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Ancestral hymenopteran queen pheromones do not share the broad phylogenetic repressive effects of honeybee queen mandibular pheromone.

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Abstract

Queen pheromones effect the reproductive division of labour, a defining feature of eusociality. Reproductive division of labour ensures that one, or a small number of, females are responsible for the majority of reproduction within a colony. Much work on the evolution and function of these pheromones has focussed on Queen Mandibular Pheromone (QMP) which is produced by the Western or European honeybee (*Apis mellifera*). QMP has phylogenetically broad effects, repressing reproduction in a variety of arthropods, including those distantly related to the honeybee such as the fruit fly *Drosophila melanogaster*. QMP is highly derived and has little chemical similarity to the majority of hymenopteran queen pheromones which are derived from cuticular hydrocarbons. This raises the question of whether the phylogenetically widespread repression of reproduction by QMP also occurs with more basal saturated hydrocarbon-based queen-pheromones. Using *D. melanogaster* we show that saturated hydrocarbons, are incapable of repressing reproduction, unlike QMP. We also show no interaction between the four saturated hydrocarbons tested or between the saturated hydrocarbons and QMP, implying that there is no conservation in the mechanism of detection or action between these compounds. We propose that the phylogenetically broad reproductive repression seen in response to QMP is not a feature of all queen pheromones, but unique to QMP itself, which has implications for our understanding of how queen pheromones act and evolve.

Introduction

Reproductive division of labour is a key feature of social insect societies, requiring a small number of females, indeed often a single female, to be reproductively dominant, and her subordinate workers to have their reproduction repressed (Oster and Wilson, 1978). To achieve this in the Hymenoptera, a clade containing many eusocial species, a mixture of behavioural aggression and chemical inhibition of reproduction is used (Le Conte and Hefetz, 2008; Padilla et al., 2016). Chemical inhibition occurs via queen pheromones (Matsuura et al., 2010; Vargo and Laurel, 1994; Winston and Slessor, 1992). These queen pheromones are produced by the reproductively dominant female and are thought to signal her fecundity to subordinates (Keller and Nonacs, 1993). Queen pheromones have been thought to be complex- both in function and composition (Brockmann et al., 1998; Slessor et al., 1988). They are theorised to be the product of an evolutionary arms race between the dominant female repressing reproduction, and the subordinate attempting to escape that repression (Le

Conte and Hefetz, 2008; Symonds and Elgar, 2008). This 'escape' could be achieved through behavioural alterations or genetic changes that overcome reproductive repression, for example through decreased sensitivity to or avoidance of the queen pheromone. It is hypothesised that this may lead to the evolution of increasingly more elaborate pheromones or mechanisms of repression, to accomplish reproductive dominance and eusociality (Katzav-Gozansky, 2006).

Cuticular hydrocarbons (CHCs) are produced in the exocrine glands of insects, and secreted into the cuticle (Howard and Blomquist, 2005). CHCs have various functions including acting as contact pheromones to convey information both between and within species. CHCs vary by species, sex, genotype, behavioural status, group within a society, reproductive state and physiology in both solitary and social insects (Howard and Blomquist, 2005). Queen signals within the social Hymenoptera are commonly CHCs, particularly long-chain linear and methyl branched alkanes, although the nature of these compounds varies between species (Van Oystaeyen et al., 2014). Van Oystaeyen *et al.* showed that that saturated hydrocarbons may act as sterility-inducing cues, or as indicators of fertility, in 57 out of 64 social Hymenoptera, implying evolutionary conservation in the class of compound used to control reproduction. This is remarkable as it indicates that CHCs have repeatedly become co-opted into maintaining worker sterility in five independent origins of eusociality. The widespread use of saturated hydrocarbons implies that these molecules may have functioned as fertility cues in the ancestor of extant bees, ants and wasps (Van Oystaeyen et al., 2014). A subset of these saturated hydrocarbons were the linear alkanes; pentacosane (*n*-C_{25}), heptacosane (*n*-C_{27}), octacosane (*n*-C_{28}) and nonacosane (*n*-C_{29}) which were identified in a bumblebee, a wasp and an ant species (*Bombus terrestris, Vespula vulgaris* and *Cataglyphis iberica).* These compounds were functionally demonstrated to act as sterility – inducing queen pheromones (Van Oystaeyen et al., 2014). It is important to note, however, that *n*-C_{25} is found on the cuticle, as well as in most exocrine glands of both reproductive and non-reproductive bumble bees (Amsalem et al., 2014; Amsalem et al., 2009) which is not consistent with it functioning as a queen pheromone. No single linear alkane molecule repressed reproduction in all three species, however, all tested species had their reproductive capacity reduced by at least one of these compounds. This is consistent with the idea that linear alkanes in particular may have convergently evolved a role as queen pheromones in the eusocial Hymenoptera. It is proposed these signalling molecules may have evolved from chemical cues of a solitary ancestor (Van Oystaeyen et al., 2014) ~180 million years ago (Peters et al., 2017).

Despite the importance of saturated hydrocarbons as hymenopteran queen pheromones, Queen Mandibular Pheromone (QMP), produced by queen honeybees (*Apis mellifera*), is still the most studied social insect queen pheromone (Keeling et al., 2003; Pankiw et al., 1994; Slessor et al., 1988). Although tergal gland secretions, which contain alkene hydrocarbons, have been implicated in repressing worker reproduction (Wossler and Crewe, 1999) the effect is generally much smaller than observed for QMP (Holman, 2018). QMP is, however, highly derived and distinct from other social hymenopteran queen pheromones (Van Oystaeyen et al., 2014). It is a complex pheromone with five major components - none of which are saturated hydrocarbons. QMP has been shown to repress oogenesis in virgin *D. melanogaster* females, producing ovaries with fewer mature oocytes when exposed (Camiletti et al., 2013; Sannasi, 1969). This is surprising given the evolutionary distance between honeybees and *Drosophila* is ~340 million years (Misof et al., 2014). QMP also represses reproduction in a variety of other arthropods, including a species of ant (Carlisle and Butler, 1956), termite (Hrdý et al., 1960), house fly (Nayar, 1963) and even a prawn (Carlisle and Butler, 1956), spanning evolutionary distances of more than ~530 million years (Misof et al., 2014).

We hypothesise that there are two possible scenarios for the broad phylogenetic range over which QMP represses reproduction. The first is that QMP has evolved to target highly conserved pathways to repress reproduction. This scenario is consistent with our current molecular understanding of how QMP acts to control reproduction in the honeybee ovary (Duncan et al., 2016; Ronai et al., 2016). These studies demonstrate that QMP modulates highly conserved processes within the honeybee ovary (Duncan et al., 2016; Ronai et al., 2016) and Notch signalling in particular is known to be environmentally responsive (Hsu and Drummond-Barbosa, 2011). This may imply that QMP has evolved to target ancient mechanisms for responding reproductively to environmental stimuli. QMP has evolved over the last ~55 million years (Peters et al., 2017). However, despite evolving relatively recently it is capable of repressing reproduction in species 530 million years diverged (Carlisle and Butler, 1956; Misof et al., 2014). If QMP has evolved to modulate conserved pathways controlling reproduction other, less derived, queen pheromones might not be expected to share this effect. Therefore, the broad range of arthropods repressed by QMP would not be similarly repressed by other social hymenopteran queen pheromones.

The second option is that all of the cuticular hydrocarbons, identified as putative basal queen pheromones in the Hymenoptera, are capable of repressing reproduction in a phylogenetically

broad range of animals, similar to QMP. This would mean the repression of reproduction observed by Van Oystaeyen *et al.* may be attributed to conserved mechanisms being targeted by all hymenopteran queen pheromones, not only QMP. If *D. melanogaster* reproduction is impaired by these ancestral-like queen pheromones then this would imply that this broader class of compounds are also targeting conserved mechanisms of reproductive repression, and that this property is not unique to QMP. If linear alkanes do not repress reproduction in *D.melanogaster*, then this implies these queen pheromones are specialised to act in the insect groups in which sociality evolved and as such have a narrower phylogenetic span than that of QMP.

In this study, we test the ability of saturated hydrocarbons to repress reproduction in *D. melanogaster*. We also test for synergistic interactions between the linear alkanes and also with honeybee QMP. This has implications for the pathways through which they act. If they disrupt or potentiate the action of each other, it would suggest the derived QMP shares mechanisms of detection or action with the ancestral social hymenopteran pheromones, further informing us about their evolutionary trajectories. If they were acting through the same mechanisms to cause reproductive repression it would indicate that selective pressure or drift has acted to change the inputs (pheromones) into this pathway. If they do not interact, it would indicate that the mechanisms through which QMP is detected or acts are different from those used by linear alkanes. We conclude that the wide phylogenetic span of arthropod species on which the repressive function of QMP acts is a derived evolutionary novelty, not a feature of the broader class of queen pheromones from which QMP evolved.

Methods

D. melanogaster stocks and maintenance

The Oregon-R modENCODE line (Stock #25211 from the Bloomington *Drosophila* stock centre) was used for all *D. melanogaster* work in this study. Stocks were maintained at 25 °C on a 12 h : 12 h light/dark cycle. Flies were raised on a sugar/yeast medium; of 3L 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.

Virgin collection

Only virgin female *D. melanogaster* were used for this study. These were anaesthetised with CO_2, and observed under a GXM-XTL stereomicroscope (GT Vision, UK), with phenotypically virgin females being isolated based upon the characteristics of enlarged

abdomens, pale colouration and presence of the meconium. Virgins were collected within one hour of emergence, isolated with other virgin females, and stored at room temperature for 24 hours.

Pheromone dilutions

QMP dilutions for concentration gradient

Queen Mandibular Pheromone (QMP) from queen honeybees (*A. mellifera)* is quantified in Queen equivalents (Qe). One Qe is the amount a mated queen will produce in a 24 hours (Pankiw et al., 1996). QMP contains five major components (Slessor et al., 1988), that make up 1 Qe for a European mated queen in the following amounts; 200μg 9-keto-(*E*)-2-decenoic acid (ODA), 80μg 9-hydroxy-(*E*)-2-decenoic acid (9-HDA), 20μg methyl*p*-hydroxybenzoate (HOB), and 2 µg 4-hydroxy-3-methoxyphenylethanol (HVA) (Pankiw et al., 1996). QMP (Intko Supply Ltd, Canada) was dissolved in absolute ethanol to the concentrations of 3.25 Qe, 6.5 Qe, 13 Qe and 26 Qe, and stored at -20 °C until use.

Linear alkane dilutions and mixtures

The linear alkanes pentacosane (*n*-C_{25}), heptacosane (*n*-C_{27}), octacosane (*n*-C_{28}) and nonacosane (*n*-C_{29}) were used based on experimentally determined doses identified in the study by Van Oystaeyen et al., 2014. These compounds had been identified as components queen signals, from the bumblebee, wasp and ant species *B. terrestris*, *V. vulgaris* and *C. iberica*. The values previously calculated were used to determine 1 Qe in the Van Oystaeyen study, this was based on the absolute amount present upon the queen's cuticle. 26 Qe was used for the treatment in this study (based on high QMP doses in *D. melanogaster* exposure studies (Camiletti et al., 2013). In order to maximise the chances of finding similar biologial effects of linear alkanes we treated *D. melanogaster* with levels of the linear alkanes 26 fold higher than those found in relevant queens from Van Oystaeyen et al. (2014). One Qe of each alkane for use in *D.melanogaster* was defined as the highest amount produced by one of the three species in Van Oystaeyen et al. (2014) *B. terrestris*; 232.5 µg *n*-C_{25}, *V. vulgaris*; 118 µg *n*- C_{27}, 6.1 µg *n*-C_{28}, 19 µg *n*-C_{29}. The alkane blend was a combination of all four linear alkanes discussed each at 26 Qe. The linear alkanes were dissolved in HPLC grade pentane, and stored at -20 °C. Pentane was used as the control treatment.

Pheromone exposure in *D. melanogaster*

QMP concentration gradient exposure in D. melanogaster

Modified vials were made from 50 ml centrifuge tubes. Tubes were heated and the collection end was removed. Two layers of Whatman number 1 filter paper shaped to fit the inside of

the lid and these were screwed into place. A cotton ball was used to plug to cut end of the tube. Virgin *D. melanogaster* were aged for 24 hours, before being put in modified vials, and 500 µl of a liquid diet was added. This liquid diet was made fresh on the day of use in 5 ml aliquots. The diet contains 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 diet, 20 µl of QMP solution was added. The virgin *D. melanogaster* were anaesthetised with CO_2, and 10 were added to the vial lying on its side, and allowed to recover before the vial was incubated upright at 25 °C for 48 hours. Each treatment consisted of seven replicates and each replicate included 10 individuals (n = 70).

Linear alkane exposure in D. melanogaster

Diet and vial set up were as described for the concentration gradient above. On top of this liquid diet, 100 µl of the linear alkane solutions were added. The virgin *D. melanogaster* were anaesthetised with CO_2, and 10 were added to the vial lying on its side, and allowed to recover before the vial was incubated upright at 25 °C for 48 hours. *D. melanogaster* were exposed to each of the linear alkanes individually, as well as the mixture of all four. The positive control for ovary repression was 26 Qe of *A. mellifera* QMP, dissolved in absolute ethanol. Each treatment had five replicates of 10 individuals (n=50).

Linear alkane and QMP combined exposure in D. melanogaster

Virgin *D. melanogaster* were exposed to a combined exposure of linear alkanes and a low dose of honeybee QMP. QMP was diluted to a dose of 3.25 Qe. This low dose was designed to induce minor repression, allowing for further reduction in mature oocyte number should the linear alkane mix interact synergistically interact with QMP. Ethanol was used as the solvent control for QMP. There were three control combinations used to test the interaction between the alkane mix and QMP; ethanol and pentane, linear alkane mix and ethanol, 3.25 Qe QMP and pentane. Exposure method and timing was carried out as described for single linear alkanes. The only difference was the addition of two treatments to the top of the liquid food, as opposed to the one addition described above.

D. melanogaster ovary dissection and fixation

Ovary dissections were performed using a GXM-XTL stereomicroscope (GT Vision, UK) after *D. melanogaster* had been briefly anaesthetised with CO_2. Ovaries were dissected into a petri dish containing ice-cold PBS. Any ovaries that were damaged or lost oocytes in the dissection process were discarded. These were stored in 400 µl PBS on ice until all dissections were complete (less than 30 min).

PBS was removed from the microcentrifuge tube containing dissected ovaries, down to 50 µl. To the tube added 900 µl PBS and 4% formaldehyde. Ovaries were rocked at room temperature for 10 minutes. Fixative was removed, and ovaries were washed four times with PTx (PBS with 0.1% TritonX). Fixed ovaries were stored in 70 % ultrapure glycerol at 4 °C in the dark until slide mounting. Ovaries were stored in glycerol for at least 24 hours before being bridge-mounted for microscopy. The number of mature (vitellogenic) oocytes was determined by manual counting under a GXM-XTL stereomicroscope (GT Vision, UK) and was used as a measure of fecundity (King, 1970).

Statistical analysis

The number of mature oocytes per ovary in the *D. melanogaster* 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 Poisson error structure were used using lme4, in all cases treatment was treated as a fixed effect 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. 1).

Results:

D. melanogaster are reproductively repressed by honeybee QMP in a dose-dependent manner

As previously reported (Camiletti et al., 2013) the number of mature oocytes in a *D. melanogaster* ovary is decreased in a dose-dependent manner by exposure to QMP for 48 h (Fig. 1) (AOD $\chi^2 = 56.142$, df = 4, $p = 1.87 \times 10^{-11}$). At the lowest dose of QMP tested (3.25 Qe) the number of mature oocytes was repressed by 36% (Ethanol mean = 17.56, 3.25 Qe mean = 11.27 $p = 0.0140$). The highest exposure tested (26 Qe) reduced the number of mature oocytes by 71% (Ethanol mean = 17.56, 26 Qe mean = 5.02 $p = < 0.001$) (Fig. 1).

D. melanogaster are not reproductively repressed by putative basal hymenopteran queen pheromones

To determine whether *D. melanogaster* are reproductively repressed by the putative conserved social insect queen signals, virgin females were exposed to the linear alkanes pentacosane (*n*-C_{25}), heptacosane (*n*-C_{27}), octacosane (*n*-C_{28}) and nonacosane (*n*-C_{29}). The 26 Qe dose of QMP was included as a positive control for reproductive repression. QMP

induced the expected repression (AOD χ^2 = 52.597, df = 7, $p = 4.26 \times 10^{-9}$) (Fig. 2), reducing the number of mature oocytes by 59% (Ethanol mean = 8.14, 26 Qe mean = 3.36 p = < 0.001). Note that this is a slightly lower magnitude of repression than observed in Fig. 1, likely due to differences in protein sources used for diet preparation. Consistent with this the solvent only controls in Fig. 1 have fewer mature oocytes (Fig. 1, Ethanol mean = 17.56) than the solvent only controls in Fig. 2 (Fig. 2, Ethanol mean = 8.14).

The high dose QMP positive control (26 Qe) was the only significant reproductive repression observed, and none of the single linear alkanes tested altered the number of mature oocytes produced (n-C_{25} p = 0.987, n-C_{27} p = 1.000, n-C_{28} p=1.000, n-C_{29} p=1.000). To test whether these compounds interact to repress reproduction we treated *D. melanogaster* with a blend of all four linear alkanes. This blend also did not cause reproductive repression and did not alter the number of mature oocytes produced (p = 1.000).

Linear alkanes and honeybee QMP do not act synergistically

To test whether there was any synergistic interaction between QMP and the linear alkanes, the high dose alkane mix (26 Qe) was given as well as a low dose of QMP (3.25 Qe) (Fig. 3). This dose of QMP was chosen to induce minor repression (Fig. 1), but not to the same extent observed as a result of exposure to 26 Qe (Fig. 1). By inducing a small reduction in the number of oocytes, we sought to observe synergistic or antagonistic effects on reproduction between QMP and the linear alkanes.

There was no effect of any of these treatments on the number of mature oocytes in this experiment (AOD χ^2 = 3.45, df = 3, p = 0.3273) (Fig. 3). Consistent with Fig. 2 there was no statistically significant repression induced by the high-dose alkane mix. The low-dose QMP with pentane control acted as anticipated, where there was a small reduction in the number of mature oocytes produced (decreasing the number of mature oocytes by ~23%, Pentane + ethanol mean = 7.59, 3.25 Qe mean = 5.82), but this was not statistically significant.

Treatment with 3. 25 Qe QMP and the blend of linear alkanes did not affect the number of mature oocytes produced. This demonstrates that there is no synergistic interaction between the linear alkanes and honeybee QMP and that QMP was not able to potentiate the effects of these linear alkanes to cause reproductive repression.

Discussion

As in previous studies, we have shown that *D. melanogaster* are reproductively repressed by honeybee QMP (Fig.1) (Camiletti et al., 2013). Initially, this appears an unusual phenomenon,

as *Drosophila* are not eusocial and are not closely related to honeybees (they are separated by ~340 million years of evolution (Misof et al., 2014)). *Drosophila* would also be unlikely to come into contact with QMP in their natural environment as these species occupy very different habitats to the honeybee. As such, they presumably have not evolved to specifically repress reproduction in response to QMP. Additionally, QMP mediated repression of reproduction in non-target species is well established (Camiletti et al., 2013; Carlisle and Butler, 1956; Hrdý et al., 1960; Nayar, 1963); this suggests QMP may have evolved to target conserved pathways to repress reproduction. What remains unknown is the evolutionary history of this response- namely, is this wide phylogenetic span of repression a feature that is derived and novel to honeybee QMP? Or is it a feature of the ancestral queen pheromones from which QMP has presumably evolved?

Linear alkanes have been identified to act as a conserved class of repressive cues in the Hymenoptera by Van Oystayaen *et al.* In this study, we tested the ability of these queen pheromones to repress reproduction in *D. melanogaster*. We show that the linear alkanes pentacosane (*n*-C_{25}), heptacosane (*n*-C_{27}), octacosane (*n*-C_{28}) and nonacosane (*n*-C_{29}) do not reduce the number of mature oocytes produced in the *D. melanogaster* ovary (Fig. 2). We also tested the hypothesis that these compounds may interact additively or synergistically to repress reproduction, but a blend of all four alkanes also showed no reduction in the number of mature oocytes (Fig.2). Determining appropriate doses of individual queen-pheromones to test (Holman et al., 2017), particularly in cross species comparisons as presented here, is challenging. In this study we verified that maximal repression of ovary activity by QMP was observed with 26 qe of QMP, similar to that previously reported (Camiletti et al., 2013). Similarly, we chose to treat *Drosophila* with 26 qe of the individual linear alkanes, calculating qe based on the highest levels of individual linear alkanes found in either *B. terrestris, V. vulgaris* or *C. iberica* queens (Van Oystaeyen et al., 2014), rather than test idential microgram quantites of each linear alkane. Our rationale was that these linear alkanes are present in *B. terrestris, V. vulgaris* and *C. iberica* at different levels and that this may reflect differences in biological activity of these compounds. To maximise the likelihood of finding a physiological effect of these compounds, but remain within the realm of physiologically relevant doses that workers of these species might be exposed to, we treated *D. melanogaster* with doses of linear alkane 26 fold higher than produced by queens of *B. terrestris, V. vulgaris* and *C. iberica*. It is also important to note that *D. melanogaster* has a smaller biomass than either *B. terrestris* and *V. vulgaris* workers and is similar to *C.*

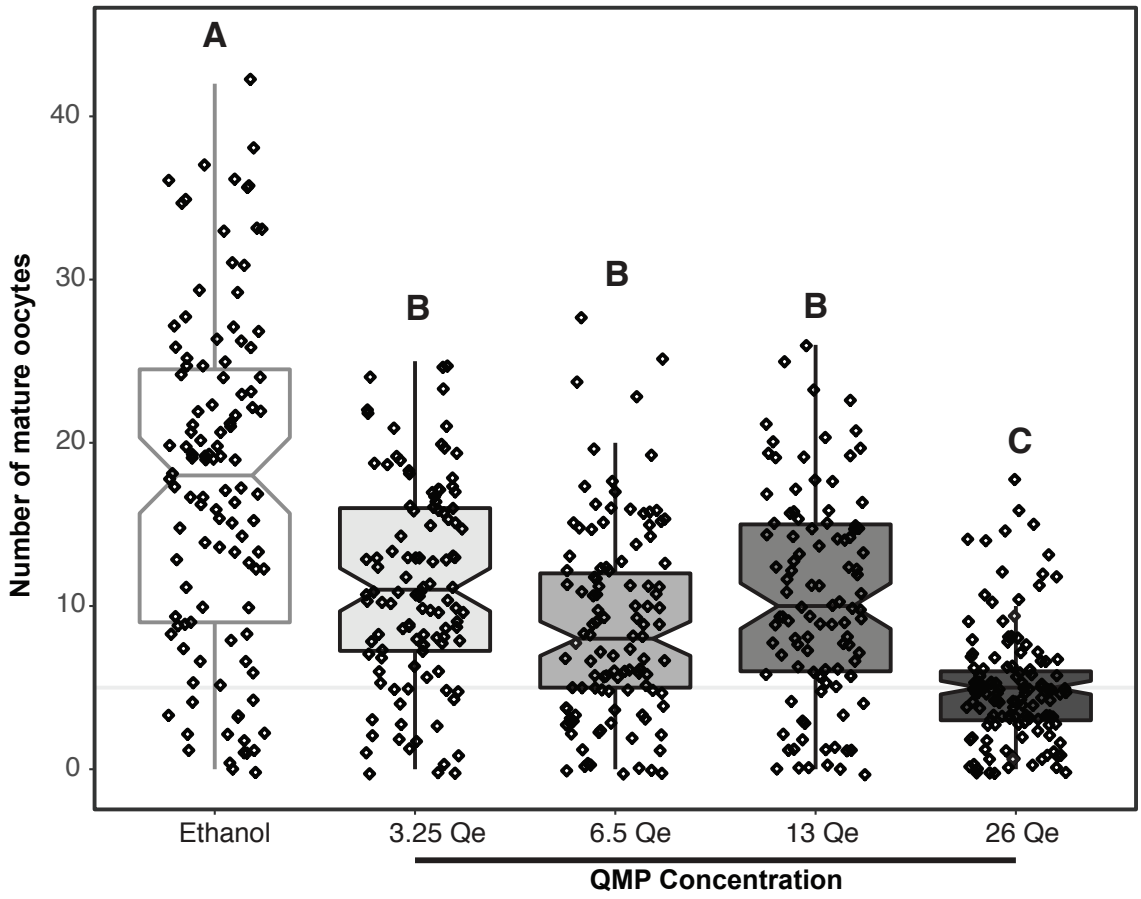
iberica so that the relative dose *D. melanogaster* were exposed to in this study is potentially higher than 26 fold and higher than workers of these species would be exposed to. That *D. melanogaster* don't respond to these relatively high doses of linear alkanes is consistent with these compounds not having any biological activity in repressing ovary activity in *D. melanogaster.*

That these basal Hymenopteran queen-pheromones don't affect reproduction in *D. melanogaster* may be due to an inability of *D. melanogaster* to detect these compounds. These linear alkanes tested in this study are derived from cuticular hydrocarbons (Van Oystaeyen et al., 2014), which are known to vary in both quantity and identity between species (Blomquist and Bagnères, 2010) so much so that even sister species can have distinct cuticular hydrocarbon profiles (Morrison and Witte, 2011). It could be that these compounds are unable to effect reproduction in *D. melanogaster* because they are not detected. However, all four linear alkanes tested are components of the *D. melanogaster* CHC profile, pentacosane (n-C_{25}) varies with geographical location in *D. melanogaster* populations (Rajpurohit et al., 2017) and all four of the linear alkanes tested in this study vary in male *D. melanogaster* with social group and genotype (Kent et al., 2008) suggesting that this species can detect and respond these compounds. It is also possible that these compounds may be affecting other aspects of *D. melanogaster* reproductive biology that were not examined in this study, such as courtship or mating, as CHC profiles are known to be altered by mating status (Everaerts et al., 2010). We hypothesise that rather than a lack of detection, these compounds are targeting a pathway or process to repress reproduction that isn't conserved between hymenoptera and *Drosophila*. This may be due to a loss of function of in the lineage leading to *D. melanogaster* or a gain of function in the hymenopteran lineage. To test this hypothesis we need a mechanistic understanding of how these linear alkanes are detected and how this signal is translated into reproductive repression in social hymenoptera (Holman et al., 2019).

We also tested whether there was a synergistic interaction between the linear alkanes and QMP (Fig. 3). The linear alkane blend neither potentiated nor disrupted the minor repressive effect of low dose QMP (Fig. 3). This implies that QMP is acting through a different mechanism to the linear alkanes and as such can repress reproduction in *D. melanogaster*. The response to QMP in *D. melanogaster,* and other non-target species, therefore is a derived feature of QMP, not a reflection of a conserved class of 'insect pheromone'.

Surprisingly, QMP is capable of seemingly ubiquitously repressing highly diverged, non-target species- whereas the other social hymenopteran queen pheromones cannot. QMP has been evolving for ~ 55 million years (Peters et al., 2017), yet represses species that diverged ~ 475 million years prior to the start of QMP evolving (Misof et al., 2014). This makes QMP capable of targeting conserved pathways more ancient than QMP itself. The evolutionary origins of QMP are unclear, but it has been hypothesised that an increase in social complexity would be accompanied by an increase in the complexity of pheromones potentially as the result of an arms race between queens and workers over worker reproduction where workers evolve resistance to reproductive repression and queens evolve new pheromone components that overcome that resistance (Bourke, 1988; Holman, 2018; Katzav-Gozansky, 2006; Kocher and Grozinger, 2011). One consequence of such an arms race might be the evolution of pheromones that target conserved pleiotropic pathways that are difficult to evolve resistance to - as escape would come with high fitness costs. One such example is the previously identified Notch cell signalling pathway (Duncan et al., 2016), which is key for QMP mediated reproductive repression in honeybees but also has pleiotropic and conserved roles in other fundamental processes including neurogenesis. These fundamental and pleiotropic roles mean that there is selective pressure to retain a functioning Notch signalling pathway. If such pathways are also evolutionary conserved, then targeting this system may cause responses in a wide range of species- just as we see with QMP. Thus the broad effect of QMP in arthropods is not a feature of ancestral queen pheromones, but instead may be a derived feature unique to QMP itself. This may reflect that QMP has evolved to target an evolutionarily conserved mechanism, possibly derived from an environmental signal linked to temperature or nutrition, for repressing reproduction.

377 Figure Legends



378

379 **Figure 1.** A jittered box and whisker plot showing the number of mature oocytes from newly
380 emerged virgin female *D. melanogaster* that were exposed to honeybee QMP in a
381 concentration gradient from 3.25 – 26 Qe, with the ethanol solvent control. Exposure was for
382 48 hours. Significant repression ($p<0.05$) was induced at all concentrations tested relative to
383 controls.

384

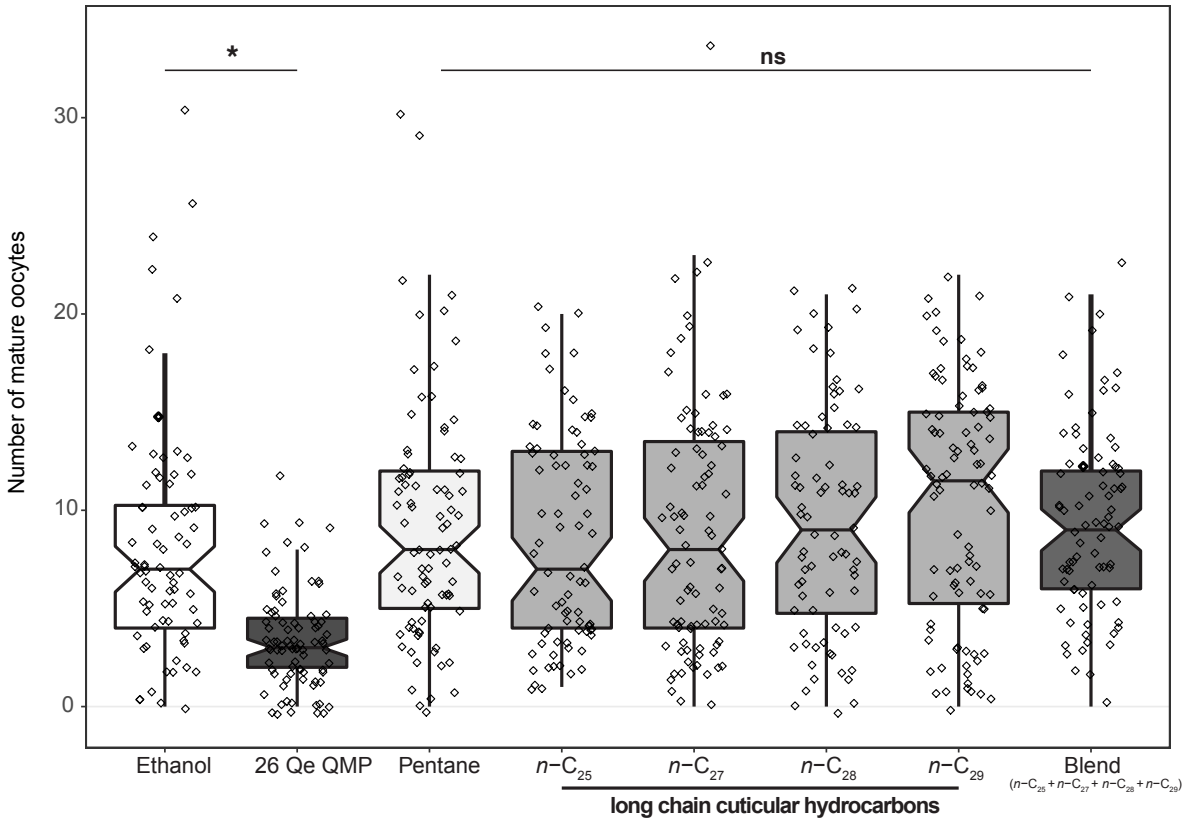


Figure 2. A jittered box and whisker plot showing the number of mature oocytes from newly emerged virgin female *D. melanogaster* that were exposed to the linear alkanes pentacosane (*n*-C_{25}), heptacosane (*n*-C_{27}), octacosane (*n*-C_{28}) and nonacosane (*n*-C_{29}) at dose of 26 Qe singularly, or as a blend of all four linear alkanes. Pentane was used as solvent control. Exposure was for 48 hours. 26 Qe of QMP from honeybees was used as a positive pheromone control, with the associated ethanol solvent control for QMP. The only statistically significant repression ($p<0.05$) was induced by the high dose QMP.

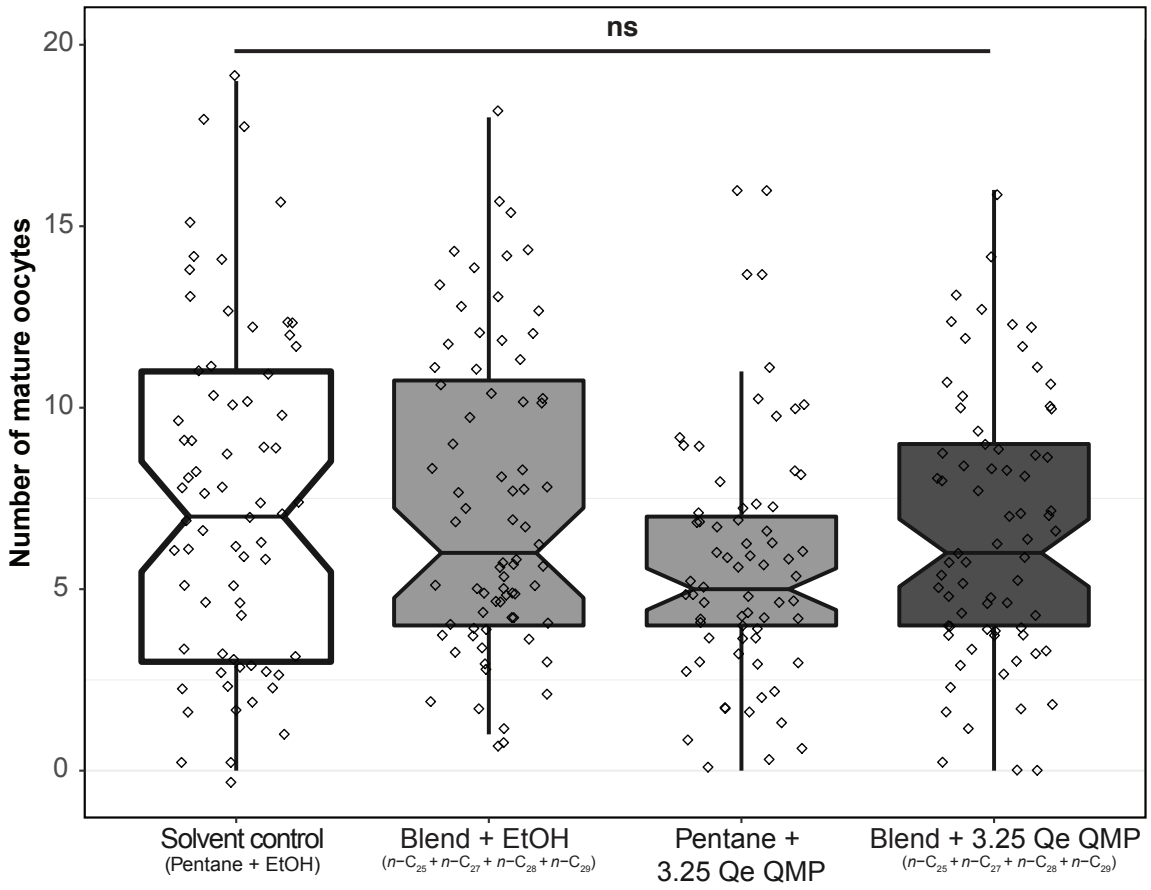
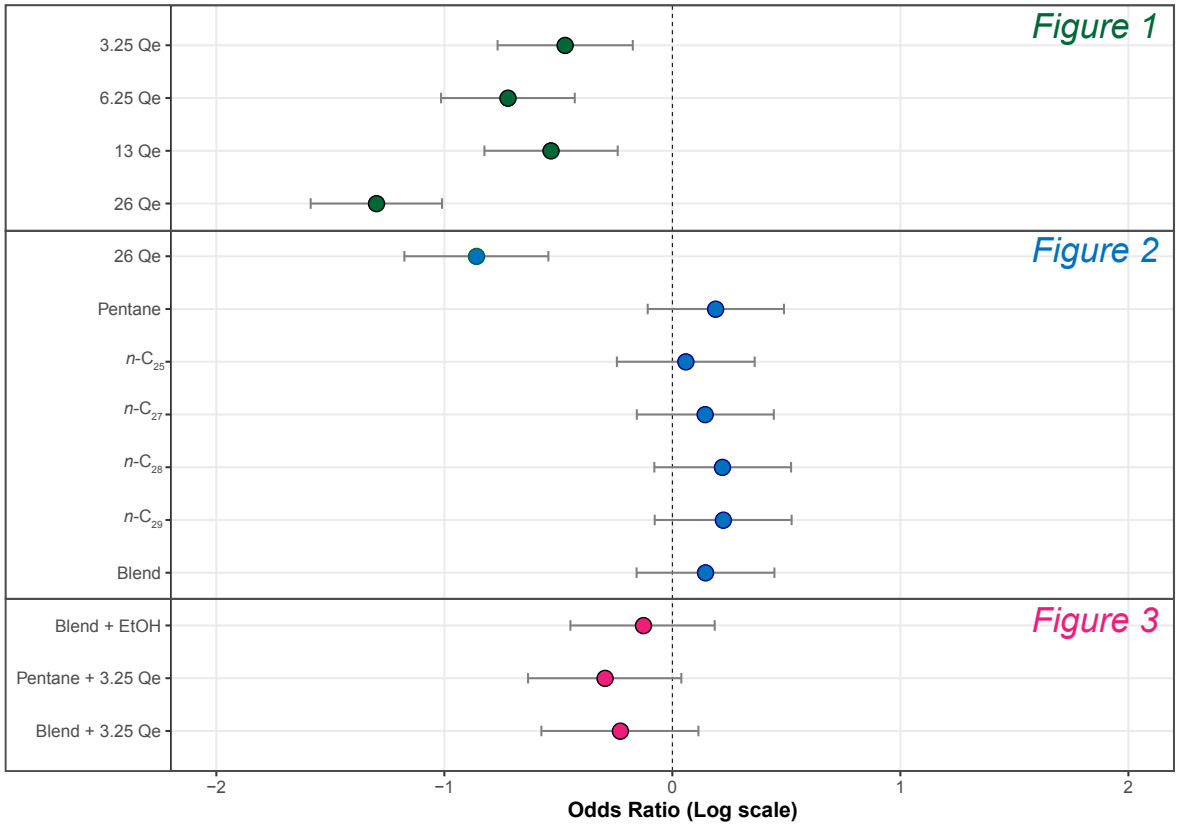


Figure 3.

A jittered box and whisker plot showing the number of mature oocytes from newly emerged virgin female *D. melanogaster* that were exposed to a blend of the four linear alkanes pentacosane (*n*-C_{25}), heptacosane (*n*-C_{27}), octacosane (*n*-C_{28}) and nonacosane (*n*-C_{29}) at dose of 26 Qe. Pentane was used as a solvent control for the alkanes. 3.25 Qe of QMP from honeybees was used to induce low levels of ovary repression, with the associated ethanol solvent control for QMP. Exposure was for 48 hours. There was no statistically significant repression induced by any of the treatments.



Supplementary Figure 1: Effect sizes and 95% confidence intervals for data presented in Fig 1-3 of the 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).

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Author contributions

MRL, PKD and EJD designed the study, MRL carried out the experimental work with assistance from EJD. EJD and MRL performed the data analysis, prepared the figures and drafted the manuscript with assistance from PKD. MRL, PKD, EJD edited and approved the final manuscript.

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