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Honeybee queen mandibular pheromone induces a starvation response in *Drosophila melanogaster*

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ABSTRACT

Eusocial insect societies are defined by the reproductive division of labour, a social structure that is generally enforced by the reproductive dominant(s) or 'queen(s)'. Reproductive dominance is maintained through behavioural dominance or production of queen pheromones, or a mixture of both.

Queen mandibular pheromone (QMP) is a queen pheromone produced by queen honeybees (*Apis mellifera*) which represses reproduction in worker honeybees. How QMP acts to repress worker reproduction, the mechanisms by which this repression is induced, and how it has evolved this activity, remain poorly understood. Surprisingly, QMP is capable of repressing reproduction in non-target arthropods.

Here we show that in *Drosophila melanogaster* QMP treatment mimics the starvation response, disrupting reproduction. QMP exposure induces an increase in food consumption and activation of checkpoints in the ovary that reduce fecundity and depresses insulin signalling. The magnitude of these effects is indistinguishable between QMP-treated and starved individuals. As QMP triggers a starvation response in an insect diverged from honeybees, we propose that QMP originally evolved by co-opting nutrition signalling pathways to regulate reproduction.

1. Introduction

In eusocial societies, reproductively dominant individuals (queens) are the primary reproductive individuals whereas workers carry out other, non-reproductive, tasks (Michener, 1974; Oster and Wilson, 1978). Eusociality evolved independently at least 16 times in the insects (Crespi, 1992; Grimaldi and Engel, 2005; Inward et al., 2007; Kent and Simpson, 1992; Tanaka and Itô, 1994), and up to 11 times in Hymenoptera (Brady et al., 2006; Cameron and Mardulyn, 2001; Crozier, 2008; Danforth et al., 2013; Hines et al., 2007; Hughes et al., 2008; Moreau et al., 2006; Peters et al., 2017).

In many eusocial species, queens regulate their colony using chemical cues (queen pheromones) which repress the reproduction of female subordinate workers. The most well-studied queen pheromone is queen mandibular pheromone (QMP) produced by the queen honeybee, *Apis mellifera* (Keeling et al., 2003; Pankiw et al., 1996; Pham-Delègue et al., 1993; Princen et al., 2019).

QMP can not only repress honeybee worker reproduction, but it also can repress reproduction in other non-target arthropods. QMP has been shown to reduce reproduction in *Drosophila melanogaster* (Camiletti et al., 2016; Lovegrove et al., 2019; Sannasi, 1969), a housefly (*Musca domestica*) (Nayar, 1963), an ant (*Formica fusca*) (Carlisle and Butler, 1956), termite (*Kalotermes flavicollis*) (Hrdy et al., 1960) and a prawn (*Leander serratus*) (Carlisle and Butler, 1956); species that shared a common ancestor more than 530 million years ago (dos Reis et al., 2015). In contrast, other hymenopteran queen pheromones (primarily linear alkanes) do not repress reproduction in *D. melanogaster* (Lovegrove et al., 2019).

The mixture of chemicals that make up Honeybee QMP differs markedly from other hymenopteran queen pheromones (Van Oystaeyen et al., 2014). The five major components of QMP are chemically distinct compounds, consisting of a medium chain fatty acid (9-keto-(E)-2-decanoic acid), carbonyl compounds (*cis*- and *trans*-9--hydroxy-(E)-2-decanoic acid), a 4-hydroxybenzoate ester (methyl-hydroxybenzoate) and a methoxy-phenol compound (4-hydroxy-3-methoxyphenylethanol (Shelley et al., 2003)). In contrast

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multiple species of ants, wasps and bees have been shown to use cuticular hydrocarbons as queen pheromones (linear alkanes, methylalkanes and alkenes) (Holman, 2018; Van Oystaeyen et al., 2014). The differences in both the complexity and chemical composition of the queen pheromones are even apparent when comparing the bumblebee with the honeybee (55 million years diverged (Peters et al., 2017)), implying relatively rapid diversification and evolution of honeybee QMP. That this pheromone evolved over the last 55 million years is not consistent with its broad repressive activity on multiple species that have not shared a common ancestor for more than 530 million years, implying QMP may have evolved to take advantage of pathways that are conserved across arthropods.

Here we examine the route by which QMP exposure leads to the repression of *D. melanogaster* reproduction.

2. Materials and methods

2.1. D. melanogaster stocks and maintenance

D. melanogaster (Oregon-R modENCODE line (Bloomington Stock #25211)) were maintained at 25 °C on a 12h:12h light/dark cycle on a solid yeast/sugar medium of; 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.

2.2. QMP dilution

QMP is measured in Queen equivalents (Qe), with one Qe being the amount a mated queen will produce in a 24 h period (Pankiw et al., 1996). One Qe for a European mated queen *Apis mellifera* contains; 200 mg 9-keto-(E)-2-decanoic acid (ODA), 80 mg 9-hydroxy-(E)-2-decanoic acid (9-HDA) and 20 mg methyl-hydroxybenzoate (HOB) and 2 mg 4-hydroxy-3-methoxyphenylethanol (HVA) (Pankiw et al., 1996). QMP (Intko Supply Ltd, Canada) was dissolved in absolute ethanol to a concentration of 26 Qe/20 ml and stored at −20 °C until use.

2.3. QMP exposure

Female virgin *D. melanogaster* were exposed to QMP in modified 50 ml centrifuge tubes as previously described (Lovegrove et al., 2019). *D. melanogaster* were fed 500 ml of liquid diet per day (made fresh daily; 4.75 ml dH_2O, 5% absolute ethanol, 0.15 g sugar and 0.1 g brewer's yeast (Camiletti et al., 2013)). For starvation conditions, the diet was 500 μl of 5% ethanol solution in dH_2O. For QMP treatment, 20 μl of 26 Qe QMP or 20 μl of ethanol solvent control was added to these diets. Concentrations of QMP, ranging from 3.25 to 26 Qe all induce repression in *D. melanogaster* (Camiletti et al., 2013; Lovegrove et al., 2019). The 24 h old virgin females (n = 10 per vial, each treatment having at least 5 replicates) were anesthetised with CO_2 and added to the tube, and the end was blocked with a cotton ball. The tube was left on its side until all individuals had recovered from CO_2 narcosis. *D. melanogaster* were incubated at 25 °C for 12–48 h (experiment dependent).

2.4. Ovary collection and fixation

D. melanogaster were anesthetised with CO_2, and ovaries were dissected into ice-cold PBS (phosphate-buffered saline). Ovaries were fixed using 4% formaldehyde in PBS at room temperature for 10 min. Ovaries were washed 4x with 1 ml of PTx (0.1% Triton X-100 in PBS). During the last wash, 1 μl of DAPI (4′,6-diamidino-2- phenylindole) was added and incubated in the dark for 15 min. Ovaries were then washed with PTx twice and bridge-mounted in 70% glycerol. The number of stage 14 mature (vitellogenic) oocytes were counted manually under a Leica L2 dissection microscope. This was used as an indicator of fecundity (King, 1970). DAPI staining was visualised under an Olympus BX61 Fluoview FV100 confocal microscope with FV10-ASW 3.0 imaging. For measurement of ovarian width and length ovaries were imaged without fixing using a GXM-XTL stereomicroscope with GXCAM-U3 Series 5 MP camera and GX Capture Software (GT Vision, UK). Ovary width and length was measured using Fiji (v. 2.1.0/1.53c) (Schindelin et al., 2012).

2.5. Food intake assay

Liquid diets supplemented with food colouring were used to visualise the food intake of *D. melanogaster*. The diet consisted of 5% brewer's yeast and 5% sugar (dissolved) in dH_2O with food colouring added to both solutions to a final concentration of 5%. Virgin females were exposed to 26 Qe QMP or solvent control for 12 or 48 h. Also included was a group that had been exposed to starvation conditions for 48 h, along with the solvent control. The flies were then transferred to a Petri dish containing 3% agar, on top of which 3 × 100 μl drops of yeast solution were alternated around the edge of the plate with 3 × 100 μl of sugar solution. The *D. melanogaster* were anesthetised with CO_2 before being transferred to the plate and incubated at room temperature until they had recovered. The plates were incubated in the dark for 2 h at 25 °C. After 2 h flies were frozen at – 20 °C to prevent further feeding. This was carried out on 10 vials (n = 10 individuals per vial) for QMP treatment and controls.

Food intake was quantified by inspecting each *D. melanogaster* after freezing and looking for evidence of coloured food within their abdomen. They were classified based on the scale described in (Jiang et al., 2018). Individuals which had not consumed food scored a 0. Those that consumed enough to colour their abdomen only lightly or fill less than 25% were classified as 1. Those that had darker abdomens and had filled 25–50% of their abdomens were scored as 2, and those that filled over 50% of their abdomen were classified as 3 (see Fig. 3A).

2.6. Determination of ovarian checkpoint activation

In *D. melanogaster* there are checkpoints where oocyte production is suppressed in adverse environmental or nutritional conditions (Pritchett et al., 2009). At stage 2a/b (Huynh and St Johnston, 2004) there is a checkpoint that reduces the output of oocytes for maturation, slowing the rate of reproduction (Drummond-Barbosa and Spradling, 2001a). At stage 9 a checkpoint occurs that, when activated, causes stage 9 oocytes to undergo cell death (McCall, 2004).

D. melanogaster oocytes were staged by DAPI staining (Jia et al., 2016; King, 1970) (Fig. 2A). The number of germaria, healthy stage 9 oocytes, degenerating stage 9 oocytes and stage 10 oocytes were counted. Stage 9 degradation was observed as a loss of structural integrity, coupled with bright, fragmented nuclei (Fig. 2A).

Evidence of early ovarian checkpoint activation in response to QMP was determined by calculating the ratio of the number of oocytes which reach or successfully pass the second checkpoint (stage 9 (S9), stage 9 degenerating (S9d), stage 10(S10) per germarium (e.g., containing the germline stem cell niche).

$$\frac{(S9 + S9d + S10)}{g}$$

If every germarium is producing oocytes that will pass through the 2a/b ovarian checkpoint, and progress to the stage 9 checkpoint we would expect a ratio of approximately 1:1, shown numerically as 1.0. However, if the stage 2a/b checkpoint is activated then the number of oocytes passing through to the stage 9 checkpoint will be reduced, lowering reproductive output below that of the anticipated theoretical value. This is reflected in a ratio significantly lower than 1.0. For example, if the 2a/b checkpoint activation reduces the number of oocytes reaching that stage 9 checkpoint by 50%, then the ratio will be 0.5.

2.7. Calculation of stage 9 oocyte checkpoint activation

The proportions of stage 9, stage 9 degenerating and stage 10 oocytes were calculated from count data. Any activity of the stage 9 checkpoint should lead to an increase in the proportion of oocytes at stage 9 which show degradation. This is associated with a reduction of oocytes that pass through this checkpoint at stage 9 and successfully reach stage 10.

2.8. RT-qPCR methods

RNA was extracted from whole *D. melanogaster* that had been snap-frozen at −80 °C using the Zymo Research Direct-zol RNA MicroPrep kit (Zymo Research). RNA was extracted from five whole flies, ovaries or heads per replicate and there were five biological replicates for each treatment group. Genomic DNA contamination was eliminated with on-column digestion with DNase (Zymo Research). The concentration of RNA was measured on a Nanodrop ND-2000 spectrophotometer (Nanodrop) and 1 μg of total RNA as a template to perform cDNA synthesis using the RevertAid cDNA Synthesis Kit (Thermo Scientific) as per the manufacturer's protocol. cDNA was diluted at 1:10 for the RT-qPCR reactions. Controls with no reverse transcriptase were used to assess the possibility of genomic DNA contamination in RT-qPCR. qRT–PCR was carried out on a BioRad CFX Real-Time PCR detection system with SsoFast Advanced PCR master mix, 5 ng of cDNA and 300 nM of each primer (sequences provided in SuppFile 2). For each condition gene expression was measured for five biological replicates and each measurement was made in duplicate. The expression of target genes was normalized by the geometric mean of the relative quantities for three reference genes that we had determined were stably expressed in each tissue and at each time point amongst our samples using GeNorm implemented in R using the ctrlGene package: (SuppFile 2).

2.9. Statistical analysis

Data were analysed using R Studio version 1.2.5033 running R version 3.6.2. Assessment of whether the data fit a normal distribution was carried out using a Shapiro-Wilk test, all data showed a non-normal distribution. Data were analysed using Generalised Linear Mixed Models (GLMMs) using lme4 (Bates et al., 2015). In all cases, treatment and time were treated as fixed effects and the slide number or replicate as a random factor. Where an effect of treatment was found, pairwise comparisons between treatments were carried out using emmeans and a Tukey posthoc test, to correct for multiple testing. The number of mature oocytes (Fig. 1A) was analysed using a negative binomial error structure after a model fitted with the Poisson error structure was shown to have higher than expected residual variance (over-dispersed). Ovarian length (Fig. 1B) was analysed using a Gaussian error structure with an identity link and Ovarian width (Fig. 1C) was analysed using a Gamma error structure with an inverse link. Ovariole numbers (Fig. 1A) were analysed using a Poisson error structure with a log link. The ratio of oocytes reaching the second ovarian checkpoint was analysed with a Gaussian error structure (Fig. 2A). Because we expect the numbers of ovarioles, stage 9 oocytes, degenerating oocytes and stage 10 oocytes to be dependent variables, we analysed the data in Fig. 2B by performing a principal component analysis (PCA), which indicated that the majority of variation was observed in the first principle component. This component was extracted and a GLMM with a Gaussian error structure was used to determine the effect of treatments at different time points. Differences in food consumption (Fig. 3) were assessed using a Fisher's exact test. Gene expression data (Fig. 4) was analysed using GLMMs; *4EBP/Thor*, *Ilp5*, *Inr*, and *Pepck* expression was analysed using a Gamma error structure with an inverse link and *fbp, Ilp2, Ilp3, Ilp6* with a Gaussian error structure with an identity link.

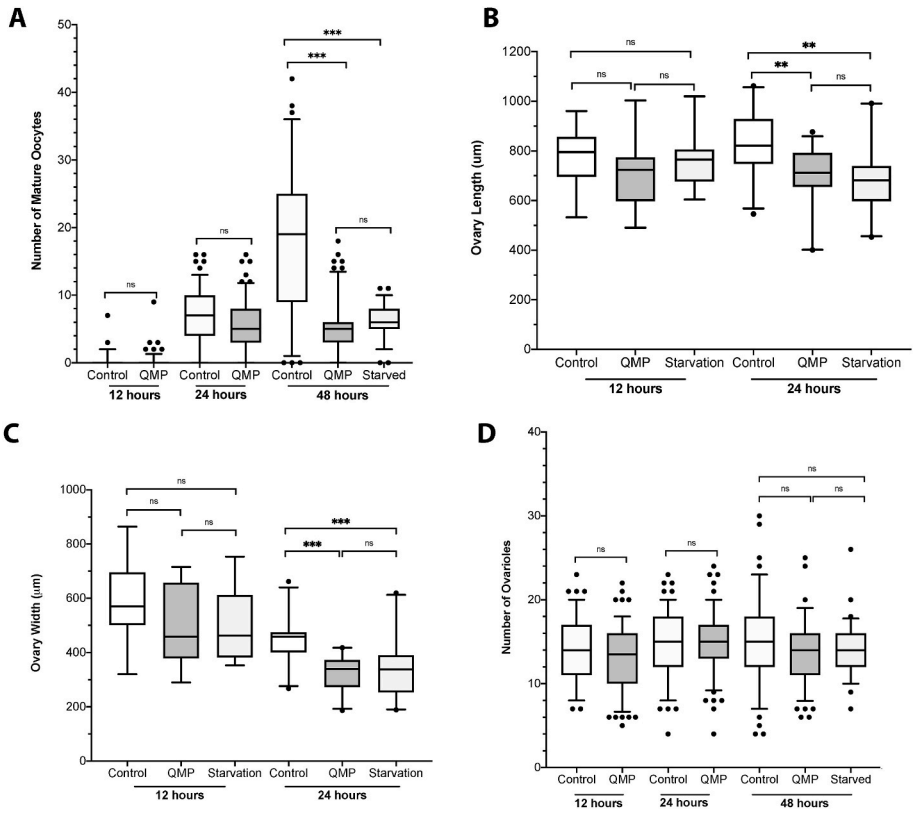

Fig. 1. QMP induces repression in the number of mature oocytes in *Drosophila* ovaries. A) Box and whisker plot showing the average number of mature (stage 14 oocytes) per ovary in *D. melanogaster* exposed to either 26 Qe QMP or an ethanol solvent control for 12, 24, 36 or 48 h. Also included was a 48 h starvation control. Differences in mature oocyte number were determined using a GLMM with a negative binomial distribution followed by Tukey posthoc analysis, statistically significant differences are indicated by asterisks (***, $p < 0.001$). B) Box and whisker plot showing the length of ovarioles in *D. melanogaster* that were exposed to either 26 Qe QMP, an ethanol solvent control, or starvation conditions, for 12 or 24h. Statistical analysis was carried out using a Gamma error structure with an identity link. Statistically significant differences are indicated by asterisks (**, $p < 0.01$). C) Box and whisker plot showing the width of the ovary in *D. melanogaster* exposed to either 26 Qe QMP, an ethanol solvent control, or starvation conditions, for 12 or 24h. Statistical analysis was carried out using a Gamma error structure with an inverse link. Statistically significant differences are indicated by asterisks (***, $p < 0.001$). D) Box and whisker plot showing the number of ovarioles per ovary in *D. melanogaster* that were exposed to either 26 Qe QMP or an ethanol solvent control for 12, 24 or 48 h. Also included was a 48 h starved positive control. Statistical analysis was carried out using a generalised linear mixed model (GLMM) with a Poisson distribution, there were no statistically significant differences in ovariole number between any of the treatments. In each panel the box is defined by the 25th percentile, median and 75th percentile, whiskers extend to 5% and 95%; outliers are represented by individual points. For all treatments, $n \geq 50$ individuals.

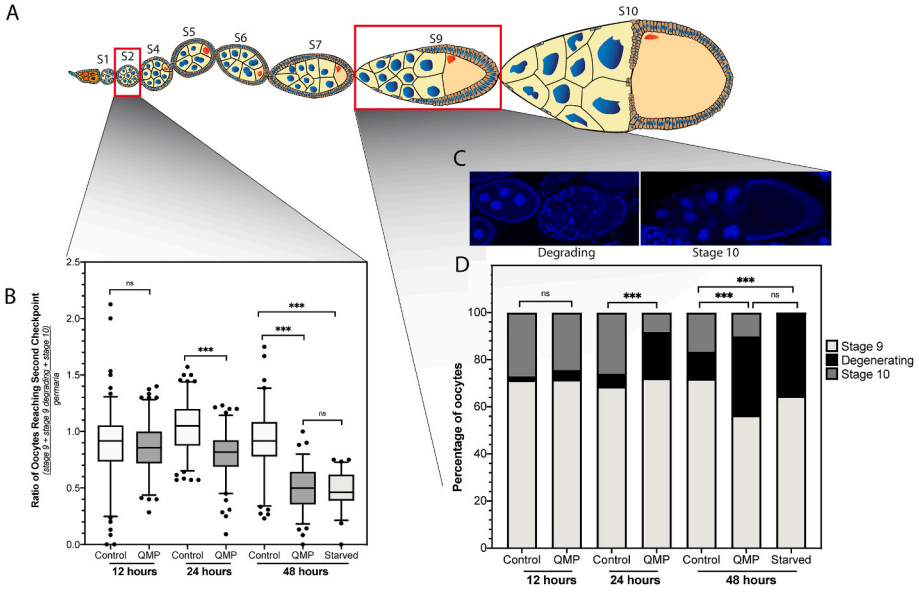

Fig. 2. QMP activates the 2a/b ovarian checkpoint to reduce fecundity consistent with a starvation response. A) Schematic of a *Drosophila* ovariole with stages and positions of the two ovarian checkpoints marked B) Ratio of germaria compared to the number of oocytes that are reaching or passing through the stage 9 ovarian checkpoint. This indicates the rate at which presumptive oocytes are passing through the stage 2a/b ovarian checkpoint. *D. melanogaster* were exposed to either a control or 26 Qe QMP for 12, 24 or 48 h. Also included was a 48 h starved control. The box is defined by the 25th percentile, median and 75th percentile, whiskers extend to 5% and 95%; the outliers are represented with circles. Differences in oocytes that had passed the Stage 2 checkpoint were determined using a GLMM with a Gaussian distribution followed by Tukey posthoc analysis, statistically significant differences are by asterisks (***, $p < 0.001$). C) Representative images of stage 9 degenerating and stage 10 oocytes were used to calculate the effect of the stage 9/10 checkpoint. D) Stacked bar chart shows the proportion of oocytes in *D. melanogaster* ovaries at stage 9, stage 9 degenerating and stage 10. Individuals were exposed to 26 Qe QMP or an ethanol solvent control for 12, 24 or 48 h. Also included is a positive control for 48 h of starvation. As the number of ovarioles, stage 9 oocytes, degenerating oocytes and stage 10 oocytes are likely dependent on each other, data was analysed by performing a principal components analysis (PCA), which indicated that the majority of variation was observed in the first principal component. This component was extracted and a GLMM with a Gaussian error structure and a Tukey post-hoc test was used to determine the effect of treatments at different time points. Statistically significant differences are by asterisks (***, $p < 0.001$). For all treatments, $n \geq 50$ individuals.

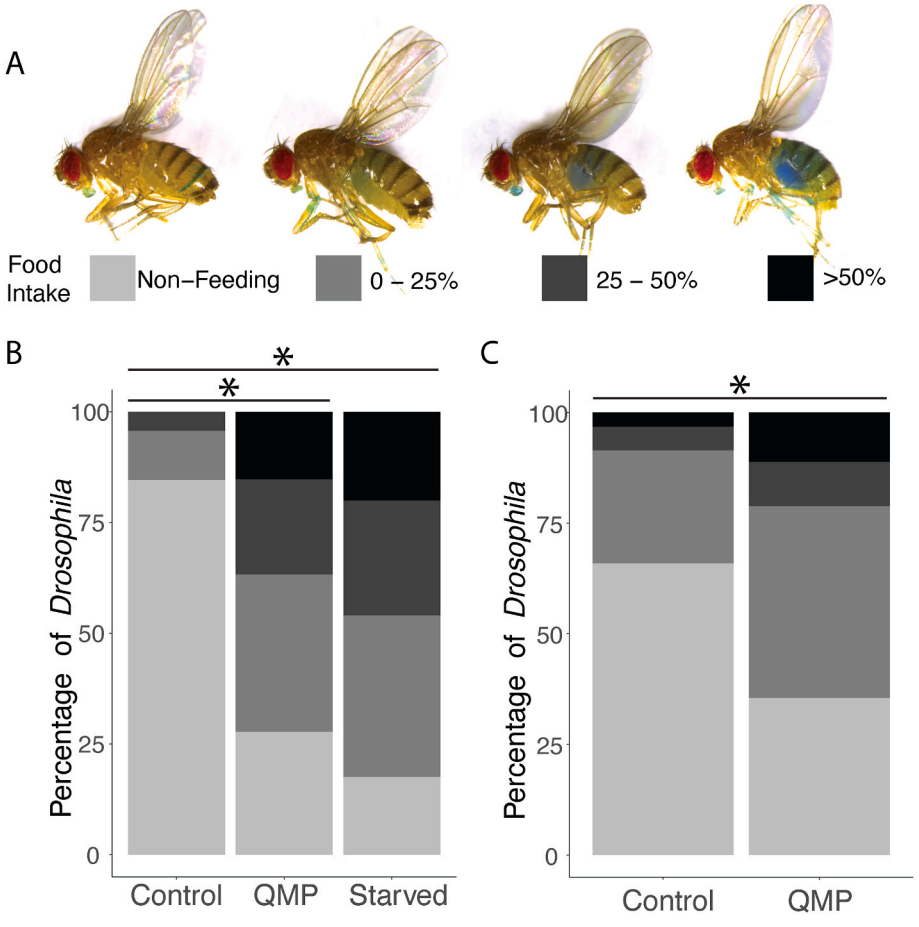

Fig. 3. QMP causes increased feeding in adult *Drosophila*. A) Representative images of feeding assays showing categorisation of food intake based on assays described in (Jiang et al., 2018). Food intake is categorised based on the percentage of the abdomen which showed the presence of coloured food. B) Food consumption in *D. melanogaster* exposed to either 26 Qe QMP (QMP) or a solvent control while on a standard liquid diet for 48 h (CONTROL) or starved for 24 h before exposure to solvent control (STARVED). **C)** Food consumption in *D. melanogaster* exposed to 26 Qe QMP (QMP) or solvent control (CONTROL) while on a standard liquid diet for only 12 h. A Fisher's exact test was carried out, with significance being determined by an alpha value of <0.05. For all treatments, $n = 100$ individuals.

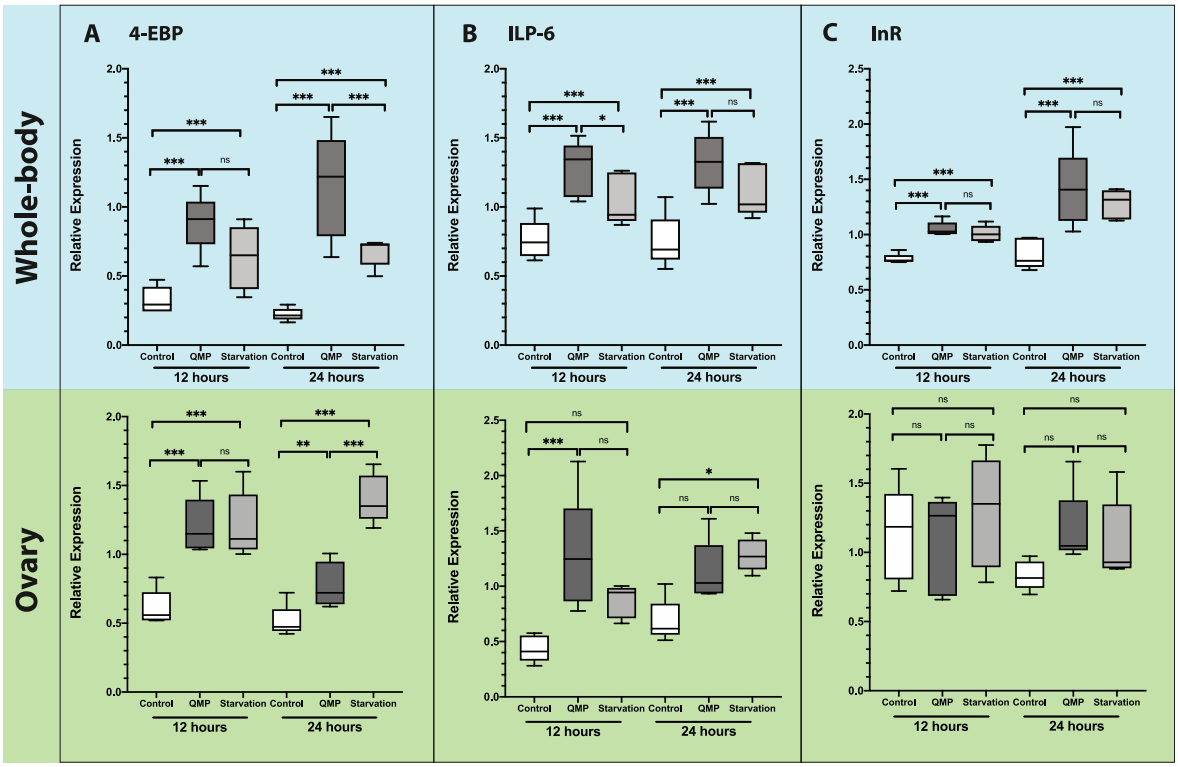

Fig. 4. QMP and starvation induce the expression of genes that indicate repression of insulin signalling (A–C). RT-qPCR analysis of FOXO responsive genes (indicating repression of insulin signalling) in each case expression is assayed in the whole-body- upper panel and isolated ovary-lower panel. A) *4EBP (Thor)* B) *Ilp6* and C) *InR* in *Drosophila* either starved or treated with QMP. Differences in expression were established using GLMMs with a Tukey posthoc test as described in the methods. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. RT–qPCR data is the mean of transcript levels in five biological samples for each time point and treatment (n = five flies per replicate).

3. Results

3.1. QMP represses ovary activity but does not cause degeneration of the ovary

Consistent with previous studies (Camiletti et al., 2013; Lovegrove et al., 2019, 2020) treatment with QMP causes a significant reduction in the number of mature oocytes after 48 h (AOD $\chi^2 = 17.689$, df = 2, $p = 0.1441 \times 10^{-3}$) (Fig. 1A) consistent with the reduction in the number of mature oocytes seen in response to starvation (Fig. 1A, SuppFile 1). Although there was no difference in the number of mature oocytes at 12 hor 24 h exposed ovaries we did see a significant reduction in both ovary length (Fig. 1B, (AOD $\chi^2 = 16.176$, df = 2, $p = 0.3072 \times 10^{-3}$) SuppFile 1) and ovary width (Fig. 1C, AOD $\chi^2 = 5.8672$, df = 2, $p = 0.05321$) SuppFile 1) in response to both QMP exposure and starvation at 24 h. There were no significant differences in ovary length ($p = 1.0000$) or width ($p = 0.9990$) between QMP-treated and starvation-treated flies. Neither QMP treatment nor starvation caused any reduction in the number of ovarioles present in the ovary (Fig. 1D, AOD $\chi^2 = 1.2457$, df = 2, $p = 0.5364$).

3.2. QMP reduces fecundity by activating the 2a/b ovarian checkpoint

We next tested if the loss of mature oocytes was due to the activation of reproductive checkpoints, well described in *Drosophila* (Pritchett et al., 2009), in oogenesis.

We found that QMP progressively reduces the number of oocytes which advance past the first checkpoint (stage 2a/b) to the second checkpoint (Fig. 2A and B, AOD $\chi^2 = 33.265$, df = 2, $p = 5.977 \times 10^{-8}$). No significant reduction was observed after only 12 h of QMP exposure (, $p = 1$), but after 24 h of QMP exposurethere was a significant difference as controls had, on average, 1.032 oocytes reaching stage 9 per germarium, whereas the QMP-treated flies had only 0.797, a 23% reduction ($p = 0.0005$). This difference became more pronounced by 48 h of exposure, where controls produced 0.945 stages 9 oocytes per germaria, and the QMP exposed had 0.505 ($p = <0.0001$). QMP exposure reduces the number of oocytes passing through the 2a/b checkpoint by 46.56% after 48 h of exposure. There was no significant difference between individuals who had been exposed to QMP for 48 h and those starved for 48 h ($p = 1.00$). QMP acts to repress reproduction in *D. melanogaster* by reducing the flow of oocytes through the 2a/b ovarian checkpoint by half, similar to the effects of starvation.

3.3. QMP also activates the stage 9 ovarian checkpoint to reduce fecundity

We investigated whether the stage 9 checkpoint was also activated in response to QMP exposure (Fig. 2A, C, D). This was carried out by counting the number of stage 9 oocytes, stage 9 degenerating and stage 10 oocytes. This data is shown proportionally to remove the effect of activation of the stage 2a/b checkpoint. To account for the differences in activation of the early checkpoint we compare the proportion of oocytes reaching stage 9, degrading or passing the checkpoint to reach stage 10, to determine if QMP exposure affects the stage 9 checkpoint (Fig. 2D).

QMP elicits a significant effect on the numbers of stage 9, degenerating and stage 10 oocytes (Fig. 2B, AOD $\chi^2 = 17.191$, df = 2, $p = 1.849 \times 10^{-4}$). There was no significant difference observed in the proportion of oocytes after 12 h QMP exposure ($p = 0.7725$) but at 24 h and 48 h of QMP exposure, there was a significant difference in the proportions of oocyte stages ($p = <0.0001$). At 24 h and 48 h there was an increase in the proportion of oocytes degenerating at stage 9 compared with the controls (At 24 h 5.41% of control oocytes were degenerating at stage 9, versus Q 19.62% of the QMP treated. At 48 h 11.53% of the control oocytes were degenerating, versus 33.54% of the QMP treated). At 48 h

of exposure to QMP there were also fewer oocytes reaching stage 9 (Control mean = 71.83% and QMP treated mean = 56.30%) consistent with activation of the stage 2a/b ovarian checkpoint (Fig. 2A). We also observed a difference in the number of mature oocytes at stage 10 (24 h controls 26.04% and QMP treated had just 8.24%, 48 h controls 16.64% and QMP treated had 10.16%).

There was no significant difference in the effects of QMP at 48 h and the effects of starvation for 48 h ($p = 1.000$), implying that the degree of activation of the second ovarian checkpoint is similar between QMP and starvation treatments.

3.4. QMP-exposed flies consume more food in a pattern consistent with starvation

We have shown that QMP treatment causes activation of both stage 2a/b and stage 9 checkpoints (Fig. 2), indistinguishable from the phenotype caused by starvation. To test the hypothesis that QMP treatment may be inducing a starvation response more generally we carried out a food intake assay (Fig. 3A).

Drosophila exposed to QMP for 48h increased their food consumption (Fig. 3B). Only 15% of the control, untreated, population consumed food, whereas 74% of the QMP exposed population did ($p = 2.4 \times 10^{-17}$) despite no restriction in access to food up to the start of the assay. Controls that did consume food mostly filled less than 25% of their abdomen, whereas half of the QMP-exposed flies filled more than 25% of their abdomen ($p = 1.02 \times 10^{-13}$) (Fig. 3B). This implies that QMP exposure increases both the likelihood an individual will feed and the quantity they consume. This was consistent with the phenotype observed in flies starved for 24h in which, compared to the 15% of the fed control population (Fig. 3B) which consumed food, 82% of the starved flies ate. When fed individuals exposed to QMP were compared to starved controls, there was no significant difference in food intake ($p > 0.05$) (Fig. 3B). The quantities of food consumed by fed QMP and starved controls also were not different ($p > 0.05$) (Fig. 3C). Fed QMP treated flies consume as often, and as much, as starved flies.

3.5. QMP treatment changes expression of genes responsive to insulin signalling

Insulin signalling is a key pathway responding to nutrient signalling and starvation conditions in *D. melanogaster* (Ojima et al., 2018; Sudhakar et al., 2020). In particular, three genes are targets of FOXO and their expression is regulated by insulin signalling (Bai et al., 2012; Puig et al., 2003; Puig and Tjian, 2005); *4EBP* (*Thor*), *InR* and *Ilp6*. All three genes increase their expression in starved flies indicating a reduction in insulin signalling during starvation (Sudhakar et al., 2020). We assayed the expression of these genes using RT-qPCR in control, starved and QMP-treated flies, in both whole body (Fig. 4A) and ovary (Fig. 4B) RNA samples. All three genes were induced in response to QMP exposure in whole-body RNA samples, mirroring the changes in expression seen with starvation. For *4EBP* and *Ilp6,* the magnitude of induction by QMP was greater than that caused by starvation (Fig. 4) in whole-body but not ovary samples. In the ovary, QMP treatment transiently increases expression of *4EBP* and *Ilp-6*, with expression dropping at 24 h consistent with the ovary responding directly to changes in insulin signalling in response to QMP treatment and starvation. InR expression is not significantly different in the ovary between control, QMP-treated or starved flies.

To investigate if the reduction in insulin signalling due to QMP is due to low insulin levels, or insulin resistance, we examined the expression of the insulin-like peptides (ILPs) genes in the isolated heads of flies. Reduction in the expression of these genes in the head would imply

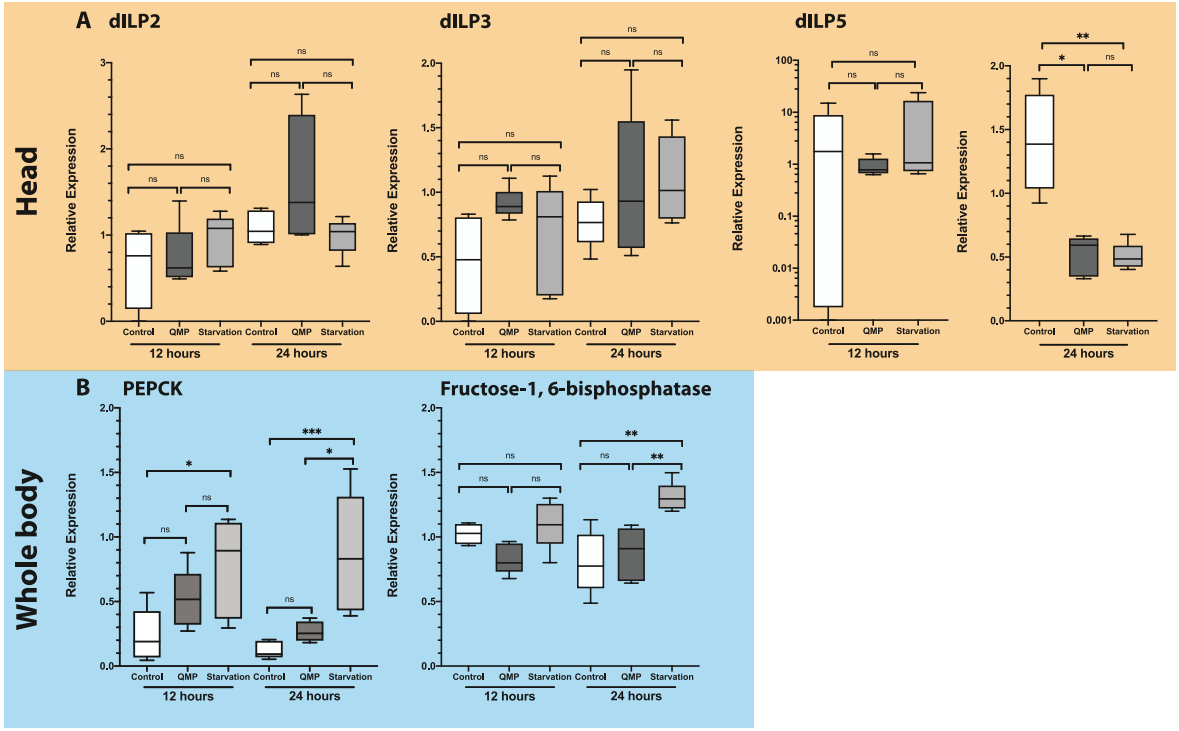

Fig. 5. A) QMP and starvation affect the expression of insulin-like peptides in the head in a similar way. Expression of *Ilp2* and *3* vary greatly in adult female head RNA, and no differences between control, QMP treated and starved flies were detected. *Ilp5* RNA is also very variably expressed in heads at 12 h, with no significant differences in expression between control, starved and control flies. At 12 h (note the difference in scale in the Y axis between 12-h and 24-h measurements), both starvation and QMP treatment lead to a significant reduction in *Ilp5* expression. B) Expression of enzymes involved in gluconeogenesis is affected by starvation but not QMP treatment. Expression of *Pepck1* and *fbp* are both significantly increased by starvation for 12 and 24 h (*Pepck1*) or just 24 h (*fbp*). No similar increase in expression is seen after QMP treatment. Differences in expression were established using GLMMs with a Tukey posthoc test as described in the methods. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. qRT–PCR data is the mean of transcript levels in five biological samples for each time point and treatment (n = five flies per replicate).

lower circulating ILPs in the hemolymph (Ikeya et al., 2002). *Ilp5* mRNA expression is reduced 24 h post-exposure to QMP or starvation in *Drosophila* heads (Fig. 5A), which is consistent with data previously published for *Drosophila* males (Sudhakar et al., 2020). Expression of *Ilp2*, *3* and *5* is highly variable at 12 h post-exposure and we were not able to detect any consistent differences in the expression of these ILPs (Fig. 5A).

QMP may act on flies by reducing their ability to take up nutrients from food. QMP doesn't require the antenna or maxillary palps to have its action in *Drosophila* (Lovegrove et al., 2020), so QMP may act directly on the gut; blocking nutrition uptake. However, starved flies, with reduced nutrition uptake, increase the expression of *Phosphoenolpyruvate carboxykinase 1* (*Pepck1*) (Fig. 5B), which encodes the rate-limiting step in gluconeogenesis, and *Fructose-1,6-bisphosphatase* (*fbp*), a key enzyme in gluconeogenesis (Chatterjee et al., 2014; Sudhakar et al., 2020). QMP-treated flies, in contrast, do not show a significant increase in either *Pepck1* or *fbp* (Fig. 5B), consistent with our finding that QMP-treated flies feed more than non-treated, and contrary to the idea that QMP might be blocking nutrition uptake. These data imply that QMP is triggering a neuroendocrine starvation response, leading to decreased insulin signalling, but that the flies are not physiologically starving, as suggested by the lack of upregulation of key gluconeogenesis enzymes. QMP-treated flies, while being well-fed, act as if they are starving by increasing food uptake, and, crucially, by shutting off female reproduction.

3.6. The starvation behaviours caused by QMP are established before the repression of the ovary

We have established that 48 h of QMP exposure induced starvation-like behaviour (Fig. 3B). Also, after 48 h of QMP exposure, there is a reproductive repressive phenotype already established within the ovary at the 2a/b checkpoint, stage 9 checkpoint, and the number of mature oocytes (Fig. 2, B, D and 1A respectively). To determine if the differences in feeding and insulin signalling are upstream of ovarian repression, or if ovarian repression stimulates the differences in feeding response, we conducted the food consumption assay after only 12 h of QMP exposure. As shown in Fig. 1A–C, as well as 2B and D, there is no ovarian repression established in QMP-treated flies after 12 h of treatment. However, after only 12 h of QMP exposure, a significant difference in food intake was already established (Fig. 3C) and consistent with this we saw an increase in the expression of genes in the whole body (*4EBP*, *InR* and *Ilp6*), and in the ovary (*4EBP* and *Ilp6*) indicating repression of insulin signalling at 12h and 24h of treatment (Fig. 4). At 12h the QMP-exposed population was more likely to consume food than the controls ($p = 9.09 \times 10^{-5}$). Of the QMP-exposed group, 64% of the population ate, compared to 34% of the controls. We conclude that QMP induces an increase in food intake and a decrease in insulin signalling rapidly after exposure (within 12h) and that this occurs before any physiological change in the ovary.

4. Discussion

We have shown that repression of reproduction by QMP in *D. melanogaster* is acting through insulin signalling, with honeybee QMP inducing sustained ovarian repression, and repression of insulin signalling, similar to that seen with starved individuals (Figs. 3 and 4, (Burn et al., 2015; Pritchett et al., 2009; Pritchett and McCall, 2012). *Ilp5* expression in the brain is reduced in starving and QMP-treated flies and key insulin-responsive genes have lower expression in the whole body, and specifically in the ovary, in both starved and QMP-treated flies (Figs. 4 and 5).

Reproduction in *D. melanogaster* is regulated by a complex interplay between insulin, biogenic amines, juvenile hormone and 20-hydroxyecdysone signalling (Knapp et al., 2022; Roy et al., 2018) and is responsive to nutrition (reviewed in (Mirth et al., 2019). In *D. melanogaster,* starvation conditions result in a temporary pause of reproduction (Burn et al., 2015), a state known as reproductive dormancy. Insulin signalling has a general role in regulating reproductive dormancy in *D. melanogaster* in response to environmental cues. But more specifically, insulin signalling mediates the germline response to starvation conditions (Drummond-Barbosa and Spradling, 2001b) and the ovary requires an intact insulin signalling pathway to allow oogenesis to proceed (Kubrak et al., 2014). Our data imply that QMP acts by triggering starvation pathways that lead to reduced reproduction.

Consistent with this, individuals that are exposed to QMP exhibit a significant increase in food consumption, indistinguishable from that caused by starving *D. melanogaster* (Fig. 3). However, QMP flies are not actually starving or nutrient deprived. Therefore, QMP induces a perceived state of nutritional deficit, leading to increased food intake, decreased signalling and ovary repression, despite higher amounts of feeding.

The perceived nutritional deficit induced by QMP exposure is established before any phenotype of repression is observed in the ovary-indicating that this is upstream process and that QMP may be acting directly on either nutrient sensing, hunger, or satiety signalling to induce this response. When an ovarian response is observed, both the 2a/b checkpoint and the stage 9 checkpoint in the ovary are activated, a phenotype also consistent with starvation (Pritchett et al., 2009). Based on *Pepck1* and *fbp* expression, QMP isn't causing a physiologically induced state of starvation. Flies treated with QMP don't upregulate gluconeogenesis, while starved flies do.

This QMP-induced starvation-like mechanism may have similarities to the effects of long-term high-sugar diets, which, in larvae, lead to insulin resistance and reduced reproduction. It is important to note, however, that QMP's effects on reproduction occur within 24 h of exposure, rather than the weeks used in high-sugar-diet experiments. More long-term experiments (Morris et al., 2012) may be required to determine if insulin resistance does occur with QMP exposure.

Repression of reproduction by QMP is not limited to *D. melanogaster* (Camiletti et al., 2013; Lovegrove et al., 2019; Sannasi, 1969), but also occurs in a wide range of arthropods (Carlisle and Butler, 1956; Hrdy et al., 1960; Nayar, 1963; Sannasi and George, 1972). It thus seems likely that QMP is disrupting conserved nutrition perception or signalling pathways, mediated by insulin signalling to disrupt reproduction in these species.

Nutrition and nutrient-sensing pathways have been implicated in establishing and maintaining reproductive skew in social and eusocial arthropods (Kapheim, 2017). In particular, social signals and reproductive state in ants (Chandra et al., 2018), reproductive dominance in paper wasps (Markiewicz and O'Donnell, 2001; O'Donnell et al., 2018) (Tibbetts, 2007) *Polistes metricus* (Toth et al., 2009) and even social spider colonies (Salomon et al., 2008). Nutrition is also associated with reproductive rate and reproductive diapause in solitary species (Mirth et al., 2019; Ojima et al., 2018), raising the possibility that QMP, and possibly other queen pheromones, have evolved to co-opt ancient mechanisms involved in environmental-responsive reproduction. Does then the nutrient-sensing- reproductive repression mechanism induced by QMP in *Drosophila* reflect what is happening in honeybees?

Our findings in *Drosophila* are superficially consistent with what is known in the honeybee ovarian response to QMP. In honeybees, QMP acts within the germarium to regulate oogenesis (Duncan et al., 2016) and is suggested to induce apoptosis at later stages of oocyte maturation (Ronai et al., 2015), both of these are similar to our findings that QMP activates ovarian checkpoints in *D. melanogaster* (Fig. 2). QMP exposed, 4-day old worker bees are more able to resist starvation than those not exposed to QMP, a phenotype linked to higher lipid stores in their fat body (Fischer and Grozinger, 2008) implying some aspects of a starvation response might be in place. QMP does not, however, increase food consumption in honeybees, as it does in *D. melanogaster* (Duncan et al., 2016, 2020). Worker bees treated across the first 10 days after emergence with QMP and without QMP show no differences in food

consumption (Duncan et al., 2016, 2020). This implies that if an ancient 'arthropod-wide' mechanism linking nutrition with reproduction has been co-opted in the evolution of QMP that this link has been decoupled in the honeybee lineage. Consistent with this, there have been changes in the regulatory interactions linking nutrition, neuroendocrine signalling and reproduction in honeybees and other eusocial insects (Kapheim, 2017; Rodrigues and Flatt, 2016).

QMP is a derived pheromone, consisting of five major semiochemicals (Slessor et al., 1988) that are chemically distinct from the less derived and more broadly used queen pheromones from other eusocial insects (Van Oystaeyen et al., 2014). These less-derived queen pheromones do not induce ovary repression in *D. melanogaster* (Lovegrove et al., 2019), suggesting that QMP may be unique in its ability to control reproduction in non-target species. Given the chemical complexity of QMP, that it has evolved over the last 55 million years (Peters et al., 2017), the broad range of the response to QMP outside eusocial insects (Carlisle and Butler, 1956; Hrdy et al., 1960; Nayar, 1963; Sannasi, 1969), and the data we present here linking QMP with manipulation of nutrient-sensing we propose that QMP has evolved, as a result of an evolutionary 'arms race' over worker reproduction, to target deeply conserved essential and pleiotropic pathways such as Notch signalling (Duncan et al., 2016) and neuroendocrine signalling.

Our data links QMP with nutrition signalling in flies, and perhaps, by extension, the broad range of species where QMP has a similar effect (Carlisle and Butler, 1956; Hrdy et al., 1960; Nayar, 1963; Sannasi, 1969). That nutrition signalling is implicated in eusociality across a range of arthropods, and multiple independent evolutions of eusociality imply a deep role for these pathways in the evolution of eusociality. We propose that these data point to 'queen pheromones' evolving to manipulate nutrient-sensing pathways in non-reproductive individuals (Okada et al., 2017; Toth, 2017).

Author contributions

MRL: Assisted with experimental design, carried out *D. melanogaster* experiments and statistical analysis (Fig. 3) drafted and edited the manuscript.

EJD: Assisted with experimental design, performed the RT-qPCR and carried out statistical analysis (Figs. 1, 2, 4 and 5), assisted with the preparation of figures and edited manuscript.

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

Data availability

All data for this work is reported in the text, or available in the supplemental materials.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2023.103908.

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