the presence of QMP, and were not repressed (Camiletti et al., 2016). However, these individuals were approximately 50 % less reproductively active than their controls with the same genetic background. This indicates that the loss of *Orco* causes impaired reproduction irrespective of QMP exposure. This is potentially a confounding factor in the analysis as it may not have been possible to detect further reduction in reproduction in response to QMP exposure on top of the significantly impaired reproduction caused by the loss of *Orco*. Here we remove the antennae and maxillary palps abolishing all sources of ORs, without reducing the reproductive capacity of these individuals. It should also be noted that in the removal of the entirety of these tissues other non-olfactory receptors were also removed. These included ionotropic receptors and gustatory receptors on the antennae, and GRs on the maxillary palps. That loss of these receptors doesn't alter the reproductive repression induced by QMP implies that these receptors are not required for this response. However, IRs and GRs remain intact in other tissues of the *D. melanogaster*- including the taste pegs, leg sensilla, midgut cells, wing hair margins and oviduct (Joseph and Carlson, 2015). These results, taken together, suggest that olfaction and ORs are not required to mediate reproductive repression in response to QMP exposure in *D. melanogaster*.

This result therefore raises the question of how *D. melanogaster* are susceptible to QMP. A previous study (Camiletti et al., 2016) carried out a no-touch assay, where the flies were physically prevented from interacting with the QMP. Eliminating physical interaction with the QMP induced an intermediate phenotype- the QMP was less effective at repressing reproduction in individuals that could not physically touch the QMP as compared to those with full access. This is indicative of physical interaction being vital for the sensing of QMP

379 in *D. melanogaster* and would suggest a non-olfactory based sensing method. Combined with
380 the data in this study, it appears that there may be multiple redundant mechanisms by which
381 *D. melanogaster* are susceptible to QMP.
382
383 It is likely that ionotropic or gustatory receptors are key QMP sensing mechanisms in *D.*
384 *melanogaster*. Gustatory receptors require physical contact, a trait which is consistent with
385 the findings of this study. Further work into determining the expression and function of
386 ionotropic and gustatory receptors in other tissues of the fly would be useful in answering this
387 question. In particular, these receptors are known to be present on the leg sensilla,
388 mouthparts, wing margins and also in the oviduct of *Drosophila* (Joseph and Carlson, 2015).
389 Disruption of these sensing systems in these tissues will be useful to reveal which tissue(s)
390 and mechanisms(s) are critical for QMP detection and response in *D. melanogaster.*
391
392 This finding in *Drosophila* can be used to inform research into the action of QMP in
393 honeybees. If ORs are not vital for *D. melanogaster* to be susceptible to QMP, then are they
394 required for the honeybees to be? Our data suggests that olfaction is not used in honeybees,
395 as workers were not subjected to reproductive repression without direct contact with QMP
396 (Fig. 3). This is consistent with previous work which has shown that workers without access
397 to QMP have their reproduction repressed within a hive, but only if trophallaxis is possible
398 between workers which can access QMP, and those which cannot (Katzav-Gozansky et al.,
399 2004). This implies that QMP may be passed directly between individuals, and likely may be
400 sensed through gustatory receptors within the mouth parts. It should be noted, however, that
401 previous work has shown that QMP is inducing neuronal responses in the antennal lobe of
402 honeybees (Roussel et al., 2014), implying a clear role for olfaction. In the Roussel et al.,
403 study, the phenotype being investigated was that of behavioural changes, as opposed to the
404 physiological change of ovarian response measured here. It is possible that had we observed
405 behaviour, we may have noticed these changes, in the absence of an ovarian response. The
406 question must also be asked as to whether QMP is volatile enough to be sensed through the
407 mesh barrier. There are two possibilities here- one being that the QMP was not volatile
408 enough for detection without touch, or alternatively that QMP is reaching the individuals, but
409 is simply not being detected by an olfactory mechanism. However, if a distance of 5 cm of
410 separation from the source of QMP is sufficient to prevent detection, it is likely that olfaction
411 is not the only sensing mechanism. There may, however, be a sex specific role of olfaction in

QMP detection in honeybees. For instance, it has been shown that male drone honeybees sense 9-ODA (one of the major components of QMP) using AmOr11 (Wanner et al., 2007).

There is evidence that olfaction is key in sensing social cues in other insects. For instance, disrupting *orco* in various ant species disrupts social structure (Trible et al., 2017; Yan et al., 2017). More work is required to further our understanding of the role of olfaction in social insects, in particular in detection of queen pheromones. Carrying out *orco* mutagenesis in honeybees would provide insight into how crucial olfaction may be for the detection of QMP.

This study has highlighted a non-olfactory receptor mediated-mechanism for QMP detection in *D. melanogaster*, indicating that ionotrophic or gustatory receptors may be key to sensing of QMP in this species. Further, we demonstrate that olfaction is not sufficient for worker honeybees to be susceptible to QMP, thereby indicating a non-olfactory mechanism in both the target and non-target species. This furthers our understanding of how species are susceptible to QMP, and will help to inform future work into how worker honeybees are susceptible to the presence of the queen.

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Author contributions

MRL: Assisted with experimental design, carried out *D. melanogaster* experiments, assisted with honeybee experiments, edited manuscript.

RAK: Assisted with honeybee experiments, blind scored honeybee ovary images, edited manuscript.

EJD: Assisted with experimental design, supervised honeybee experiments, blind-scored honeybee ovary images, carried out statistical analysis, assisted with preparation of figures and edited manuscript.

PKD: Assisted with experimental design, supervised *Drosophila* experiments, assisted with preparation of figures and manuscript.

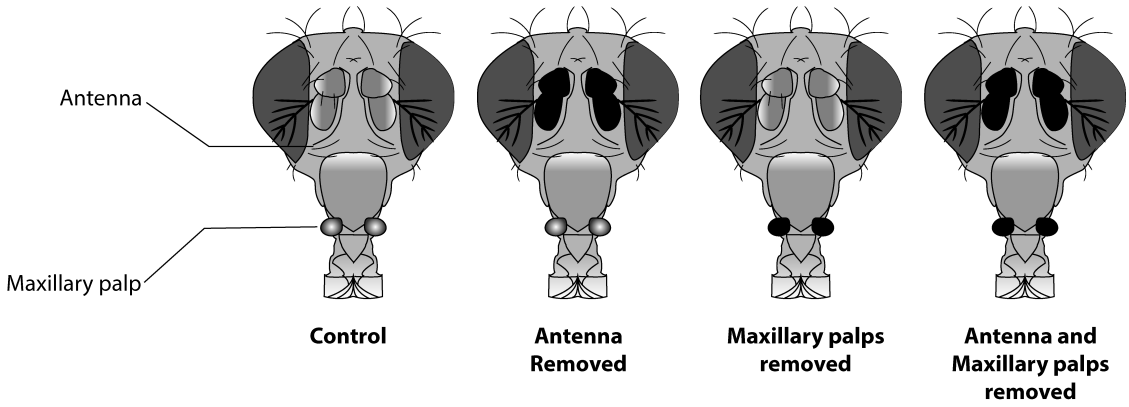


Figure 1 Image shows a stylised *D. melanogaster* head. Arrows indicate the sensory organs which were removed in this study.

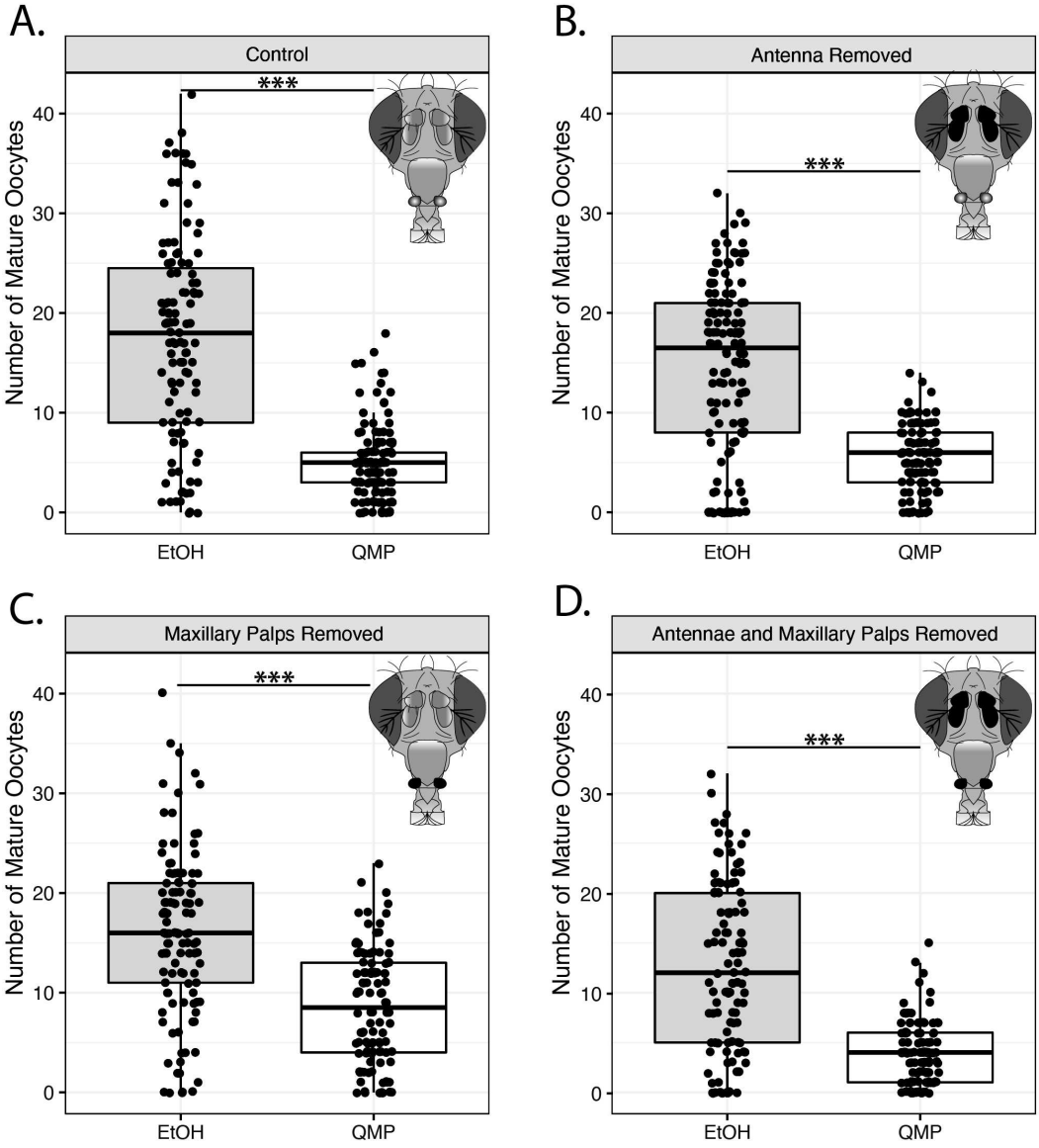


Figure 2 Surgical ablation of antennae, maxillary palps or both tissues has no effect on reproductive repression induced by exposure to QMP. Jittered box and whisker plots showing the number of mature oocytes from virgin female *D. melanogaster* which were

exposed to 26 Qe QMP or a solvent control. Exposure was for 48 h with each group having a n = 108 – 130). A) Intact individuals did not have any tissues removed B) individuals had their antennae removed. C) Individuals had their maxillary palps removed and D) Both antennae and maxillary palps were removed. Significant repression (***, $p < 0.001$) was induced by QMP treatment irrespective of whether the antennae, maxillary palps or both had been removed.

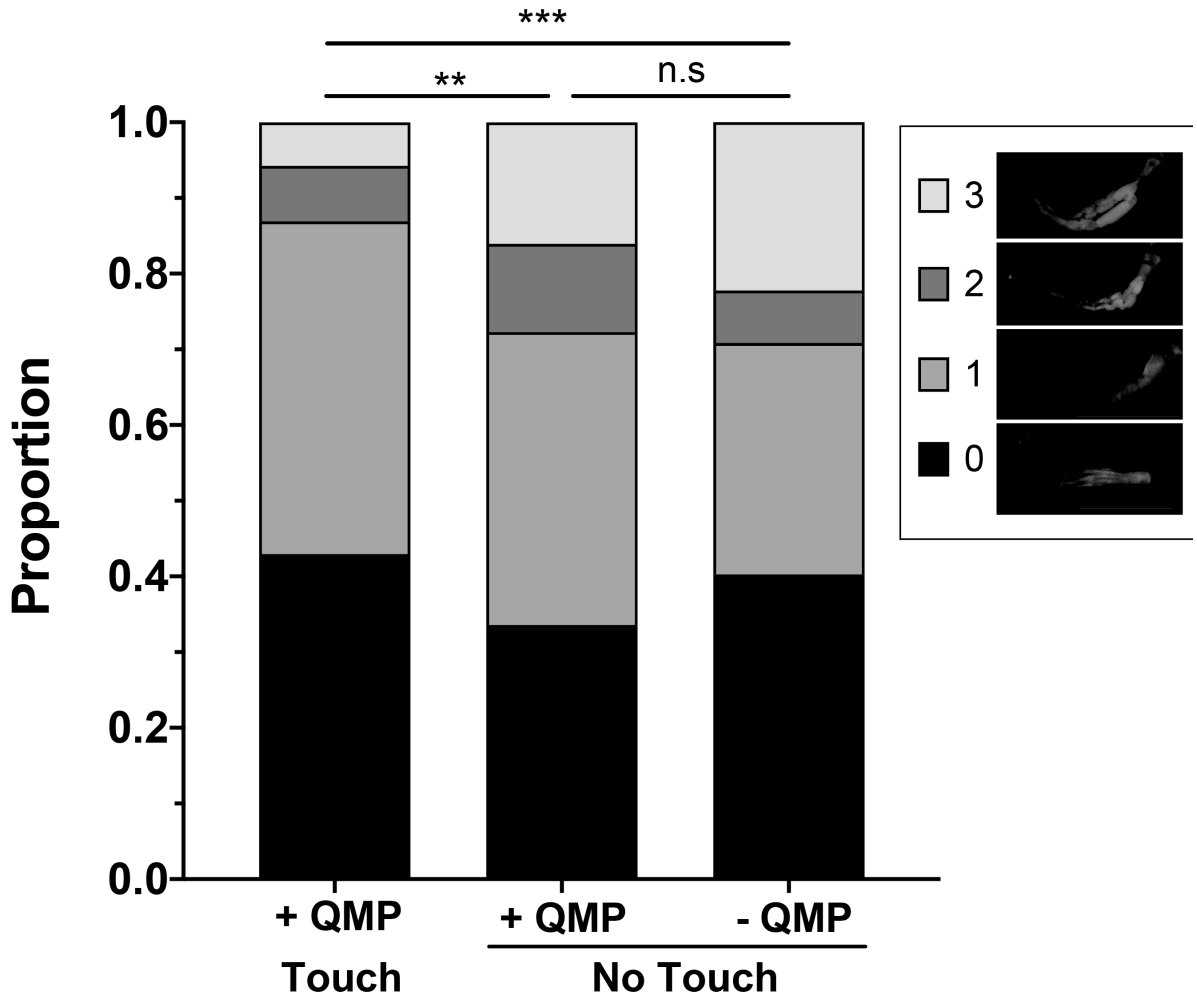
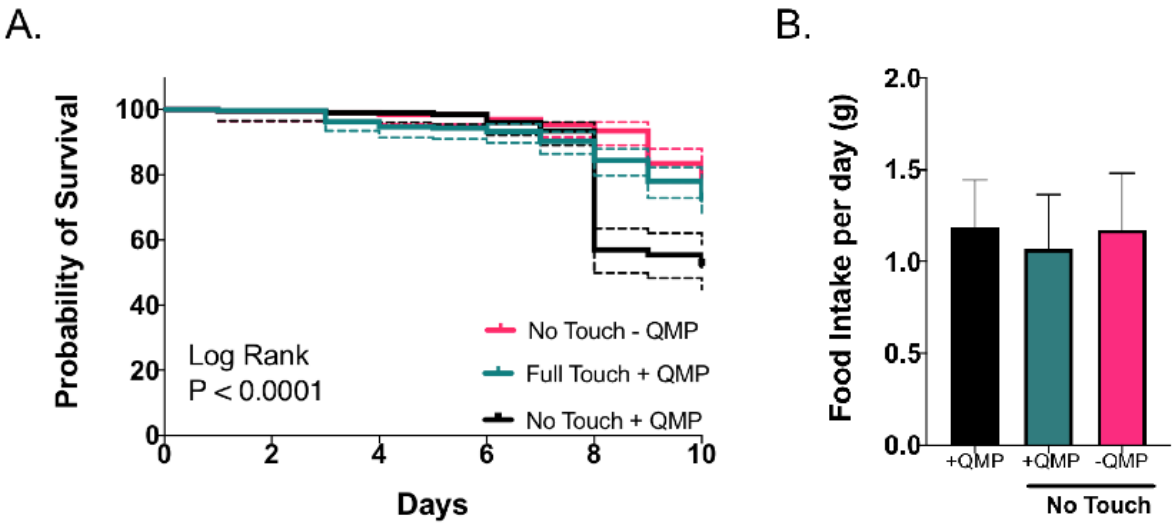
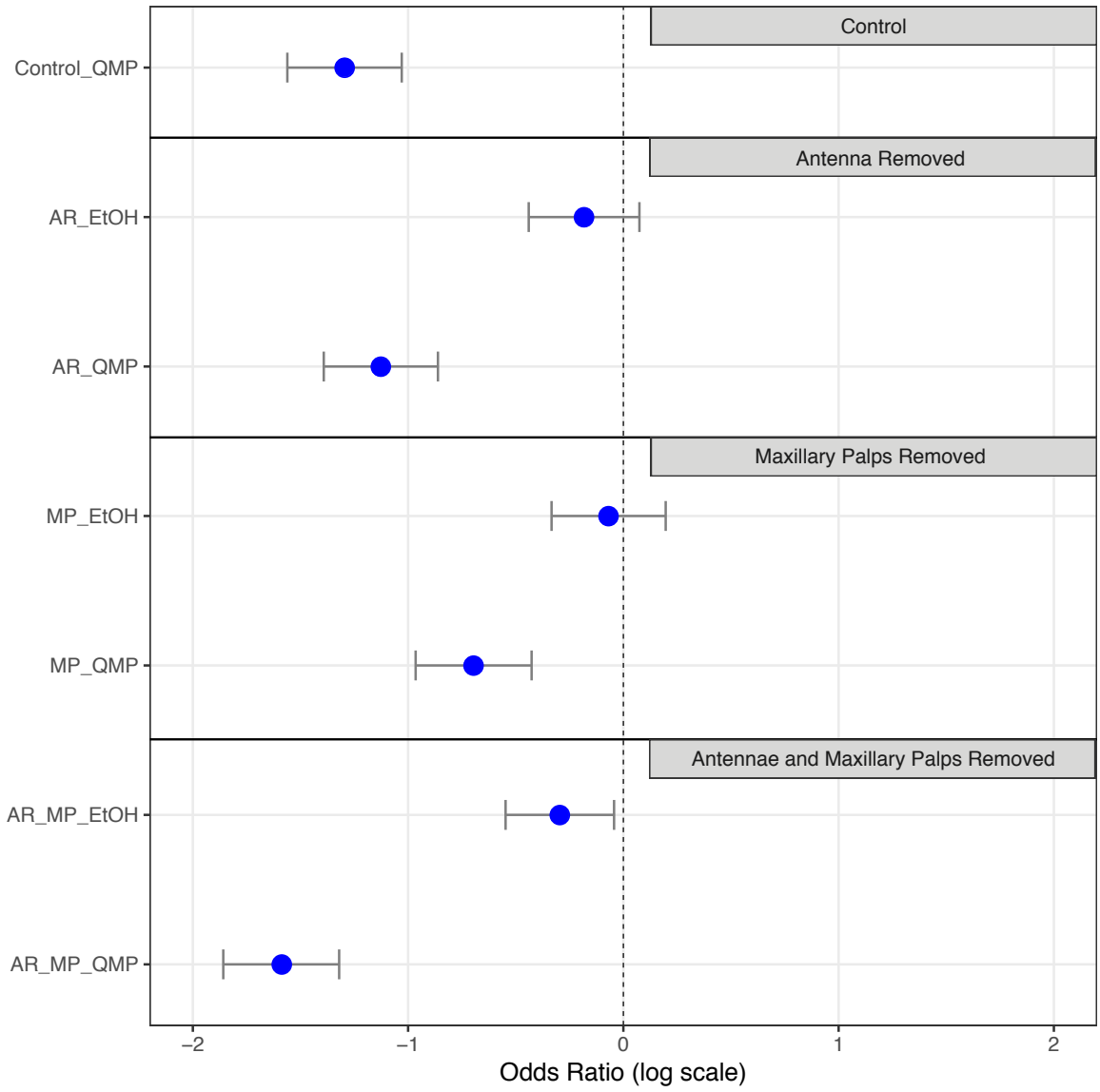


Figure 3 Repression of reproduction by QMP in worker honeybees requires direct physical interaction. Cages of workers were exposed to QMP with unimpeded access

(+QMP, n=191), QMP with a mesh barrier (No touch +QMP, n=137) or no QMP with a barrier (No touch -QMP, n=144). QMP was provided at a biologically relevant dose of 1 queen equivalent (Qe) per day. Cages were initially established with n = 100 newly emerged worker *A. mellifera*. Scores range from 0 (inactive) to 3 (having at least one fully mature egg) as shown in the inset. Statistical significance between treatments was determined using a Fisher's exact test, n.s. = not significant, ** $p < 0.01$, *** $p < 0.001$, showing that the proportion of activation differs between groups.



Supplementary Fig. 1: Kaplan-Meier survival curves and food intake for *Apis mellifera* presented in Fig. 3 of main text. A) Kaplan-Meir survival analysis indicates that individually QMP treatment and the inclusion of a mesh barrier inside the cages ("No Touch") had no effect on survival. However, combining these treatments "No Touch + QMP" caused a significant decrease in survival (Log Rank $P < 0.0001$). B) None of the treatments had any impact on food intake.



Supplementary Figure 2: Effect sizes and 95% confidence intervals for data presented in Fig 2 of main text. Effect sizes (Log odds) and 95% confidence intervals were calculated from the GLMMs using R Studio version 3. 5. 2. The only significant effects on *D. melanogaster* reproduction are for repression of reproduction by QMP (effect sizes do not overlap zero) except that removal of both the antennae and maxillary palps causes a slight reduction in the number of oocytes (the effect size does not overlap zero). However, this was not significant when the pairwise comparisons were corrected for multiple testing (Tukey post-hoc test, p= 0.28304) and similar effect sizes for all manipulations were seen with QMP treatment irrespective of manipulation.

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